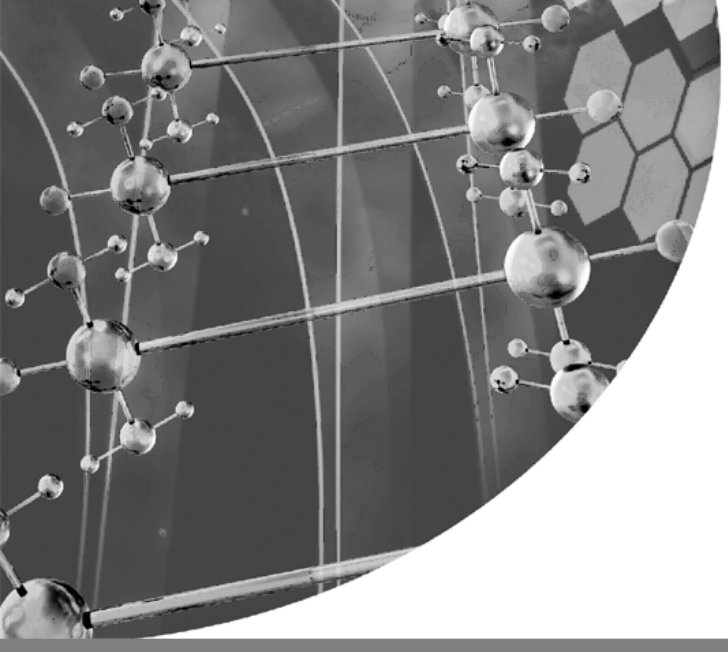


PerfectPro Detection and Assay Manual

For detection and assays using 6xHis tag recombinant proteins

PerfectPro Detection and Assay Manual, September 2010

Contents

Product specifications

Product description

PerfectPro Ni-NTA Detection Systems are designed for simple and sensitive detection of recombinant 6xHis-tagged proteins. The reagents have been developed to allow detection of almost any 6xHis-tagged protein in applications such as western blot analysis. PerfectPro Ni-NTA detection reagents include high-affinity, high-specificity monoclonal Anti·His Antibodies and antibody conjugates. These antibodies can be used for assays, as well as with the Ni-NTA HisPrime Strips and plates of the PerfectPro Ni-NTA Detection Systems.

Product limitations

The PerfectPro System is developed, designed, and sold for research purposes only. It is 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 the materials described in this text.

Materials supplied

PerfectPro Detection Kits

Contents	Penta·His HRP Conjugate Kit
Ordering no.	2400410
Anti·His HRP Conjugate	125 µl, for 250 ml working solution
Blocking Reagent	5 g
Blocking Reagent Buffer (10x Concentrate)	50 ml

Contents	Anti·His Antibody Selector Kit
Ordering no.	2400300
Penta·His Antibody, BSA free	3 µg, lyophilized for 30 ml working solution
RGS·His Antibody	3 µg, lyophilized for 30 ml working solution
Tetra·His Antibody, BSA free	3 µg, lyophilized for 30 ml working solution

Contents	Tetra·His Antibody BSA-free – 100 µg	Penta·His Antibody BSA-free – 100 µg	RGS Anti·His Antibody – 100 µg
Ordering no.	2400310	2400320	2400330
Antibody	100 µg, lyophilized	100 µg, lyophilized	100 µg, lyophilized
	for 1000 ml working solution each		

Ni-NTA HisPrime Strips and Plates

Contents	Ni-NTA HisPrime Strips (24)	Ni-NTA HisPrime Plates - 5	Ni-NTA HisPrime Plates, white - 5
Order/ref. no.	2400700	2400720	2400730
Racks of 12 x Ni-NTA–coated-well strips	2 racks (for 192 assays)		
Ni-NTA–coated, transparent 96-well plates		5 plates (for 480 assays)	
Ni-NTA–coated, opaque, white 96-well plates			5 plates (for 480 assays)

Additional materials

For convenience, additional materials to be supplied by the user are listed at the beginning of the protocol for which they are required.

Storage and reconstitution conditions

The PerfectPro System components should be stored as described below.

Component	Storage instructions
Penta·His HRP Conjugates	Store at 2–8°C for up to one year. Do not freeze the conjugate solution.
Blocking Reagent and Blocking Reagent Buffer Concentrate	Store at room temperature (15–25°C) for up to one year.
Anti·His Antibodies	Store lyophilized Anti·His Antibody at 2–8°C until use. Under these conditions, the antibodies are stable for 1 year. Once dissolved, store for 3 months at 2–8°C or in aliquots at –20°C for up to 6 months. Avoid repeated freezing and thawing. Dissolve 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).
Ni-NTA HisPrime Strips and Plates	Store dry at room temperature. Under these conditions Ni-NTA HisPrime Strips and Plates are stable for 1 year.

Safety information

All due care and attention should be exercised in the handling of this product. We recommend all users of 5 PRIME products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines. Specifically, always wear a suitable lab coat, disposable gloves, and protective goggles when working with chemicals.

Additional safety information is available from www.5PRIME.com in material safety data sheets (MSDSs) for 5 PRIME products and 5 PRIME product components.

Quality assurance

5 PRIME products are manufactured using quality chemicals and materials that meet our high standards. All product components are subjected to rigorous quality assurance testing process:

- **Component testing**: each component is tested to ensure the composition and quality meet stated specifications.
- **Performance testing**: each product is tested to ensure it meets the stated performance specification.

Additional quality information is available from www.5PRIME.com including certificate of analysis sheets for 5 PRIME products and 5 PRIME product components.

Product warranty

5 PRIME is committed to providing products that improve the speed, ease-of-use and quality of enabling technologies.

5 PRIME 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.

This warranty is in place of any other warranty or guarantee, expressed, or implied, instituted by law or otherwise. 5 PRIME provides no other warranties of any kind, expressed or implied, including warranties of merchantability and fitness for a particular purpose. Under no circumstance shall 5 PRIME be responsible for any direct, indirect, consequential or incidental damages or loss arising from the use, misuse, results of use or inability to use its products, even if the possibility of such loss, damage or expense was known by 5 PRIME.

Protocols

Introduction

The use of small affinity tags has simplified the expression, purification, detection, and assay of recombinant proteins. PerfectPro Protein Expression and Purification Systems are based on the remarkable selectivity and affinity for proteins tagged with 6 consecutive histidine residues (6xHis tag) of patented nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices (Figure 1). The features and benefits of the PerfectPro System are given in Table 1.

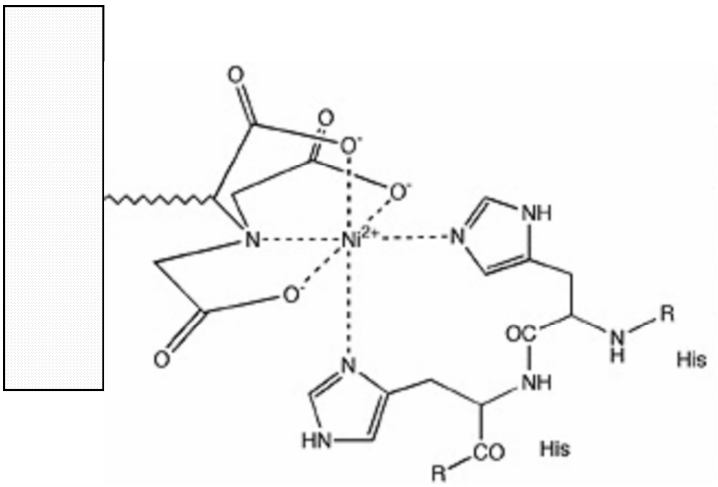


Figure 1. Interaction between neighboring residues in the 6xHis tag and PerfectPro matrix.

Table 1. Overview of the PerfectPro System

Feature	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	The 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 usefulness of the 6xHis affinity tag is based on its binding to Ni-NTA.

Based on observations made with tagged enzymes, transcription factors, and engineered antibodies, the 6xHis tag does not usually interfere with the structure or function of the tagged protein.

In spite of the great demand for highly specific antibodies against the 6xHis tag, no satisfactory antibodies are available due to the poor antigenicity of the tag. Therefore, the PerfectPro Anti·His HRP Conjugates and Anti·His Antibodies are used to specifically recognize the 6xHis tag.

Ni-NTA technology

In 1975[1], the chelating ligand iminodiacetic acid (IDA) was used to purify proteins with immobilized-metal affinity chromatography (IMAC). IDA cannot tightly bind metal ions with its 3 metal-chelating sites. Proteins that bind the metal ions are weakly bound to the metal-chelating matrix as the metal ions leach from the chromatography matrix. Ni-NTA, developed at Hoffmann-La Roche, binds metal ions far more stably than other available chelating resins[2,3]. Since it occupies four of the six ligand binding sites in the co-ordination sphere of the nickel ion, the remaining two sites are free to interact with the 6xHis tag. In addition, NTA retains the ions under a wide variety of stringent conditions.

The strong binding of the 6xHis tag to the immobilized nickel ion allows one-step protein purification with Ni-NTA chromatography matrices. Ni-NTA HisPrime Strips and Plates, based on this principle, are highly suited for the detection and assay of 6xHis-tagged proteins.

Anti·His Antibodies

The mouse monoclonal IgG1 antibodies that have high affinity and specificity for the 6xHis tag, PerfectPro Anti·His Antibodies, are obtained from serum-free hybridoma cell cultures, ensuring preparations free of viruses, mycoplasmas, and contaminating immunoglobulins. A peptide library has been used to determine the exact epitopes that are recognized by each of the Anti·His Antibodies (Table 2).

Preparations with the highest purity and activity are obtained by adsorption chromatography performed at physiological pH without using protein A or protein G. Because they allow detection of all 6xHis-tagged proteins, using Anti·His Antibodies does not require prior purification of each individual protein, animal immunization, and testing and processing of serum. All three Anti·His HRP Conjugates and Anti·His Antibodies have very high specificity and are suitable for all expression systems.

