Fourth Edition

QIA*express*® Detection and Assay Handbook

For

Anti·His Antibodies

Anti·His HRP Conjugates

Penta·His™ Alexa Fluor® Conjugates

Penta·His Biotin Conjugate

Ni-NTA Conjugates

Tag·100™ Antibody

Streptavidin–R-PE

6xHis Protein Ladder

Ni-NTA HisSorb™ Strips and Plates

July 2015

QIAGEN

www.qiagen.com

Contents

QIA*express* Protocols in This Handbook

Kit Contents

Anti·His HRP Conjugates	RGS·His™ HRP Conjugate Kit	Penta·His™ HRP Conjugate Kit	Tetra·His™ HRP Conjugate Kit
Catalog No.	**34450**	**34460**	**34470**
Anti·His HRP Conjugate	125 µl, for 250 ml working solution	125 µl, for 250 ml working solution	125 µl, for 250 ml working solution
Blocking Reagent	5 g	5 g	5 g
Blocking Reagent Buffer (10x Concentrate)	50 ml	50 ml	50 ml
Handbook	1	1	1

Ni-NTA Conjugate	Ni-NTA AP Conjugate	Ni-NTA HRP Conjugate
Catalog No.	**34510**	**34530**
Conjugate	Lyophilized, for 500 ml working solution	Lyophilized, for 500 ml working solution
Handbook	1	1

Anti·His Antibody	RGS·His Antibody (100 µg)	RGS·His Antibody, BSA-free (100 µg)
Catalog No.	**34610**	**34650**
Antibody	100 µg, lyophilized for 1000 ml working solution	100 µg, lyophilized for 1000 ml working solution
Handbook	1	1

Anti·His Antibody	Penta·His Antibody, BSA-free (100 µg)	Tetra·His Antibody, BSA-free (100 µg)
Catalog No.	**34660**	**34670**
Antibody	100 µg, lyophilized for 1000 ml working solution	100 µg, lyophilized for 1000 ml working solution
Handbook	1	1

Tag·100™ Antibody (100 µg)	
Catalog No.	**34680**
Antibody	100 µg, lyophilized (BSA-free) for 1000–2500 ml working solution
Handbook	1

Anti·His Antibody Selector Kit	
Catalog No.	**34698**
Penta·His Antibody	3 µg, lyophilized for 30 ml working solution
RGS·His Antibody	3 µg, lyophilized for 30 ml working solution
Tetra·His Antibody	3 µg, lyophilized for 30 ml working solution
Handbook	1

Ni-NTA HisSorb Strips	**(24)**	**Ni-NTA HisSorb Plates**	**(5)**
Catalog No.	**35023**	**Catalog No.**	**35061**
Racks of 12 x Ni-NTA–coated 8-well strips (for 192 assays)	2	Ni-NTA–coated, transparent 96-well plates (for 480 assays)	5
Handbook	1	Handbook	1

Ni-NTA HisSorb Plates, white	**(5)**
Catalog No.	**35081**
Ni-NTA–coated, opaque, white 96-well plates (for 480 assays)	5
Handbook	1

6xHis Protein Ladder	
Catalog No.	**34705**
6xHis-tagged molecular weight marker (15–100 kDa)	Lyophilized, for 50–100 lanes on western blots
Reference card	1
Handbook	1

Kit Contents are continued on the next page

Kit Contents

Penta·His Alexa Fluor® Conjugates	
Catalog Nos.	**35310, 35330, 35350, 35370**
Penta·His Alexa Fluor Conjugate	125 µl, 200 µg/ml
Product sheet	1

Penta·His Biotin Conjugate	
Catalog No.	**34440**
Penta·His Alexa Fluor Conjugate	125 µl, 200 µg/ml
Product sheet	1

Streptavidin–R-PE	
Catalog No.	**922721**
Streptavidin–R-PE	250 µg, 1 mg/ml solution
Product sheet	1

Storage Conditions

Anti·His HRP Conjugates are supplied as a stabilized stock solution and can be stored for up to one year at 2–8°C. Do not freeze the conjugate solution. Blocking Reagent and Blocking Reagent Buffer Concentrate can be stored for up to one year at room temperature.

Anti·His and Tag·100™ Antibodies should be stored lyophilized until they are to be used. They can be stored lyophilized for 1 year at 2–8°C. In solution they can be stored for 3 months at 2–8°C or for up to 6 months in aliquots at –30 to –15°C. Avoid repeated freezing and thawing. Dissolve the lyophilized Anti·His Antibody (100 µg) in 500 µl water per vial (final concentration, 0.2 mg/ml). Dissolve Anti·His Antibody Selector Kit antibodies (3 µg) in 15 µl water per tube (final concentration, 0.2 mg/ml).

Penta·His Alexa Fluor Conjugates and **Streptavidin–R-PE** can be stored undiluted at 2–8°C for 1 year. Protect from light.

Penta·His Biotin Conjugate can be stored undiluted at 2–8°C for 1 year.

Ni-NTA AP Conjugate can be stored lyophilized for 6 months at 2–8°C or for 1 year at –30 to –15°C. In solution it can be stored for 1 month at 2–8°C or for 1 year at –30 to –15°C. Recommended storage is in aliquots of stock solution at –30 to –15°C. Dissolve the lyophilized conjugate in 500 µl water per vial.

Ni-NTA HRP Conjugate can be stored lyophilized for 1 year at 2–8°C or at –30 to –15°C. In solution it can be stored for 3 months at 2–8°C or for 1 year at –30 to –15°C. Recommended storage is in aliquots of stock solution at –30 to –15°C. Dissolve the lyophilized conjugate in 500 µl water per vial.

6xHis Protein Ladder can be stored lyophilized for 6 months at 2–8°C or in solution for up to 6 months in aliquots at –30 to –15°C. Avoid repeated thawing and freezing.

Ni-NTA HisSorb™ Strips and Plates should be stored dry at room temperature. Under these conditions they are stable for 1 year.

Product Use Limitations

QIA*express*® products are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN® product does not meet your expectations, simply call your local Technical Service Department. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor.

Safety Information

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

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. They are always available to discuss any general or specific questions you may have. If you have any questions or experience any problems regarding components of the QIA*express* Detection and Assay Systems 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 also 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).

Introduction to The QIA*express* System

The expression, purification, detection, and assay of recombinant proteins has been made much more simple and powerful by the use of small affinity tags. The well-established QIA*express* Protein Expression and Purification Systems are based on the remarkable selectivity and affinity of patented nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices for proteins tagged with 6 consecutive histidine residues (6xHis tag, Figure 1) available exclusively from QIAGEN.

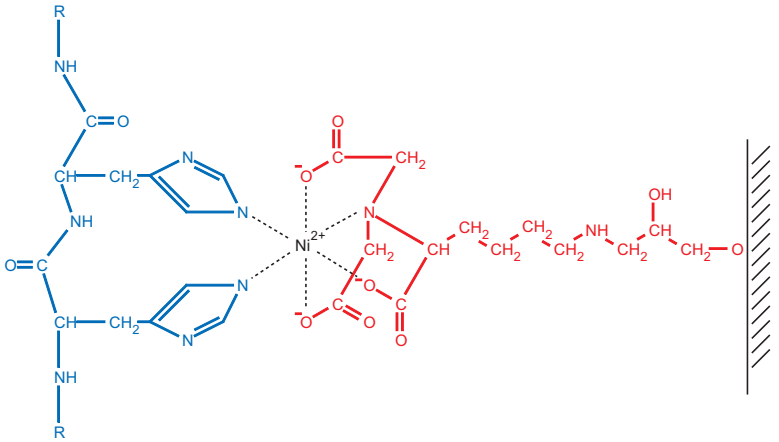


Figure 1. *Interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix.*

QIA*express* provides a complete system for the expression, purification, detection, and assay of 6xHis-tagged proteins. This new *QIA*express *Detection and Assay Handbook* gives detailed protocols and guidelines for the detection and assay of 6xHis-tagged proteins and can also be used as a guide for the assay of other 6xHis-tagged biomolecules, such as peptides or nucleic acids. Detailed instructions and protocols for the expression and purification of 6xHis-tagged proteins are given in *The QIA*expressionist™, *a handbook for high-level expression and purification of 6xHis-tagged protein*.

Table 1. Features and benefits of the QIA*express* System

Features	Benefits
The interaction of the 6xHis tag with Ni-NTA is conformation independent.	Ni-NTA products can be used for purification, detection, and assay under native or denaturing conditions.
The 6xHis tag is much smaller than other commonly used tags.	Tag does not interfere with the structure or function of the recombinant protein.
	Tag removal by protease cleavage is not necessary.
	6xHis tags can be used in any expression system — conversion of vectors requires only the inclusion of a small oligonucleotide.
	The 6xHis tag provides a general method for purification, detection, and assay of all recombinant proteins.
The 6xHis tag is poorly immunogenic.	The recombinant protein, without prior removal of the tag, can be used as an antigen to generate antibodies against the protein of interest.

The 6xHis tag

The 6xHis affinity tag facilitates binding to Ni-NTA. It is poorly immunogenic, meaning that antibodies raised to 6xHis-tagged proteins will normally recognize only the protein of interest and not the 6xHis tag. This feature has meant, however, that antibodies have been unavailable, in spite of the great demand for highly specific antibodies against the 6xHis tag. This problem has been solved after extensive research by the introduction of the QIA*express* Anti·His HRP Conjugates and Anti·His Antibodies that specifically recognize the 6xHis tag (1). In most cases, the 6xHis tag does not interfere with the structure or function of the tagged protein as demonstrated for a wide variety of proteins, including enzymes (2), transcription factors (3), and engineered antibodies (4).

Ni-NTA technology

Immobilized-metal affinity chromatography (IMAC) was first used to purify proteins in 1975 (5) using the chelating ligand iminodiacetic acid (IDA, Figure 2). IDA has only 3 metal-chelating sites and cannot tightly bind metal ions. This results in considerable leaching of metal ions from the chromatography matrix reducing the efficiency of protein purification (6). Therefore proteins that bind the metal ions are weakly bound to the metal-chelating matrix. A new, improved ligand for metal-chelate chromatography, Ni-NTA, was developed at Hoffmann-La Roche and is exclusively available from QIAGEN. NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag (Figure 2). NTA binds metal ions far more stably than other available chelating resins (7, 8) and retains the ions under a wide variety of stringent conditions. The 6xHis tag binds strongly to the immobilized nickel ion allowing optimum separation. For optimal performance, both the 6xHis tag–nickel and the nickel–NTA interactions are important (Figure 3). These properties, which allow powerful one-step protein purification with Ni-NTA chromatography matrices (9), are exploited by Ni-NTA Conjugates and Ni-NTA HisSorb Strips and Plates, making them ideal tools for the detection and assay of 6xHis-tagged proteins.

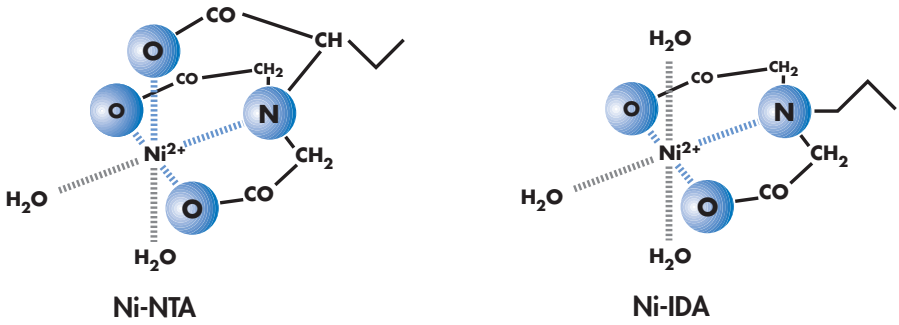


Figure 2. *Comparison of the interactions of different metal chelate matrices with nickel ions.*

Figure 3. *The capture of 6xHis-tagged proteins by metal-chelate affinity matrices relies on two interactions. Both are important for optimal performance. If interaction A is weak then there is no binding of the 6xHis-tagged proteins. If interaction A is strong, but interaction B 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.*

Anti·His Antibodies

QIA*express* Anti·His Antibodies are mouse monoclonal IgG1 antibodies that have high affinity and specificity for the 6xHis tag (for background information about antibodies, see Appendix, page 86). These antibodies are obtained from serum-free hybridoma cell cultures, ensuring preparations free of viruses, mycoplasmas, and contaminating immunoglobulins. Purification by adsorption chromatography is performed without using protein A or protein G, and entirely at physiological pH, to yield preparations with the highest purity and activity. Because they allow detection of all 6xHis-tagged proteins Anti·His Antibodies eliminate the need for prior purification of each individual protein, animal immunization, and testing and processing of serum. The exact epitopes that are recognized by each of the Anti·His Antibodies have been determined using a peptide library (Table 2).

RGS·His™ Antibody recognizes the RGS(His)$_4$ epitope that is found on proteins encoded by the pQE-9, pQE-30, pQE-31, pQE-32, pQE-40, pQE-80L, pQE-81L, pQE-82L, and pQE-100 vectors. The epitope is also found encoded by the pRSETA, pRSETB, pRSETC, pBlueBacHis2A, pBlueBacHis2B, and pBlueBacHis2C vectors available from Invitrogen. RGS·His Antibody has been used to identify 6xHis-tagged proteins on western blots and to study protein–protein interactions (10, 11) and protein–DNA binding (12).

Penta·His™ Antibody and Tetra·His™ Antibody do not require amino acids additional to the polyhistidine sequence in the 6xHis tag. Therefore, they can bind to 6xHis-tagged proteins expressed from any vector. Penta·His Antibody recognizes five consecutive histidine residues while Tetra·His Antibody binds to four consecutive histidine residues regardless of the surrounding amino-acid context. Therefore, Penta·His and Tetra·His Antibodies can bind to even partially hidden 6xHis tags that other anti-His antibodies do not recognize. All three Anti·His HRP Conjugates and Anti·His Antibodies have very high specificity and are suitable for all expression systems.

This handbook provides protocols for their use in detecting 6xHis-tagged proteins by western, dot, or colony blotting. In addition, QIA*express* Anti·His Antibodies can be used for localization of 6xHis-tagged proteins in situ using immunohistochemical and immunocytochemical procedures (13, 14, see "Immunolocalization of 6xHis-tagged Proteins" page 56). The very high affinity of the Anti·His Antibodies for their epitopes (Table 3) means that they are suitable for immunoprecipitation (15), and immunoaffinity chromatography. A protocol is also provided for coating 96-well microplates to allow highly specific, high-density immobilization of 6xHis-tagged proteins for efficient ELISA protocols and other assays.

Anti·His Antibody Selector Kit

Individual 6xHis-tagged proteins are often recognized better by one Anti·His Antibody than by the others, possibly because of subtle differences in the exact conformation of the 6xHis tag and other parts of the protein in the vicinity of the tag. To choose the optimal antibody for your specific 6xHis-tagged protein and application, QIAGEN offers the Anti·His Antibody Selector Kit. The kit includes 3 µg of each of the three antibodies for direct comparison and economical selection of the one that gives the best results.

Table 2. Determination of epitopes recognized by QIA*express* Anti·His HRP Conjugates and Anti·His Antibodies

Peptide	Penta·His	Tetra·His	RGS·His
X**HHHHHH**X	+	+	–
XX**HHHHHH**X	+	+	–
X**H**X**HHHH**X	–	+	–
X**HHHH**X**H**X	–	+	–
XRGS**HHHH**X	–	+	+
XRGS**HHHHHH**X	+	+	+
X**H**X**HH**X**H**X	–	–	–

*The specificity of the antibodies was determined by epitope mapping using libraries of peptides synthesized directly on nitrocellulose (1 µg per peptide). "X" signifies any amino acid other than histidine. Note that the presence of specific residues N- or C-terminal to the epitope are not required. Filters were washed in TBS (20 mM Tris·Cl; 150 mM NaCl, pH 8), blocked in 1% casein in TBS, and washed further in TBS. Blots were probed with QIA*express *Anti·His Antibody (0.1 µg/ml) followed by secondary antibody and chromogenic detection with AP-conjugated anti-mouse IgG and NBT/BCIP.*

Table 3. Anti·His Antibody epitopes and dissociation constants

Antibody	Epitope	Dissociation constant K_d (M)*
RGS·His Antibody	RGSHHHH	$1 \times 10^{-8} – 5 \times 10^{-8}$
Penta·His Antibody	HHHHH	$5 \times 10^{-8} – 1 \times 10^{-9}$
Tetra·His Antibody	HHHH	$1 \times 10^{-8} – 5 \times 10^{-8}$

** Dissociation constants were measured using surface plasmon resonance (BIACORE®) technology. The exact value of K_d is dependent on the individual 6xHis-tagged protein.*

Anti·His HRP Conjugates

Anti·His HRP Conjugates consist of mouse monoclonal IgG1 Anti·His Antibodies coupled to horseradish peroxidase. They can be used for direct detection of any protein with an accessible 6xHis tag by chromogenic or chemiluminescent methods. They eliminate the need for secondary antibodies, thereby saving time and expense in blotting and ELISA procedures. The conjugates are available in highly specific Penta·His, Tetra·His, and RGS·His forms (see Anti·His Antibodies section above). Anti·His HRP conjugates are supplied with a specially formulated Blocking Reagent and Blocking Reagent Buffer to ensure optimal specificity and sensitivity.

Penta·His Alexa Fluor Conjugates

Proven Penta·His monoclonal antibodies are available conjugated to a range of Alexa Fluor fluorescent dyes for use in immunofluorescence procedures. The mouse monoclonal antibodies are available as Alexa Fluor 488, 532, 555, and 647 conjugates, giving a range of highly specific reagents whose emission wavelengths cover a wide portion of the visible spectrum (Table 4). The Penta·His Antibody recognizes an epitope comprising five consecutive histidine residues. The conjugates can be used in all immunofluorescence procedures, such as fluorescence-activated cell sorting (FACS®), flow cytometry, confocal laser microscopy, immunocytochemistry and immunohistochemistry, protein localization, and targeting studies.

Table 4. Spectral characteristics of Alexa Fluor dyes and R-PE

Dye	Excitation maximum (nm)	Emission maximum (nm)	Laser/light sources
Alexa Fluor 488	494	519	Ar, Xe, ZnCdSe
Alexa Fluor 532	531	554	Ar, Xe, Nd-YAG
Alexa Fluor 555	555	565	Xe, HeNe, Hg-arc
Alexa Fluor 647	650	668	Kr, HeNe
R-phycoerythrin	565 (480, 546)	578	Ar, Xe, Hg-arc

Ni-NTA Conjugates

Ni-NTA Conjugates consist of Ni-NTA coupled to calf intestinal alkaline phosphatase (Ni-NTA AP Conjugate) or horseradish peroxidase (Ni-NTA HRP Conjugate). They can be used for direct, straightforward detection of any recombinant protein with an accessible 6xHis tag, in a simplified procedure without the need for a secondary antibody. Detection is based on the affinity of Ni-NTA for affinity tags consisting of consecutive histidine residues; the same principle that underlies purification of 6xHis-tagged proteins using Ni-NTA Agarose. They eliminate the need for secondary antibodies, thereby saving time in blotting procedures. Ni-NTA Conjugates are recommended for use with chromogenic substrates, and for *E. coli* expression systems.

Penta·His Biotin Conjugate and Streptavidin–R-PE

The Penta·His Biotin Conjugate can be used in conjunction with any streptavidin or avidin conjugate, delivering high flexibility in immunodetection procedures. The Penta·His Antibody recognizes an epitope comprising five consecutive histidine residues. For highly sensitive immunofluorescent detection using the Penta·His Biotin Conjugate, QIAGEN offers streptavidin conjugated to the intensely fluorescing algal pigment R-phycoerythrin (Streptavidin–R-PE).

QIA*express* Tag·100 Antibody

The Tag·100 Antibody is a mouse monoclonal antibody that recognize proteins expressed from the pQE-100 DoubleTag™ vector (see *The QIA*expressionist for further details). These proteins have an N-terminal 6xHis tag and a C-terminal Tag·100 to allow oriented binding of the protein by its 6xHis tag to Ni-NTA supports and subsequent detection of the well exposed Tag·100 by the Tag·100 antibody. The system is ideal for ELISA procedures including drug screening and accurate protein quantitation.

6xHis Protein Ladder

The 6xHis Protein Ladder serves as a molecular weight standard, and as a positive control for western blotting. The 6xHis Protein Ladder consists of five 6xHis-tagged proteins ranging from 15 to 100 kDa. Each of the proteins that make up the 6xHis Protein Ladder is tagged at the N-terminus with the RGS$(His)_6$ sequence, therefore they can be detected by any of the QIA*express* detection reagents including the RGS·His Antibody. The 6xHis Protein Ladder is simply dissolved in SDS-PAGE sample buffer, heated, and loaded onto a gel (Protocol 2). SDS-PAGE and blotting are performed in the usual way (Protocols 1 and 3). Bands appear directly on blot or film to provide a permanent record with no extra work.

Ni-NTA HisSorb Strips and Plates

Ni-NTA HisSorb Strips and Plates utilize the power of Ni-NTA in a solid-phase, multiwell format for assays using 6xHis-tagged proteins or other biomolecules with a 6xHis tag. Tagged biomolecules are bound to the strips and plates in a uniform orientation and are not denatured. This means they remain conformationally active giving optimal presentation to detection reagents, typically antibodies or any interacting biomolecule, used in subsequent assay steps. This results in the increased sensitivity of Ni-NTA HisSorb Strips and Plates compared to polystyrene plates where biomolecules are randomly adsorbed in nonuniform orientations and denatured by immobilization. Since 6xHis-tagged proteins are selectively bound to the Ni-NTA–coated surface, the strips and plates can be used to assay proteins even in crude cell lysates and dilute solutions. Ni-NTA HisSorb Strips and Plates can be used to quantitate 6xHis-tagged biomolecules, to establish ELISA or RIA procedures or diagnostic assays, to study biomolecular interactions, and to screen expression clones, engineered enzymes, pharmaceuticals, antibodies, or serum samples. Ni-NTA HisSorb Plates are available in either a transparent format for colorimetric assays, or an opaque, white format for luminescence- and fluorescence-based assays.

