Direct Detection of His-tagged Proteins Using Nickel-NTA Conjugates

For Products:

HisDetector™ Western Blot Kits	Catalog No.
HisDetector™ Western Blot Kit, HRP Colorimetric	24-00-01
HisDetector™ Western Blot Kit, HRP Chemiluminescent	24-00-02
HisDetector™ Western Blot Kit, AP Colorimetric	25-00-01

HisDetector™ Nickel Conjugates	Catalog No.
HisDetector™ Nickel-HRP	24-01-01
HisDetector™ Nickel-AP	25-01-01

Store HisDetector™ Nickel-HRP at -20˚C Immediately Upon Arrival

KPL

TABLE OF CONTENTS

Section **Page**

PRODUCT CONTENTS

WESTERN BLOT KITS

24-00-01, HisDetector™ Western Blot Kit, HRP Colorimetric
Size: 40 Blots

Kit Component	Part Number	Volume	Quantity
HisDetector™ Nickel-HRP	24-01-02	0.1 mL	4
BSA Powder	51-61-00	10 grams	1
20X TBST	50-63-16	100 mL	2
TMB 1-Component Membrane Substrate	50-77-02	100 mL	4

24-00-02, HisDetector™ Western Blot Kit, HRP Chemiluminescent
Size: 40 Blots

Kit Component	Part Number	Volume	Quantity
HisDetector™ Nickel-HRP	24-01-02	0.1 mL	1
BSA Powder	51-61-00	10 grams	1
20X TBST	50-63-16	100 mL	2
LumiGLO Substrate, Solution A	50-59-00	120 mL	1
LumiGLO Substrate, Solution B	50-60-00	120 mL	1

25-00-01, HisDetector™ Western Blot Kit, AP Colorimetric
Size: 40 Blots

Kit Component	Part Number	Volume	Quantity
HisDetector™ Nickel-AP	25-01-02	1 mL	1
5X Detector Block Solution	71-83-01	120 mL	2
20X TBST	50-63-16	100 mL	2
BCIP/NBT 1-Component Membrane Substrate	50-81-08	100 mL	4

HisDetector Western Blot Kits provide sufficient reagents to test approximately 4000 cm^2 of membrane (forty 10 cm x 10 cm blots) when using recommended volumes. If desired, increased working volumes may be used; however, additional reagents will be necessary. See Related Products on page 22 for information on additional reagents.

PRODUCT CONTENTS (cont.)

Ni-NTA CONJUGATES

24-01-01, HisDetector™ Nickel-HRP
Size: 0.1 mL

Component	Part Number	Volume	Quantity
HisDetector™ Nickel-HRP	24-01-02	0.1 mL	1

25-01-01, HisDetector™ Nickel-AP
Size: 1 mL

Component	Part Number	Volume	Quantity
HisDetector™ Nickel-AP	25-01-02	1.0 mL	1
Detector™ Block Solution (5X)	71-83-01	120 mL	1

STORAGE AND STABILITY

Storage

HisDetector™ Nickel-AP products (25-00-01, 25-01-01) are shipped at ambient temperature. Upon receipt, store HisDetector™ Nickel-AP kits at 2-8˚C.

HisDetector™ Nickel-HRP products (24-01-01, 24-00-01, 24-00-02) are shipped with cold pack. Upon receipt, store HisDetector™ Nickel-HRP (24-01-02) at -20°C and other kit components at 2-8˚C.

Stability

HisDetector™ Western Blot Kits and Conjugates are stable for a minimum of one year from date of receipt when stored at recommended temperatures.