RGS·His Antibody recognizes the RGS(His)$_4$ epitope found on proteins encoded by the following vectors:

- pPP- 9, pPP- 30, pPP- 31, pPP- 32, pPP- 40 and pPP- 100 Double Tag.
- pRSETA, pRSETB, pRSETC, pBlueBacHis2A, pBlueBacHis2B and pBlueBacHis2C (Life Technologies)

Protein–DNA binding and protein–protein interactions have been studied using RGS·His Antibody to identify 6xHis-tagged proteins on western blots.

Penta·His Antibody and Tetra·His Antibody can bind to 6xHis-tagged proteins expressed from any vector and do not require any additional amino acids. Regardless of the surrounding amino-acid context, Penta·His Antibody recognizes five consecutive histidine residues, and Tetra·His Antibody recognizes four, allowing them to bind to even partially hidden 6xHis tags that other anti-His antibodies do not recognize.

The PerfectPro Anti·His Antibodies can be used for the following:

- RGS·His Antibody localization of 6xHis-tagged proteins in situ using immunohistochemical and immunocytochemical procedures
- Immunoprecipitation
- Immunoaffinity chromatography
- Efficient enzyme-linked immunosorbent assay (ELISA) protocols
- Other assays

See protocols for more details.

Anti·His Antibody Selector Kit

The Anti·His Antibody Selector Kit should be used to choose the optimal antibody for your specific 6xHis-tagged protein and application (Table 2 and Table 3). The kit is provided as individual 6xHis-tagged proteins are often recognized better by one Anti·His Antibody than by the others. Each of the three antibodies is provided to determine which give the best results.

Table 2. Epitopes recognized by PerfectPro Anti·His Antibodies and Penta·His HRP Conjugates

Peptide	Penta·His	Tetra·His	RGS·His
XHHHHHHX	+	+	–
XXHHHHHX	+	+	–
XHXHHHHX	–	+	–
XHHHHXHX	–	+	–
XRGSHHHHX	–	+	+
XRGSHHHHHHX	+	+	+
XHXHHXHX	–	–	–

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, pH 8; 150 mM NaCl), blocked in 1% casein in TBS, and washed further in TBS. Blots were probed with PerfectPro Anti·His Antibody (0.1 µg/ml) followed by secondary antibody and chromogenic detection with alkaline phosphatase-conjugated anti-mouse IgG and NBT/BCIP.

Table 3. Anti·His Antibody epitopes and dissociation constants

Antibody	Epitope	Dissociation constant Kd (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 Kd is dependent on the individual 6xHis-tagged protein.

Penta·His HRP Conjugates

Mouse monoclonal IgG1 Penta·His Antibodies are coupled to horseradish peroxidase (HRP) to create Penta·His HRP conjugates and are used for direct detection of any protein with an accessible 6xHis tag by chromogenic or chemiluminescent methods. Secondary antibodies are not needed, allowing fast, cost-effective blotting and ELISA procedures. The highly specific Penta·His conjugates are supplied with a specially formulated Blocking Reagent and Blocking Reagent Buffer to ensure optimal specificity and sensitivity.

PerfectPro 6xHis-tagged Protein Ladder

The PerfectPro 6xHis-tagged Protein Ladder mixture of five highly purified recombinant proteins ranging in size from 15 to 100 kDa containing a N-terminal RGS (His)6 sequence. Each protein can be detected by any of the PerfectPro detection reactions, including the RGS His Antibody. It yields five clear bands of similar intensity on western blots, thus can be used for precise molecular weight determination of proteins on western blot membranes.

Ni-NTA HisPrime Strips and Plates

Ni-NTA HisPrime Strips and Plates provide Ni-NTA in a solid-phase, multiwell format for 6xHis-tag assay of proteins or other molecules. Tagged molecules are not denatured and are bound to the strips and plates in a uniform orientation. They are conformationally active while remaining available to detection reagents (antibodies or any interacting molecule). Ni-NTA HisPrime Strips and Plates exhibit increased sensitivity compared with polystyrene plates, where molecules are randomly adsorbed in non-uniform orientations and can be denatured by immobilization. The strips and plates can be used to assay proteins in crude cell lysates and dilute solutions as 6xHis-tagged proteins are selectively bound to the Ni-NTA–coated surface. Ni-NTA HisPrime Plates are available in either a transparent format for colorimetric assays, or an opaque, white format for luminescence- and fluorescence-based assays. Ni-NTA HisPrime Strips and Plates can be used for the following:

- Quantifying 6xHis-tagged molecules
- Establishing ELISA or radioimmunoassay (RIA) procedures or diagnostic assays
- Studying molecular interactions
- Screening expression clones, engineered enzymes, pharmaceuticals, antibodies, or serum samples

Ni-NTA Magnetic Agarose Beads

Ni-NTA Magnetic Agarose Beads are designed for a wide range of magnetocapture assays using 6xHis-tagged proteins, as well as rapid, high-throughput protein purification. Visit our homepage at www.5PRIME.com or your local 5PRIME distributor for further details.

Protocols for detection of 6xHis-tagged proteins

PerfectPro Detection Systems allow detection of recombinant proteins fused to a 6xHis tag; for example, after immobilization to a membrane. Proteins can be immobilized directly from the following:

- Bacterial colonies (colony blotting: Protocol 5, page 29)
- Crude cell lysates or solutions (dot blotting: Protocol 4, page 28)
- After separation of proteins by SDS-PAGE (western blotting: Protocols 1–3, pages 22–27)

In combination with chromogenic substrates Anti·His HRP Conjugates can be used for simple, direct detection of 6xHis-tagged proteins. Secondary binding reagents, such as enzyme-conjugated antibodies, are unnecessary. As little as 2–5 ng protein can be quickly and easily detected in western-blot analysis. When a higher level of specificity and sensitivity is required, PerfectPro Anti·His Antibodies and Anti·His HRP Conjugates can be used: RGS·His, Penta·His, or Tetra·His Antibody recognizing the RGS(His)4, (His)5, and (His)4 epitopes, respectively.

For direct detection by chemiluminescent or chromogenic methods without a secondary antibody, Anti·His HRP Conjugates contain a HRP moiety. When analyzing samples containing complex mixtures of proteins (such as some eukaryotic expression systems, Anti·His Antibodies and Anti·His HRP Conjugates can be particularly useful.

Regardless of the surrounding amino-acid context, Penta·His and Tetra·His Antibodies bind to 6xHis tags — even when the tag is partially hidden. The antibodies exhibit differential sensitivity to some tagged proteins. Comparison of these antibodies should be performed using the Anti·His Antibody Selector Kit for new applications and proteins.

See Table 4 for an overview of the PerfectPro detection and assay systems.

Table 4. Overview of PerfectPro detection and assay systems

Product	Epitope detected	Substrates for blot detection	Protocols for detection	Substrates for assay procedures	Protocol for assays
Penta·His Antibody	HHHHH	Dependent on secondary antibody	6 or 7	Dependent on secondary antibody	14
Tetra·His Antibody	HHHH	Dependent on secondary antibody	6 or 7	Dependent on secondary antibody	14
RGS·His Antibody	RGSHHHH[1]	Dependent on secondary antibody	6 or 7	Dependent on secondary antibody	14
Penta·His HRP Conjugate	HHHHH	Colorimetric: 4C1N[2] Chemiluminescent ECL (GE Life Sciences)	6 or 7	o-Phenylene-diamine (OPD)[2]	15

[1] Recombinant proteins expressed from pPP- 9, pPP- 30, pPP- 31, pPP- 32, pPP- 40, pPP-100 DoubleTag®, pRSET (Life Technologies) and pBlueBacHis (Life Technologies) encode this epitope.

[2] Further substrates are listed on page 68.

Immobilizing proteins with blotting procedures

Overview

By transferring proteins to a membrane from a polyacrylamide gel after size separation with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), the size of the expressed 6xHis-tagged protein can be confirmed by comparison with molecular-weight markers. The presence of degradation products and formation of complexes can also be detected. 6xHis-tagged proteins can be identified in crude cell lysates, which is particularly useful when the protein migrates anomalously in SDS-PAGE.

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. Dilutions of a solution or crude cell lysate containing the 6xHis-tagged protein are pipetted directly onto a nitrocellulose membrane. Detection may be improved by denaturing proteins to expose the 6xHis tags. Dot blotting is preferred for functional testing, as problems with transferring the protein are avoided. Any components that interfere with binding or bind non-specifically, 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 4, page 28.

Colony blots

Colony blotting allows rapid, simultaneous screening of numerous colonies to identify expressing clones and even to distinguish semi-quantitatively between expression rates of individual colonies. 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 5, page 29.

Bacterial colonies are transferred to nitrocellulose membrane that is laid on the surface of an agar plate. After protein expression is induced, cells are lysed with alkali, exposing proteins which bind to the membrane.

Step	Task
Master plate (original transformants without IPTG)	Make replica with nitrocellulose membrane
Selection plate (with IPTG)	Transfer nitrocellulose membrane to a new plate and induce expression (4 h)
Positive signal detection	Nitrocellulose membrane subjected to alkaline lysis and western blot procedures
Pick positive clones	Use the membrane to identify and pick the positive clones from master plate

Separation of proteins by SDS-PAGE

PAGE is the standard electrophoretic technique used for the separation of proteins. Polymerization of monomeric acrylamide polymerize into polyacrylamide chains is initiated by the addition of ammonium persulfate and tetramethylenediamine (TEMED). A network of polyacrylamide chains that contain pores through which the proteins migrate is formed by polyacrylamide chains that are cross-linked by N,N'-methylenebisacrylamide. The acrylamide concentration and the proportion of bisacrylamide determine the pore size.