Ni-NTA Magnetic Agarose Beads

Ni-NTA Magnetic Agarose Beads combine all of the benefits of Ni-NTA with the convenience and speed of magnetic-bead technology. The beads are designed for a wide range of magnetocapture assays using 6xHis-tagged proteins as well as rapid, high-throughput protein purification. They are supplied with a comprehensive handbook containing background information and protocols — contact QIAGEN for further details.

Detection of 6xHis-tagged Proteins

QIA*express* Detection Systems allow recombinant proteins fused to a 6xHis tag to be detected in a variety of ways. Probably the most commonly used procedure is the detection of proteins after immobilization to a membrane. Proteins can be immobilized directly from bacterial colonies (colony blotting: Protocol 6, page 38), from crude cell lysates or solutions (dot blotting: Protocol 5, page 37), or after separation of proteins by SDS-PAGE (western blotting: Protocols 1–4, pages 30–36).

Ni-NTA Conjugates (in combination with chromogenic substrates) and Anti·His HRP Conjugates can be used for simple, direct detection of 6xHis-tagged proteins. They make secondary binding reagents such as enzyme-conjugated antibodies unnecessary, and allow quick and easy detection of as little as 2–5 ng protein in western-blot analysis.

QIA*express* Anti·His Antibodies and Anti·His HRP Conjugates can be used in applications where a higher level of specificity and sensitivity is required. They comprise the RGS·His, Penta·His, and Tetra·His Antibody that recognize the RGS$(His)_4$, $(His)_5$, and $(His)_4$ epitopes respectively. Anti·His HRP Conjugates also contain a horseradish peroxidase (HRP) moiety that allows direct detection by chemiluminescent or chromogenic methods, without the requirement of a secondary antibody. Anti·His Antibodies and Anti·His HRP Conjugates are particularly useful when analyzing samples containing complex mixtures of proteins (such as some eukaryotic expression systems; Figures 4 and 5).

6xHis-tagged DNA Polymerase Detected by Tetra·His Antibody in Huh7 Cells

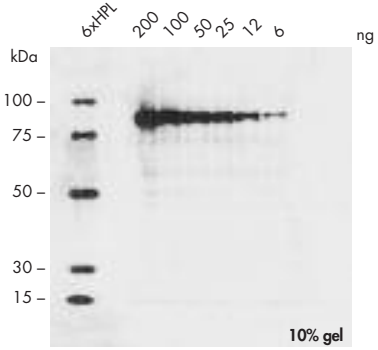


Figure 4. *The indicated amounts of purified 6xHis-tagged DNA polymerase mixed with crude extract from Huh7 cells (5 µg total protein per lane) were applied to a 10% SDS-polyacrylamide gel. After electrophoresis and western transfer, 6xHis-tagged proteins were detected with Tetra·His Antibody followed by chemiluminescent detection with AP-conjugated rabbit anti-mouse IgG and CDP-Star™ detection reagent.* ***6xHPL****: 6xHis Protein Ladder (2.5 µl).*

6xHis-tagged Thioredoxin Detected by Penta·His Antibody in Yeast Cells

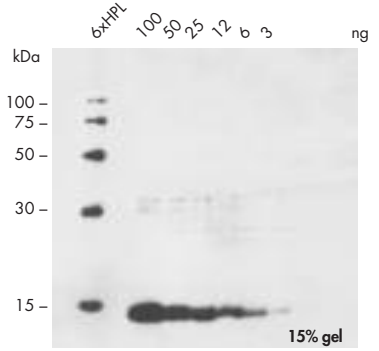
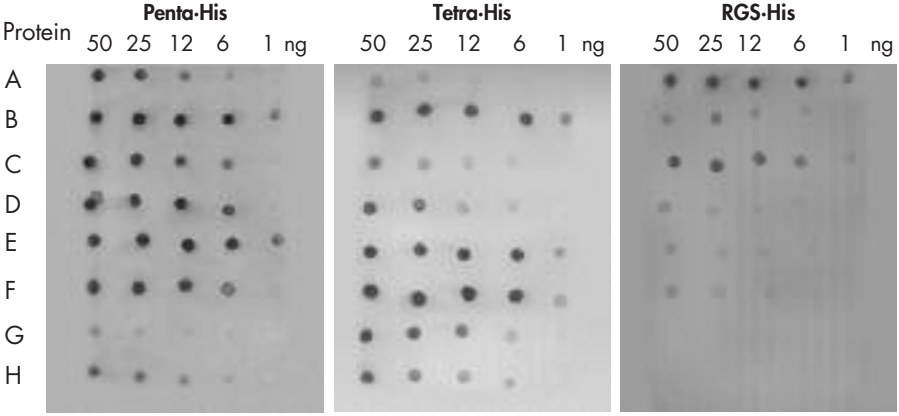


Figure 5. *The indicated amounts of purified 6xHis-tagged thioredoxin mixed with crude extract from yeast cells (5 µg total protein per lane) were applied to a 15% SDS-polyacrylamide gel. After electrophoresis and western transfer, 6xHis-tagged proteins were detected with Penta·His Antibody followed by chemiluminescent detection with HRP-conjugated rabbit anti-mouse IgG and ECL™ detection reagent.* ***6xHPL****: 6xHis Protein Ladder (2.5 µl).*

Penta·His and Tetra·His Antibody bind to proteins that have 6xHis tags regardless of the surrounding amino-acid context and even when the tag is partially hidden. Subtle differences in the antigen recognition sites of these antibodies mean that some 6xHis-tagged proteins are detected with higher sensitivity by one Anti·His Antibody than by the others (Figure 6). Therefore, to achieve optimum results, we recommend comparison of these antibodies in parallel for new applications and proteins. QIAGEN offers the Anti·His Antibody Selector Kit for this purpose (see page 15). The 6xHis Protein Ladder provides a molecular weight standard and positive control for western blotting (see page 17).

Sensitivity of QIA*express* Anti·His Antibodies



	Protein	Tag location	Sequence
A	DHFR	N-term	MRGSHHHHHHGSVRPLNCIV.....
B	DHFR	C-term	.....VYEKKDGSRSHHHHHH
C	Thioredoxin	N-term	MRGSHHHHHHGSSDKIIHLT.....
D	TNF-α	N-term	MRHHHHHHQSTESMIRDV.....
E	TFIIAγ	N-term	MGSSHHHHHHSSGLVPRGSH.....
F	Chaperonin	C-term	..... AKIAKDGSRSHHHHHH
G	DNA polymerase	C-term	..... EDWLSAKERSHHHHHH
H	TFIIAαβ	Internal	.....QQQHQPQQQQHHHHHHHQQAQPQQTVP.....

Figure 6. *Detection of 6xHis-tagged proteins (**A–H**, see table) with QIA*express *Anti·His Antibodies. Indicated amounts of pure 6xHis-tagged protein were applied to a nitrocellulose membrane, and detection was carried out with the Anti·His primary antibody indicated diluted 1/2000, followed by chromogenic detection with AP-conjugated rabbit anti-mouse IgG and NBT/BCIP.*

Table 5. Overview of QIA*express* detection and assay systems

Product	Epitope detected	Substrates for blot detection	Protocol(s) for detection	Substrates for assay procedures	Protocol for assays
Penta·His Antibody	HHHHH	Dependent on secondary antibody	Protocol 7 or 8	Dependent on secondary antibody	Protocol 16
Tetra·His Antibody	HHHH	Dependent on secondary antibody	Protocol 7 or 8	Dependent on secondary antibody	Protocol 16
RGS·His Antibody	RGSHHHH*	Dependent on secondary antibody	Protocol 7 or 8	Dependent on secondary antibody	Protocol 16
Penta·His HRP Conjugate	HHHHH	Colorimetric: 4C1N† Chemiluminescent: Amersham Pharmacia Biotech ECL	Protocol 7 or 8	*o*-Phenylenediamine (OPD)	Protocol 16
Tetra·His HRP Conjugate	HHHH		Protocol 7 or 8	*o*-Phenylenediamine (OPD)	Protocol 16
RGS·His HRP Conjugate	RGSHHHH*		Protocol 7 or 8	*o*-Phenylenediamine (OPD)	Protocol 16
Ni-NTA AP Conjugate	n.a.	Colorimetric: BCIP/NBT	Protocol 9	*p*-Nitrophenylphosphate (pNPP)	Protocol 17
Ni-NTA HRP Conjugate	n.a.	Colorimetric: 4C1N†	Protocol 9	*o*-Phenylenediamine (OPD)	Protocol 17
Tag·100 Antibody	12 aa of ERK‡	Dependent on secondary antibody	see page 67	Dependent on secondary antibody	see page 67

** Recombinant proteins expressed from pQE-9, pQE-30, pQE-31, pQE-32, pQE-40, pQE-80L, pQE-81L, pQE-82L, pQE-100 DoubleTag™, pRSET (Invitrogen), and pBlueBacHis (Invitrogen) encode this epitope. † Further substrates are listed on page 90. ‡ Recombinant proteins expressed from pQE-100 DoubleTag Vector encode this epitope.*

Immobilization onto membrane — western, dot, or colony blotting

Overview

In the western blot, proteins are transferred to a membrane from a polyacrylamide gel after separation according to size by SDS-PAGE (SDS polyacrylamide gel electrophoresis). This allows confirmation of the size of the expressed 6xHis-tagged protein by comparison with molecular-weight markers (6xHis Protein Ladder, see page 17) and detection of any degradation products that may be present. Western blotting also allows identification of 6xHis-tagged proteins in crude cell lysates — invaluable if the protein migrates anomolously in SDS-PAGE. Native proteins that form complexes may also be identified (16).

The simplest and fastest immobilization method is the dot blot: dilutions of a solution or crude cell lysate containing the 6xHis-tagged protein are pipetted directly onto a nitrocellulose membrane. Native proteins can be directly dot blotted but detection may be improved if proteins are denatured to ensure exposure of their 6xHis tags. We recommend dot blotting for the functional testing of the detection system and protein, in order to avoid possible problems associated with the transfer of proteins from polyacrylamide gels during western blotting.

Colony blotting is recommended as a troubleshooting procedure when rapid identification of 6xHis-tagged protein expressing bacterial colonies is necessary. In the colony-blot procedure, bacterial colonies are transferred to a nitrocellulose membrane that is laid on the surface of an agar plate. Protein expression is induced, and after incubation, the cells are lysed with alkali. The proteins that are released bind to the membrane in the position of the colony. This allows rapid, simultaneous screening of numerous colonies to identify expressing clones and even to distinguish semi-quantitatively between expression rates of individual colonies.

Separation of proteins by SDS-PAGE

The standard electrophoretic technique used for the separation of proteins is polyacrylamide-gel electrophoresis (PAGE). Polyacrylamide gels form after polymerization of monomeric acrylamide into polyacrylamide chains. Polymerization is initiated by the addition of ammonium persulfate, which in solution dissociates to sulfate radicals, and tetramethylenediamine (TEMED), which catalyzes the formation of free radicals. The polyacrylamide chains are crosslinked by *N,N'*-methylenebisacrylamide leading to a network of polyacrylamide chains that contain pores through which the proteins migrate. The pore size is dependent on the acrylamide concentration and the proportion of bisacrylamide in the total acrylamide amount.

The most widely used system to separate proteins in a polyacrylamide gel is the denaturing, discontinuous system with a separating and a stacking gel described by Laemmli (17). Protein samples are heated in the presence of a reducing agent and SDS, which binds to the polypeptide chains giving a constant charge to mass ratio. The gel is

cast in two parts: the lower, separating gel and the upper, stacking gel. The stacking gel has large pores and is prepared with a slightly acidic buffer (pH 6.7); whereas the separating gel has a higher pH (8.9), a higher ion concentration, and smaller pores to facilitate protein separation. The electrophoresis buffer contains glycine, and its pH is similar to that of the separating gel.

After protein samples are loaded into wells in the stacking gel, an electric field is applied at a constant current, and anions (chloride, SDS-coated proteins, and glycine) begin to migrate toward the anode. Once the glycine ions (also called trailing ions) reach the sample buffer and stacking gel their mobility is retarded as their net charge changes with the pH-shift. Chloride ions (leading ions) and proteins remain mobile and continue to move toward the anode. The retarded glycine ions and the faster-moving chloride ions create a zone of lower conductivity in the stacking gel between themselves and, as a result, a higher voltage gradient. This allows the proteins to migrate faster until they reach the zone of leading ions, where they concentrate, forming a tight band. Once the proteins reach the separating gel with much smaller pores, their movement is slowed. Glycine ions regain their charge and now pass the proteins; the electric field becomes constant in the complete gel allowing separation of proteins in the separating gel.

Since proteins in SDS-PAGE migrate as a function of their size, it is possible to determine the molecular weight of a protein by comparing its mobility with those of reference proteins. There is a logarithmic relationship between the molecular weight of a protein and its relative mobility, i.e., the distance it migrates relative to the distance the bromophenol blue in the sample buffer migrates (R_f value).

The percentage of acrylamide in the separating gel is chosen according to the size of the proteins to be separated (see Table 6). The protocol described on page 27 for a 12% polyacrylamide gel has been extensively used in the QIA*express* R&D laboratories to separate proteins from ~15 to ~120 kDa. Although out of the range where there is a strictly logarithmic relationship between molecular weight and migration distance (Table 6), the separation of the larger proteins is sufficient for routine work. There are many alternative SDS-PAGE protocols in current use that work just as well (18–22).

Table 6. Sizes of proteins separated by SDS-PAGE

Acrylamide concentration (%)	Linear range of separation (kDa)
15.0	12–43
10.0	16–68
7.5	36–94
5.0	57–212

Adapted from Sambrook et al. (1989)

Western transfer

Following electrophoresis, proteins in a polyacrylamide gel can be transferred to nitrocellulose membrane (e.g., Schleicher and Schuell BA85) in a buffer-tank-blotting apparatus or by semi-dry electroblotting as described in Protocol 3 (page 34).

With the semi-dry electroblotting method, stacks of filter paper are prewetted in electrotransfer buffer and placed at the cathode and the anode. The polyacrylamide gel and membrane are sandwiched between two stacks of filter paper. The gel is placed near the cathode (negatively charged) and the membrane is placed near the anode (positively charged). Proteins are transferred from the gel onto the membrane when an electrical field is applied.

With the tank-blotting method, the blotting cassette is submerged in a tank reservoir for electroblotting. Tank blotting can be performed over extended periods as the buffer capacity is far greater than that with semi-dry transfer systems. Results with the tank-blotting method are typically better, providing more efficient transfer, particularly of large proteins (Figure 7). The difference between the two methods can be as much as ten-fold, since the semi-dry method never results in complete transfer of proteins. Larger proteins are transferred less efficiently than smaller proteins — with larger proteins it may help to increase transfer times or use a lower percentage of acrylamide in the gel. With smaller proteins attention should be paid to the pore size of the membrane used. It may help to use a second membrane to bind proteins that pass through the first membrane. Proteins may then be detected on this second membrane. Hydrophobic proteins may also be less efficiently transferred — for these proteins it may help to increase the percentage of methanol in the transfer buffer. Transfer efficiency should be checked by staining proteins on the membrane (Protocol 4, page 36).

We recommend using the 6xHis Protein Ladder as a positive control for western blotting (see page 17). Use of the 6xHis Protein Ladder shows whether proteins of different sizes have been uniformly transferred and detected. After staining with Ponceau S the proteins of the 6xHis Protein Ladder should be at least weakly stained, and visible as faint bands.

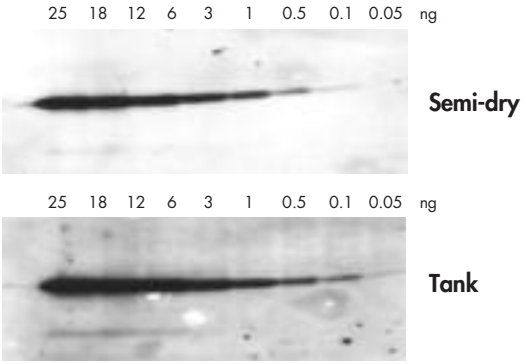


Figure 7. *The indicated amounts of purified 6xHis-tagged DHFR mixed with crude extract from HeLa cells (8 µg total protein) were applied to an SDS-polyacrylamide gel. Western blots were prepared in an electrophoretic transfer apparatus according to the method indicated. 6xHis-tagged protein was detected with Penta·His Antibody diluted 1/2000 followed by HRP-conjugated rabbit anti-mouse IgG and ECL™ detection system.*

Staining proteins after transfer

After electrotransfer, proteins should be visualized on the membrane by staining. This allows the efficiency of transfer to be estimated. Strips of membrane can be cut out to allow different subsequent treatment — for example, detection with different antibodies/detection reagents. There are several methods for staining membrane-bound proteins, but Ponceau S is recommended because the membrane is easily destained prior to immunological detection of the 6xHis-tagged protein and therefore will not interfere with the subsequent detection procedure. See Protocol 4, page 36.

Dot blots

Dot blotting is a simple, convenient method for detection of 6xHis-tagged proteins in crude lysates or solutions without the need for separation by SDS-PAGE. This method is especially useful as a simple control because it avoids problems that may be due to the western transfer process. Any components that interfere with binding or bind nonspecifically, however, will not be spatially separated from the 6xHis-tagged protein and will interfere with the intensity of signals. Suitable controls should always be employed to compensate for this. See Protocol 5, page 37.

Colony blots

We recommend the colony-blot procedure (Figure 8) to identify clones expressing a 6xHis-tagged protein and to distinguish semi-quantitatively between expression rates. This can be an advantage for selecting clones after transformation since freshly transformed colonies may differ significantly in their expression rates.

Using this method, colonies subsequently found to be expressing 6xHis-tagged proteins at rates as low as 0.1 to 0.5 mg/liter are easily distinguished from colonies that do not express 6xHis-tagged protein. See Protocol 6, page 38.

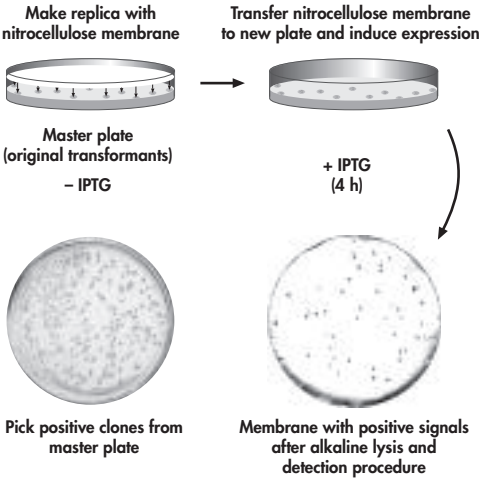


Figure 8. *Detection of positive expression clones by colony blotting.*

Blocking of membrane and nonspecific binding

After transfer of proteins (including the 6xHis-tagged protein), the remaining protein-free sites on the membrane must be blocked so as to prevent high background due to binding of the primary or secondary antibody, or Anti·His HRP or Ni-NTA Conjugate directly to the membrane. Blocking reagents are included in all binding steps to minimize nonspecific interactions of detection reagents with proteins bound to the membrane as well as exchange of blocking reagents bound to the membrane by the subsequent detection reagents.

Several different blocking reagents are in common use, including nonfat dried milk, gelatin, BSA, casein, serum, and Tween® 20. In the majority of protocols described in this handbook BSA is recommended for blocking. BSA gives consistently good, reproducibly high signal-to-noise ratios and is the reagent of choice for all applications using chromogenic substrates. When using chemiluminescent substrates with Anti·His Antibodies, use of BSA can lead to increased background because blocking may not be sufficient for such highly sensitive detection methods. Therefore, an additional blocking reagent is used to block nonspecific binding: we recommend nonfat dried milk, but only during incubation with secondary antibodies. Milk leads to reduced sensitivity if present before the Anti·His Antibody binds to its antigen. Therefore milk should only be used in the last incubation step. Alternatively, if alkali-soluble casein (Merck, Cat. No. 1.02241) is available in your country, it can be used as blocking reagent throughout the chemiluminescent detection protocol.

Note: If using Anti·His HRP Conjugates the specially formulated Blocking Reagent and Blocking Reagent Buffer should be used for blocking the membrane.

Secondary antibodies

When using Anti·His Antibodies for detection, the position of the Anti·His Antibody bound to immobilized 6xHis-tagged protein is visualized using secondary anti-mouse IgG antibodies conjugated to either alkaline phosphatase or horseradish peroxidase together with appropriate enzyme substrates. These antibodies are available from many suppliers. We have obtained consistently good results with rabbit anti-mouse IgG–AP-conjugate from Pierce (Cat. No. 31332) or goat anti-mouse IgG–HRP-conjugate from Jackson Immunoresearch (Cat. No. 115-035-003). Ensure that secondary antibodies used recognize mouse IgG1, and use the highest recommended dilution to avoid nonspecific signals. Detection of 6xHis-tagged proteins with Anti·His HRP and Ni-NTA Conjugates does not require the use of secondary antibodies.

Chromogenic or chemiluminescent substrates

Chromogenic substrates give rise to insoluble, colored products that form a precipitate on the membrane at the location where the enzyme is situated. Therefore the membrane must be kept stationary during color development (see Table 12, Appendix, for suitable chromogenic substrates). The result is obtained quickly and easily, but the membrane must be photographed or scanned to provide a permanent record. The reaction must be monitored and stopped at the optimal time to prevent overdevelopment and high background.