HisDetector™ PRODUCT DESCRIPTION

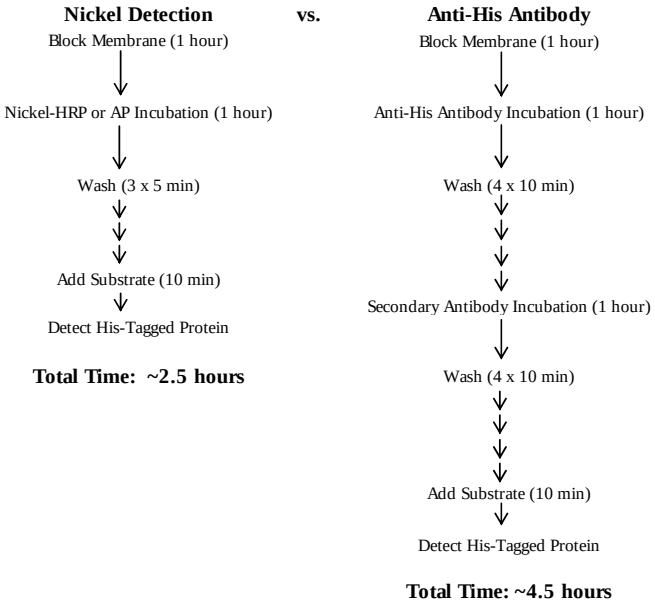
Using His-Tagged Proteins

His-tagged fusion is commonly employed in protein analysis to facilitate the production, purification, and detection of a protein of interest. The first step in this process is typically inserting a DNA sequence coding for four to ten consecutive histidines into the coding region flanking the gene of the protein sequence being expressed. The protein containing the His-tag fusion can then be expressed in large quantities and easily purified over immobilized metal affinity chromatography (IMAC) resins. In addition, the His-tag can be further used in downstream applications to determine level of protein expression, protein function, and especially protein-protein interactions.

Nickel Detection of His-Tagged Proteins

His-tagged proteins have a high affinity for nickel (Ni^{2+}) ions. Nickel-NTA (nitrilotriacetic acid) conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) will therefore bind directly to the His-tag, eliminating the need for primary and secondary antibodies. This results in assay simplification and reduced detection time. Direct detection with nickel-NTA also offers excellent sensitivity and low background in applications such as Western blotting. Nickel-NTA conjugates can detect proteins with a His-tag at either their N- or C-terminus.

Western Blot Detection of His-tagged Proteins



HisDetector Nickel Conjugates

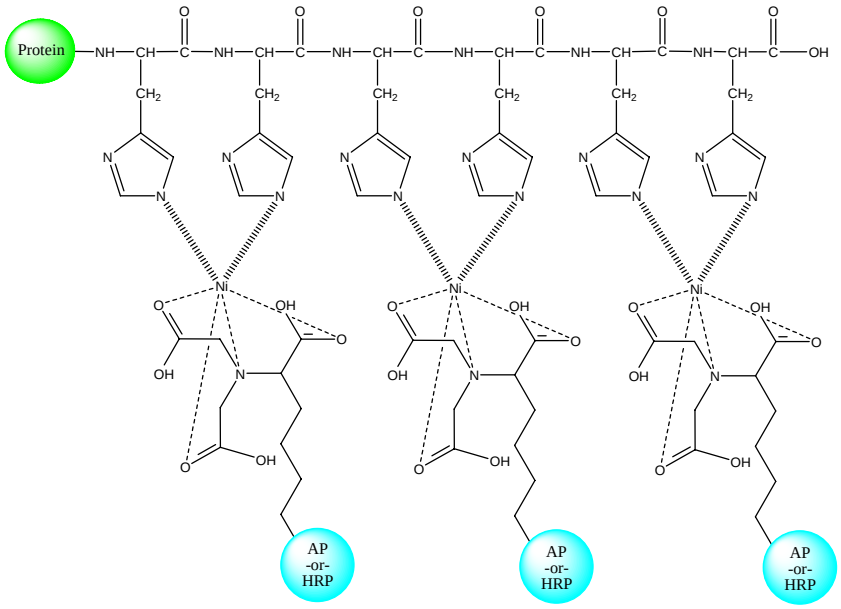
HisDetector Nickel-HRP and Nickel-AP conjugates are tools for detection of His-tagged recombinant proteins. These products are made by covalently conjugating nickel-NTA to the reporter enzymes horseradish peroxidase (HRP) and alkaline phosphatase (AP), respectively. Each conjugate is manufactured to provide high sensitivity and low background in Western blotting applications using both chemiluminescent and colorimetric substrates. HisDetector nickel conjugates may also be used in dot blotting, ELISA and IHC applications.

HisDetector Western Blot Kits

HisDetector Western Blot Kits provide convenience and flexibility for researchers by offering HisDetector nickel conjugates, assay reagents (blocker, wash solutions), and substrates within a fully optimized kit system. HRP Kits are offered for colorimetric and chemiluminescent detection. The AP HisDetector™ Western Blot Kit offers colorimetric detection.

His-tagged Protein Interaction with Ni-NTA Conjugated to HRP/AP:

Nickel ions (Ni^{2+}) can form six electron coordination bonds. NTA binds to four of these bonds. Two ligand binding sites are then available to readily coordinate with a 6X His-tagged protein as shown in the schematic below.