The denaturing, discontinuous system with a separating and a stacking gel described by Laemmli[4] is the most widely used system to separate proteins in a polyacrylamide gel. A reducing agent and SDS, which binds to the polypeptide chains giving a constant charge-to-mass ratio, are added to protein samples, which are then heated.

The gel is prepared with two sections. The stacking gel (upper portion of the gel) has large pores and is prepared with a slightly acidic buffer (pH 6.7). The separating gel (lower portion of the 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.

Protein samples are loaded into the stacking gel, and an electric field is applied at a constant current. Anions (chloride, SDS-coated proteins, and glycine) begin to migrate toward the anode. The mobility of glycine ions is retarded when they reach the sample buffer and stacking gel, as their net charge changes with the pH-shift. Proteins and chloride ions continue to move toward the anode.

The combined effects of the glycerin ions and the boundary of the stacking and separating gels cause the proteins to form a tight band. Upon entering the separating gel, movement of the proteins is slowed and the electric field becomes constant allowing separation of proteins based on size. The molecular weight of a protein can be determined by comparing its mobility with those of reference proteins.

The size of the proteins to be separated determines the percentage of acrylamide to use in the separating gel (see Table 5). In general, a 12%

polyacrylamide gel is used to separate 15–120 kDa proteins. Separation of large proteins is sufficient for routine work with this gel although it is out of the range where there is a strictly logarithmic relationship between molecular weight and migration distance (Table 5). Many alternative SDS-PAGE protocols are currently available[5-7].

Table 5. 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

After size separation, a buffer-tank-blotting apparatus or by semi-dry electroblotting is used to transfer proteins in the polyacrylamide gel to nitrocellulose membrane (as described in Protocol 2, page 25)

With the semi-dry electroblotting method, proteins are transferred from the gel onto the membrane with an electrical field. The polyacrylamide gel and membrane are sandwiched between two stacks of filter paper pre-wetted in electrotransfer buffer. An electrical field is applied: the gel at the cathode (negatively charged); the membrane is placed at the anode (positively charged).

The blotting cassette is submerged in a tank reservoir in the tank-blotting method.

Results with the tank-blotting method can be up to 10-fold better than the semi-dry method as it provides more efficient transfer, particularly of large proteins, and results in complete transfer of proteins. In addition, the buffer capacity is far greater than that with semi-dry transfer systems and, thus, tank blotting can be performed over extended periods.

The following guidelines improve performance of the tank-blotting method:

- Increase transfer times or use a lower percentage of acrylamide in the gel when transferring large proteins.
- Adjust the pore size of the membrane used when using small proteins
- A second membrane can be used to bind and detect proteins that pass through the first membrane
- Increase the percentage of methanol in the transfer buffer for hydrophobic proteins
- Check transfer efficiency by staining proteins on the membrane (Protocol 3, page 27).

For western blotting methods it is recommended to use a positive control for uniform transfer and detection of proteins of different sizes. Protein size markers including a His-tagged protein, such as the PerfectPro 6xHis-tagged Protein Ladder, enable direct detection on western blots using anti-his antibody.

Staining proteins after transfer

Proteins transferred to the membrane can be visualized by staining to estimate the efficiency of transfer. Strips of membrane can be cut out for use in different treatments, such as detection using different antibodies/detection reagents. Ponceau S is recommended over other protein-staining methods because the membrane can be easily destained before detection procedures (see Protocol 3, page 27).

Blocking membrane and non-specific binding

If the primary or secondary antibody, or Penta·His HRP Conjugate, bind directly to the membrane, high background will be the result. To prevent this, protein-free sites on the membrane must be blocked after transfer of proteins (including the 6xHis-tagged protein).

Blocking reagents minimize non-specific interactions of detection reagents with proteins bound to the membrane. Blocking reagents are included in all binding steps.

The following are commonly used blocking reagents:

- Non-fat dried milk
- Gelatin
- BSA (bovine serum albumin)
- Casein
- Serum
- Tween® 20

Most protocols use BSA for blocking, as it gives consistently good, reproducibly high signal-to-noise ratios and is the reagent of choice for all applications using chromogenic substrates. BSA increases background when using chemiluminescent substrates with Anti·His Antibodies because blocking may not be sufficient for such highly sensitive detection methods. In this case, it is recommended to use non-fat dried milk an additional blocking reagent, but only during incubation with secondary antibodies. If present before the last incubation step, milk can reduce the sensitivity of Anti·His Antibody binding. Alternatively, alkali-soluble casein can be used as blocking reagent throughout the chemiluminescent detection protocol, when available (Merck Food, order/reference number 102241).

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

Using secondary antibodies

Secondary anti-mouse IgG antibodies conjugated to either alkaline phosphatase (AP) or HRP together with appropriate enzyme substrates are used to visualize the position of the Anti·His Antibody bound to immobilized 6xHis-tagged protein.

Rabbit anti-mouse IgG–AP-conjugate (Pierce [Thermo Fisher Scientific, Inc], order/reference number 31332) or goat anti-mouse IgG–HRP-conjugate (Jackson Immunoresearch, order/reference number 115-035-003) have been used successfully. Use the highest recommended dilution to avoid non-specific signals. Detection of 6xHis-tagged proteins with Anti·His HRP Conjugates does not require the use of secondary antibodies. However, when used, the secondary antibodies must recognize mouse IgG1.

Using chromogenic or chemiluminescent substrates

Results are quickly and easily obtained using chromogenic substrates (see Table 6, page 21). An insoluble, colored product forms a precipitate on the membrane where the enzyme is located. To prevent overdevelopment and high background, the reaction must be monitored and stopped at the optimal time. The membrane must be kept stationary during color development. To make a permanent record of the results, the membrane is photographed or scanned.

Chemiluminescent substrates allow more sensitive detection than chromogenic substrates. Products of chemiluminescent substrates spontaneously emit light,

indicating the location of the enzyme. To make a permanent record of the results, the membrane is photographed.

Table 6 provides guidelines for choosing the detection substrate according to the conjugated enzyme and the desired sensitivity.

Table 6. PerfectPro detection system specifications

Product	Penta·His Antibody	RGS·His Antibody	Tetra·His Antibody	Penta·His HRP Conjugates
Epitope	HHHHH	RGSHHHH*	HHHH	HHHHH §
Dissociation constant, Kd (M)	5×10^{-9} to 1×10^{-8}	1×10^{-8} to 5×10^{-8}	1×10^{-8} to 5×10^{-8}	5×10^{-9} to 1×10^{-8}
Sensitivity in dot blots (2 mm dots)†	10 pg	10 pg	10 pg	10 pg
Sensitivity in dot blots (2 mm dots)‡	0.5 ng	0.5 ng	0.5 ng	n.d.
Sensitivity in western blots†	50 pg	50 pg	50 pg	50 pg
Sensitivity in western blots†	2 ng	2 ng	2 ng	n.d.
Direct detection on blots or in ELISA	Secondary antibody required	Secondary antibody required	Secondary antibody required	Yes
Conjugated enzyme	None	None	None	HRP

n.a.: not applicable; n.d.: not determined; HRP: horseradish peroxidase.

* Recombinant proteins expressed from pPP- 9, pPP- 30, pPP- 31, pPP- 32, pPP- 40, pPP- 100 DoubleTag, pRSET (Life Technologies) and pBlueBacHis (Life Technologies) encode this epitope.

† Detection using chemiluminescent substrate.

‡ Detection using AP 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

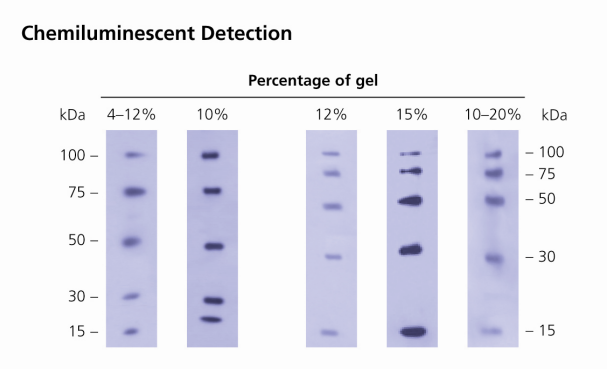
A cell lysate or extract from material that is similar to the test sample but lacking the 6xHis-tagged protein can be used as a negative control. A cell lysate of an *E. coli* strain which harbors only the vector (without the 6xHis-tagged protein-encoding insert), for example, could be used.

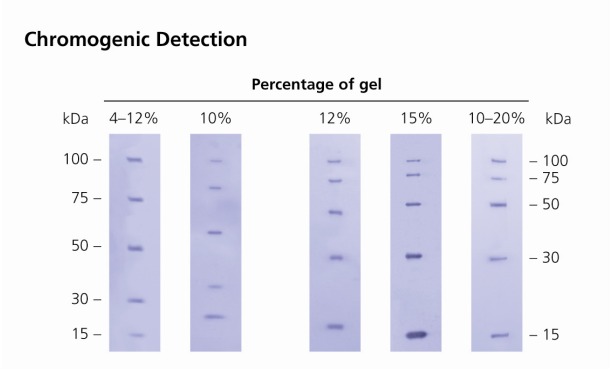
A purified 6xHis-tagged protein may be added to a negative control sample to generate a positive control. Alternatively, a sample known to contain a 6xHis-tagged protein may be used.

A suitable positive control protein can be prepared from 6xHis-tagged DHFR expressed from PerfectPro 40 (supplied in PerfectPro Kits). The protein is recognized by any of the PerfectPro Anti·His HRP Conjugates or Anti·His Antibodies as it contains has the RGS·His Antibody epitope (RGS(His)4) in addition to the N-terminal 6xHis tag.