Chemiluminescent substrates give rise to products that spontaneously emit light at the enzyme's location. This can be recorded using photographic film, with the advantage that a permanent record is made and multiple exposures are possible, allowing optimization of the result obtained. Chemiluminescent substrates are far more sensitive than chromogenic substrates.

The substrate used for detection will depend on the conjugated enzyme and the desired sensitivity. Table 7 on the next page can act as a guideline.

Table 7. QIA*express* Detection System specifications

Product	Penta·His Antibody	RGS·His Antibody	Tetra·His Antibody	Anti·His HRP Conjugates	Ni-NTA Conjugates
Epitope	HHHHH	RGSHHHH*	HHHH	As relevant antibody§	n.a.
Dissociation constant, kd (M)	5 x 10^{-9} – 1 x 10^{-8}	1 x 10^{-8} – 5 x 10^{-8}	1 x 10^{-8} – 5 x 10^{-8}	As relevant antibody§	n.d.
Sensitivity in dot blots (2 mm dots)†	10 pg	10 pg	10 pg	10 pg	n.a.
Sensitivity in dot blots (2 mm dots)‡	0.5 ng	0.5 ng	0.5 ng	n.d.	1–2 ng
Sensitivity in western blots†	50 pg	50 pg	50 pg	50 pg	n.a.
Sensitivity in western blots†	2 ng	2 ng	2 ng	n.d.	2–5 ng
Direct detection on blots or in ELISA	Secondary antibody required	Secondary antibody required	Secondary antibody required	YES	YES
Conjugated enzyme	None	None	None	Horseradish peroxidase	Alkaline phosphatase/ horseradish peroxidase

n.a.: not applicable; n.d.: not determined

** Recombinant proteins expressed from pQE-9, pQE-30, pQE-31, pQE-32, pQE-40, pQE-80L, pQE-81L, pQE-82L, pQE-100 DoubleTag, pRSET (Invitrogen), and pBlueBacHis (Invitrogen) encode this epitope.*

† Detection using chemiluminescent substrate.

‡ Detection using alkaline phosphatase chromogenic substrate. The apparent sensitivity on western blots is lower than on dot blots due to loss of protein during transfer.

§ Anti·His HRP Conjugates are available as Penta·His, Tetra·His, and RGS·His variants, and have the same 6xHis tag recognition and binding properties as the corresponding antibody.

Controls

The use of suitable negative and positive controls is essential for most blotting and detection experiments. As a negative control, usually a cell lysate or extract from material that is similar to the test sample but lacking the 6xHis-tagged protein can 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, 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. For western blotting procedures, the 6xHis Protein Ladder provides five 6xHis-tagged proteins with sizes of 15 to 100 kDa. The 6xHis Protein Ladder acts as a positive control for transfer and detection, and enables convenient size determination of 6xHis-tagged proteins (see page 17). 6xHis-tagged DHFR expressed from pQE-40 (which is supplied as a component of QIA*express* Kits — see page 98) provides a suitable positive control protein. This N-terminally 6xHis-tagged protein also has the RGS·His Antibody epitope (RGS(His)$_4$) and can therefore be recognized by any of the QIA*express* Anti·His HRP Conjugates, Anti·His Antibodies or the Ni-NTA Conjugates.

Protocol 1. Separation of proteins by SDS-PAGE

Materials

Gel apparatus and electrophoresis equipment

30% acrylamide/0.8% bis-acrylamide stock solution* — This can also be conveniently purchased as a ready-to-use solution from several companies, e.g., Rotiphorese Gel 30 (Roth, Cat. No. 3029.1)

2.5x separating gel buffer

5x stacking gel buffer

TEMED (*N,N,N',N'*-tetramethylethylenediamine)

10% ammonium persulfate

Butanol

5x electrophoresis buffer

Protein samples

5x SDS-PAGE sample buffer

Note: Use only high-quality reagents and water for SDS-PAGE.

For buffer and reagent compositions, see Appendix, page 87.

Procedure

1. **Assemble gel plates with spacers according to the manufacturer's instructions.**
2. **Mark the level to which the separating gel should be poured — a few millimeters below the level where the wells will be formed by the comb.**

   The size of the gel apparatus used will determine the volumes of gel solutions necessary. The following are used for a 12% acrylamide 8 x 8 or 8 x 10 cm, 1mm thick, minigel.
3. **For a 12% acrylamide gel, mix the following in a beaker or similar vessel.**

   2.2 ml 30% acrylamide/0.8% bis-acrylamide stock solution

   2.2 ml separating gel buffer

   1.1 ml distilled water

   5 µl TEMED

   The volume of acrylamide solution and water should be adjusted according to the percentage acrylamide required (dependent on the size of 6xHis-tagged protein to be separated, see Table 6, page 23).

** Acrylamide is a potent neurotoxin and is absorbed through the skin. Take appropriate safety measures particularly when weighing solid acrylamide/bisacrylamide, but also when working with the solutions and gels.*

4. **Add 50 µl 10% ammonium persulfate, and mix well, just before pouring between the assembled gel plates to the level marked in step 2. Overlay with butanol.**

   As soon as ammonium persulfate is added the gel should be poured quickly before the acrylamide polymerizes.

   Water can be used instead of butanol when using apparatus that may be damaged by the use of butanol — see the manufacturer's instructions.

5. **After polymerization is complete, pour off butanol, rinse with water and dry.**

   Water remaining on the plates can be removed using pieces of filter paper.

6. **For the stacking gel, mix the following:**

   0.28 ml 30% acrylamide stock solution

   0.33 ml stacking gel buffer

   1 ml distilled water

   2 µl TEMED

7. **Add 15 µl 10% ammonium persulfate, and mix well, just before pouring on top of separating gel. Insert comb, avoiding introduction of air bubbles.**

   As soon as ammonium persulfate is added the stacking gel should be poured quickly before the acrylamide polymerizes.

8. **After the stacking gel polymerizes, the gel can be placed in the electrophoresis chamber. Fill the chamber with 1x electrophoresis buffer, remove comb, load samples, and run the gel. For electrophoresis conditions refer to the recommendations provided by the manufacturer of the apparatus.**

Protocol 2. Preparation of 6xHis Protein Ladder

Table 8 gives the sizes and amounts of proteins in the 6xHis Protein Ladder.

Table 8. Sizes and amounts of proteins in the 6xHis Protein Ladder

Molecular weight	Amount per lane (ng)*
100	75
75	60
50	50
30	50
15	75

**When run on a mini gel as described below (2.5 µl per lane)*

Materials

6xHis Protein Ladder (lyophilized)

1x SDS-PAGE sample buffer

For buffer composition, see Appendix, page 87.

Procedure

1. **Add 250 µl 1x SDS-PAGE sample buffer to the lyophilized 6xHis Protein Ladder.**

   It is important to use the buffer recommended. The buffer used must at least contain a suitable reducing agent (e.g., DTT).

2. **Allow proteins to dissolve for 30 min at room temperature.**

3. **Transfer to a microcentrifuge tube and heat for 10 min at 98°C. Store suitable aliquots at –20°C. Always heat the required aliquot immediately before loading the gel.**

   It is important to perform this heating step for the time and at the temperature recommended.

   Insufficient heating leads to detection of protein aggregates as extra bands (Figure 9).

4. **For western blots using mini gels (8 x 8 cm or 10 x 10 cm) and ECL detection, load 2.5–5 µl of the 6xHis Protein Ladder per lane.**

   When using larger gels load correspondingly larger volumes, for example, for gels of 20 x 20 cm use 10–20 µl of 6xHis Protein Ladder.

   To obtain 6xHis Protein Ladder bands of similar signal intensity to protein(s) of interest, load approximately equal amounts of each protein per lane. For example, if detection of 100 ng 6xHis-tagged protein per sample is expected, load 5 µl of dissolved 6xHis Protein Ladder corresponding to approx. 100 ng of each protein (see Table 8).

   If necessary, the dissolved 6xHis Protein Ladder can be diluted with 1x SDS-PAGE sample buffer just before loading the gel.

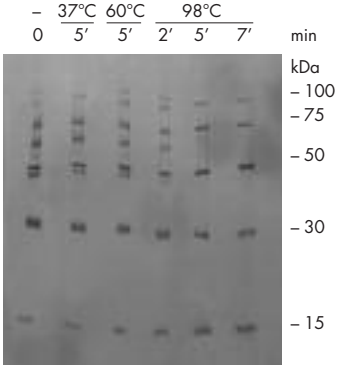


Figure 9. *Effect of insufficient heating. The 6xHis Protein Ladder was dissolved as described above and heated at different temperatures for the different times shown. After electrophoresis and western transfer, 6xHis-tagged proteins were detected using Penta·His Antibody followed by AP-conjugated anti-mouse antibody and chromogenic substrate (BCIP/NBT).*

Protocol 3. Western transfer

Materials

Transfer apparatus

Filter paper

Nitrocellulose membrane (e.g., Schleicher and Schuell BA85)

SDS polyacrylamide gel containing separated proteins (Protocol 1)

Transfer buffer (semi-dry or tank-blotting)

For buffer and reagent compositions, see Appendix, page 88.

Semi-dry–transfer procedure

1. **Cut 8 pieces of filter paper and a piece of membrane to the same size as the gel.**

   To avoid contamination, always handle the filter paper, gel, and membrane with gloves.
2. **Incubate membrane for 10 min in semi-dry–transfer buffer.**
3. **Soak filter paper in semi-dry–transfer buffer.**
4. **Avoiding air bubbles, place 4 sheets of filter paper on the cathode (negative, usually black), followed by the gel, the membrane, 4 sheets of filter paper, and finally the anode (positive, usually red). See Figure 10.**

   Air bubbles may cause localized nontransfer of proteins. They can be removed by gently rolling a Pasteur pipette over each layer in the sandwich.
5. **For current, voltage, and transfer times consult the manufacturer's instructions.**

   Time of transfer is dependent on the size of the proteins, percentage acrylamide, and gel thickness. Transfer efficiency should be monitored by staining (Protocol 4).

   The field strength required is determined by the surface area and thickness of the gel: 0.8 mA/cm^2 is a useful guide (1 h transfer).
6. **After transfer, mark the orientation of the membrane on the gel.**

Tank-blotting procedure

1. **Cut 8 pieces of filter paper and a piece of membrane to the same size as the gel.**

   To avoid contamination, always handle the filter paper, gel, and membrane with gloves.
2. **Incubate membrane for 10 min in tank-blotting transfer buffer.**
3. **Soak filter paper and membrane in tank-blotting transfer buffer.**
4. **Avoiding air bubbles, place 4 sheets of filter paper on the fiber pad, followed by the gel, the membrane, 4 sheets of filter paper, and finally the other fiber pad (see Figure 10).**

   Air bubbles may cause localized nontransfer of proteins. They can be removed by gently rolling a Pasteur pipette over each layer in the sandwich.

5. **For current, voltage, and transfer times consult the manufacturer's instructions.**

   Time of transfer is dependent on the size of the proteins, percentage acrylamide, and gel thickness. Transfer efficiency should be monitored by staining (Protocol 4).

   The field strength required is determined by the surface area and thickness of the gel: 0.8 mA/cm^2 is a useful guide (1 h transfer).

6. **After transfer, mark the orientation of the gel on the membrane.**

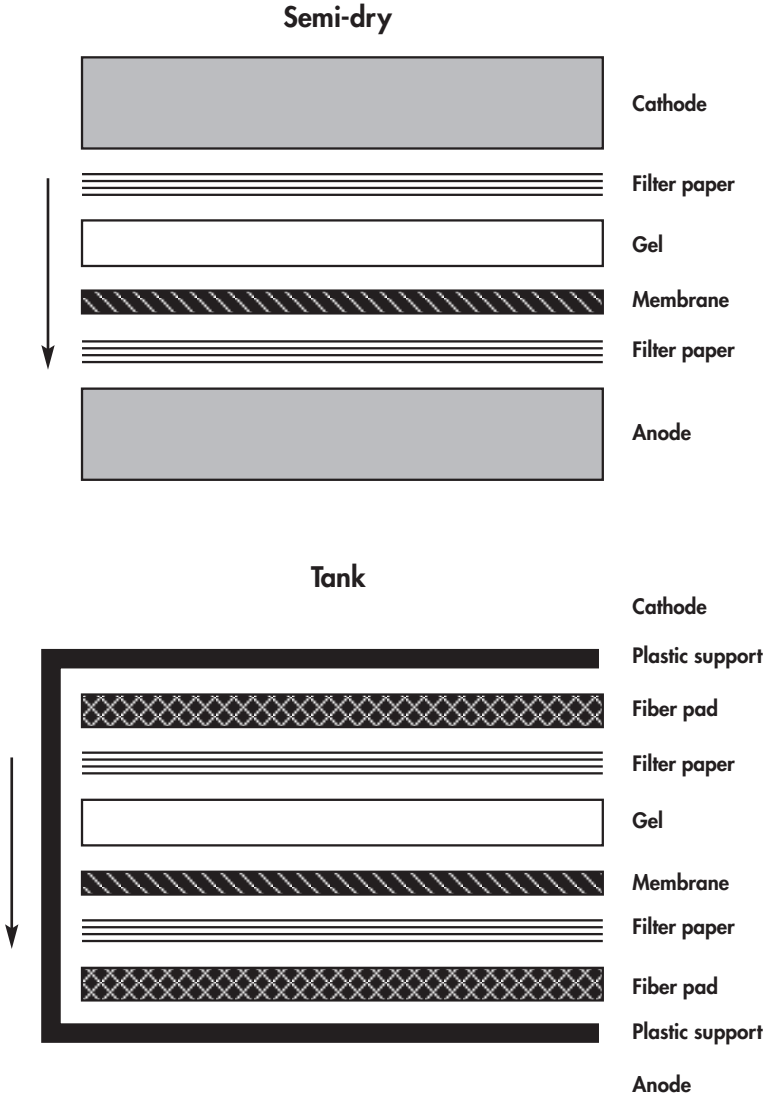


Figure 10. *Schematic drawing of indicated blotting methods. Arrows show direction of transfer.*

Protocol 4. Staining proteins after western transfer

Materials

Staining solution: 0.5% Ponceau S, 1% acetic acid

Western blot (from Protocol 3)

Procedure

1. **Incubate membrane in staining solution (0.5% Ponceau S, 1% acetic acid) with gentle agitation for 2 min.**
2. **Destain in distilled water until bands are visible.**

   If the 6xHis Protein Ladder has been used as a positive control, the bands should be weakly visible after this staining procedure. Check that the proteins of different sizes have been transferred uniformly to the membrane.
3. **Mark membrane or cut as desired.**

   The orientation of the membrane on the gel should be marked. It is not necessary to mark the positions of bands of the 6xHis Protein Ladder. They will be clear after the 6xHis detection procedure employed.
4. **Proceed with Protocol 7, 8, or 9.**

   The blot will be destained in the washing or blocking solution at the beginning of the immunological detection protocol. If the membrane is to be stored at this stage it should be blocked and washed (step 1–4, Protocol 7 or 8; step 1–3, Protocol 9), dried, and then stored at 4°C. The length of time that the blot can be stored is dependent on the samples on the blot.

Protocol 5. Preparation of dot blots

Materials

Nitrocellulose membrane (e.g., Schleicher and Schuell BA85)

Protein samples

Dilution buffer for native or denaturing conditions

For buffer and reagent compositions, see Appendix, page 88.

Procedure

1. **Dilute protein samples in buffer to final protein concentrations of 1–100 ng/µl.**

   The protein of interest is diluted in dilution buffer for denaturing conditions, dilution buffer for native conditions, or another preferred buffer.

2. **Wet membrane with water or follow manufacturer's instructions for preparation. Apply 1 µl samples of diluted protein directly onto membrane.**

   It is also possible to use crude cell lysate and apply 1 µl samples with an estimated concentration of 1–100 ng/µl 6xHis-tagged protein.

   Please note that especially under native conditions, the 6xHis tag must be at least partially exposed to allow antibody binding. In most cases diluting the protein with buffer containing denaturing reagents will increase tag exposure and give better results.

   To differentiate between nonspecific and positive signals, an extra sample containing 1 µl of a cell extract of the host strain without plasmid (or other suitable control) should also be applied to the membrane and treated together with the protein of interest.

   After applying the samples, the membrane should be dried shortly at room temperature before proceeding with the detection process.

   For larger sample volumes suitable equipment is available from several suppliers.

3. **Proceed with Protocol 7, 8, or 9.**

Protocol 6. Preparation of colony blots

Chemiluminescent substrates are not recommended for use with colony blots.

Important note before starting

The colony-blot procedure described can distinguish clones that express a 6xHis-tagged protein from those that express the short peptide sequence encoded by pQE plasmids lacking an insert. Small peptides (<30 amino acids) expressed from QIA*express* vectors pQE-9, -30, -31, -32, -60, -70, -80L, -81L, and -82L without an insert are degraded within the cells and will not yield a positive signal in the detection procedure. Other commonly used vectors that encode larger peptides consisting of polyhistidine tags with additional amino acid sequences (e.g., protease recognition sites) may lead to expression of a small, but stable and detectable translation product even without an insert. This will lead to positive signals from colonies that harbor the expression vector without insert that may be indistinguishable the signals from colonies expressing the desired 6xHis-tagged protein.

Materials

LB plates with appropriate antibiotics

LB plates with appropriate antibiotics and 250 µM IPTG

Nitrocellulose membrane discs (e.g., Millipore, Cat. No. HATF 085 25)

Blunt-ended forceps

Syringe needle

Polystyrene dishes

SDS solution

Denaturing solution

Neutralization solution

20x SSC

For buffer and reagent compositions, see Appendix, page 88.

Procedure

1. **Plate freshly transformed cells on LB plates containing the appropriate antibiotics, and incubate overnight.**

   After spreading the transformation mix, dry the plates inverted with the lids slightly open until small wrinkles develop on the surface of the agar. To prevent smearing, incubation should not be started until all of the liquid has absorbed into the agar.

   To avoid expression of toxic proteins in the absence of IPTG (a result of "leaky" promoters) and to maintain plasmid stability, incubation can be carried out at 30°C. If the expressed protein is not toxic and the plasmids are stable, incubation can be carried out at 37°C, but care should be taken that the colonies do not become too large.

2. **Remove the plates from the incubator, open lids slightly, and allow any condensation to dry for 10 min.**
3. **Place a dry, numbered nitrocellulose filter on the agar surface in contact with the colonies, taking care not to introduce air bubbles.**

   Number filters with a water-resistant marking pen or pencil. Hold the filter on opposite sides with blunt-ended forceps, and lower gently onto the agar surface, making contact first along the middle and then lowering (but not dropping) the sides.
4. **Using a syringe needle, pierce the filter and agar at asymmetric positions to facilitate proper alignment following detection. Grip filter on the sides with blunt-ended forceps, and peel it off in one movement.**
5. **Transfer filter (colonies up) to a fresh LB plate containing antibiotics and 250 µM IPTG, as described above. Avoid introducing air bubbles.**

   Hold the filter on opposite sides with blunt-ended forceps, and lower gently onto the agar surface, making contact first along the middle and then lowering (but not dropping) the sides.
6. **Incubate plates for 4 h at 37°C. Place master plates in a 30°C incubator for 4 h to allow colonies to regrow.**
7. **Prepare a set of polystyrene dishes for colony lysis and binding of protein to the filters. Each dish should contain a sheet of 3MM paper soaked with one of the following solutions:**

   1) SDS solution

   2) Denaturing solution

   3) Neutralization solution

   4) Neutralization solution

   5) 2x SSC

   Note: Discard excess fluid so that paper is moist but not wet. Excess liquid promotes colony swelling and diffusion which will result in blurred signals.
8. **Place the nitrocellulose filters (colony side up) on top of the paper in each of these dishes, taking care to exclude air bubbles (colonies above air bubbles will not lyse properly and will generate a higher background in the final staining step).**

   Incubate at room temperature as follows:

   1) SDS solution 10 min

   2) Denaturing solution 5 min

   3) Neutralization solution 5 min

   4) Neutralization solution 5 min

   5) 2x SSC 15 min

9. **Continue with one of the protocols for immunodetection using an Anti·His Antibody or Ni-NTA Conjugate with a chromogenic substrate (Protocol 8 or 9).**

   Due to the problem of high background, protocols using chemiluminescent substrates are not recommended for detection after colony blotting.

   Note: At times there is only a slight difference between colonies which express 6xHis-tagged protein and those that do not. Shorter staining times are required with this procedure. 2–3 min is usually sufficient, but it is very important to monitor color development at this stage.

   If it is still difficult to differentiate between positive clones and background, the cause of the high background should be determined. The following controls should be included:

   1. A plate of host bacteria without the expression plasmid
   2. A plate of host bacteria harboring the expression plasmid without the insert
   3. A colony-blot treated only with secondary antibody prior to detection
   4. A positive control expressing 6xHis-tagged DHFR from pQE-40, if possible

Protocol 7. Immunodetection with Anti·His Antibodies or Anti·His HRP Conjugates (chemiluminescent method)

Materials*

Western Blot (Protocol 1–4) or dot blot (Protocol 5)

TBS Buffer

TBS-Tween/Triton Buffer

Blocking buffer

Anti·His HRP Conjugate stock solution or Anti·His Antibody stock solution

Anti-mouse secondary antibody conjugate (not required if using Anti·His HRP Conjugate)

Secondary antibody dilution buffer (not required if using Anti·His HRP Conjugate)

The solutions required depend on the detection method employed.