BEFORE YOU BEGIN

Safety and Handling

- Read MSDS and all instructions thoroughly before using product.
- Wear appropriate personal protective equipment when handling all reagents.
- Caution should be used when preparing or handling polyacrylamide gels; monomeric acrylamide is a neurotoxin.

Required Supplies and Equipment

- Nitrocellulose, PVDF or Nylon membrane
- Polyacrylamide gels
- Electrophoresis equipment
- ß-mercaptoethanol or DTT
- Incubation trays or tubes for reagent incubation
- Platform shaker or rocker
- Coomassie blue for gel staining (optional)
- Protein stain such as Ponceau S (optional)
- Protein standards (optional)
- X-ray film (double emulsion such as Kodak BioMax® Light) or a Chemiluminescent Imaging System (for chemiluminescent detection only)
- Film developing chemicals and equipment (for chemiluminescent detection only)

Important Product Information

- His-tag segments of proteins may fold in a manner that reduces interaction between the His-tag and the nickel-NTA conjugate. Denaturing the sample by reducing all available disulfide bonds may enhance exposure of buried histidine residues and increase the probability of His-tag interaction with nickel-NTA. See Sample Preparation on page 10 for information on reducing samples.
- Use of certain buffers or reagents may inhibit interaction of HisDetector Nickel conjugates with the His-tagged segment of the protein. The most notable examples are:
    - Imidazole will bind to various divalent metal cations including nickel. It is commonly used to elute His-tagged proteins when bound to nickel resins.
    - EDTA and other divalent metal chelators will strip the nickel from the reporter enzyme. All divalent metal chelators should be avoided in nickel detection systems.

REAGENT PREPARATION

- **1% BSA Blocking Solution**

Dissolve BSA Powder in 1X TBST Wash Solution to a final concentration of 1% (w/v), i.e., 1 gram of BSA Powder in 100 mL 1X TBST Wash Solution. Freshly prepare solution for each use and discard any unused portion. **Alternative blocking solutions may significantly reduce sensitivity, especially if they contain milk-based or phosphorylated proteins.**

- **1X Detector Block Solution**

Dilute Detector Block Solution (5X) 1/5 v/v in reagent quality water. Freshly prepare solution for each use and discard any unused portion**. Do not use an alternative blocking solution in applications using Nickel-AP.**

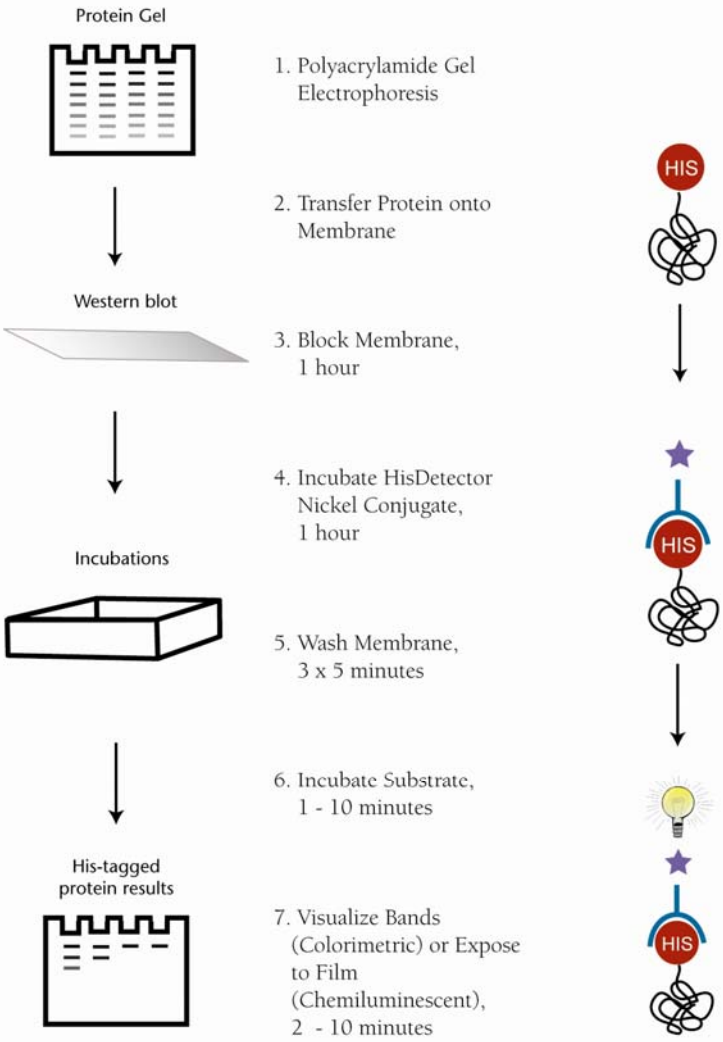
- **1X TBST Wash Solution**

Dilute 20X TBST Wash Solution Concentrate 1/20 with reagent quality water (*i.e.*, 10 mL Wash Solution Concentration + 190 mL H_2O). Store at room temperature or at 2 - 8°C. **Do not use wash solution with EDTA or imidazole.**