PerfectPro 6xHis-tagged Protein Ladder as control for blotting applications

The PerfectPro 6xHis-tagged Protein Ladder (2500300) is a lyophilized mixture of five highly purified recombinant proteins ranging in size from 15 to 100 kDa containing a N-terminal RGS $(His)_6$ sequence. Each protein can be detected by any of the PerfectPro detection reactions, including the RGS His Antibody. It yields five clear bands of similar intensity on western blots, thus can be used for precise molecular weight determination of proteins on western blot membranes. PerfectPro 6xHis-tagged Protein Ladder can be directly dissolved in SDS-PAGE sample buffer, heated, and loaded onto a gel (see the *PerfectPro 6xHis-tagged Protein Ladder Manual*). SDS-PAGE and blotting are performed in the usual way (see Protocol 1, page 23 and Protocol 2, page 25). Bands appear directly on blot or film to provide a permanent record, with no extra work.





Protocol 1: Separation of proteins by SDS-PAGE materials

Additional materials required

- Gel apparatus and electrophoresis equipment
- 30% acrylamide/0.8% bis-acrylamide stock solution[1]. This can be purchased as a ready-to-use solution from several companies, e.g., Rotiphorese® Gel 30 (Carl Roth, order/reference number 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
- Optional: PerfectPro 6xHis-tagged Protein Ladder (5 PRIME, 2500300)

Note: use only high-quality reagents and water for SDS-PAGE. For buffer and reagent compositions, see page 64.

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, 1 mm thick, minigel.

[1] 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.

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 5, page 18).
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 (optionally use one lane for 2.5–5 µl PerfectPro 6xHis-tagged Protein Ladder), and run the gel. For electrophoresis conditions, refer to the recommendations provided by the manufacturer of the apparatus.

Protocol 2: Western transfer

Additional materials required

- Transfer apparatus
- Filter paper
- Nitrocellulose membrane (e.g., Schleicher and Schuell BA85)
- SDS polyacrylamide gel containing separated proteins (from Protocol 1)
- Transfer buffer (semi-dry or tank-blotting)

For buffer and reagent compositions, see page 64.

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 using 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).
5. Air bubbles may cause localized non-transfer of proteins. They can be removed by gently rolling a Pasteur pipet over each layer in the sandwich.
6. 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).
7. 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. Air bubbles may cause localized non-transfer of proteins. They can be removed by gently rolling a Pasteur pipet 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 3, page 27).

   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.

Protocol 3: Staining proteins after western transfer

Additional materials required

- Staining solution: 0.5% Ponceau S, 1% acetic acid
- Western blot (from Protocol 2)

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.
3. Mark membrane or cut as desired.

   The orientation of the membrane on the gel should be marked.
4. Precede with Protocol 6 or 7.

   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 (steps 1–4, Protocol 6 or 7), 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 4: Preparation of dot blots

Additional materials required

- Nitrocellulose membrane (e.g., Schleicher and Schuell BA85)
- Protein samples
- Dilution buffer for native or denaturing conditions

For buffer and reagent compositions, see page 64.

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.

   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 non-specific 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 6 or 7.

Protocol 5: Preparation of colony blots

Chemiluminescent substrates are not recommended for use with colony blots.

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 PerfectPro plasmids lacking an insert. Small peptides (<30 amino acids) expressed from PerfectPro vectors pPP-9, -30, -31, -32, -60 and 70 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.

Additional materials required

- LB plates with appropriate antibiotics
- LB plates with appropriate antibiotics and 250 µM IPTG
- Nitrocellulose membrane discs
  (e.g., Millipore, order/reference number HATF 08250)
- Blunt-ended forceps
- Syringe needle
- Polystyrene dishes
- SDS solution
- Denaturing solution
- Neutralization solution
- 20x SSC

For buffer and reagent compositions, see page 64.

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 the filter on the sides using 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 3 mm paper soaked with one of the following solutions:
   - SDS solution
   - Denaturing solution
   - Neutralization solution
   - Neutralization solution
   - 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:
   - SDS solution 10 min
   - Denaturing solution 5 min
   - Neutralization solution 5 min
   - Neutralization solution 5 min
   - 2x SSC 15 min

9. Continue with the protocols for immunodetection using an Anti·His Antibody with a chromogenic substrate (Protocol 7).

   Due to 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 minutes 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:
   - A plate of host bacteria without the expression plasmid
   - A plate of host bacteria harboring the expression plasmid without the insert
   - A colony-blot treated only with secondary antibody prior to detection
   - A positive control expressing 6xHis-tagged dihydrofolate reductase (DHFR) from pPP-40, if possible.

Protocol 6: Immunodetection with Anti·His Antibodies or Penta·His HRP Conjugates (chemiluminescent method)

Additional materials required

- Western Blot (Protocols 1–3) or dot blot (Protocol 4)
- 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. This is not required if using the Penta·His HRP Conjugate; either AP- or HRP conjugated anti-mouse IgG may be used. Rabbit-anti-mouse IgG/AP-conjugate (Pierce, order/reference number 31332) or goat-anti-mouse IgG/HRP-conjugate (Jackson Immunoresearch, order/reference number 115-035-003) yield good results.
- Secondary antibody dilution buffer. This is not required if using Anti·His HRP Conjugate.

The solutions required depend on the detection method employed.

If using Penta·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 64. 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 non-specific 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 Food, order/reference number 102241) 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 7.

Table 7. 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[1]
Anti·His Antibody binding	3% BSA in TBS	1% Casein in TBS	–
Anti·His HRP conjugate binding	–	–	Blocking Reagent in Blocking Reagent Buffer[1]
Secondary antibody binding	10% milk powder in TBS	1% Casein in TBS	–

[1] For compositions and preparation of buffers and reagents, see page 64.

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 GE Healthcare 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 PerfectPro 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[1], 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.

[1] If alkali-soluble casein (Merck Food, order/reference number 102241) 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 64.

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[1], 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.
8. Incubate with secondary antibody solution for 1 h at room temperature.

   Either AP- or HRP-conjugated anti-mouse IgG may be used. Rabbit-anti-mouse IgG/AP-conjugate (Pierce, order/reference number 31332) or goat-anti-mouse IgG/HRP-conjugate (Jackson Immunoresearch, order/reference number 115-035-003) yield good results. Dilute according to the manufacturer's recommendations. Use the lowest recommended concentration to avoid false signals.

   10% non-fat dried milk in TBS[2] is used for incubation with secondary antibody when using chemiluminescent detection.

   Milk powder is required to reduce background since 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.

[1] If alkali -soluble casein (Merck Food, order/reference number 102241) is available in your country a 1% (w/v) solution in TBS buffer may be used for this protocol step.

[2] For compositions and preparation of buffers and reagents, see page 64.

Protocol 7: Immunodetection with Anti·His Antibodies or Penta·His HRP Conjugates (chromogenic method)

Additional materials required

- Western Blot (Protocol 1–3), dot blot (Protocol 4) or colony blot (protocol 5)
- TBS Buffer
- TBS-Tween/Triton Buffer
- Anti·His Antibody or Penta·His HRP Conjugate stock solution
- Anti-mouse secondary antibody AP or HRP
- (HRP) conjugate
- Blocking buffer
- Secondary antibody dilution buffer for chemiluminescent detection
- Staining solutions for AP or HRP

For compositions and preparation of buffers and reagents, see page 64.

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 64. 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.

The reagents used are described in Table 8.

Table 8. Reagents used in chromogenic detection of 6xHis-tagged proteins

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

[1] For compositions and preparation of buffers and reagents, see page 64.

Procedure

1. Wash the membrane twice for 10 min 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 Penta·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 the membrane twice for 10 min each 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 Penta·His HRP Conjugate solution (1/1000–1/2000 dilution of antibody or conjugate stock solution in blocking buffer) at room temperature for 1 h. Membranes can be sealed in plastic bags.

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

   3% BSA (w/v) in TBS buffer, or complete Penta·His HRP Conjugate blocking buffer is used for blocking throughout the procedure when using chromogenic detection. If using Penta·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 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 AP- or HRP-conjugated anti-mouse IgG may be used. Rabbit-anti-mouse IgG/AP-conjugate (Pierce, order/reference number 31332) or goat-anti-mouse IgG/HRP-conjugate (Jackson Immunoresearch, order/ number 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 in TBS-Tween/Triton buffer at room temperature.
10. Stain with AP or HRP staining solution (when using Penta·His HRP Conjugates, be sure to use an HRP staining solution) until the signal is clearly visible (approx. 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.

Protocols for immunolocalization of 6xHis-tagged proteins

Introduction

Using Anti·His Antibodies for immunolocalization

The location of recombinant proteins expressed in particular cells, as well as the subcellular localization of the proteins, is determined using immunohistochemical methods. In mammalian cells, exclusive detection of recombinant 6xHis-tagged proteins is possible with the high specificity and affinity of PerfectPro Anti·His Antibodies. In addition, the location of cellular proteins that interact with 6xHis-tagged proteins can also be detected.

Localization of 6xHis-tagged protein by indirect immunofluorescent detection

Localization of a cellular 6xHis-tagged protein by indirect immunofluorescence generally begins with fixation and subsequent permeabilization of cells. This treatment allows penetration of antibodies. After non-specific interactions have been blocked, proteins are labeled with Anti·His Antibody and fluorophore-conjugated secondary antibody. The sample is mounted and subjected to microscopic analysis.

Fixation and permeabilization

To preserve cell morphology as well as antigen structure and accessibility, samples must be fixed with formaldehyde, methanol/acetone or other fixative appropriate for indirect immunofluorescent procedures.

Formaldehyde, which acts as a mild cross-linker, is preferred when examining the localization of proteins in cultured cells. Cell membranes must be subsequently permeabilized to allow access to intracellular components by solubilizing the phospholipid membranes with the non-ionic detergent Triton X-100.