If using Anti·His HRP Conjugates, use the specially formulated Blocking Reagent and Blocking Reagent Buffer supplied with the conjugates for the blocking and conjugate incubation protocol steps. For preparation of complete Anti·His HRP Conjugate blocking buffer using Blocking Reagent, Blocking Reagent Buffer, and Tween 20, see page 89. Although complete Anti·His HRP Conjugate blocking buffer is stable for several weeks when stored at 2–8°C, we recommend preparing fresh blocking buffer each time it is required. Approximately 20 ml of complete blocking buffer is required for processing of one 8 x 10 cm minigel.

For chemiluminescent detection, BSA does not sufficiently block nonspecific binding of the secondary antibody to the membrane, and milk powder should be used to dilute the secondary antibody. Buffer containing milk powder should not be used for Anti·His Antibody dilution, however, as this will reduce sensitivity. Alternatively, if alkali-soluble casein (Merck, Cat. No. 1.02241) is available in your country it can be used as a blocking reagent throughout the chemiluminescent detection protocol. The reagents used are shown in Table 9.

Table 9. Reagents used in chemiluminescent detection of 6xHis-tagged proteins

Step	Anti·His Antibody	(Alternative method)	Anti·His HRP Conjugate
Blocking	3% BSA in TBS	1% casein in TBS	Blocking Reagent in Blocking Reagent Buffer
Anti·His Antibody binding	3% BSA in TBS	1% casein in TBS	–
Anti·His HRP Conjugate binding	–	–	Blocking Reagent in Blocking Reagent Buffer
Secondary antibody binding	10% milk powder in TBS	1% casein in TBS	–

** For compositions and preparation of buffers and reagents, see Appendix, page 88–89.*

Chemiluminescent substrates

Please refer to manufacturer's recommendations.

CDP-Star from Tropix, Inc. can be used with AP-conjugated secondary antibodies, and the ECL system from Amersham Pharmacia Biotech can be used in combination with HRP-conjugated secondary antibodies and Anti·His HRP conjugates. The blocking reagents supplied with the CDP-Star system are compatible with QIA*express* Anti·His Antibodies and can be used, according to the manufacturer's instructions, instead of the blocking buffers and secondary antibody dilution buffers described in the following protocol.

Procedure

1. **Wash membrane twice for 10 min each time with TBS buffer at room temperature.**
2. **Incubate for 1 h in blocking buffer at room temperature.**

   3% BSA (w/v) in TBS buffer*, or complete Anti·His HRP Conjugate blocking buffer† is used for blocking until incubation. If using Anti·His HRP Conjugate blocking buffer, be sure that you have added 0.1% (v/v) Tween 20 (final concentration) to complete the blocking buffer.
3. **Wash membrane twice for 10 min each time in TBS-Tween/Triton buffer at room temperature.**
4. **Wash membrane for 10 min with TBS buffer at room temperature.**
5. **Incubate in Anti·His Antibody or Anti·His HRP Conjugate solution (1/1000–1/2000 dilution of antibody or conjugate stock solution in blocking buffer) at room temperature for 1 h.**

   Membrane can be sealed in plastic bags.

   Note: Do not use buffer containing milk powder for Anti·His Antibody or Anti·His HRP Conjugate dilution. This will reduce sensitivity.

   3% BSA (w/v) in TBS buffer*, or complete Anti·His HRP Conjugate blocking buffer† is used for this blocking step when using chemiluminescent detection. If using Anti·His HRP Conjugate blocking buffer, be sure that you have added 0.1% (v/v) Tween 20 (final concentration) to complete the blocking buffer.
6. **Wash twice for 10 min each time in TBS-Tween/Triton buffer at room temperature.**
7. **Wash for 10 min in TBS buffer at room temperature. If using Anti·His HRP Conjugates proceed directly to protocol step 10.**

* *If alkali -soluble casein (Merck, Cat. No. 1.02241) is available in your country a 1% (w/v) solution in TBS buffer may be used for this protocol step.*

† *0.5% (w/v) Blocking Reagent dissolved in 1x Blocking Reagent Buffer; 0.1% (v/v) Tween 20. For preparation of buffer see page 89.*

8. **Incubate with secondary antibody solution for 1 h at room temperature.**

   Either alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated anti-mouse IgG may be used. Rabbit-anti-mouse IgG/AP-conjugate from Pierce (Cat. No. 31332) or goat-anti-mouse IgG/HRP-conjugate from Jackson Immunoresearch (Cat. No. 115-035-003) yield good results. Dilute according to the manufacturer's recommendations. Use the lowest recommended concentration to avoid false signals.

   10% nonfat dried milk in TBS* is used for incubation with secondary antibody when using chemiluminescent detection.

   Milk powder is needed to reduce background because BSA does not block sufficiently for the very sensitive chemiluminescent detection method.

9. **Wash 4 times for 10 min each time in TBS-Tween/Triton buffer at room temperature.**

10. **Perform chemiluminescent detection reaction and expose to X-ray film according to the manufacturer's recommendations.**

** If alkali -soluble casein (Merck, Cat. No. 1.02241) is available in your country a 1% (w/v) solution in TBS buffer can be used for this protocol step.*

Protocol 8. Immunodetection with Anti·His Antibodies or Anti·His HRP Conjugates (chromogenic method)

Materials*

Western Blot (Protocol 1–4), dot blot (Protocol 5) or colony blot (protocol 6)

TBS Buffer

TBS-Tween/Triton Buffer

Anti·His Antibody or Anti·His HRP Conjugate stock solution

Anti-mouse secondary antibody alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugate

Blocking buffer

Secondary antibody dilution buffer for chemiluminescent detection

Staining solutions for alkaline phosphatase (AP) or horseradish peroxidase (HRP)

The solutions required depend on the antibody and detection method used. For the chromogenic detection method, 3% (w/v) BSA in TBS (Anti·His Antibodies) or complete Anti·His HRP Conjugate blocking buffer is used as a blocking reagent throughout the whole procedure. For preparation of complete Anti·His HRP Conjugate blocking buffer using Blocking Reagent, Blocking Reagent Buffer, and Tween 20 (see page 89). Although complete Anti·His HRP Conjugate blocking buffer is stable for several weeks when stored at 2–8°C, we recommend preparing fresh blocking buffer each time it is required. Approximately 20 ml of complete blocking buffer is required for processing of one 8 x 10 cm minigel. Buffer containing milk powder should not be used for Anti·His Antibody or Anti·His HRP Conjugate dilution however, as this will reduce sensitivity.

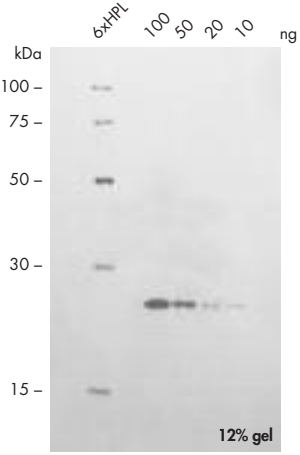


Figure 11. *The indicated amounts of purified 6xHis-tagged DHFR mixed with crude extract from* E. coli *cells (8 µg total protein per lane) were applied to a 12% SDS-polyacrylamide gel. After electrophoresis and western transfer, 6xHis-tagged proteins were detected with Penta·His Antibody followed by chromogenic detection with AP-conjugated rabbit anti-mouse IgG and NBT/BCIP detection reagent.* ***6xHPL****: 6xHis Protein Ladder (2.5 µl).*

** For compositions and preparation of buffers and reagents, see Appendix, page 88–90.*

Procedure

1. **Wash membrane twice for 10 min each time with TBS buffer at room temperature.**
2. **Incubate for 1 h in blocking buffer at room temperature.**

   3% BSA (w/v) in TBS buffer, or complete Anti·His HRP Conjugate blocking buffer* is used for blocking throughout the procedure when using chromogenic detection. If using Anti·His HRP Conjugate blocking buffer, be sure that you have added 0.1% (v/v) Tween 20 (final concentration) to complete the blocking buffer.
3. **Wash membrane twice for 10 min each time in TBS-Tween/Triton buffer at room temperature.**

   Use of TBS-Tween/Triton buffer has been found empirically to result in optimal signal-to-noise ratios.
4. **Wash membrane for 10 min with TBS buffer at room temperature.**
5. **Incubate in Anti·His Antibody or Anti·His HRP Conjugate solution (1/1000–1/2000 dilution of antibody or conjugate stock solution in blocking buffer) at room temperature for 1 h.**

   Membrane can be sealed in plastic bags.

   Note: Do not use buffer containing milk powder for Anti·His Antibody or Anti·His HRP Conjugate dilution. This will reduce sensitivity.

   3% BSA (w/v) in TBS buffer, or complete Anti·His HRP Conjugate blocking buffer* is used for blocking throughout the procedure when using chromogenic detection. If using Anti·His HRP Conjugate blocking buffer, be sure that you have added 0.1% (v/v) Tween 20 (final concentration) to complete the blocking buffer.
6. **Wash twice for 10 min each time in TBS-Tween/Triton buffer at room temperature.**
7. **Wash for 10 min in TBS buffer at room temperature. If using Anti·His HRP Conjugates proceed directly to protocol step 10.**
8. **Incubate with secondary antibody solution diluted in 3% BSA (w/v) in TBS for 1h at room temperature.**

   Either alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated anti-mouse IgG may be used. Rabbit-anti-mouse IgG/AP-conjugate from Pierce (Cat. No. 31332) or goat-anti-mouse IgG/HRP-conjugate from Jackson Immunoresearch (Cat. No. 115-035-003) yield good results. Dilute according to the manufacturer's recommendations. Use the lowest recommended amounts to avoid false signals.
9. **Wash 4 times for 10 min each time in TBS-Tween/Triton buffer at room temperature.**

** 0.5% (w/v) Blocking Reagent dissolved in 1x Blocking Reagent Buffer containing 0.1% (v/v) Tween 20. For preparation of blocking buffer see page 89.*

10. **Stain with AP or HRP staining solution (when using Anti·His HRP Conjugates, be sure that you use an HRP staining solution) until the signal is clearly visible (approximately 5–15 min).**

    Do not shake blots during color development.

11. **Stop the chromogenic reaction by rinsing the membrane twice with water.**

12. **Dry the membrane and photograph as soon as possible.**

    The colors will fade with time. The product formed when using HRP is particularly unstable.

Protocol 9. Detection with Ni-NTA Conjugates

This protocol uses standard western (Protocols 1–4), dot (Protocol 5), or colony blotting (Protocol 6) procedures. Chemiluminescent substrates are not recommended for use with Ni-NTA Conjugates. Best results will be obtained if all steps are carried out on a shaker or rocker platform unless otherwise indicated.

This protocol can be performed with either Ni-NTA AP Conjugate or Ni-NTA HRP Conjugate. Only the substrate used to localize the alkaline-phosphatase or horseradish-peroxidase activity is specific for each Ni-NTA Conjugate.

Note: Ni-NTA Conjugates also form a complex with a 31 kDa molecular-weight standard, bovine carbonic anhydrase, a metalloenzyme with one zinc ion per protein molecule (23).

Materials

Western, dot, or colony blot (Protocols 1–4, 5, or 6)

TBS buffer

TBS-Tween buffer

3% BSA in TBS

Ni-NTA Conjugate: Dissolve the lyophilized conjugate in 500 µl distilled water per vial.

Alkaline-phosphatase **or** horseradish-peroxidase staining solutions

For buffer and reagent compositions, see Appendix, pages 88–90.

Procedure

1. **Wash western or dot blot membrane twice for 10 min each time with TBS buffer.**
2. **Incubate for 1 h in 3% BSA in TBS at room temperature.**
3. **Wash 3 times for 10 min with TBS-Tween buffer.**
4. **Incubate the membrane for 1 h at room temperature in TBS-Tween buffer containing a 1/1000 dilution of Ni-NTA Conjugate stock solution.**

   Membranes can be sealed in plastic bags.

   Do not incubate in the presence of BSA, milk, or other proteinaceous blocking reagents, chelating reagents, or electron donating groups.
5. **Wash 3 times for 10 min in TBS-Tween buffer at room temperature.**
6. **Stain with AP or HRP staining solution until the signal is clearly visible (approximately 5–15 min for AP, 1–5 min for HRP).**

   Do not shake blots during color development.
7. **Stop the reaction by rinsing the membrane twice in water.**
8. **Dry the membrane and photograph as soon as possible.**

   The colors will fade with time. The product when using horseradish peroxide substrate is particularly unstable.

Troubleshooting Guide — Detection

The following troubleshooting guide may be helpful in solving any problems that may arise with the detection protocols. Both positive and negative controls should normally be included in all detection experiments. They are often essential to enable correct interpretation of results and can help identify the cause of problems. The scientists at QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications.

	Comments and suggestions
Weak or no signal	
6xHis Protein Ladder visible	
a) Recombinant protein of interest does not have a 6xHis tag	Check construct by DNA sequencing. **N-terminal tag:** Ensure that the cloned insert does not contain an extra ribosome binding site and that the ORF contains no putative internal translational start sites. An additional ribosome binding site or internal start site close to the N-terminus of the insert could result in an over-expressed, nearly full-size protein lacking a 6xHis tag. **C-terminal tag:** Premature translation termination will result in a protein lacking a 6xHis tag. Ensure that the ORF contains no additional, unwanted translational stop sites. Check codon usage — for example the arginine codons AGG and AGA are the least frequently used in *E. coli* and the tRNAs that recognize them are the least abundant. If present in expression constructs, these codons can lead to truncated protein products that do not have the 6xHis tag.
b) Insufficient recombinant protein present	If the protein has been stored for any length of time and was successfully detected immediately after purification, it may be unstable. Add a protease inhibitor such as 1 mM PMSF and/or store at –70°C instead of 4°C or –20°C. In the presence of urea or guanidine hydrochloride and/or at high protein concentrations, aggregates may form upon storage, leading to precipitation of the protein. Resuspend thoroughly before use. Increase amount of protein loaded onto gel or applied to dot blots. Clones used for colony blotting may express insufficient protein to be detected above background

	colonies not expressing 6xHis-tagged protein. Check by including suitable positive-control colonies. Check construct.
c) Suboptimal recognition of 6xHis-tagged protein of interest by Anti·His HRP Conjugate or Anti·His Antibody	Individual 6xHis-tagged proteins are often recognized better by one of the Anti·His HRP Conjugates or Anti·His Antibodies than the others, possibly because of subtle differences in the exact conformation of the 6xHis tag and other parts of the protein in the vicinity of the tag. To identify the optimal antibody for your specific 6xHis-tagged protein and application, compare the Anti·His Antibodies using the Anti·His Antibody Selector Kit. If using the RGS·His Antibody remember that it requires the RGS(His)$_4$ epitope for recognition. Expression vectors encoding this epitope include pQE-9, pQE-30, pQE-31, pQE-32, pQE-40, pQE-80L, pQE-81L, pQE-82L, pQE-100, and some versions of the pRSET and pBlueBacHis vectors (Invitrogen). A positive control for expression should be included whenever possible. QIA*express* Kits include pQE-40 (encoding 6xHis-tagged DHFR) for this purpose. 6xHis-tag recognition on dot blots may be improved by denaturing proteins prior to application on the membrane — this may make the 6xHis tag more accessible to the Anti·His HRP Conjugate or Anti·His Antibody.

6xHis Protein Ladder not visible

a) Exposure/development time suboptimal (chemiluminescent detection)	Refer to manufacturer's instructions. The kinetics of chemiluminescence are different for the different substrates available. Longer exposure/development times may help, but some substrates are not stable for longer than 30 min.
b) Incomplete transfer (western blotting)	Ensure that appropriate transfer conditions have been used. Check for sufficient transfer by staining membrane with Ponceau S (Protocol 4). Efficiency can be as low as 10% with the semi-dry transfer method. Increase transfer time for larger 6xHis-tagged proteins. Use a second membrane for smaller 6xHis-tagged proteins to bind any that may have passed through the first layer. Carry out detection procedures on both

	Comments and suggestions
	membranes. For hydrophobic proteins, increasing the methanol content of the transfer buffer from 10% to 20% may help. Check the efficiency of the detection system with a dot blot.
c) Suboptimal incubation with Ni-NTA Conjugate	The conjugate should not be incubated in the presence of blocking reagents. Even a slight affinity for these proteins will have an effect on sensitivity. EDTA, other chelating reagents, or a too acidic pH (< 6·5) will interfere with the Ni-NTA complex formation and should be avoided when using Ni-NTA Conjugates.
d) Inappropriate wash conditions	The interactions between 6xHis-tagged proteins and detection reagent (Anti·His Antibody, or Anti·His HRP or Ni-NTA Conjugate) are sensitive to the type and concentration of detergents used in the buffers. Deviation from the conditions specified in the protocols could lead to reductions in signal intensity. When using Ni-NTA Conjugates for detection, blots should not be washed too extensively (not more than 3 times for 10 minutes each time in TBS-Tween buffer). Longer washes could result in a weaker signal, especially when the binding kinetics may be low (e.g., under native conditions). EDTA, other chelating reagents, or a too acidic pH interfere with the Ni-NTA complex formation and should be avoided when using Ni-NTA Conjugates.
e) Inappropriate secondary antibodies	QIA*express* Anti·His Antibodies are mouse IgG1 monoclonal antibodies. Ensure that the secondary antibody has the appropriate species specificity (e.g., goat anti-mouse IgG antibody) and that the IgG1 isotype is recognized. This can be checked by performing a dot blot with the Anti·His Antibody applied directly to the membrane or by a suitable ELISA procedure. Check that the appropriate substrate has been used for the specific conjugate in the detection system.

f) Enzyme conjugated to secondary antibody is inactive	Check activity of secondary antibody conjugate by incubation directly with the appropriate substrate.
g) Membrane shaken during color development	Do not shake membrane during color development (chromogenic method) because the color precipitate will disperse.
h) Blocking Buffer Reagent was not prepared correctly	When using Anti·His HRP Conjugates, sensitivity is reduced when Blocking Reagent concentration is too high, or Tween 20 has not been added to the solution prior to use. Ensure that the Blocking Reagent Buffer has been correctly prepared according to the instructions given in the Appendix on page 89.

Problems with the 6xHis Protein Ladder — too many bands visible

a) Proteins were not dissolved properly	It is important to allow proteins to dissolve for 30 min at room temperature.
b) Proteins were not heated sufficiently	Heat for 10 min at 98°C. See Protocol 2.
c) No reducing agent in sample buffer	Prepare sample buffer containing reducing agent.

Problems with the 6xHis Protein Ladder — too few bands visible

a) Largest bands missing	Transfer of the larger bands may be suboptimal when using semi-dry transfer. Try using a tank-blotting transfer procedure. Check buffers. Ensure that air bubbles are removed before transfer.
b) Smallest band missing	If the smallest band is not detected, it may have run out of the gel. Do not allow gel to run too far.

Bands on western blot are diffuse

a) Membrane was not held firmly on gel during western transfer	Check transfer setup. If too loose, use additional layers of filter paper.

Comments and suggestions	
Bands on western blot have small spots with no signal	
a) Presence of bubbles between the gel and membrane during transfer	Ensure that all bubbles are removed after placing on gel prior to transfer. This may be conveniently achieved by rolling a Pasteur pipet over the membrane to force bubbles from between membrane and gel.
Nonspecific signals	
a) System considerations	Expression of a 6xHis-tagged protein in mammalian cell lines, yeast, baculovirus systems, or *E. coli* should not result in nonspecific secondary signals with Anti·His HRP Conjugates or Anti·His Antibodies. Ni-NTA Conjugates are best used with *E. coli*.
b) Secondary antibody concentration	Secondary antibody–enzyme conjugates are often a major source of nonspecific interactions. Refer to the manufacturer's recommendations: use the dilution with the lowest antibody concentration resulting in good detection levels. The secondary antibody should not be stored for more than 6 months. Suitable positive and negative controls should always be included to enable unequivocal identification of the 6xHis-tagged protein target. Try detection with secondary antibody alone, without the primary antibody.
c) Crossreacting proteins of different size than the protein of interest	**Smaller proteins:** Protein degradation results in cross-reacting proteins smaller than the protein of interest. This may be a result of inadequate or prolonged storage. Freshly prepared protein should be compared with stored protein. In addition, add a protease inhibitor such as 1 mM PMSF and/or store at –70°C instead of 4°C or –20°C. Protein degradation may also occur during sample preparation. A variety of protease inhibitors (e.g., PMSF) included in lysis buffers may help. **Larger proteins:** Multimers of the expressed proteins are the most common source of larger, crossreacting proteins. They are almost exactly double or triple the size of the monomer. Try preparing samples for SDS-PAGE without the 95°C heating step. Instead incubate e.g., at 60°C. Aggregation of proteins is dependent on the time and temperature used for sample preparation.

	Comments and suggestions
d) Inappropriate wash conditions	The interactions between 6xHis-tagged proteins and detection reagent (Anti·His Antibody or Anti·His HRP or Ni-NTA Conjugate) are sensitive to the type and concentration of detergents used in the buffers. Deviation from the conditions specified in the protocols described could lead to nonspecific interactions.
e) Insufficient washing after antibody or conjugate incubation	Ensure that the membrane is washed extensively after incubation with antibody. It may help to increase the number of, duration of, or volume of buffer used in, wash steps. Background signals may also be eliminated by increasing the detergent concentration.
f) Improper blocking reagents and/or blocking time	Nonspecific signals are often a result of incomplete saturation of the membrane with blocking protein. Use the lowest concentration of secondary antibody recommended. Follow the appropriate protocol for the detection method and substrate combination.
High background	
a) Development too long	Excessively long development times are often the cause of high background. Monitor development closely, and always include a suitable negative control without a 6xHis-tagged protein.
b) Secondary antibody concentration	Secondary antibody–enzyme conjugates are often a major source of high background. Refer to the manufacturer's recommendations. Use the lowest antibody concentration resulting in good detection levels. The secondary antibody should not be stored for more than 6 months. Try detection with secondary antibody alone, without the primary antibody.
c) Improper blocking reagents and/or blocking time	High background is often the result of incomplete saturation of the membrane with blocking protein. Use the lowest concentration of secondary antibody recommended. Follow the appropriate protocol for the detection method and substrate combination. Pay particular attention to the blocking requirements when using chemiluminescent substrates.
d) Insufficient washing prior to detection with chemiluminescent substrates	Some chemiluminescent substrates require very thorough washing after incubation with the secondary antibody conjugate. Refer to the supplier's recommendations.