- **LumiGLO Chemiluminescent Substrate**

Mix equal parts of LumiGLO Solution A and LumiGLO Solution B (*i.e.*, 5 mL Solution A + 5 mL Solution B). The LumiGLO mixture is stable for up to 24 hours at 2 - 8°C.

HisDetector™ Western Blot Protocol at a Glance



SAMPLE PREPARATION AND GEL ELECTROPHORESIS

1. Prepare protein extract for electrophoresis. Add 5% ß-mercaptoethanol or 1% DTT (final concentration) to reduce protein samples. Incubate protein samples at 90-100°C for 3 minutes prior to loading on gel. The recommended amount of sample to load in each lane is 1-10 µg of crude lysate or 500 pg - 1 µg of purified protein. Optimization may be required depending on the expression of the His-tagged protein and substrate used.

   Avoid overloading protein samples as it will adversely affect membrane detection results.

   Recommended Control: It is advisable to run control samples containing protein extract from the expression strain that does not contain the recombinant His-tagged protein.

2. Electrophoresis samples and standards until tracking dye approaches the bottom of the gel.

   Conditions for electrophoresis will vary depending on the type of gel and the molecular weight of the protein off interest. Check the apparatus manufacturer for recommendations.

3. Transfer proteins on to nitrocellulose, PVDF, or nylon membrane. Run transfer according to equipment manufacturer's instructions.

   HisDetector™ products have been optimized with nitrocellulose.

   Optimal transfer time should be determined experimentally depending on the size and abundance of the protein of interest.

4. a) Optional**:** Stain the gel post-transfer with Coomassie™ Blue to determine transfer efficiency.

   b) Optional**:** Stain proteins on a different membrane with Ponceau-S for 10 minutes at room temperature with shaking. Use a sufficient volume of stain to cover the membrane. Remove membrane from stain and rinse with reagent quality water to remove excess stain. Protein bands will appear as background diminishes. Do not continue to rinse or specific protein staining will diminish.

   The presence of proteins on the gel indicates sub-optimal transfer. Pre-stained protein markers can be used to reliably monitor the efficiency of transfer.

   Ponceau-S for total protein stain is compatible with downstream detection, since it can be removed by washing the membrane in reagent quality water.

5. Proceed to detection on pages 11 and 12.

HRP WESTERN BLOT DETECTION

Volumes indicated are for a 10 x 10 cm^2 blot and may be adjusted for other sizes or multiple blots. Perform all steps at room temperature.

1. Block the membrane by completely immersing in 20 mL of freshly prepared 1% BSA Block Solution for 1 hour at room temperature with gentle agitation or overnight at 2-8°C without agitation. **Alternative blocking solutions may significantly reduce sensitivity, especially if they contain milk-based or phosphorylated proteins.**

2. Add HisDetector™ Nickel-HRP conjugate.

   Colorimetric Assay: Dilute HisDetector Nickel-HRP 1/2,000-1/10,000 directly into the 20 mL of 1% BSA Block Solution used to block the membrane. Incubate for 1 hour at room temperature with gentle agitation.

   Chemiluminescent Assay: Dilute HisDetector Nickel-HRP 1/10,000-1/40,000 directly into the 20 mL of 1% BSA Block Solution used to block the membrane. Incubate for 1 hour at room temperature with gentle agitation.

   Recommended dilutions are provided as starting points. It may be necessary to titrate the concentration of Nickel-HRP to receive optimal results with your system.

3. Wash the membrane by immersing in 1X TBST, 3 times for 5 minutes each with gentle rocking.

4. **Colorimetric Detection:** Add 10 mL TMB Membrane Substrate directly to the membrane and allow to develop for 5-15 minutes. Rinse the membrane for 10-30 seconds in reagent quality water to stop the reaction. Allow the membrane to air dry.

   A digital photograph is recommended if a permanent record is required.