Cold methanol and acetone act quickly to fix and permeabilize cells at the same time. As these reagents precipitate proteins and carbohydrates, they might alter the localization pattern of the recombinant 6xHis-tagged protein. In addition the integrity of membranes and organelles is destroyed during localization.

Glutaraldehyde is a strong fixative. However, its use is not recommended, as it can alter epitopes and prevent binding of the antibody probe.

Blocking non-specific binding

Blocking is required after fixation and permeabilization to prevent high background from non-specific binding of primary and secondary antibody. BSA results in reproducible low signal intensities of non-transfected cells compared to transfected cells. Alternatively, normal serum obtained from the species from which the secondary antibody was produced can be used.

Using primary and secondary antibodies

The localization of Anti·His Antibody bound to immobilized 6xHis-tagged protein is visualized using secondary anti-mouse IgG antibodies conjugated to a fluorophore. Popular fluorophores, such as fluorescein isothiocyanate (FITC), rhodamine, and Texas Red®, are available conjugated to antibodies. FITC-conjugated goat anti-mouse IgG (H+L) antibody performs well (Jackson Immunoresearch Laboratories, order/reference number 115-095-062). To obtain the best signal-to-noise ratio, the antibody should be diluted according to the supplier's instructions.

Mounting

Slides containing samples are overlaid with a cover slip after labeling is complete. Most mounting media available is based on buffered glycerol. Mounting Medium (Sigma, order/reference number M1289) and Kaiser's glycerol gelatine from (Merck Food, order/reference number 109242) have been used successfully.

Protocol 8: 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 6 and 8 (see References, page 85).

Additional materials required

- Chamber slides (Lab-Tek™ TC Chamber Slides, Thermo Fisher Scientific, Inc.)
- PBS-IF
- 2% paraformaldehyde in PBS-IL
- 0.25% Triton X-100 in PBS-IL
- Anti·His Antibody stock solution
- Blocking buffer IL
- Antibody dilution buffer IL
- Anti-mouse secondary antibody conjugate (e.g., FITC-conjugated goat anti-mouse IgG (H+L) antibody (Jackson Immunoresearch Laboratories, order/reference number 115-095-062)
- Mounting medium

For buffer and reagent compositions, see page 64.

13. Grow transfected cells in chamber slides under standard growth conditions.
14. Wash cells with PBS-IL for 30 s at room temperature.

    Do not shake samples during this step or any subsequent steps.
15. Fix the cells in 2% paraformaldehyde in PBS-IL for 10 min at room temperature.
16. Remove the fixation mixture and wash 3 times with PBS-IL 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.
17. Permeabilize the cells with 0.25% Triton X-100 in PBS-IL 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 pre-cooled methanol at –20°C for 20 min. This quicker method also has some disadvantages, see page 37).

18. Wash 3 times with PBS-IL for 30 s at room temperature.
19. Incubate for 1 h in 5% BSA in PBS-IL at room temperature.

    Note: all incubation steps should be performed in a sealed, humid container to prevent the slides from drying out.
20. Wash 3 times for 10 min each time in PBS-IL at room temperature.
21. 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.
22. Wash 3 times for 10 min each time in PBS-IF at room temperature.
23. 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 (Jackson Immunoresearch, order/reference number 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.
24. Wash 4 times for 10 min each time in PBS-IL at room temperature.
25. Mount the cells with mounting medium.

    For short-term preservation of specimens we have obtained good results with Mounting Medium (Sigma, order/reference number M1289) and also with Kaiser's glycerol gelatine (Merck Food, order/reference number 109242). Follow the supplier's recommendations.

Immunoprecipitation of 6xHis-tagged proteins

Introduction

Immunoprecipitation with Anti·His Antibodies

Recombinant 6xHis-tagged proteins can be selectively precipitated using PerfectPro Anti·His Antibodies.

Immunoprecipitation of 6xHis-tagged proteins can be used for the following:

- In vitro translation products
- Radiolabeled proteins
- Studying protein–protein interactions
- Small-scale protein purification

For more details and background information about immunoprecipitation please refer to Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

In immunoprecipitation, protein A or protein G binds to the second and third constant regions of antibody heavy chains. When bound to a solid-phase matrix such as cross-linked agarose beads, these proteins can be used to capture antibody–antigen complexes from solution. The procedure involves the following:

- Preparation of the sample in suitable buffer
- Preadsorption with the solid-phase matrix
- Binding Anti·His Antibody to 6xHis-tagged protein
- Binding antibody–6xHis-tagged-protein complexes to protein A or protein G on the solid-phase matrix
- Washing
- Preparation of samples for SDS-PAGE

Preparing samples and preadsorption

Cell lysis should not be carried out using lysozyme when using Penta·His Antibody or Tetra·His Antibody for immunoprecipitation as the antibodies weakly cross-react. Contamination from high concentrations of lysozyme in the cleared lysate may reduce in immunoprecipitation efficiency. Preadsorption of the samples followed by centrifugation removes proteins which bind non-specifically to the solid-phase matrix.

Binding and washing

Before complexes are immobilized to the solid-phase matrix, Anti·His Antibody is bound to the 6xHis-tagged protein. Performing the steps in this order minimizes steric hindrance while maximizing the accessibility of the 6xHis-tagged. Wash steps remove proteins that do not have a 6xHis tag (or, in protein–protein interaction studies, that are not bound to the 6xHis-tagged protein).

Preparing for SDS-PAGE

After washing, SDS-PAGE sample buffer is added and samples are heated to release the heavy and light chains of the Anti·His Antibody as well as the immobilized, immunoprecipitated 6xHis-tagged protein.

As they are covalently bound to the cross-linked agarose beads, protein A or protein G is removed together with the beads before SDS-PAGE by centrifugation.

Protocol 9: Immunoprecipitation

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., Sigma, order/reference number P3391), protein G agarose beads (e.g., Sigma, order/reference number P7700), or inactivated *Staphylococcus* cells (e.g., Sigma, order/reference number P7155). If *Staphylococcus* cells are used, background signals may be higher, see below.
- PerfectPro 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 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.

Additional materials required

- 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 page 64.

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 the 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. Pre-incubate 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.

   Non-tagged proteins present in the sample that bind to protein A or protein G are removed after this pre-incubation and centrifugation in step 3. This prevents subsequent co-precipitation of non-tagged 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 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, and incubate 5 min at 95–100°C. Centrifuge, and then 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).

Protocols for detection and assay using the PerfectPro Tag·100

Introduction

Antibody

The pPP-100 DoubleTag vector encodes an N-terminal 6xHis tag and a second epitope, the Tag·100, at the C-terminus (see the *PerfectPro Ni-NTA System Manual* for further details). The proteins expressed by the vector are recognized by Tag 100 Antibody mouse monoclonal antibody from QIAGEN (cat. no. 34680)

The Tag·100 Antibody can be used for the following:

- Immunodetection on dot blots
- Immunodetection on western blots
- ELISA

The antibody is detected by secondary anti-mouse IgG antibodies conjugated to either AP or HRP together with appropriate enzyme substrates (see page 20). Consistently good results have been obtained with rabbit anti-mouse IgG-AP–conjugate (Pierce, (order/reference number 31332) or goat anti-mouse IgG-HRP-conjugate (Jackson Immunoresearch, order/reference number 115-035). The highest recommended dilution should be used for the secondary antibodies that recognize mouse IgG1 to avoid non-specific signals.

Western-blot and dot-blot protocols

BSA does not sufficiently block non-specific-binding of the primary and secondary antibody to the membrane for chemiluminescent detection. Instead, use 10% milk powder to dilute the Tag·100 Antibody and secondary antibody. Alternatively, alkali-soluble casein can be used as blocking reagent at a concentration of 1% throughout the chemiluminescent detection protocol for a slight increase in sensitivity (Merck Food, order/reference number 1.02241). 3% BSA is sufficient for blocking for the chromogenic method.

Tag·100 Antibody should be used at concentrations of 0.1–0.04 µg/ml (a 1/2000–1/5000 dilution of the antibody stock solution). Use a 1/5000 dilution of the antibody stock solution for chemiluminescent detection (see Protocol 6 on page 32.) See Protocol 7 on page 35 for chromogenic detection.

ELISA protocols

In ELISA, the 6xHis tag is used to immobilize the protein and the Tag·100 epitope is used for detection. Ni-NTA HisPrime Strips and Plates can be used for immobilization (see Protocol 10, page 48). The Tag·100 Antibody should be used at concentrations of 0.1–0.04 µg/ml (a 1/2000–1/5000 dilution of the antibody stock solution).

Assaying 6xHis-tagged proteins

Capture assays with Ni-NTA HisPrime Strips and Plates

The metal-chelating properties of NTA are used in Ni-NTA HisPrime Strips and Plates to bind nickel ions. 6xHis-tagged proteins or other molecules, such as peptides or nucleic acids, bind with high specificity, even when present in cleared lysates or other complex mixture. The inner surfaces of the 8-well strips and 96-well microplates are coated with a spacer bearing a Ni-NTA group. 6xHis-tagged proteins are bound in a directed manner, allowing fully functional proteins to be immobilized.

Ni-NTA HisPrime Strips and Plates are ready to use as NTA groups are pre-charged with nickel ions and the polystyrene surface is pre-blocked with BSA to prevent non-specific binding. Ni-NTA HisPrime plates are available in either transparent format for colorimetric assays, or opaque, white format for luminescence- and fluorescence-based assays.

The following applications can be performed using Ni-NTA HisPrime Strips or Plates:

- Assay activity of bound proteins
- Assay of binding, interacting molecules
- ELISA or RIA
- Quantitation of 6xHis-tagged proteins
- Antibody screening
- Diagnostic assays
- Expression screening
- Screening of engineered enzymes
- Drug screening

Assaying 6xHis-tagged proteins in crude cell lysates is greatly facilitated by Ni-NTA HisPrime Strips and Plates.