Immunolocalization of 6xHis-tagged Proteins

Immunolocalization using Anti·His Antibodies

Immunohistochemical methods can be used to investigate the location of recombinant proteins expressed in particular cells within tissues or organs, as well as for determination of the subcellular localization of proteins after targeting to particular cell compartments. The high specificity and affinity of QIA*express* Anti·His Antibodies allow exclusive detection of recombinant 6xHis-tagged proteins in mammalian cells. The RGS·His Antibody has, for example, been successfully used to localize 6xHis-tagged *Xenopus* hepatocyte nuclear factor 4 in the nuclei of transfected human renal carcinoma cells (13). Penta·His and Tetra·His Antibody have been used to detect 6xHis-tagged aggrecan in the perinuclear Golgi and the endoplasmic reticulum of transiently transfected CHO cells (24). The technique can also be used to localize cellular proteins that interact with 6xHis-tagged proteins. This was demonstrated by Zamorano and coworkers (14), who localized a leptin receptor in rat brain by binding 6xHis-tagged leptin followed by RGS·His Antibody.

Localization of 6xHis-tagged protein by indirect immunofluorescent detection

Typically the overall scheme for localization of a cellular 6xHis-tagged protein by indirect immunofluorescence involves four steps:

- Fixation and subsequent permeabilization of cells to allow penetration of antibodies
- Blocking sites prone to nonspecific interactions
- Labeling with Anti·His Antibody followed by fluorophore-conjugated secondary antibody
- Mounting the sample for microscopic analysis

Fixation and permeabilization

Sample fixation is one of the most crucial steps regarding preservation of cell morphology as well as antigen structure and accessibility. Formaldehyde and methanol/acetone are the most commonly used fixatives in indirect immunofluorescent procedures. Formaldehyde, prepared from solid paraformaldehyde, is the fixative of choice to examine the localization of proteins in cultured cells, and acts as a mild crosslinker. To gain access to intracellular components, cell membranes must be subsequently permeabilized. This can be achieved using the nonionic detergent Triton X-100 to solubilize the phospholipid membranes. More rapid fixatives are cold methanol and acetone which fix and permeabilize cells at the same time. However, because methanol and acetone work by precipitating proteins and carbohydrates they are firstly more likely to alter the localization pattern of the recombinantly expressed 6xHis-tagged protein and secondly they do not maintain the integrity of membranes and organelles during localization. The use of glutaraldehyde as a stronger fixative is not recommended, because epitopes are frequently sufficiently altered to prevent binding of the antibody probe.

Blocking nonspecific binding

After fixation and permeabilization, nonspecific binding sites of the cells must be blocked so as to prevent high background due to binding of the primary and secondary antibody to nonspecific cell epitopes. Bovine serum albumin (BSA) and normal serum obtained from the species from which the secondary antibody was produced are commonly used blocking agents. Blocking reagents are included in all binding steps to minimize nonspecific interaction of detection reagents with epitopes presented by the cells. The protocol described here uses BSA for blocking, because BSA results in reproducible low signal intensities of nontransfected cells compared to transfected cells. However normal serum can be used as an alternative.

Primary and secondary antibodies

When using Anti·His Antibodies for immunofluorescent detection, the localization of Anti·His Antibody bound to immobilized 6xHis-tagged protein is visualized using secondary anti-mouse IgG antibodies conjugated to a fluorophore. Antibodies conjugated to popular fluorophores such as fluorescein isothiocyanate (FITC), rhodamine, and Texas red are available from many suppliers. Secondary antibodies to be used in combination with Anti·His Antibodies have to recognize mouse IgG1. Using FITC as fluorophore, we have obtained good results with FITC-conjugated goat anti-mouse IgG (H+L) antibody from Jackson Immunoresearch Laboratories (Cat. No. 115-095-062). We recommend optimization of antibody dilution following the supplier's recommendations, to obtain high signal intensity with low nonspecific background fluorescence.

Mounting

After the labeling procedure, slides are overlaid with a coverslip. Mounting media, usually based on buffered glycerol, are available from a number of commercial sources. For short-term preservation of specimens we obtained good results with Mounting Medium from Sigma (Cat. No. 1000-4) and also with Kaiser's glycerol gelatine from Merck (Cat. No. 109242).

Protocol 10. Immunolocalization using Anti·His Antibodies

The method described below has been established with NIH3T3 cells using FITC conjugated secondary antibody. It could be used as the starting point for developing a protocol, but optimization will be necessary for different 6xHis-tagged proteins expressed in other cells. We recommend optimization of fixation conditions, antibody concentrations, and blocking reagents, as well as incubation and wash times. Further details about immunolocalization techniques can be found in references 19 and 25.

Materials

Chamber slides (Lab-Tek®, TC Chamber Slides, Nunc Inc.)

PBS-IF

2% paraformaldehyde in PBS-IF

0.25% Triton X-100 in PBS-IF

Anti·His Antibody stock solution

Blocking buffer IF

Antibody dilution buffer IF

Anti-mouse secondary antibody conjugate (e.g., FITC-conjugated goat anti-mouse IgG (H+L) antibody from Jackson Immunoresearch Laboratories, Cat. No. 115-095-062)

Mounting medium

For buffer and reagent compositions, see Appendix, page 90.

1. **Grow transfected cells in chamber slides under standard growth conditions.**
2. **Wash cells with PBS-IF for 30 s at room temperature.**

   Do not shake samples during this step or any subsequent steps.
3. **Fix the cells in 2% paraformaldehyde in PBS-IF for 10 min at room temperature.**
4. **Remove the fixation mixture and wash 3 times with PBS-IF for 30 s at room temperature.**

   If fixation is not sufficient, paraformaldehyde concentration can be increased (up to 4%) and/or incubation time can be increased.
5. **Permeabilize the cells with 0.25% Triton X-100 in PBS-IF for 5 min at room temperature.**

   Longer incubation times can lead to partial disruption of cell structure.

   Note: If the protein under investigation is expressed on the cell surface, this permeabilization step can be omitted.

   Alternatively, for more rapid fixation, fix the cells in precooled methanol at –20°C for 20 min. This quicker method also has some disadvantages — see "Fixation and Permeabilization", page 54).

6. **Wash 3 times with PBS-IF for 30 s at room temperature.**
7. **Incubate for 1 h in 5% BSA in PBS-IF at room temperature.**

   Note: All incubation steps should be performed in a sealed, humid container to prevent the slides from drying out.
8. **Wash 3 times for 10 min each time in PBS-IF at room temperature.**
9. **Incubate in Anti-His Antibody solution (1/20 – 1/50 dilution of antibody stock solution in Antibody Dilution Buffer) at room temperature for 1 h.**
10. **Wash 3 times for 10 min each time in PBS-IF at room temperature.**
11. **Incubate the cells with a fluorophore-conjugated secondary anti-mouse IgG1 antibody solution (diluted in Antibody Dilution Buffer) for 1h at room temperature.**

    We have obtained good results with FITC-conjugated goat anti-mouse IgG (H+L) antibody from Jackson Immunoresearch Laboratories, Cat. No. 115-095-062).

    Dilute fluorophore-conjugated antibody according to the supplier's recommendations.

    Note: For this and all subsequent steps, avoid excessive exposure of samples to light in order to prevent fluorescence fading of the fluorophore.
12. **Wash 4 times for 10 min each time in PBS-IF at room temperature.**
13. **Mount the cells with mounting medium.**

    For short-term preservation of specimens we have obtained good results with Mounting Medium from Sigma (Cat. No. 1000-4) and also with Kaiser's glycerol gelatine from Merck (Cat. No. 109242). Follow the supplier's recommendations.

Troubleshooting Guide — Immunolocalization

This troubleshooting guide may be helpful in solving any problems that may arise with the immunolocalization protocol. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see inside front cover for contact information).

	Comments and suggestions
Weak or no signal	
a) Recombinant protein does not have a 6xHis tag	Check protein by western blotting or construct by DNA sequencing. C-terminal tag: premature translation termination will result in a protein lacking a 6xHis tag. Ensure that the ORF contains no additional, unwanted translation stop sites.

	Comments and suggestions
b) Insufficient recombinant protein present	Check the expression of the recombinant 6xHis-tagged protein. Lyse transfected cells and perform a dot or western blot. If recombinant 6xHis-tagged protein is expressed it should be detected with Anti·His Antibodies.
c) 6xHis tag is partially hidden	Anti·His Antibody binding may be hindered by a partially hidden 6xHis tag. Try using higher antibody concentrations (up to 1/20 dilution) and longer binding times (e.g., overnight at 4°C).
d) 6xHis epitope is altered during fixation	Overfixation may alter the 6xHis epitope so that the binding site for the Anti·His Antibody is lost. Try reducing the fixative concentration or incubation time, or use an alternative reagent.
e) Suboptimal recognition of 6xHis-tagged protein by Anti·His Antibody	Individual 6xHis-tagged proteins are often recognized better by one of the Anti·His Antibodies than the others, possibly because of subtle differences in the exact conformation of the 6xHis tag, and other parts of the protein in the vicinity of the tag. To identify the optimal antibody for your specific 6xHis-tagged protein, compare the Anti·His Antibodies using the Anti·His Antibody Selector Kit. If using the RGS·His Antibody remember that it requires the RGS(His)$_4$ epitope for recognition.
f) Inappropriate secondary antibodies	QIA*express* Anti·His Antibodies are mouse IgG1 monoclonal antibodies. Ensure that the secondary antibody has the appropriate species specificity (e.g., anti-mouse IgG antibody) and that the IgG1 isotype is recognized.
g) Fluorescence fading	Light emission intensity of some fluorophores during fluorescence microscopy is sensitive to light exposure. Ensure that the fluorophore-conjugated secondary antibody is not exposed extensively to light during storage and the immunostaining procedure.
Nonspecific signals	
a) Secondary antibody concentration too high	Secondary antibody fluorophore conjugates are often a major source of non-specific interactions. Refer to the manufacturer's recommendations: use the dilution with the lowest antibody concentration resulting in good detection levels. Suitable negative controls should always be included. Try detection with secondary antibody alone without primary antibody, as well as with nontransfected cells.

	Comments and suggestions
b) Crossreacting cell proteins other than the protein of interest	Expression of 6xHis tagged proteins in mammalian cell lines should not, in general, result in nonspecific secondary signals with Anti·His Antibodies. To examine whether endogenous proteins are detected, perform the immunolocalization procedure with nontransfected cells. If this problem arises it can be avoided by tagging your protein with the RGS(His)$_6$ tag and immunostaining with RGS·His Antibody, which specifically recognizes the RGS(His)$_4$ epitope.
c) Overfixation	Overfixation of the cells might lead to signal artifacts. Try to reduce the fixative concentration and/or incubation time.
High background	
a) Blocking conditions	High background is often the result of incomplete saturation of nonspecific cell epitopes with blocking protein. Try increasing the BSA concentration during blocking and antibody incubation steps. Alternatively try normal serum (e.g., goat normal serum) as blocking reagent.
b) Wash conditions	Nonspecifically bound primary and secondary antibody will increase nonspecific background. More extensive washing times after primary and secondary antibody incubation steps should decrease background signals.
Poor morphological preservation	
a) Insufficient fixation	Insufficient fixation of samples leads to poor morphological preservation. If using methanol or acetone try fixation with paraformaldehyde. If 2% paraformaldehyde is insufficient, increase fixative concentration to 4% and increase incubation time. Keep in mind that too harsh fixation can lead to functional alteration of the 6xHis epitope and loss of recognition by the Anti·His Antibodies.

Immunoprecipitation of 6xHis-tagged Proteins

Immunoprecipitation with Anti·His Antibodies

Immunoprecipitation is a useful method for isolating proteins of interest from solutions containing a mixture of proteins. The high specificity and affinity of QIA*express* Anti·His Antibodies allows exclusive precipitation of recombinant 6xHis-tagged proteins, as determined by the presence of a single band, after SDS-PAGE and silver staining (Figure 12).

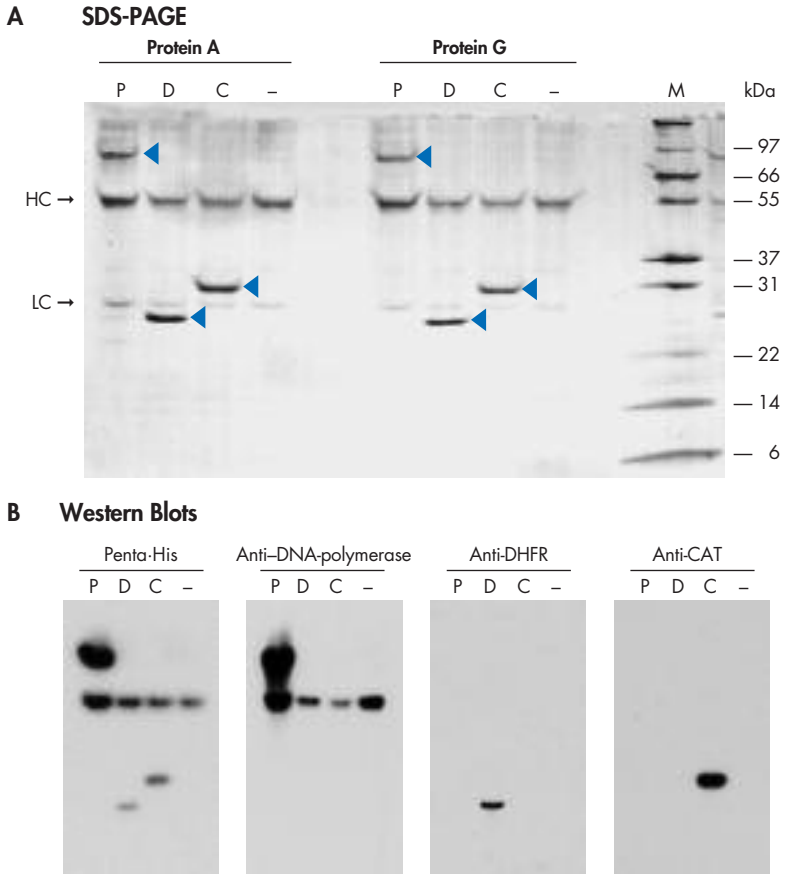


Figure 12. *Proteins immunoprecipitated with protein A or protein G agarose, as indicated, from crude cell extracts of* E. coli *expressing* ***P****: DNA polymerase;* ***D****: DHFR; or* ***C****: CAT. –: lysate without 6xHis-tagged protein;* ***M****: markers. Proteins were visualized by silver staining* ***(A)****. The bands indicated with arrows correspond to the* ***HC****: heavy and* ***LC****: light chains of the Penta·His Antibody released from protein A/protein G by heating in SDS-PAGE sample buffer. Immunoprecipitated 6xHis-tagged proteins are indicated by blue triangles. Western blots were performed using primary antibodies indicated* ***(B)****. The heavy chain of the Penta·His Antibody released from the protein A/protein G agarose (see Figure 14) was detected by the secondary anti-mouse antibody used for the western blots with Penta·His Antibody and anti-DNA polymerase antibody. The mouse heavy chain was not detected by the secondary anti-rabbit antibody used for the western blots with anti-DHFR and anti-CAT antibodies.*

The method should be useful for isolation of 6xHis-tagged in vitro translation products (as demonstrated by Mo and Holland, ref. 26, with RGS·His Antibody), radiolabeled proteins, and the study of protein–protein interactions. Immunoprecipitation is suitable for small-scale protein purification, and a number of samples can be conveniently processed in parallel. For more details and background information about immunoprecipitation please refer to reference 27.

Ni-NTA Magnetic Agarose Beads can be used to perform similar experiments — call QIAGEN for more information.

The immunoprecipitation procedure exploits the ability of protein A or protein G to bind to the second and third constant regions of antibody heavy chains. Protein A or protein G bound to a solid-phase matrix such as cross-linked agarose beads is used to capture antibody–antigen complexes from solution (Figure 13). The procedure involves 1) the preparation of the sample in suitable buffer and preadsorption with the solid-phase matrix, 2) binding of the Anti·His Antibody to 6xHis-tagged protein, 3) binding of antibody–6xHis-tagged-protein complexes to protein A or protein G on the solid-phase matrix, 4) washing, and 5) preparation of samples for SDS-PAGE (see Figure 13).

Preparation of samples and preadsorption

If using Penta·His Antibody or Tetra·His Antibody for immunoprecipitation, cell lysis should not be carried out using lysozyme. These antibodies cross-react weakly with lysozyme. Presence of high concentrations of lysozyme in the cleared lysate may lead to contamination with lysozyme and a reduction in efficiency of immunoprecipitation of the 6xHis-tagged protein. Proteins which bind nonspecifically to the solid-phase matrix are removed by preadsorption of the samples followed by centrifugation.

Binding and washing

In this protocol, binding of Anti·His Antibody to the 6xHis-tagged protein is carried out prior to immobilization of these complexes to the solid-phase matrix (rather than binding of Anti·His Antibody to the solid-phase matrix followed by 6xHis-tagged protein). In this way steric hindrance is minimized and accessibility of the 6xHis-tagged protein for the Anti·His Antibody is maximized. Proteins that do not have a 6xHis tag (or are not bound to the 6xHis-tagged protein when carrying out protein–protein interaction studies) are removed by several wash steps.

Preparation of samples for SDS-PAGE

After several wash steps to remove proteins that are not tightly bound to the matrix, the samples are heated in SDS-PAGE sample buffer. This releases the heavy and light chains of the Anti·His Antibody as well as the immobilized, immunoprecipitated 6xHis-tagged protein. Protein A or protein G is covalently bound to the cross-linked agarose beads, is not released from the matrix by heating, and is removed together with the beads by centrifugation before the samples are analyzed by SDS-PAGE.

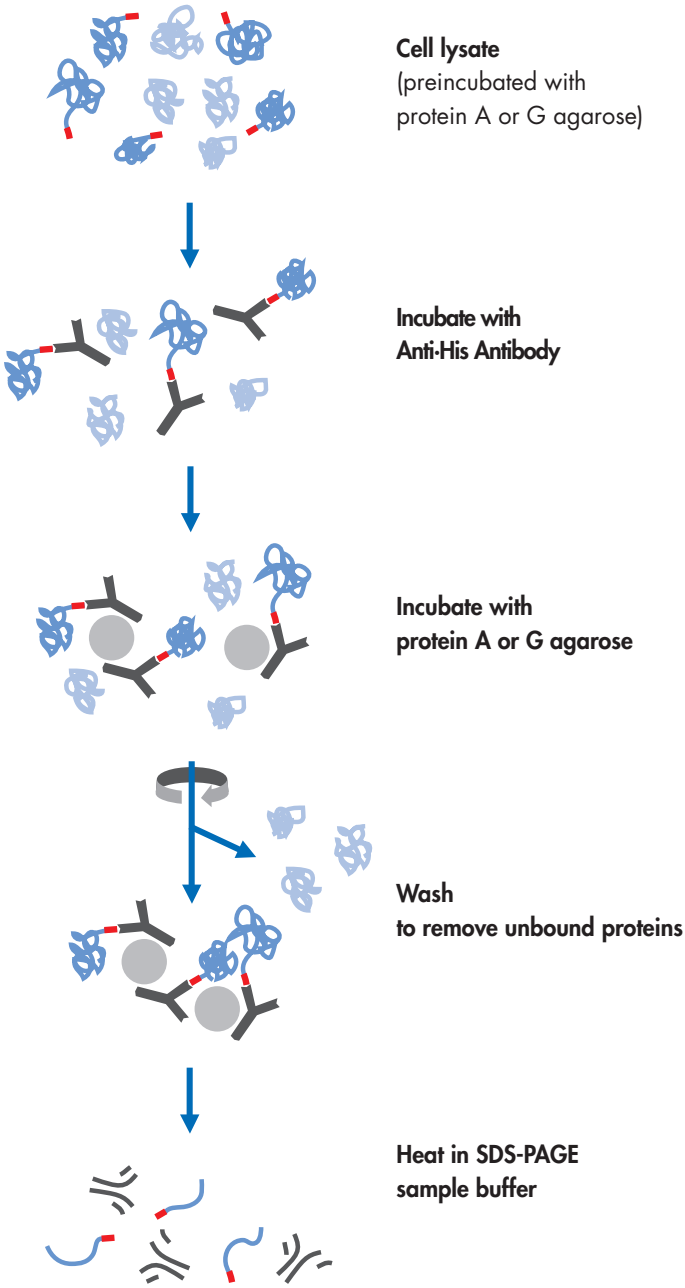


Figure13. *Immunoprecipitation using Anti-His Antibody.*

Protocol 11. Immunoprecipitation

Important notes before starting

- This procedure can be performed with RGS·His Antibody, Penta·His Antibody, or Tetra·His Antibody.
- The protocol can be used with protein A agarose (e.g., Cat. No. P 3391, Sigma), protein G agarose beads (e.g., Cat. No. P 7700, Sigma), or inactivated *Staphylococcus* cells (e.g., Cat. No. P 7155, Sigma). If *Staphylococcus* cells are used, background signals may be higher — see below.
- QIA*express* mouse monoclonal Anti·His Antibodies belong to the subclass IgG1, which have a higher affinity for protein G than for protein A. Nevertheless, immunoprecipitation can be successfully performed with either protein G or protein A agarose.
- Calculate the amount of protein A or protein G agarose needed for efficient precipitation of the amount of antibody used according to the manufacturer's instructions. Note that the affinity of protein A or protein G for mouse IgG is significantly lower than its affinity for human IgG. Its capacity for mouse IgG is approximately 1/10 that for human IgG, so larger amounts must be used for efficient precipitation.
- Please refer to the manufacturer's instructions for preparation of the protein A or protein G agarose prior to use and for binding capacities.
- The choice of matrix used for binding the Anti·His Antibody for immunoprecipitation will depend on the downstream applications. For instance, if inactivated *Staphylococcus* cells are used for immunoprecipitation and are solubilized in SDS-PAGE sample buffer after the wash steps, then *Staphylococcus* proteins will be released from the cells and revealed by silver staining of a gel after SDS-PAGE. Therefore specific detection of proteins of interest will be necessary — for instance by western blotting or detection of radiolabeled proteins. If protein A agarose or protein G agarose is used for immunoprecipitation, then the Anti·His Antibody released from the agarose may be detected in subsequent analyses — for instance, especially the heavy chain may be detected if using anti-mouse IgG antibodies in detection procedures (Figure 12).