   Chemiluminescent Detection: Apply 5 mL LumiGLO® working solution directly to the membrane for 1 minute. Remove the membrane from substrate and touch the corner to a piece of filter paper to remove excess liquid. Seal the membrane in clear plastic and expose to X-ray film for 2 - 10 minutes. An initial exposure of 2 minutes is recommended.

AP WESTERN BLOT DETECTION

Volumes indicated are for a 10 x 10 cm^2 blot and may be adjusted for other sizes or multiple blots. Perform all steps at room temperature.

1. Block the membrane by completely immersing in 20 mL 1X Detector Block Solution for 1 hour at room temperature with gentle agitation or overnight at 2-8°C without agitation. **Do not use alternative blocking solutions.**

2. Add HisDetector™ Nickel-AP conjugate.

   Colorimetric Assay: Dilute HisDetector Nickel-AP 1/1,000-1/2,000 directly into the 20 mL of 1X Detector Block Solution used to block the membrane. Incubate for one hour at room temperature with gentle agitation.

   Chemiluminescent Assay: Dilute HisDetector Nickel-AP 1/10,000 to 1/40,000 directly in the 20 mL 1X Detector Block Solution used to block the membrane. Incubate for one hour at room temperature with gentle agitation.

   Recommended dilutions are provided as starting points. It may be necessary to titrate the concentration of Nickel-AP to receive optimal results with your system.

3. Wash the membrane by immersing in 1X TBST, 3 times for 5 minutes each with gentle rocking.

4. **Colorimetric Detection:** Incubate membrane in 10 mL BCIP/NBT and allow to develop for 5-15 minutes. Rinse the membrane for 10-30 seconds in reagent quality water to stop the reaction. Allow the membrane to air dry.

   A digital photograph is recommended if a permanent record is required.

   Chemiluminescent Detection: Apply 5 mL AP Chemiluminescent Substrate directly to the membrane and incubate for 1 minute. Remove the membrane from substrate and touch the corner to a piece of filter paper to remove excess liquid. Seal the membrane in clear plastic and expose to X-ray film. For most applications, 1-10 minutes exposure is sufficient.

   KPL recommends PhosphaGLO AP Substrate (Part No. 55-60-01 and 55-60-02) for chemiluminescent detection of AP conjugates in Western blotting.

DOT BLOT DETECTION

The "Dot Blot" is a useful semi-quantitative technique for detecting and characterizing proteins. It is similar to Western blotting. However, the protein samples are directly spotted onto the membrane, instead of being transferred from a gel after electrophoresis. The Dot Blot can also be used to troubleshoot or optimize your Western blot. The sensitivity of detection is comparable in a Dot Blot and a Western Blot. However, the sensitivity tends to be slightly higher in Western blots. Dot Blots are especially useful when titrating the amount of conjugate or comparing various block solutions.

1. Using a pencil, draw a grid to indicate the region you are going to blot. For 1µL spots, draw squares at least ¼"x¼". We recommend using nitrocellulose membranes for dot blots.
2. For each protein, spot 1µL of the serially diluted protein samples onto the membrane. Use TBS as the diluent. In general, eight two-fold dilutions from 1µg/µL will yield good results.

   *Recommended Controls**:** It is advisable to run a positive control containing the 6xHis Protein Ladder (Qiagen), where 1µL corresponds to 20-25 ng of protein. The recommended negative control is protein extract that does not contain the recombinant His-tagged protein.*
3. Allow the membranes to air dry at least 2 hours before blocking the membrane.
4. Refer to detection on pages 11 and 12. Note that dot blots do not typically require an entire 10 x 10 cm^2 membrane, so the reagent volumes can be reduced.