Immobilization takes place at physiological pH, allowing conformationally active immobilized proteins with exposed binding domains to be bound. Assays using Ni-NTA HisPrime Strips or Plates are more sensitive and reproducible than protocols cause denaturation of the proteins.

Many well-established protocols can be modified for use with Ni-NTA HisPrime Strips and Plates. Assay components that might interfere with the interaction of the 6xHis tag with Ni-NTA should be avoided, especially EDTA which is used in many standard ELISA protocols.

Capture assays with Anti·His Antibody-coated plates

Anti·His Antibodies can be coated onto 96-well microplates, which can then be used in a manner similar to Ni-NTA HisPrime Strips and Plates.

ELISA can be performed on the coated plates with suitable antibodies. However, anti-mouse secondary antibodies will bind to the Anti·His Antibodies coated on the plate and mouse primary antibodies can not be used. To use mouse primary antibodies, use Ni-NTA HisPrime Strips or Plates or couple the primary antibody directly to AP or HRP.

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

6xHis-tagged protein can be detected directly using one of the Anti·His HRP Conjugates. Such assays can be used for quantitation of 6xHis-tagged proteins that have already been purified (usually by Ni-NTA chromatography) but should not be used with cell lysates or other complex mixtures of proteins.

Immobilization is not specific and all proteins in the sample bind to the plate. As the 6xHis-tagged protein must compete for binding, assay sensitivity is greatly reduced. Even when using purified proteins, signal is low and less reproducible than with Ni-NTA HisPrime Strips and Plates. In addition, 6xHis-tagged proteins might loose activity as they bind in random orientation, further reducing assay efficiency.

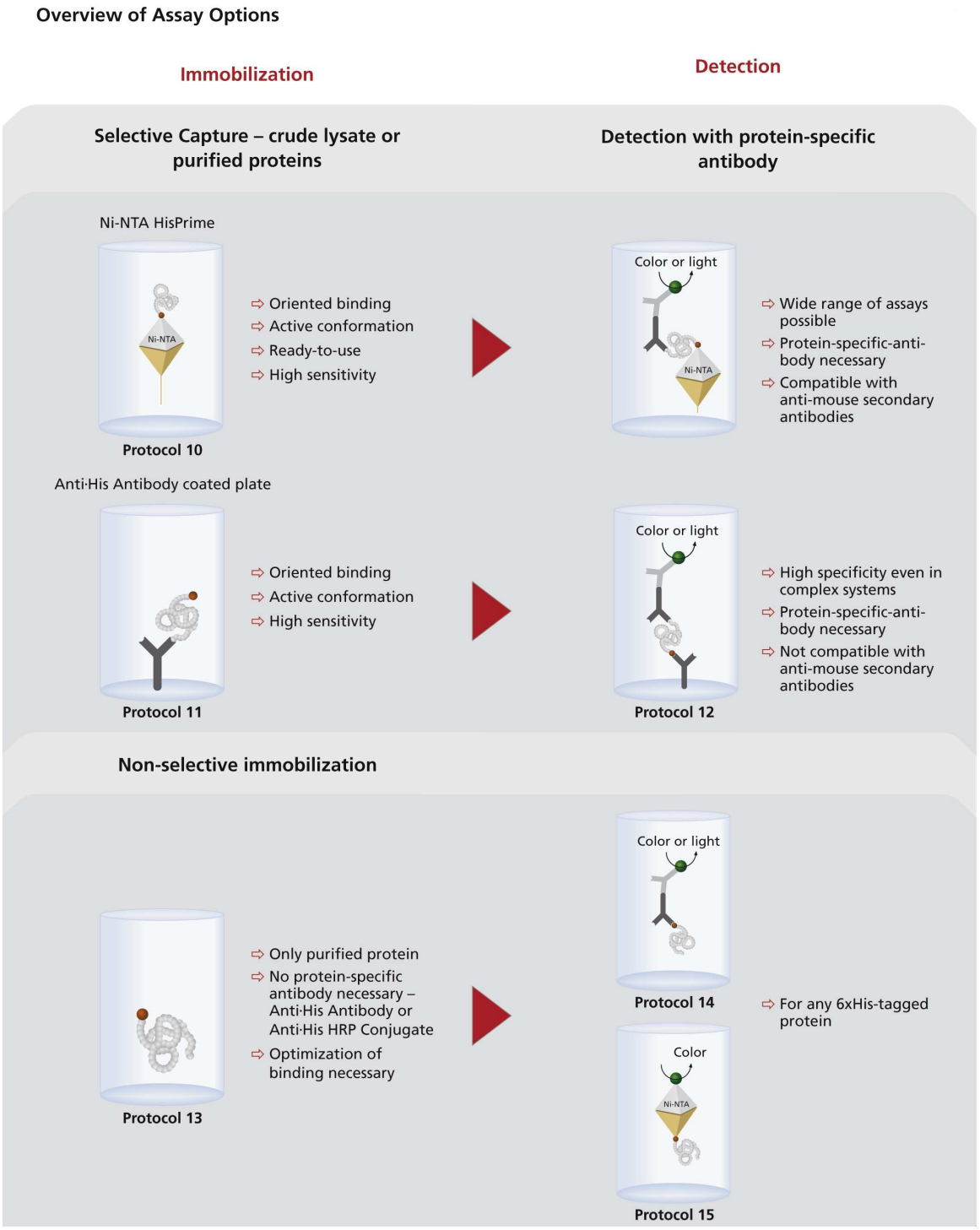
Bound 6xHis-tagged proteins (Protocol 13, page 58) are assayed with one of the following:

- Anti·His Antibodies (Protocol 14, page 59)
- Ni-NTA or Anti·His HRP Conjugates (Protocol 15, page 61).

The second method may be necessary when no additional antibody is available for your 6xHis-tagged protein.

The protocols are provided as guidelines. Each assay must be designed and optimized according to the characteristics of the proteins in the procedure.

The following chart provides an overview on the different assay options.



Protocol 10: ELISA with Ni-NTA HisPrime Strips or Plates

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 Magnet 96-Well or Magnet 12-Tube (for more details, visit www.5PRIME.com or contact your local distributor).
- 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 HisPrime Strips and Plates are pre-blocked 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 (e.g., NH^+), or ionic detergents (e.g., SDS) interfere with binding.

Additional materials required

- Ni-NTA HisPrime Strips or Plates
- PBS
- PBS/BSA
- PBS-Tween
- 1 M Tris·Cl, pH 8.0
- Phosphate-citrate buffer, pH 5.0
- Substrate for the AP or HRP or one of the alternative substrates for HRP (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 page 64.

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.

   Proteins 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 HisPrime 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 HisPrime Strips and Plates are pre-blocked 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 HRP or AP 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 11: 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 HisPrime Strips and Plates then this protocol can be followed.
A sandwich ELISA can be performed by following Protocol 14.

Before starting

- This procedure is used to immobilize PerfectPro Anti·His Antibodies onto the inner surfaces of suitable 96-well microplates (for example MaxiSorp™ plates, Thermo Fisher Scientific, Inc., order/reference number 442404 or strips order/reference number 468667).
- For this procedure, RGS·His Antibody (100 µg) with order/reference number 2400330 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 AP or HRP). 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.

Additional materials required

- 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, order/reference number 2400330, which is lyophilized with BSA)
- Microplate blocking buffer
- PBS
- Microplate shaker platform

For buffer and reagent compositions, see page 64.

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 12: 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

- 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 AP or HRP. Otherwise, Ni-NTA HisPrime 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.

Additional materials required

- PBS PBS/BSA
- Anti·His-Antibody–coated strips or plates (from Protocol 13)
- Substrate for AP or HRP or one of the alternative substrates for HRP
- 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 page 64.

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 HRP or AP 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 13: 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 PerfectPro Anti·His Antibodies followed by a suitable anti-mouse enzyme-conjugated secondary antibody and substrate (Protocol 14), or directly and conveniently with Anti·His HRP Conjugates (Protocol 15).

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.

Additional materials required

- 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 page 64.

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 14) or Anti·His HRP Conjugates (Protocol 15).

Protocol 14: Assay of 6xHis-tagged proteins with Anti·His Antibody

Anti·His Antibodies can be used to assay 6xHis-tagged proteins 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.

Additional materials required

- 96-well microplates coated with 6xHis-tagged protein (from Protocol 13)
- PBS/BSA
- PBS
- Anti·His Antibody
- Secondary-antibody conjugate: either AP- or HRP-conjugated anti-mouse IgG (from rabbit or goat) may be used. Rabbit-anti-mouse IgG–AP conjugate from (Pierce, order/reference number 31332) or goat-anti-mouse IgG–HRP conjugate (Jackson Immunoresearch, order/reference number 115-035-003) yield good results.
- Substrate for AP or HRP or one of the alternative substrates for HRP
- 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 page 64.

Procedure

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

   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 AP- or HRP-conjugated anti-mouse IgG may be used. Rabbit-anti-mouse IgG–AP conjugate (Pierce, order/reference number 31332) or goat-anti-mouse IgG–HRP conjugate (Jackson Immunoresearch, order/reference number 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 15: Assay of 6xHis-tagged proteins with Penta·His HRP Conjugate

Penta·His HRP Conjugates can be used to assay conveniently and directly 6xHis-tagged proteins that are immobilized on a polystyrene plate (Protocol 13, page 58). The sensitivity of assays performed in this way will largely depend on the particular 6xHis-tagged protein to be assayed.

Additional materials required

- 96-well microplates coated with 6xHis-tagged protein (from Protocol 13, page 58)
- PBS/BSA
- PBS
- Penta·His HRP Conjugate stock solution
- Substrate for AP or HRP.
- Stopping reagent (optional)

Note: when using Penta·His HRP Conjugates please ensure that you use a HRP substrate for detection.