Materials

Sodium phosphate buffer

1x SDS-PAGE sample buffer

Protein A agarose **or** protein G agarose **or** inactivated *Staphylococcus* cells

For buffer and reagent compositions, see Appendix, page 91.

Procedure

1. **Prepare the sample containing the 6xHis-tagged protein in sodium phosphate buffer.**

   Other buffers can also be used, but the buffer should be pH 7.0–8.5 and have similar ionic strength to enhance affinity of protein A or protein G for mouse IgG. If necessary, dialyze sample to adjust the ion concentration. Samples in denaturing buffers are not suitable — urea, guanidine hydrochloride, and other denaturants would inactivate the Anti·His Antibody.

2. **Preincubate the sample with the calculated amount of protein A or protein G agarose for 1 h at 4°C on an end-over-end shaker.**

   Nontagged proteins present in the sample that bind to protein A or protein G are removed after this preincubation and centrifugation in step 3. This prevents subsequent coprecipitation of nontagged proteins with 6xHis-tagged proteins.

3. **Centrifuge at maximum speed for 2 min in a microcentrifuge, and transfer supernatant to a fresh microcentrifuge tube.**

4. **Add Anti·His Antibody to the supernatant to a final concentration of 5 µg/ml, and incubate 1 h at 4°C on an end-over-end shaker.**

   The amount of Anti·His Antibody added may be adjusted to give an excess of Anti·His Antibody or at least an equimolar mixture of Anti·His Antibody and 6xHis-tagged protein.

   If necessary, binding can be extended overnight.

5. **Add protein A or protein G agarose, and incubate for 1–2 h at 4°C.**

   Add the amount of protein A or protein G agarose calculated according to the manufacturer's instructions.

6. **Centrifuge at maximum speed for 2 min, and carefully discard supernatant.**

7. **Add 100 µl sodium phosphate buffer and resuspend.**

8. **Centrifuge at maximum speed for 2 min in a microcentrifuge, and transfer supernatant to a fresh microcentrifuge tube.**

9. **Repeat steps 7 and 8 twice.**

10. **Add 20 µl 1x SDS-PAGE sample buffer, mix, incubate 5 min at 95–100°C, centrifuge, and load supernatant onto a gel for further analysis by SDS-PAGE.**

    In addition to the 6xHis-tagged protein, the Anti·His Antibody will be released from the protein A or protein G agarose and separated on the SDS polyacrylamide gel. This will not interfere with most further analysis, such as autoradiographic detection of immunoprecipitated, radiolabeled 6xHis-tagged proteins or western-blot analysis (although bands of 25 and 55 kDa, from the light and heavy chains respectively, may be detected when using anti-mouse antibodies for immunodetection).

Troubleshooting Guide — Immunoprecipitation

The following troubleshooting guide may be helpful in solving any problems that may arise with the immunoprecipitation protocol. We recommend using suitable positive and negative controls for immunoprecipitation studies. They are often essential to enable correct interpretation of results and may help to identify the cause of problems. The scientists at QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications.

	Comments and suggestions
Nonspecific background proteins	
a) Inappropriate wash conditions	Ensure that matrix used for immunoprecipitation is properly resuspended between wash steps. Increase the number of washes, the duration of the wash steps, or the volume of buffer used for washing.
b) Proteins of different size than the protein of interest	**Smaller proteins:** Protein degradation results in 6xHis-tagged proteins smaller than the protein of interest. This may be a result of inadequate or prolonged storage. Freshly prepared protein should be compared with any stored protein. In addition, add a protease inhibitor such as PMSF and/or store at –70°C instead of 4°C or –20°C. Protein degradation may also occur during sample preparation. A variety of protease inhibitors (e.g., PMSF) included in lysis buffers may help. **Larger proteins:** Multimers of the expressed proteins are the most common source of larger crossreacting proteins. They are almost exactly double or triple the size of the monomer. Try preparing samples for SDS-PAGE without the 95°C heating step. Instead incubate, for example, at 60°C. Aggregation of proteins is dependent on the sample preparation method used.
c) Nonspecific binding of proteins to the matrix used for immunoprecipitation	If using *Staphylococcus* cells for immunoprecipitation, try using protein A or protein G agarose instead. Try adding competitor proteins to act as blocking reagents. BSA or gelatin (0.5–1%) may be helpful.
d) Proteins have precipitated out of the lysates and are retained throughout the immunoprecipitation procedure	Clear lysate by centrifugation at 13,000 x *g* for 30 min before adding Anti·His Antibody.

Protein is not immunoprecipitated

As an aid to identification of the cause of this problem the presence or absence of the Anti·His Antibody chains should be established by staining an SDS polyacrylamide gel or by western-blot analysis.

a) Antibody heavy and light chains are visible on stained gel or detected by western-blot analysis	Recombinant protein does not have a 6xHis tag: see "Troubleshooting Guide – Detection", page 48. Suboptimal binding of the 6xHis-tagged protein: Individual 6xHis-tagged proteins are often recognized better by one of the Anti·His Antibodies than the others, possibly because of subtle differences in the exact conformation of the 6xHis tag and other parts of the protein in the vicinity of the tag. To identify the optimal antibody for your specific 6xHis-tagged protein and application, compare the Anti·His Antibodies using the Anti·His Antibody Selector Kit. If using the RGS·His Antibody remember that it requires the RGS(His)$_4$ epitope for recognition. Expression vectors encoding this epitope include pQE-9, pQE-30, pQE-31, pQE-32, pQE-40, pQE-80L, pQE-81L, pQE-82L, pQE-100, and some versions of the pRSET and pBlueBacHis vectors (Invitrogen). A positive control for expression should be included whenever possible. QIA*express* Kits include pQE-40 (encoding 6xHis-tagged DHFR) for this purpose.
b) Antibody heavy and light chains are not visible on stained gel or detected by western-blot analysis	Check with the supplier's instructions that the correct amount of protein A agarose, protein G agarose, or *Staphylococcus* cells has been used to bind the Anti·His Antibody. Extending the incubation time for the binding steps may help.

Detection and assay using the QIA*express* Tag·100 Antibody

The Tag·100 Antibody is a mouse monoclonal antibody that recognizes proteins expressed from the pQE-100 DoubleTag vector, which encodes for an N-terminal 6xHis tag and a second epitope, the Tag·100, at the C-terminus (see *The QIA*expressionist for further details). Protocols using Tag·100 Antibody for immunodetection on dot blots and western blots as well as for ELISA procedures are analogous to those described in this handbook using Anti·His Antibodies. In general, the Tag·100 Antibody is detected by secondary anti-mouse IgG antibodies conjugated to either alkaline phosphatase or horseradish peroxidase together with appropriate enzyme substrates (see page 27). These antibodies are available from many suppliers. We have obtained consistently good results with rabbit anti-mouse IgG-AP–conjugate from Pierce (Cat. No. 31332) or goat anti-mouse IgG-HRP-conjugate from Jackson Immunoresearch (Cat. No. 115-035). Ensure that secondary antibodies used recognize mouse IgG1, and use the highest recommended dilution to avoid nonspecific signals.

Western-blot and dot-blot protocols

The solutions required depend on the detection method used. For chemiluminescent detection, BSA does not sufficiently block nonspecific-binding of the primary and secondary antibody to the membrane. 10% milk powder should be used to dilute the Tag·100 Antibody and secondary antibody. Alternatively, if alkali-soluble casein (Merck, Cat. No. 1.02241) is available in your country it can be used as blocking reagent at a concentration of 1% throughout the chemiluminescent detection protocol resulting in slightly increased sensitivity. Throughout the chromogenic method 3% BSA is sufficient for blocking.

- Use Tag·100 Antibody diluted to concentrations of 0.1–0.04 µg/ml (a 1/2000–1/5000 dilution of the antibody stock solution).
- For chemiluminescent detection refer to Protocol 7 on page 41. Use a 1/5000 dilution of the antibody stock solution.
- For chromogenic detection refer to Protocol 8 on page 44.

ELISA protocols

The protocols make use of the 6xHis tag for immobilization of the protein and the Tag·100 epitope for detection. With two tags located at opposite ends of the protein, the 6xHis tag can be used for directed immobilization of the protein to HisSorb Strips or Plates or Ni-NTA Agarose Magnetic Beads, while the Tag·100 remains easily accessible to the Tag·100 Antibody.

- Use Tag·100 Antibody diluted to concentrations of 0.1–0.04 µg/ml (a 1/2000–1/5000 dilution of the antibody stock solution).
- For ELISA procedures using Ni-NTA HisSorb Strips and Plates refer to Protocol 12 "ELISA with Ni-NTA HisSorb Strips or Plates" in this handbook on page 73.

Assay of 6xHis-tagged Proteins

QIA*express* Assay Systems have been designed to allow assays using 6xHis-tagged biomolecules. Many different assays are possible — for an overview see Figure 14 below.

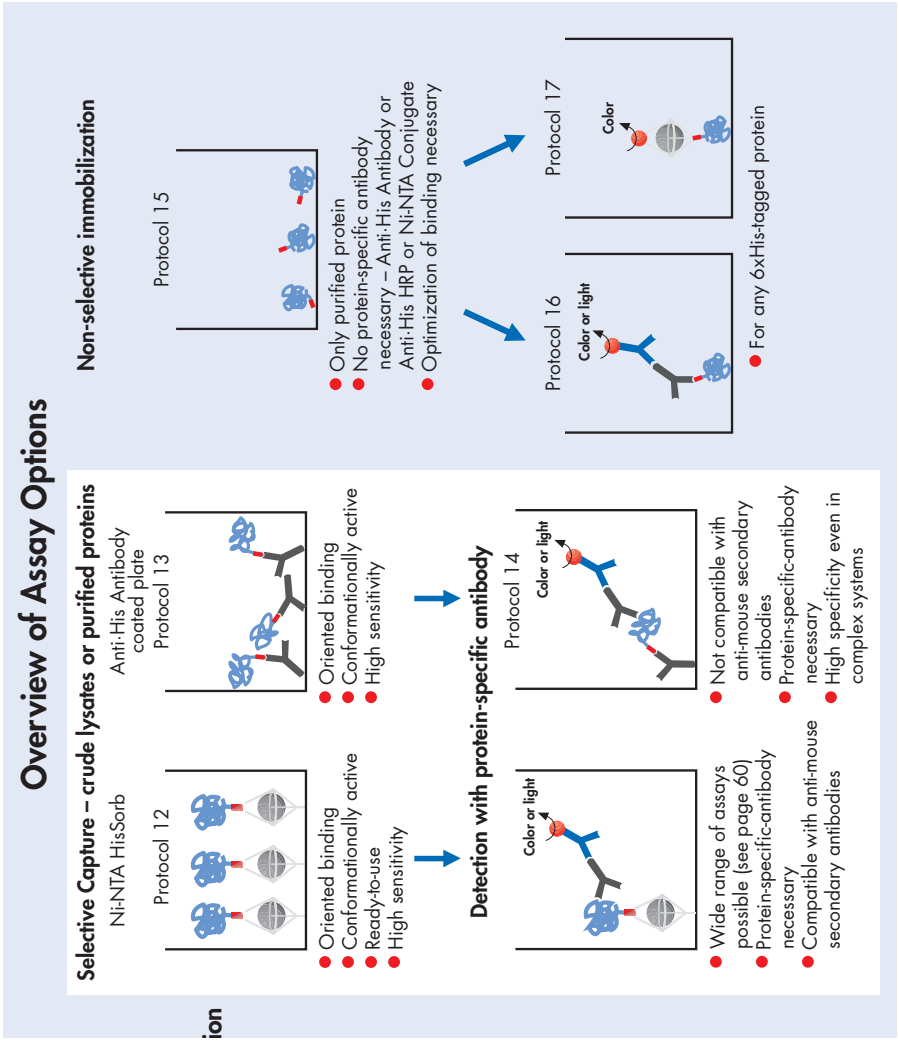


Figure 14. *Overview of assay options.*

Capture assays with Ni-NTA HisSorb Strips and Plates

Ni-NTA HisSorb Strips and Plates utilize the unique metal-chelating properties of nitrilotriacetic acid (NTA) to bind nickel ions and subsequently 6xHis-tagged proteins or other biomolecules, such as peptides or nucleic acids, with high specificity, even from complex mixtures, such as cleared lysates. They consist of high-quality 8-well strips and 96-well microplates whose inner surfaces are coated with a spacer bearing a Ni-NTA group.

The plates and strips allow binding of 6xHis-tagged proteins in a directed manner via their 6xHis tags, meaning that fully functional proteins are immobilized. The activity of bound proteins can be assayed directly using suitable tests (Figure 15 A), or binding, interacting biomolecules can be assayed (Figure 15 B and C). ELISA can be performed using primary antibodies that bind to the 6xHis-tagged protein and secondary antibodies conjugated to an enzyme (usually alkaline phosphatase or horseradish peroxidase) (Figure 15 D).

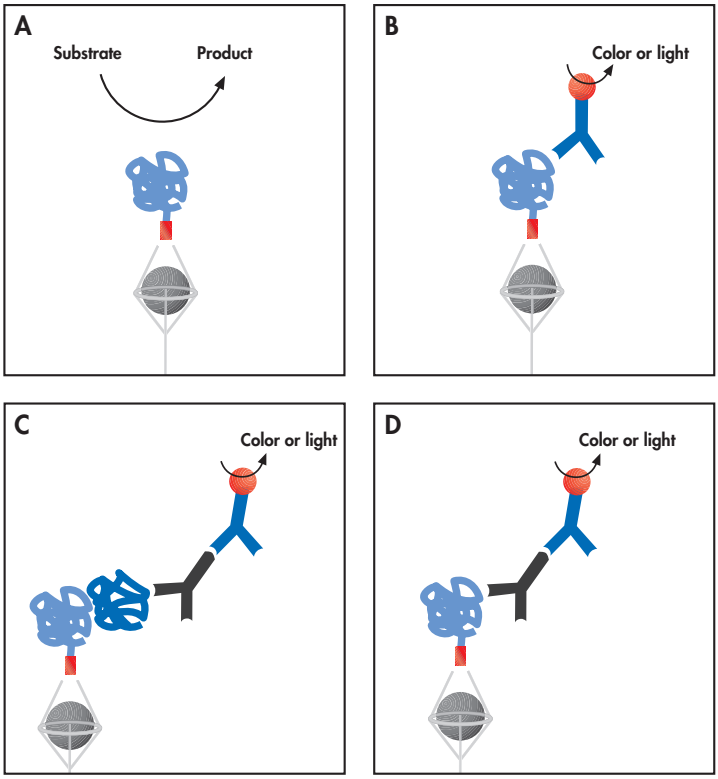


Figure 15. *Ni-NTA HisSorb Assay design.*
A. *Enzyme screening;* ***B.*** *ELISA with antibody conjugate specific for 6xHis-tagged protein;* ***C.*** *Protein-protein interaction assay with antibody conjugate specific for interacting protein;* ***D.*** *ELISA using primary and secondary antibodies.*

Ni-NTA HisSorb Strips and Plates are particularly useful for assaying 6xHis-tagged proteins in crude cell lysates (Figure 16) for example, for the determination of expression rates or in enzyme engineering. Immobilization of 6xHis-tagged biomolecules to Ni-NTA HisSorb Strips or Plates is oriented and takes place at physiological pH, meaning that native protein conformation can be retained. This feature leads to conformationally active immobilized proteins with exposed binding domains, meaning that Ni-NTA HisSorb Strips or Plates allow more sensitive, reproducible assays than protocols which rely on direct binding of proteins to be assayed to polystyrene or similar plates that cause denaturation of the proteins (Figure 17). Krasnykh and coworkers (28) used Ni-NTA HisSorb Strips, in combination with conformation-specific antibodies, to confirm that a recombinant adenovirus fiber protein was in a trimeric form after expression in and purification from insect cells. A range of applications that can be performed using Ni-NTA HisSorb Strips or Plates is given in Table 10 on page 71.

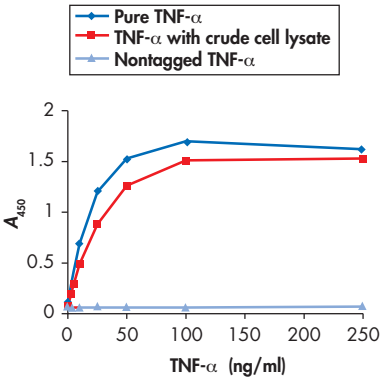


Figure 16. *Purified 6xHis-tagged protein was bound to Ni-NTA HisSorb Strips for 1 h at room temperature, either alone or mixed with crude cell lysate at 5 mg/ml. As a control, nontagged protein was also applied. Bound TNF-α was determined using anti-TNF-α–peroxidase conjugate for 1 h at room temperature followed by determination of enzyme activity using OPD as substrate.*

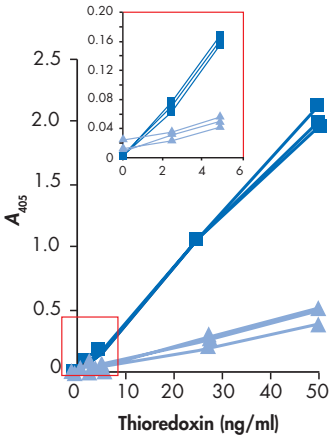


Figure 17. *6xHis-tagged thioredoxin was diluted in PBS, pH 7.2, applied to either polystyrene plates (▲) or to Ni-NTA HisSorb Strips (■), and incubated overnight at 4°C. Three separate standard curves were prepared using each type of plate. The amount of immobilized protein was determined by incubating with rabbit anti-thioredoxin polyclonal antibody for 2 h at room temperature followed by anti-rabbit–alkaline-phosphatase conjugate for 1 h at room temperature and measuring AP activity using pNPP as substrate.*

If a well-established protocol already exists for your assay system, then usually this can be used with Ni-NTA HisSorb Strips and Plates. Care should be taken to avoid the use of assay components that might interfere with the interaction of the 6xHis tag with Ni-NTA — especially EDTA wich is used in many standard ELISA protocols. The NTA groups are precharged with nickel ions, and the polystyrene surface is preblocked with BSA to prevent nonspecific binding: therefore, Ni-NTA HisSorb Strips and Plates are ready to use. Ni-NTA HisSorb plates are available in either transparent format for colorimetric assays, or opaque, white format for luminescence- and fluorescence-based assays.

Table 10. Applications with Ni-NTA HisSorb Strips and Plates

Quantitation of 6xHis-tagged proteins
ELISA or RIA
Antibody screening
Diagnostic assays
Expression screening
Protein interaction studies
Screening of engineered enzymes
Drug screening

Capture assays with Anti·His Antibody-coated plates

As an alternative to Ni-NTA HisSorb Strips or Plates, Anti·His Antibodies can be coated onto 96-well microplates. The immobilized Anti·His Antibody can then selectively capture 6xHis-tagged biomolecules similar to capture by Ni-NTA in the ready-to-use Ni-NTA HisSorb Strips and Plates. ELISA protocols can be designed using suitable antibodies. Mouse primary antibodies, however, are not compatible with this assay setup as anti-mouse secondary antibodies will bind to the Anti·His Antibodies coated on the plate. If only mouse primary antibodies are available, assays should be designed using Ni-NTA HisSorb Strips or Plates, or the primary antibody should be coupled directly to alkaline phosphatase or horseradish peroxidase.

Assays with 6xHis-tagged proteins bound directly to well surfaces

Assays can also be performed by coating 96-well microplates with 6xHis-tagged protein and detecting them using one of the Anti·His Antibodies followed by an anti-mouse IgG secondary antibody conjugated to alkaline phosphatase or horseradish peroxidase. Alternatively, assays can be performed directly using one of the Anti·His HRP or Ni-NTA Conjugates. These protocols are suitable for quantitation of 6xHis-tagged proteins that have already been purified (usually by Ni-NTA chromatography) but are not recommended with cell lysates or other complex mixtures of proteins. The lack of a 6xHis-tagged protein–specific immobilization step means that all proteins in the sample will become coated onto the plate, competing for binding with the 6xHis-tagged protein. This results in greatly reduced assay sensitivity, and positive signals may not be significantly higher than background. It should also be noted that even with purified proteins the signals will be lower and are likely to be less reproducible than those that would be obtained using Ni-NTA HisSorb Strips and Plates. The 6xHis-tagged proteins are bound in random orientation and are likely to lose their native conformation when binding to the well surfaces, resulting in less efficient assays. 6xHis-tagged proteins bound to the plate (Protocol 15, page 80) may then be assayed using one of the Anti·His Antibodies (Protocol 16, page 81) or Ni-NTA or Anti·His HRP Conjugates (Protocol 17, page 83). This method may be necessary if an antibody specific for your 6xHis-tagged protein (in addition to the Anti·His Antibodies) is unavailable.

The assays presented here are given as examples and can be used as guidelines but each specific assay that is designed will depend on the characteristics of the individual proteins or other biomolecules involved in the procedure. Protocol optimization will be necessary for each specific assay.

Protocol 12. ELISA with Ni-NTA HisSorb Strips or Plates

Important notes before starting

- The interaction of 6xHis-tagged proteins with immobilized nickel ions is pH dependent; binding should therefore be carried out at pH 7.2–7.5.
- The binding capacity is approximately 20 pmol/well for small peptides (20–30 amino acids in length) and approximately 10 pmol/well for proteins. 10 pmol of a 25 kDa protein corresponds to 250 ng. However, for each protein assayed, different dilutions should be tested to determine the linear range of detection. The detection limit for each protein depends on the assay system used (e.g., primary and secondary antibody, incubation times, detection reagent), the accessibility of the 6xHis tag, and the size of the protein (very large proteins will bind at lower densities because of steric hindrance). If more 6xHis-tagged protein binding capacity is necessary, Ni-NTA Magnetic Agarose Beads can be used in conjunction with the 96-Well or 12-Tube Magnet (for more details, contact QIAGEN).
- Binding can be performed under native or denaturing conditions, but urea concentrations exceeding 4 M or guanidine hydrochloride concentrations exceeding 1 M should be avoided. An assay in the presence of even higher concentrations of urea or guanidine hydrochloride is also possible, but the protein concentration should be increased significantly.
- Binding should be carried out for at least 1 hour at room temperature. If the concentration of the 6xHis-tagged molecules is very low or if the 6xHis tag is partly hidden, incubation times of 2–4 hours or overnight may increase sensitivity.
- Best results will be obtained if all steps are carried out on a shaker. If there is no shaker available, incubation times should be increased (up to 2–3 h at room temperature or overnight at 4°C) or the incubation temperature should be raised to allow sufficient diffusion of molecules.
- Antibody dilution depends on the individual antibody used. Please refer to manufacturer's recommendations or begin at concentrations useful for western-blot or dot-blot analyses and try further dilutions. Usually primary monoclonal antibody at 0.1 µg/ml to 1 µg/ml will yield satisfactory results. Each antibody should be titrated over this range of concentrations to determine the optimal dilution.
- Ni-NTA HisSorb Strips and Plates are preblocked with BSA. Antibodies or sera that react with BSA, such as those obtained by immunization with peptide–BSA or hapten–BSA conjugates, cannot be used as they will bind to BSA on the well surfaces.
- Suitable negative controls are essential. Assays should always be performed in parallel with samples without any proteins (lysis/dilution buffer alone, reagent blank) and with samples similar to those assayed but lacking the 6xHis-tagged protein (e.g., lysate from *E. coli* transformed with vector lacking the protein-encoding insert). These controls should be incubated with antibodies and the remaining assay components.

- **Note:** This protocol is intended to be used as an example. Optimal conditions for each individual protein and antibody should be determined. If a suitable assay has already been established for your system using polystyrene plates, then similar conditions may be used for all but the initial step of binding the 6xHis-tagged protein to the Ni-NTA–coated wells, provided that the subsequent steps do not involve conditions which lead to dissociation of the 6xHis-tagged protein from Ni-NTA.
- If establishing a new assay system, the binding of the 6xHis-tagged protein should be optimized first (incubation time and amounts of 6xHis-tagged protein). Primary antibody or other secondary components of the assay should be optimized afterwards.
- **Note:** Immobilization is based on metal affinity interactions. High concentrations of chelating reagents (e.g., EDTA, EGTA), strong electron donors, or ionic detergents (e.g., SDS) interfere with binding.

Materials

Ni-NTA HisSorb Strips or Plates

PBS

PBS/BSA

PBS-Tween

1 M Tris·Cl, pH 8.0

Phosphate-citrate buffer, pH 5.0

Substrate for the alkaline phosphatase **or** horseradish peroxidase **or** one of the alternative substrates for horseradish peroxidase

- Prepare solutions for alkaline-phosphatase or horseradish-peroxidase reaction immediately before use.
- Buffers and substrates indicated for alternative use will yield higher sensitivity, but depending on the antibody system used, they can also lead to increased background signals.

For buffer and reagent compositions, see Appendix, page 91.

Procedure

1. **Prepare the 6xHis-tagged molecule at various concentrations in PBS/BSA. Alternatively, different dilutions of a cell lysate containing the 6xHis-tagged protein or peptide can be used. A control without protein should always be included (zero standard).**

   For ELISA assays, concentrations of 6xHis-tagged protein of 0.1–1 µg/ml are recommended; however, lower concentrations (1–10 ng/ml) can often be used, depending on the sensitivity of the antibodies and subsequent assay system. In some assays higher protein concentrations may be necessary. The binding capacity of each well depends on the protein used and ranges between approximately 200 and 400 ng/well.

Protein can be immobilized directly from cleared cell lysate, for example when comparing expression rates. In this case, the content of 6xHis-tagged protein in the total lysate should be estimated, and different dilutions of cell lysate, depending on the expected expression rate, should be applied. Total cellular protein up to approximately 50 µg/ml can be used. To determine the exact amount of bound 6xHis-tagged protein, a standard curve of previously purified protein with known amounts of 6xHis-tagged protein can be applied.

In addition to the protein dilutions, a negative control, containing all assay components except the 6xHis-tagged protein, and a blank, containing only the detection reagents, should always be run together in the same assay.

Ni-NTA HisSorb Strips or Plates can be used under native or denaturing conditions, but urea concentrations exceeding 4 M and guanidine concentrations exceeding 1 M should be avoided. Assays in the presence of even higher concentrations of urea or guanidine hydrochloride are also possible, but the protein concentration should be increased significantly.

The buffer used for dilution and binding of the protein should always contain BSA to prevent adsorption of the protein to the wall of the vial used for dilution.

The pH of the binding buffer should be between 7.2 and 7.5, but the optimal value must be determined for each protein.

2. **Add 200 µl protein solution to each well, and incubate for 1 h at room temperature.**

   Ni-NTA HisSorb Strips and Plates are preblocked and therefore ready for use.

   The time and temperature necessary for efficient immobilization is dependent on the protein, for example, on the degree of 6xHis tag accessibility within the buffer system. For higher assay sensitivity, protein binding for 2–4 h at room temperature or overnight at 4°C may help. If the protein is unstable, incubation should be carried out at 4°C with increased binding time.

3. **Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by tapping on the strips or plates on paper towels.**

   For higher stringency, the pH of the washing buffer can be lowered to 6.0.

4. **Add 200 µl of primary monoclonal antibody diluted in PBS/BSA, cover plate, and incubate for 1–2 h at room temperature.**

   For higher sensitivity, it may help to perform the antibody binding step overnight at 4°C.

   Antibody dilution depends on the individual antibody used. Please refer to manufacturer's recommendations, or begin at concentrations useful for western-blot or dot-blot analyses and try further dilutions. Usually primary monoclonal antibody at 0.1 µg/ml to 1 µg/ml will yield satisfactory results. Each antibody should be titrated over this range of concentrations to determine the optimal dilution.

Using a primary antibody conjugated to horseradish peroxidase or alkaline phosphatase will decrease the time for the whole assay and lead to even more reproducible results and reduced background. If you are using such a labeled primary antibody please continue with step 7.

5. **Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the strips or plates on paper towels after the wash.**

   For higher stringency, the pH of the washing buffer can be lowered to 6.0.

6. **Dilute secondary antibody in PBS/BSA, add 200 µl of the diluted antibody to each well, and incubate at room temperature for 45 min.**

   Concentration of secondary antibody should be chosen following manufacturer's recommendations.

7. **Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the strips or plates on paper towels.**

   For higher stringency, the pH of the washing buffer can be lowered to 6.0.

8. **Add 200 µl of substrate solution, and monitor color development in a microplate reader.**

   Substrate solution should always be prepared immediately before use.

   Monitor color development over a period of 45 min, or add 50 µl stopping reagent after a specific time and measure stopped product. When testing a new assay system, a time-course of color development should be carried out to determine optimal development time and temperature.

   If the reaction is stopped, the signal will increase slightly, depending on the substrate used, and the color will be stable for a period of time.

Protocol 13. Coating 96-well microplates with Anti·His Antibodies

If you wish to use Anti·His-Antibody–coated strips or plates as an alternative to Ni-NTA HisSorb Strips and Plates then this protocol can be followed. A sandwich ELISA can be performed by following Protocol 14.

Important notes before starting

- This procedure is used to immobilize QIA*express* Anti·His Antibodies onto the inner surfaces of suitable 96-well microplates (for example MaxiSorp™ plates, Nunc, Cat. No. 442404 or strips Cat. No. 468667).
- For this procedure, RGS·His Antibody (100 µg) with catalog number 34610, which is supplied lyophilized with BSA, is **not** suitable because the BSA will compete with the RGS·His Antibody binding to the well surfaces.
- The high affinity and specificity of the Anti·His Antibodies means that assays can be performed using crude cell lysates and even dilute solutions.
- It should be remembered that the Anti·His Antibodies are mouse IgG1 monoclonals and that any anti-mouse antibodies used during assay steps may bind directly to the Anti·His Antibodies bound to the plate. This prevents the use of mouse antibodies as the primary antibody to detect 6xHis-tagged proteins bound to the Anti·His Antibody coated on the plate — unless the primary antibody is directly conjugated to an enzyme (usually alkaline phosphatase or horseradish peroxidase). Use of secondary antibody conjugates that are specific for isotypes other than mouse IgG1 in combination with mouse monoclonal antibodies of isotypes other than IgG1 may also eliminate this problem.

Materials

Suitable 96-well microplates (see above)

Coating buffer for Penta·His and Tetra·His Antibody

Coating buffer for RGS·His Antibody

Anti·His Antibody stock solution (**not** RGS·His Antibody, Cat. No. 34610, which is lyophilized with BSA)

Microplate blocking buffer

PBS

Microplate shaker platform

For buffer and reagent compositions, see Appendix, page 91.

Procedure

1. **Dilute antibody in appropriate coating buffer (see above) to a final concentration of 2–5 µg/ml.**
2. **Pipet 200 µl of diluted antibody into each well of a 96-well microplate, cover plate, and incubate overnight at 4°C on a shaker platform.**
3. **Wash wells 4 times with at least 250 µl PBS per well.**
4. **Block wells with 250 µl of blocking buffer for 2 h at room temperature (20–25°C) on a shaker platform.**

   After blocking, plates can be dried overnight at 20–25°C, but sensitivity of the assay will be reduced. After drying, the plates can be stored at 4°C for at least two months before use.
5. **Proceed with Protocol 14.**

Protocol 14. Sandwich ELISA with Anti·His Antibody–coated strips or plates

With this method, immobilization of the 6xHis-tagged molecule is usually accomplished without loss of function and in a specific orientation, thereby providing optimal accessibility to the binding domain, increasing sensitivity and reproducibility, and enhancing signal-to-noise ratio.

Before starting, a few points should be considered:

- The Anti·His Antibodies coated on the microplate are mouse monoclonal antibodies. Secondary anti-mouse antibodies, used in the detection chain for the quantification of the primary antibody, will also bind to the coating antibody and **should not be used** with this system. Primary antibodies derived from mouse can only be used if they are directly conjugated to alkaline phosphatase or horseradish peroxidase. Otherwise Ni-NTA HisSorb Strips or Plates should be used for immobilization.
- Best results will be obtained if all steps are carried out on a shaker platform. If there is no shaker available, incubation times should be extended.
- This protocol is intended to be used as a guideline. Optimal conditions for each individual protein and detection system should be determined empirically.

Materials

PBS

PBS/BSA

Anti·His-Antibody–coated strips or plates (from Protocol 13)

Substrate for alkaline phosphatase **or** horseradish peroxidase **or** one of the alternative substrates for horseradish peroxidase

Buffers and substrates indicated for alternative use will yield higher sensitivity, but dependent on the antibody system used, they can also lead to increased background signals.

For buffer and reagent compositions, see Appendix, page 91.

Procedure

1. **Prepare the 6xHis-tagged molecule at various concentrations in PBS/BSA. Alternatively, different dilutions of a cell lysate containing the 6xHis-tagged protein or peptide may be used. A control without protein should always be included (reagent blank).**

   Concentrations of 6xHis-tagged protein at 0.1–1 µg/ml are recommended; however, lower concentrations (1–10 ng/ml) can often be used, depending on the sensitivity of the antibodies in the subsequent assay system.

   In addition to the protein dilutions, a negative control, containing all assay components with the exception of the 6xHis-tagged protein, and a blank, containing only the detection reagent, should always be run together in the same assay.

2. **Add 200 µl of the protein solution to each well, and incubate for 1 h at room temperature.**

   The time and temperature necessary for efficient immobilization is dependent on the protein. For example, the accessibility of the 6xHis tag in the buffer system used is an important factor. For higher assay sensitivity, it may help to incubate the protein overnight at 4°C.

3. **Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by tapping the strips or plates on paper towels.**

4. **Add 200 µl of primary antibody diluted in PBS/BSA, cover plate, and incubate for 1–2 h at room temperature.**

   For higher sensitivity, it may help to perform the antibody binding step overnight at 4°C.

   Antibody dilution depends on the individual antibody used. Please refer to manufacturer's recommendations, or begin at concentrations useful for western-blot or dot-blot analyses and try further dilutions. Usually primary antibody at 0.1 µg/ml to 1 µg/ml will yield satisfactory results. Each antibody should be titrated over this range of concentrations to determine the optimal dilution.

Using a primary antibody conjugated to horseradish peroxidase or alkaline phosphatase will decrease the time for the whole assay and lead to even more reproducible results and reduced background. If you are using such a labeled primary antibody please continue with step 7.

5. **Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the strips or plates on paper towels after the wash.**
6. **Dilute secondary antibody in PBS/BSA, add 200 µl of the diluted antibody to each well, and incubate at room temperature for 45 min.**

   Concentration of secondary antibody should be chosen following manufacturer's recommendations.

   Do not use secondary anti-mouse antibodies as they will also detect the coating antibody.
7. **Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the strips or plates on paper towels.**
8. **Add 200 µl of substrate solution, and monitor color development in a microplate reader.**

   Substrate solution should always be prepared immediately before use.

   Monitor color development over a period of 45 min, or add 50 µl stopping reagent after a specific time and measure stopped product. When testing a new assay system, a time-course of color development should be carried out to determine optimal development time and temperature.

   If the reaction is stopped the signal will increase slightly, depending on the substrate used, and the color will be stable for a certain period of time.

Protocol 15. Coating 96-well microplates with 6xHis-tagged protein

This procedure is used to immobilize 6xHis-tagged proteins onto the inner surfaces of 96-well microplates. The proteins can then be assayed using one of the QIA*express* Anti·His Antibodies followed by a suitable anti-mouse enzyme-conjugated secondary antibody and substrate (Protocol 16), or directly and conveniently with Anti·HisHRP or Ni-NTA Conjugates (Protocol 17).

Important notes before starting

- The ease with which 6xHis-tagged proteins bind to polystyrene plates is very much dependent on the particular protein. Optimization of binding conditions is necessary. Refer to the manufacturer's instructions.
- As a starting point, three buffers at different pH should be compared.
- Binding may be carried out at 4–37°C. Successful binding may depend on the stability of the 6xHis-tagged protein.

Materials

Suitable 96-well microplates

Coating buffers:

PBS, pH 7.2

50 mM sodium carbonate, pH 9.6

50 mM sodium carbonate, pH 10.6

Microplate blocking buffer

For buffer and reagent compositions, see Appendix, page 91.

Procedure

1. **Serially dilute solution containing 6xHis-tagged protein in coating buffer(s).**
2. **Add 200 µl of the protein solution to each well, and incubate overnight at 4°C.**
3. **Wash wells 4 times with PBS. Soak wells for 10–60 s per wash, and dry the wells by tapping the plate on paper towels.**
4. **Block wells with 250 µl of microplate blocking buffer for 2 h at room temperature (20–25°C) on a shaker platform.**

   After blocking, plates can be dried overnight at 20–25°C, but sensitivity of the assay will be reduced. After drying, it may be possible to store the plates at 4°C for a period of time before use, but this will depend on the specific 6xHis-tagged protein to be assayed.
5. **Wash wells 4 times with PBS. Soak wells for 10–60 s per wash, and dry the wells by tapping the plate on paper towels.**
6. **Proceed with the protocols for assay of 6xHis-tagged proteins with Anti·His Antibody (Protocol 16) or Anti·His HRP or Ni-NTA Conjugates (Protocol 17).**

Protocol 16. Assay of 6xHis-tagged proteins with Anti·His Antibody

Anti·His Antibodies can be used to assay 6xHis-tagged proteins that are immobilized directly to the well surfaces of a 96-well polystyrene microplate (Protocol 15). The sensitivity of assays performed in this way depends largely on the particular 6xHis-tagged protein to be assayed.

Materials

96-well microplates coated with 6xHis-tagged protein (from Protocol 15)

PBS/BSA

PBS

Anti·His Antibody

Secondary-antibody conjugate: Either alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated anti-mouse IgG (from rabbit or goat) may be used. Rabbit-anti-mouse IgG–AP conjugate from Pierce (Cat. No. 31332) or goat-anti-mouse IgG–HRP conjugate from Jackson Immunoresearch (Cat. No. 115-035-003) yield good results.

Substrate for alkaline phosphatase **or** horseradish peroxidase **or** one of the alternative substrates for horseradish peroxidase

Buffers and substrates indicated for alternative use will yield higher sensitivity, but dependent on the antibody system used, they may also lead to increased background signals.

For buffer and reagent compositions, see Appendix, page 91.

Procedure

1. **Add 200 µl of Anti-His Antibody diluted 1/2000 in PBS/BSA. Cover plate, and incubate for 1–2 h at RT.**

   For higher sensitivity, antibody binding can be performed overnight at 4°C.

2. **Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the plate on paper towels after the wash.**

3. **Dilute secondary antibody in PBS/BSA. Add 200 µl of the diluted antibody to each well, and incubate at room temperature for 45 min.**

   Either alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated anti-mouse IgG may be used. Rabbit-anti-mouse IgG–AP conjugate from Pierce (Cat. No. 31332) or goat-anti-mouse IgG–HRP conjugate from Jackson Immunoresearch (Cat. No. 115-035-003) yield good results. Dilute according to the manufacturer's recommendations.

4. **Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the plate on paper towels.**

5. **Add 200 µl of substrate solution, and monitor color development in a microplate reader.**

   Substrate solution should always be prepared immediately before use.

   Monitor color development over a period of 45 min, or add 50 µl stopping reagent after a specific time and measure stopped product. When testing a new assay system, a time-course of color development should be carried out to determine optimal development time and temperature.

   If the reaction is stopped the signal will increase slightly, depending on the substrate used, and the color will be stable for a certain period of time.

Protocol 17. Assay of 6xHis-tagged proteins with Anti·His HRP or Ni-NTA Conjugate

Anti·His HRP or Ni-NTA Conjugates can be used to assay conveniently and directly 6xHis-tagged proteins that are immobilized on a polystyrene plate (Protocol 15, page 80). The sensitivity of assays performed in this way will largely depend on the particular 6xHis-tagged protein to be assayed.

Materials

96-well microplates coated with 6xHis-tagged protein (from Protocol 15, page 80)

PBS/BSA

PBS

Anti·His HRP, Ni-NTA AP, or Ni-NTA HRP Conjugate stock solution

Substrate for alkaline phosphatase or horseradish peroxidase.

Stopping reagent (optional)

Note: When using Anti·His HRP Conjugates please ensure that you use a horseradish peroxidase substrate for detection.

For buffer and reagent compositions, see Appendix, pages 91–92.

Procedure

1. **Add 200 µl of Anti·His HRP or Ni-NTA Conjugate diluted in PBS/BSA to each well, cover plate, and incubate for 1–2 h at room temperature.**

   Conjugate dilution depends on which conjugate is used. For Anti·His HRP Conjugate, a 1/2000 dilution is recommended; for Ni-NTA HRP Conjugate, a 1/1000 dilution is recommended; while for Ni-NTA AP Conjugate, a 1/500 dilution should be used.

   For higher sensitivity, binding can be performed overnight at 4°C.

2. **Wash wells 4 times with PBS. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the plate on paper towels after the wash.**

3. **Add 200 µl of substrate solution, and monitor color development in a microplate reader.**

   Substrate solution should always be prepared immediately before use.

   Monitor color development over a period of 45 min, or add 50 µl stopping reagent after a specific time and measure stopped product (see Table 11, page 92, for details). When testing a new assay system, a time-course of color development should be carried out to determine optimal development time and temperature.

   If the reaction is stopped the signal will increase slightly, depending on the substrate used, and the color will be stable for a certain period of time.

Troubleshooting Guide — Assay

The following troubleshooting guide may be helpful in solving any problems that may arise with the assay protocols. We recommend using suitable positive and negative controls for all assays. They are often essential to enable correct interpretation of results and may help to identify the cause of problems. The scientists at QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications.

	Comments and suggestions
Weak or no signal	
a) Recombinant protein does not have a 6xHis tag	Check construct by DNA sequencing. **N-terminal tag:** Ensure that the cloned insert no longer contains a ribosome binding site and that the ORF contains no putative internal translational start sites. An additional ribosome binding site or internal start site close to the N-terminus of the insert could result in overexpressed, nearly full-size protein lacking a tag. **C-terminal tag:** Premature translation termination will result in a protein lacking a 6xHis tag. Ensure that the ORF contains no additional, unwanted translational stop sites. Check codon usage — for example the arginine codons AGG and AGA are the least frequently used in *E. coli* and the tRNAs that recognize them are the least abundant. If present in expression constructs, these codons can lead to truncated protein products that do not have the 6xHis tag.
b) 6xHis tag is partially hidden	Protein binding may be hindered by a partially hidden 6xHis tag. Try using higher protein concentrations (up to 5 µg/ml), longer binding times, or low concentrations of urea if this is compatible with the subsequent steps of your assay.
c) Assay reagents are no longer functional	Check primary and secondary antibody in a dot blot to determine if this is the case. Check enzyme activity of conjugates by incubation with substrate.
d) Binding of 6xHis-tagged protein is slower than usual	Increase 6xHis-tagged protein binding time (e.g., overnight). Bind protein at 4°C to prevent degradation.