For buffer and reagent compositions, see page 64.

Procedure

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

   For Penta·His HRP Conjugate, a 1/2000 dilution is recommended. 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 9, page 66 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.

Supporting information

Antibodies

The following sections provide a very basic introduction to antibodies. For more detailed information, refer to Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Antibody classification

Antibodies are classed into isotypes IgA, IgD, IgE, IgG, and IgM according to their heavy chains.

IgG antibodies are the most commonly used in immunochemical procedures and are the most abundant in serum. The immunoglobulin molecule with two antigen binding sites consist of two identical heavy chains (~55 kDa) and two identical light chains (~25 kDa) that form a Y-shape (Figure 2). The light chain consists of one constant region and one variable region. The heavy chain consists of three constant regions and one variable region.

Differences in the heavy-chain constant regions are used to further classify mouse IgG antibodies into isotypes: IgG1, IgG2a, IgG2b, and IgG3.

PerfectPro Anti·His Antibodies are IgG1.

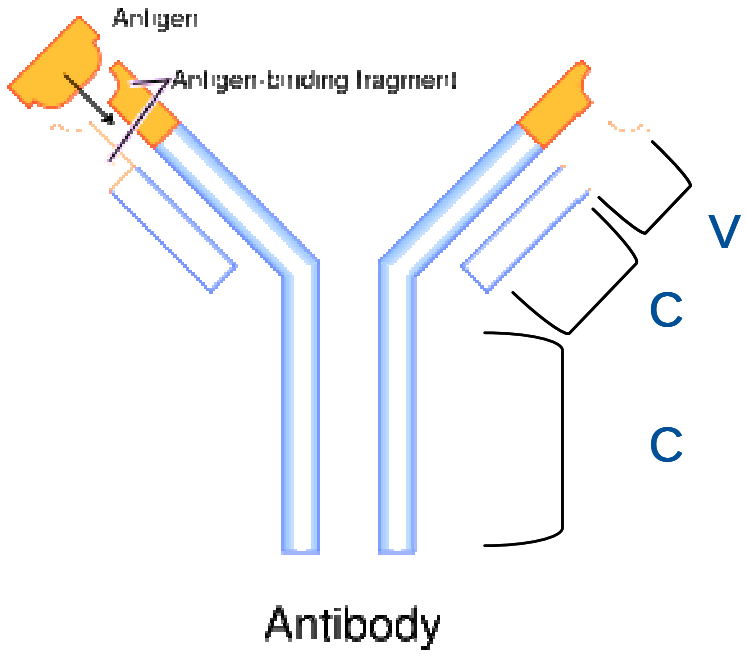


Figure 2. Schematic diagram of an IgG antibody. C: constant region; V: variable region.

Monoclonal antibodies

Antibody-secreting B cells can be fused with myeloma cells to produce hybridoma cell lines that can be cultured indefinitely in vitro and produce only one specific monoclonal antibody[9]. After injection into mice to produce ascites fluid, hybridoma cells are used to produce antibodies on a large scale in vivo. In vitro they are used for production of pure antibodies.

The purity and activity of PerfectPro antibodies are guaranteed by in vitro production. To ensure preparations free of viruses, mycoplasma, and contaminating immunoglobulins, Anti·His Antibodies are isolated from serum-free hybridoma cell cultures.

Conjugated antibodies

Detection and assay procedures often make use of species-specific antibodies conjugated to either AP or HRP. The correct secondary antibody must be used as immunoglobulin molecules vary between species.

Anti-mouse conjugates should be used for PerfectPro Anti·His Antibodies, and the conjugates must recognize the subclass IgG1.

Molecules that can bind to the different epitopes on each primary antibody (Anti·His Antibody) are conjugated to amplify signal and increase sensitivity in detection and assay procedures. Enzymes conjugated to protein A or protein G are unsuitable for detection on blots with the IgG Anti·His Antibodies. However, the Anti·His Antibodies can be used with both protein A and protein G for immunoprecipitation (see Protocol 9).

Solutions

Solutions for SDS-PAGE

30% acrylamide/0.8% bis-acrylamide	30% acrylamide stock solution: 0.8% bis-acrylamide (N,N'-methylene-bis-acrylamide) (e.g., Carl Roth, order/reference number 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

Solutions for western transfer

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

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, order/ reference number 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, order/reference number X-100)
Blocking buffer	3% (w/v) BSA (Sigma, order/reference number A7906), in TBS buffer
Blocking buffer (for alternative method)	1% (w/v) alkali-soluble casein (Merck, order/reference number 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*. 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.
Secondary antibody dilution buffer for chemiluminescent detection	10% (w/v) skim milk powder (Fluka, order/reference number 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).

Solutions for AP staining

Buffer AP	100 mM Tris·Cl, pH 9.5, 100 mM NaCl, 5mM $MgCl_2$
NBT stock solution	5% NBT (nitro blue tetrazolium, Sigma, order/reference number N5514) in 70% dimethylformamide. (Store in aliquots at –20°C)
BCIP stock solution	5% BCIP (5-bromo-4-chloro-3-indolyl phosphate, Sigma, order/reference number 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 AP (final concentration: 0.33 mg/ml NBT; 0.165 mg/ml BCIP). Alternatively, BCIP/NBT tablets can be obtained from Sigma (order/reference number B5655).

Solutions for 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, order/reference number 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-IL (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-IL (10 mM sodium phosphate, pH 7.4; 140 mM NaCl)	Dilute 10x PBS-IL 1/10 with distilled water.
2% paraformaldehyde in 1x PBS-IL	Heat 100 ml 1x PBS-IL to 60°C, add 2 g solid paraformaldehyde (Sigma, order/reference number 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-IL	0.25% Triton X-100 in 1x PBS-IL
Blocking Buffer IL	5% (w/v) BSA (Serva, order/reference number 11930) in 1x PBS-IF
Anti·His Antibody stock solution	0.2 mg/ml Anti·His Antibody (see page 7)
Antibody Dilution Buffer I	1% (w/v) BSA (Serva, order/reference number 11930) in 1x PBS-IL

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.

Details of substrates for different procedures can be found in Table 9, below and Table 10, page 69).

Substrate for AP

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

Substrate for HRP

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 HRP

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 9. 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 10. Substrates for ELISA, immunoblotting, and immunohistology

Substrate system	Abbrev.	Reaction product	Application
Horseradish peroxidase (HRP)			
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
ECL (GE Healthcare)	—	Chemiluminescence	Immunoblotting
Alkaline phosphatase (AP)			
p-Nitrophenylphosphate	pNPP	Yellow, soluble	ELISA
5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue	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

Troubleshooting guide

The following troubleshooting recommendations are designed to address unexpected or undesired results. To ensure optimal use, follow the guidelines and recommendations in the manual.

Detection

Observation	Weak or no signal; positive control visible
Possible cause	Recombinant protein of interest does not have a 6xHis tag
Avoiding	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.
Possible cause	Insufficient recombinant protein present
Avoiding	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.

Possible cause	Suboptimal recognition of 6xHis-tagged protein of interest by Penta·His HRP Conjugate or Anti·His Antibody
Avoiding	Individual 6xHis-tagged proteins are often recognized better by Penta·His HRP Conjugates or 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 pPP-9, pPP- 30, pPP- 31, pPP- 32, pPP- 40, pPP- 100 DoubleTag and some versions of the pRSET and pBlueBacHis vectors (Life Technologies). A positive control for expression should be included whenever possible. PerfectPro Kits include PerfectPro 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.

Observation	Weak or no signal; positive control not visible
Possible cause	Exposure/development time suboptimal (chemiluminescent detection)
Avoiding	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.
Possible cause	Incomplete transfer (western blotting)
Avoiding	Ensure that appropriate transfer conditions have been used. Check for sufficient transfer by staining membrane with Ponceau S (Protocol 3). 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.
Possible cause	Inappropriate wash conditions
Avoiding	The interactions between 6xHis-tagged proteins and detection reagent (Anti·His Antibody or Anti·His HRP 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.

Possible cause	Inappropriate secondary antibodies
Avoiding	PerfectPro 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.
Possible cause	Enzyme conjugated to secondary antibody is inactive
Avoiding	Check activity of secondary antibody conjugate by incubation directly with the appropriate substrate.
Possible cause	Membrane shaken during color development
Avoiding	Do not shake membrane during color development (chromogenic method) because the color precipitate will disperse.
Possible cause	Blocking Buffer Reagent was not prepared correctly
Avoiding	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 starting on page 64.

Observation	**Bands on western blot are diffuse**
Possible cause	Membrane was not held firmly on gel during western transfer
Avoiding	Check transfer setup. If too loose, use additional layers of firmly on gel during filter paper.

Observation	Bands on western blot have small spots with no signal
Possible cause	Presence of bubbles between the gel and membrane during transfer
Avoiding	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.

Observation	Non-specific signals
Possible cause	System considerations
Avoiding	Expression of a 6xHis-tagged protein in mammalian cell lines, yeast, baculovirus systems, or *E. coli* should not result in non-specific secondary signals with Anti·His HRP Conjugates or Anti·His Antibodies.
Possible cause	Secondary antibody concentration
Avoiding	Secondary antibody–enzyme 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. 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.

Possible cause	Cross-reacting proteins of different size than the protein of interest
Avoiding	**Smaller proteins**: protein degradation results in cross-reacting proteins smaller than the protein of interest. This protein 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, cross-reacting 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.
Possible cause	Inappropriate wash conditions
Avoiding	The interactions between 6xHis-tagged proteins and detection reagent (Anti·His Antibody or Anti·His HRP 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 non-specific interactions.
Possible cause	Insufficient washing after antibody or conjugate incubation
Avoiding	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.
Possible cause	Incorrect blocking reagents and/or blocking time
Avoiding	Non-specific 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.