	Comments and suggestions
e) Inhibitors of interaction between Ni-NTA and 6xHis tag are present (when using Ni-NTA HisSorb Strips and Plates or Ni-NTA Conjugates)	Binding of 6xHis-tagged biomolecules to Ni-NTA HisSorb Strips and Plates and Ni-NTA Conjugates is based on metal affinity interactions similar to those of Ni-NTA Agarose or Ni-NTA Superflow. High concentrations of chelating reagents (e.g., EDTA), strong electron donors, or ionic detergents (e.g., SDS) may interfere with binding.
f) Concentration of denaturing agent too high	Do not exceed 4 M urea or 1 M Gu·HCl. If higher concentrations are needed, increase protein content significantly.
High background	
a) Nonspecific interaction with antibodies or other reagents used in the assay	Check signal in a zero standard (wells without sample). High background is usually the result of the antibody system used, especially when antisera or polyclonal antibodies are used. Try using another, less sensitive substrate. Monitor color development. If assay is performed with crude lysates, further dilution of samples may help. Washing with PBS instead of PBS-Tween may help.
b) Stringency of wash steps is too low	Washing the wells with buffer of reduced pH may help.
c) Secondary antibody concentration too high	Try using a lower concentration of secondary antibody.
d) Ni-NTA Conjugate concentration is too high	The optimal Ni-NTA Conjugate dilution depends on the 6xHis-tagged protein and other components of the assay, such as the plates and substrate used. It may help to try a range of dilutions to determine the optimum Ni-NTA Conjugate concentration for your particular 6xHis-tagged protein and assay system.

Appendix

Background information on antibodies

The information given here is intended as a very basic introduction to antibodies, for more information please see reference 27.

Antibody classification

Antibodies serve as a powerful tool for protein research and diagnostics. They are classed into isotypes IgA, IgD, IgE, IgG, and IgM according to their heavy chains. IgG antibodies are the most abundant in serum and are the most commonly used in immunochemical procedures. They consist of two identical γ heavy chains (~55 kDa) and two identical κ or λ light chains (~25 kDa) that form a Y-shaped immunoglobulin molecule with two antigen binding sites (Figure 18). The heavy chain consists of three constant regions and one variable region, while the light chain consists of one constant region and one variable region. Mouse IgG antibodies are further classified, according to differences in their heavy-chain constant regions, into isotypes IgG1, IgG2a, IgG2b, and IgG3. All QIA*express* Anti·His Antibodies are IgG1.

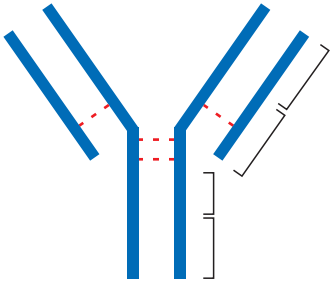


Figure 18. *Schematic diagram of an IgG antibody.* ***C****: constant region;* ***V****: variable region.*

Monoclonal antibodies

In 1975, Köhler and Milstein (29) established a method for fusing antibody-secreting B cells with myeloma cells to produce hybridoma cell lines that can be cultured indefinitely in vitro and produce only one specific monoclonal antibody. Hybridoma cells are used to produce antibodies on a large scale in vivo, following injection into mice to produce ascites fluid, or in vitro, allowing production of purer antibodies. QIA*express* antibodies are produced in vitro under conditions that guarantee the highest purity and activity. Anti·His Antibodies are isolated from serum-free hybridoma cell cultures, ensuring preparations free of viruses, mycoplasma, and contaminating immunoglobulins.

Conjugated antibodies

Species-specific antibodies conjugated to either alkaline phosphatase or horseradish peroxidase are often used in detection and assay procedures. Immunoglobulin molecules vary between species, and therefore it is important to use the correct secondary antibody (for QIA*express* Anti·His Antibodies, anti-mouse conjugates should be used; make sure that the conjugates recognize the subclass IgG1 — please refer to the individual protocols for details). Usually polyclonal antibodies are conjugated, meaning that molecules can bind to the different epitopes on each primary antibody (Anti·His Antibody). This provides an amplification of signal and increased sensitivity in detection and assay procedures in comparison with using direct detection reagents, such as enzyme-conjugated primary antibodies.

Enzymes conjugated to protein A or protein G have been found to be unsuitable for detection on blots with the IgG Anti·His Antibodies. However, both protein A and protein G agarose can be used in combination with the Anti·His Antibodies for immunoprecipitation (see Protocol 11).

Solutions for SDS-PAGE

30% acrylamide/0.8% bis-acrylamide stock solution:	30% acrylamide 0.8% bis-acrylamide (*N,N'*-methylene-bis-acrylamide) (e.g., Roth, Cat. No. 3029.1)
2.5x separating gel buffer:	1.875 M Tris·Cl, pH 8.9 0.25% SDS
5x stacking gel buffer:	0.3 M Tris-phosphate, pH 6.7 0.5% SDS
5x electrophoresis buffer:	0.5 M Tris base 1.92 M glycine 0.5% SDS should be pH 8.8. Do not adjust.
5x SDS-PAGE sample buffer:	0.225 M Tris·Cl, pH 6.8 50% glycerol 5% SDS 0.05% bromophenol blue 0.25 M DTT

Solution for dissolving 6xHis Protein Ladder

1x SDS-PAGE sample buffer:	0.045 M Tris·Cl, pH 6.8 10% glycerol 1% SDS 0.01% bromophenol blue 0.05 M DTT

Solutions for western transfer

Semi-dry transfer buffer:	25 mM Tris base 150 mM glycine 10% methanol should be pH 8.3 without adjusting
Tank-blotting transfer buffer:	25 mM Tris base 150 mM glycine 20% methanol should be pH 8.3 without adjusting

Solutions for dot-blot preparation

Dilution buffer for denaturing conditions:	8 M urea 0.1 M NaH_2PO_4 0.01 M Tris·Cl pH 8.0
Dilution buffer for native conditions:	50 mM NaH_2PO_4 300 mM NaCl pH 8.0

Solutions for colony-blot preparation

SDS solution:	10% (w/v) sodium dodecyl sulfate
Denaturing solution:	0.5 M NaOH 1.5 M NaCl
Neutralization solution:	1.5 M NaCl 0.5 M Tris·Cl, pH 7.4
20x SSC:	500 ml: 87.65 g NaCl, 50.25 g trisodium citrate·$2H_2O$

Solutions for detection procedures

TBS buffer:	10 mM Tris·Cl, pH 7.5 150 mM NaCl
TBS-Tween buffer:	20 mM Tris·Cl, pH 7.5 500 mM NaCl 0.05% (v/v) Tween 20 (Sigma, Cat. No. P1379)
TBS-Tween/Triton buffer:	20 mM Tris·Cl, pH 7.5 500 mM NaCl 0.05% (v/v) Tween 20 0.2% (v/v) Triton X-100 (Sigma, Cat. No. X-100)

Blocking buffer:	3% (w/v) BSA (Sigma, Cat. No. A7906) in TBS buffer
Blocking buffer (for alternative method):	1% (w/v) alkali-soluble casein (Merck, Cat. No. 1.02241) in TBS Alkali-soluble casein is not easily dissolved in TBS. Dissolve casein and NaCl in 10 mM Tris base, and then adjust pH if necessary.
Anti·His HRP Conjugate blocking buffer	For 20 ml, (sufficient for processing of one 8 x 10 cm minigel) add 0.1 g Blocking Reagent to 20 ml 1 x Blocking Reagent Buffer at 70°C, and stir until dissolved (final concentration 0.5 % [w/v]). Add 200 µl 10% (v/v) Tween-20 (final concentration 0.1 % [v/v]). The resulting solution is slightly opaque. Allow to cool to room temperature before use.*
Secondary antibody dilution buffer for chemiluminescent detection:	10% (w/v) skim milk powder (Fluka, Cat. No. 70166) in TBS buffer For best results, milk powder should be dissolved overnight at 4°C.
Anti·His or Tag·100 Antibody stock solution:	Dissolve the lyophilized Anti·His or Tag·100 Antibody (100 µg) in 500 µl water per vial (final concentration, 0.2 mg/ml). Dissolve Anti·His Antibody Selector Kit antibodies (3 µg) in 15 µl water per tube (final concentration, 0.2 mg/ml).
Ni-NTA Conjugate stock solution:	Dissolve the lyophilized conjugate in 500 µl water per vial.

Solutions for alkaline phosphatase (AP) staining

Buffer A:	100 mM Tris·Cl, pH 9.5 100 mM NaCl 5mM $MgCl_2$
NBT stock solution:	5% NBT (nitro blue tetrazolium, Sigma, Cat. No. N5514) in 70% dimethylformamide (Store in aliquots at –20°C)

** Although complete Blocking Reagent Buffer is stable for several weeks when stored at 2–8°C, we recommend preparing fresh Blocking Reagent Buffer each time it is required.*

BCIP stock solution:	5% BCIP (5-bromo-4-chloro-3-indolyl phosphate, Sigma, Cat. No. B0274) in 100% dimethylformamide (Store in aliquots at –20°C)
Staining solution:	**Prepare immediately before staining.** Add 66 µl NBT stock solution and 33 µl BCIP stock solution to 10 ml Buffer A (final concentration: 0.33 mg/ml NBT; 0.165 mg/ml BCIP). Alternatively, BCIP/NBT tablets can be obtained from Sigma (Cat. No. B5655).

Solutions for horseradish peroxidase (HRP) staining

10x Tris-saline:	9% (w/v) NaCl in 1 M Tris·Cl, pH 8.0
Staining solution:	**Prepare immediately before staining.** Dissolve 18 mg 4-chloro-1-naphthol (Sigma, Cat. No. C8890) in 6 ml methanol. Add 24 ml 1x Tris-saline followed by 60 µl 30% hydrogen peroxide (H_2O_2). **Note:** The final staining solution is only stable for a short period.

Solutions for immunolocalization

10x PBS-IF (100 mM sodium phosphate pH 7.4, 1.4 M NaCl):	Dissolve 7.12 g $Na_2HPO_4 \cdot 2H_2O$, 1.38 g $NaH_2PO_4 \cdot H_2O$, and 40.95 g NaCl in 500 ml distilled water.
1x PBS-IF (10 mM sodium phosphate, pH 7.4; 140 mM NaCl):	Dilute 10x PBS-IF 1/10 with distilled water
2% paraformaldehyde in 1x PBS-IF:	Heat 100 ml 1x PBS-IF to 60°C, add 2 g solid paraformaldehyde (Sigma Cat. No. P-6148) and a few drops 2N NaOH and stir, adjust pH 7.2 with HCl, filter the solution through a folded filter, and store in a dark bottle at room temperature.
0.25% Triton X-100 in 1x PBS-IF:	0.25% Triton X-100 in 1x PBS-IF
Blocking Buffer IF:	5% (w/v) BSA (Serva, Cat. No 11930) in 1x PBS-IF
Anti·His Antibody stock solution:	0.2 mg/ml Anti·His Antibody (see Storage Conditions, page 8)

Antibody Dilution Buffer IF:	1% (w/v) BSA (Serva, Cat. No. 11930) in 1x PBS-IF

Solutions for immunoprecipitation

Sodium phosphate buffer:	50 mM NaH_2PO_4, pH 8.0 300 mM NaCl
1x SDS Sample buffer:	45 mM Tris·Cl, pH 6.8 10% glycerol 1% SDS 0.01% bromophenol blue 50 mM DTT

Solutions for assay procedures

PBS (50 mM potassium phosphate, pH 7.2; 150 mM NaCl):	71.7 ml/liter 0.5 M K_2HPO_4 28.3 ml/liter 0.5 M KH_2PO_4 8.57 g/liter NaCl, pH 7.2
PBS/BSA:	0.2% BSA in PBS
PBS-Tween:	0.05% Tween 20 in PBS
1 M Tris·Cl, pH 8.0:	121.1 g/liter Tris base pH adjusted to 8.0 with HCl
Phosphate–citrate buffer, pH 5.0:	51.5 ml 0.2 M Na_2HPO_4 48.5 ml 0.1 M citric acid, pH 5.0
Coating buffer for Penta·His Antibody and Tetra·His Antibody:	50 mM sodium carbonate, pH 10.6
Coating buffer for RGS·His Antibody:	PBS, pH 7.2 (see above)
Microplate blocking buffer:	2.0% sucrose 0.1% bovine serum albumin 0.9% sodium chloride

Substrates for assay procedures

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

Buffers and substrates indicated for alternative use will yield higher sensitivity, but depending on the antibody system used, they can also lead to increased background signals.

Substrate for alkaline phosphatase

p-Nitrophenyl Phosphate (pNPP): Dissolve 50 mg pNPP in 10 ml 1 M diethanolamine; 0.01% $MgCl_2 \cdot 6\ H_2O$, pH 9.8

Substrate for horseradish peroxidase

2,2'-Azino-bis[3-Ethylbenzthiazoline-6-Sulfonic Acid] (ABTS®): Dissolve10 mg ABTS in 10 ml phosphate-citrate buffer.
Immediately before use add 2 µl 30% H_2O_2

Alternative substrates for horseradish peroxidase

o-Phenylenediamine (OPD): Dissolve 10 mg OPD in 10 ml phosphate-citrate buffer.
Immediately before use add 2 µl 30% H_2O_2

3,3',5,5'-Tetramethylbenzidine (TMB): Dissolve 1 mg TMB in 1 ml DMSO; add 9 ml phosphate-citrate buffer.
Immediately before use add 2 µl 30% H_2O_2

Table 11. Details of substrates for assay procedures

Substrate	Wavelength for monitoring color development	Stopping reagent*	Wavelength for determining stopped product
pNPP	405 nm	3 M NaOH	405 nm
ABTS	415 nm	1% SDS	415 nm
OPD	450 nm	3 M HCl or 3 M H_2SO_4	492 nm
TMB	370 nm or 650 nm	2 M H_2SO_4	450 nm

** If the reaction is stopped, the signal will increase slightly, depending on the substrate used, and the color will be stable for a period of time.*

Table 12. Substrates for ELISA, immunoblotting, and immunohistology

Substrate system	Abbrev.	Reaction product	Application
Peroxidase			
2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)	ABTS	Green, soluble	ELISA
o-Phenylenediamine	OPD	Orange, soluble	ELISA
3,3',5,5'-Tetramethyl-benzidine	TMB	Blue, soluble	ELISA
o-Dianisidine	—	Yellow-orange, soluble	ELISA
5-Aminosalicylic acid	5AS	Brown, soluble	ELISA
3,3'-Diaminobenzidine	DAB	Brown, insoluble	Immunoblotting, immunohistology
3-Amino-9-ethylcarbazole	AEC	Red, insoluble	Immunoblotting, immunohistology
4-Chloro-1-naphthol	4C1N	Blue, insoluble	Immunoblotting
Amersham Pharmacia Biotech ECL	—	Chemiluminescence	Immunoblotting
Alkaline phosphatase			
p-Nitrophenylphosphate	pNPP	Yellow, soluble	ELISA
5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium	BCIP/NBT	Blue, insoluble	Immunoblotting, immunohistology
CDP-Star	—	Chemiluminescence	Immunoblotting
Lumigen™ PPD (4-methoxy-4-(3-phosphatephenyl)spiro-(1,2-dioxetane-3,2'-adamantane))	—	Chemiluminescence	Immunoblotting

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Product	Contents	Cat. No.
QIA*express* detection reagents		
RGS·His HRP Conjugate Kit	125 µl of RGS·His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer (10x concentrate)	34450
Penta·His HRP Conjugate Kit	125 µl of Penta·His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer (10x concentrate)	34460
Tetra·His HRP Conjugate Kit	125 µl of Tetra·His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer (10x concentrate)	34470
Ni-NTA AP Conjugate	Alkaline phosphatase-conjugated Ni-NTA (lyophilized, for 500 ml working solution)	34510
Ni-NTA HRP Conjugate	Horseradish peroxidase-conjugated Ni-NTA (lyophilized, for 500 ml working solution)	34530
RGS·His Antibody (100 µg)	100 µg mouse anti-RGS(His)$_4$ (lyophilized, for 1000 ml working solution)	34610
RGS·His Antibody, BSA-free (100 µg)	100 µg mouse anti-RGS(His)$_4$ (lyophilized, BSA-free, for 1000 ml working solution)	34650
Penta·His Antibody, BSA-free (100 µg)	100 µg mouse anti-(His)$_5$ (lyophilized, for 1000 ml working solution)	34660
Tetra·His Antibody, BSA-free (100 µg)	100 µg mouse anti-(His)$_4$ (lyophilized, for 1000 ml working solution)	34670
Tag·100 Antibody (100 µg)	100 µg mouse anti·Tag 100 (BSA-free) for 1000–2500 ml working solution	34680
Anti·His Antibody Selector Kit	RGS·His Antibody, Penta·His Antibody, Tetra·His Antibody, all BSA-free, 3 µg each	34698

Please inquire for availability and pricing of bulk antibodies.

Ordering Information

Product	Contents	Cat. No.
QIA*express* detection reagents		
Penta·His Alexa Fluor 488 Conjugate	125 µl Penta·His Alexa Fluor 488 Conjugate, 200 µg/ml	35310
Penta·His Alexa Fluor 532 Conjugate	125 µl Penta·His Alexa Fluor 532 Conjugate, 200 µg/ml	35330
Penta·His Alexa Fluor 555 Conjugate	125 µl Penta·His Alexa Fluor 555 Conjugate, 200 µg/ml	35350
Penta·His Alexa Fluor 647 Conjugate	125 µl Penta·His Alexa Fluor 647 Conjugate, 200 µg/ml	35370
Penta·His Biotin Conjugate	125 µl Penta·His Biotin Conjugate, 200 µg/ml	34440
Streptavidin–R-PE	250 µl Streptavidin–R-phycoerythrin Conjugate, 1mg/ml	922721
6xHis Protein Ladder	6xHis-tagged marker proteins (lyophilized, for 50–100 lanes on western blots)	34705
Ni-NTA HisSorb Strips and Plates		
Ni-NTA HisSorb Strips (24)	2 racks of 12 x Ni-NTA-coated 8-well strips in 96-well format	35023
Ni-NTA HisSorb Plates (5)	5 Ni-NTA–coated, transparent 96-well plates	35061
Ni-NTA HisSorb Plates, white (5)	5 Ni-NTA–coated, opaque, white 96-well plates	35081
Ni-NTA magnetic bead system		
Ni-NTA Magnetic Agarose Beads (2 x 1 ml)	2 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36111
Ni-NTA Magnetic Agarose Beads (6 x 1 ml)	6 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36113
12-Tube Magnet	Magnet for separating magnetic beads in 1.5-ml or 2-ml tubes	36912
96-Well Magnet Type A	Magnet for separating magnetic beads in wells of 96-well plates, 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

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Product	Contents	Cat. No.
Ni-NTA matrices		
Ni-NTA Agarose (25 ml)	25 ml nickel-charged resin (max. pressure: 2.8 psi)	30210
Ni-NTA Agarose (100 ml)	100 ml nickel-charged resin (max. pressure: 2.8 psi)	30230
Ni-NTA Agarose (500 ml)	500 ml nickel-charged resin (max. pressure: 2.8 psi)	30250
Ni-NTA Superflow (25 ml)	25 ml nickel-charged resin (max. pressure: 140 psi)	30410
Ni-NTA Superflow (100 ml)	100 ml nickel-charged resin (max. pressure: 140 psi)	30430
Ni-NTA Superflow (500 ml)	500 ml nickel-charged resin (max. pressure: 140 psi)	30450
Ni-NTA Spin Columns (50)	50 Spin Columns, Collection Tubes	31014
Ni-NTA Spin Kit (50)	50 Ni-NTA Spin Columns, Reagents, Buffers, Collection Tubes (2 ml)	31314
QIA*express* Kits		
QIA*express* Type IV Kit	5 µg each of pQE-30, pQE-31, pQE-32 (N-terminal 6xHis), 10 ml Ni-NTA Agarose	32149
QIA*express* Type ATG Kit	5 µg each of pQE-60, pQE-70 (C-terminal 6xHis), 10 ml Ni-NTA Agarose	32169

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QIA*express* vectors		
N-Terminus pQE Vector Set	25 µg each of pQE-9, pQE-30, pQE-31, pQE-32, and pQE-40	32915
C-Terminus pQE Vector Set	25 µg each of pQE-16, pQE-60, and pQE-70	32903
cis-Repressed pQE Vector Set	25 µg each of pQE-80L, pQE-81L, and pQE-82L	32923
pQE-100 DoubleTag Vector DNA	25 µg of pQE-100	33003
QIA*express* sequencing primers		
pQE Sequencing-Primer Set	0.1 A_{260} unit each of Primer-Promoter Region, Primer-Type III/IV, and Primer-Reverse Sequencing (3.0, 2.8, and 3.1 µg, respectively; lyophilized)	34051
QIA*express* host strains		
E. coli Host Strains	One stab culture each of *E. coli* M15[pREP4] and SG13009[pREP4]	34210
QIA*express* accessories		
Polypropylene Columns (1 ml)	50/pack, 1 ml capacity	34924
Polypropylene Columns (5 ml)	50/pack, 5 ml capacity	34964

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