Observation	High background
Possible cause	Development too long
Avoiding	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.
Possible cause	Secondary antibody concentration
Avoiding	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.
Possible cause	Improper blocking reagents and/or blocking time
Avoiding	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.
Possible cause	Insufficient washing prior to detection with chemiluminescent substrates
Avoiding	Some chemiluminescent substrates require very thorough washing after incubation with the secondary antibody conjugate. Refer to the supplier's recommendations.

PerfectPro 6xHis-tagged Protein Ladder

Observation	PerfectPro 6xHis-tagged Protein Ladder shows too many bands
Possible cause	Proteins were not dissolved properly
Avoiding	It is important to allow proteins to dissolve for 30 min at room temperature.
Possible cause	Proteins were not heated sufficiently
Avoiding	Heat for 10 min at 98°C. See Protocol 2.
Possible cause	No reducing agent in sample buffer
Avoiding	Prepare sample buffer containing reducing agent.

Observation	PerfectPro 6xHis-tagged Protein Ladder shows too few bands
Possible cause	Largest bands missing
Avoiding	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.
Possible cause	Smallest band missing
Avoiding	If the smallest band is not detected, it may have run off the gel. Do not allow gel to run too far.

Immunolocalization

Observation	Weak or no signal
Possible cause	Recombinant protein does not have a 6xHis tag
Avoiding	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.
Possible cause	Insufficient recombinant protein present
Avoiding	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.
Possible cause	6xHis tag is partially hidden
Avoiding	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).
Possible cause	6xHis epitope is altered during fixation
Avoiding	Over-fixation 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.

Possible cause	Suboptimal recognition of 6xHis-tagged protein by Anti·His Antibody
Avoiding	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.
Possible cause	Inappropriate secondary antibodies
Avoiding	PerfectPro 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.
Possible cause	Fluorescence fading
Avoiding	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.

Observation	Non-specific signals
Possible cause	Secondary antibody concentration too high
Avoiding	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 non-transfected cells.
Possible cause	Cross-reacting cell proteins other than the protein of interest
Avoiding	Expression of 6xHis-tagged proteins in mammalian cell lines should not, in general, result in non-specific secondary signals with Anti·His Antibodies. To examine whether endogenous proteins are detected, perform the immunolocalization procedure with non-transfected 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.
Possible cause	Over-fixation
Avoiding	Over-fixation of the cells might lead to signal artifacts. Try to reduce the fixative concentration and/or incubation time.

Observation	High background
Possible cause	Blocking conditions
Avoiding	High background is often the result of incomplete saturation of non-specific 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.
Possible cause	Wash conditions
Avoiding	Non-specifically bound primary and secondary antibody will increase non-specific background. More extensive washing times after primary and secondary antibody incubation steps should decrease background signals.

Observation	Poor morphological preservation
Possible cause	Insufficient fixation
Avoiding	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

Observation	Non-specific background proteins
Possible cause	Inappropriate wash conditions
Avoiding	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.
Possible cause	Proteins of different size than the protein of interest
Avoiding	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 cross-reacting 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.

Possible cause	Non-specific binding of proteins to the matrix used for immunoprecipitation
Avoiding	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.
Possible cause	Proteins have precipitated out of the lysates and are retained throughout the immunoprecipitation procedure
Avoiding	Clear lysate by centrifugation at 13,000 x g for 30 min before adding Anti·His Antibody.

Observation	**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.
Possible cause	Antibody heavy and light chains are visible on stained gel or detected by western-blot analysis
Avoiding	Recombinant protein does not have a 6xHis tag: see the section starting on page 70. 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 con- formation 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 pPP- 9, pPP- 30, pPP- 31, pPP- 32, pPP- 40, pPP- 100 double tag and some versions of the pRSET, pBlueBacHis vectors (Life Technologies). A positive control for expression should be included whenever possible. PerfectPro Kits include pPP- 40 (encoding 6xHis-tagged DHFR) for this purpose.

Possible cause	Antibody heavy and light chains are not visible on stained gel or detected by western-blot analysis
Avoiding	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.

Assay

Observation	**Weak or no signal**
Possible cause	Recombinant protein does not have a 6xHis tag
Avoiding	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.
Possible cause	6xHis tag is partially hidden
Avoiding	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.

Possible cause	Assay reagents are no longer functional
Avoiding	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.
Possible cause	Binding of 6xHis-tagged protein is slower than usual
Avoiding	Increase 6xHis-tagged protein binding time (e.g., overnight). Bind protein at 4°C to prevent degradation.
Possible cause	Inhibitors of interaction between Ni-NTA and 6xHis tag are present (when using Ni-NTA HisPrime Strips and Plates)
Avoiding	Binding of 6xHis-tagged molecules to Ni-NTA HisPrime Strips and Plates is based on metal affinity interactions similar to those of PerfectPro Ni-NTA Agarose or PerfectPro Ni-NTA Superflow. High concentrations of chelating reagents (e.g., EDTA), strong electron donors (e.g., NH_4^+), or ionic detergents (e.g., SDS) may interfere with binding. 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.

Observation	**High background**
Possible cause	Non-specific interaction with antibodies or other reagents used in the assay
Avoiding	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.
Possible cause	Stringency of wash steps is too low
Avoiding	Washing the wells with buffer of reduced pH may help.
Possible cause	Secondary antibody concentration too high
Avoiding	Try using a lower concentration of secondary antibody.

References

1. Porath, J., Carlsson, J., Olsson, I., and Belfrager, G. (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. Nature 258, 598.
2. Hochuli, E., Döbeli, H., and Schacher, A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. J. Chromatogr. 41, 177.
3. Schmitt, J., Hess, H., and Stunnenberg, H.G. (1993) Affinity purification of histidine-tagged proteins. Mol. Biol. Rep. 18, 223.
4. Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680.
5. Sambrook, J. Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
6. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Sedman, J.G., Smith, J.A., and Struhl, K., eds. (1995) Current Protocols in Molecular Biology. New York: John Wiley and Sons.
7. Gallagher, S. (1995) Electrophoresis. In: Coligan, J.E., Dunn, B.M., Ploegh, H.L., Speicher, D.W., and Wingfield, P.T., eds. Current Protocols in Protein Science, Vol. 1. New York: John Wiley and Sons.
8. Spector, D.L., Goldman, R.D., and Leinwand, L.A. (1998) Cells: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press.
9. Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495.
10. Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Ordering information

Kit	Size/detail	Order/ref. no.
Anti His Antibody Selector Kit	3 µg each antibody	2400300
Tetra His Antibody, BSA free	100 µg	2400310
Penta His Antibody, BSA free	100 µg	2400320
RGS His Antibody	100 µg	2400330
Penta His HRP Conjugate Kit	Conjugate and blocking reagent for 250 ml working solution	2400410
PerfectPro 6xHis-tagged Protein Ladder	Lyophilized for 50–100 lanes on western blots	2500300
PerfectPro Ni-NTA MagBeads	2 x 1 ml	2400600
Magnet 96-Well	1	2400620
Microplates 96-Well Flat Bottom	24	2400630
Magnet 12-Tube	1	2400640
Ni-NTA HisPrime Strips	24	2400700
Ni-NTA HisPrime Plates	5 plates	2400720
Ni-NTA HisPrime Plates white	5 plates	2400730
PerfectPro N-6-His Vector Set	25 µg of each vector; pPP-16, pPP-60 pPP-70	2400830
PerfectPro C-6-His Vector Set	25 µg each vector; pPP- 9, -30, -31, -32, and 40	2400840
PerfectPro 100 Double Tag Vector	25 µg	2400860
PerfectPro 30 Xa Vector DNA	25 µg	2400870
PerfectPro Sequencing Primer Set	Primer Promoter Region (3.0µg), Primer Type III/IV(2.8µg) Primer Reverse Sequencing (3.1µg)	2400880
PerfectPro Ni-NTA Agarose	10 ml	2400000
PerfectPro Ni-NTA Agarose	25 ml	2400010
PerfectPro Ni-NTA Agarose	100 ml	2400020
PerfectPro Ni-NTA Agarose	500 ml	2400030
PerfectPro Ni-NTA Superflow	10 ml	2400060
PerfectPro Ni-NTA Superflow	25 ml	2400070

Kit	Size/detail	Order/ref. no.
PerfectPro Ni-NTA Superflow	100 ml	2400080
PerfectPro Ni-NTA Superflow	500 ml	2400090
RTS™ *E. coli* LinTempGenSet, His_6- tag	96 reactions	2401000
RTS 100 *E. coli* HY Kit	24 reactions	2401100
RTS 100 *E. coli* HY Kit	96 reactions	2401110
RTS 500 ProteoMaster™ *E. coli* HY Kit	5 reactions	2401500
RTS 500 *E. coli* HY Kit	5 reactions	2401510
RTS 9000 *E. coli* HY Kit	1 reaction	2401900
RTS 100 *E. coli* Disulfide Kit	24 x 50 µl reactions	2401120
RTS 500 *E. coli* Disulfide Kit	5 x 1 ml reactions	2401520
RTS pIVEX His_6-Tag 2nd Generation Vector Set	2 vectors, 10 µg each	2401010
RTS Wheat Germ LinTempGenSet, His_6-tag	96 reactions	2402000
RTS pIVEX Wheat Germ His_6-tag Vector Set	10 µg each	2402010
RTS 100 Wheat Germ CECF Kit	24 reactions	2402100
RTS 500 Wheat Germ CECF Kit	5 x 1 ml reactions	2402500
RTS GroE Supplement	For 5 RTS 500 reactions	2401030
RTS DnaK Supplement	For 5 RTS 500 reactions	2401020
RTS Amino Acid Sampler	For 5 RTS 500 reactions	2401530

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