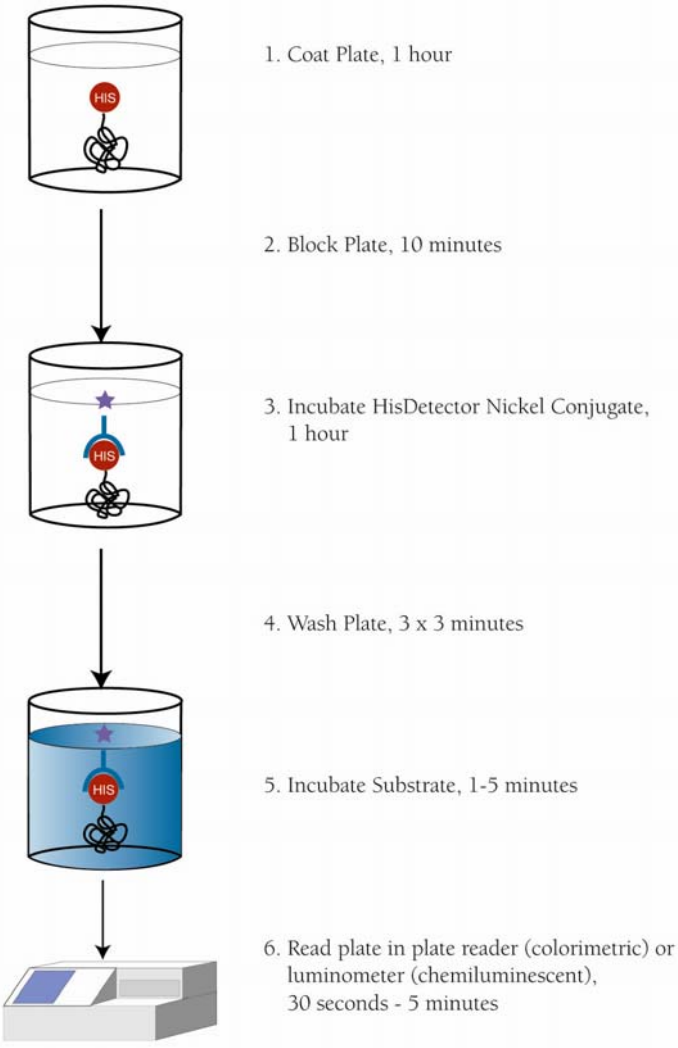
STRIPPING AND REPROBING BLOTS

It is possible to strip a membrane that has been probed with either Nickel-HRP or Nickel-AP and detected with LumiGLO or PhosphaGLO, respectively. It may be re-probed with an alternate detection method if the membrane is not allowed to dry prior to stripping. Stripping the membrane may be done several times, although a gradual increase in background and/or a decrease in sensitivity may occur.

1. Immerse blot in either 20 mL of 6M urea or 100 mM imidazole for 30 minutes with gentle shaking.
2. Carefully rinse blot in reagent quality water for 2 minutes.
3. Block membrane by immersing in 20 mL of appropriate blocking solution for 1 hour at room temperature with gentle rocking or stationary at 2 - 8°C overnight.
4. Detect membrane as described on pages 11 and 12.

ELISA PROTOCOL

HisDetector™ ELISA at a Glance
Total time: 2.5 hours



1. Coat Plate, 1 hour
2. Block Plate, 10 minutes
3. Incubate HisDetector Nickel Conjugate, 1 hour
4. Wash Plate, 3 x 3 minutes
5. Incubate Substrate, 1-5 minutes
6. Read plate in plate reader (colorimetric) or luminometer (chemiluminescent), 30 seconds - 5 minutes

ELISA PLATE COATING

Protocol describes coating purified His-tagged protein for ELISA (standard 96-well plate). Perform all steps at room temperature.

1. Dilute the His-tagged protein to 10 µg/mL in PBS. Add 100 µL per well and incubate for one hour.

   The recommendations stated here offer a starting point. Optimal protein concentration must be pre-determined prior to coating. The amount of protein coated onto the plate will depend on the expression of the target protein.

   KPL recommends the use of high binding microwell plates (i.e. Nunc MaxiSorp™ or equivalent).

2. Add 200 µL of blocking solution to each well and incubate for at least ten minutes.

   KPL recommends: 1% BSA in 1X TBST for blocking ELISA plates.

3. Remove liquid from wells.

4. Add 200 µL/well of 2% sucrose and incubate for 5 minutes.

5. Remove liquid from wells and dry for at least 2 hours. Detect his-tagged proteins as described on page 16 or 17.

   Coated plates can be stored dessicated for several months.

HRP ELISA DETECTION

Protocol is for a standard 96-well plate. Perform all steps at room temperature.

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1. Dilute HisDetector™ Nickel-HRP 1/1,000 to 1/10,000 in 1% BSA in 1X TBST. Add 100 µL diluted Nickel-HRP per well. Incubate for 30 minutes.
2. Wash plate by adding 300 µL 1X TBST Wash Solution per well. Let plate stand for 3 minutes with wash solution in the wells. Remove wash solution from wells and repeat two additional times.

   Other wash solutions may be used, however, the solution must not contain chelators of divalent metal cations, i.e., no EDTA.

3. Add 100 µL/well of ABTS Substrate for detection. Follow the product instructions for incubation time and necessity of stop solution.

   Other substrates are available for HRP detection in ELISA. KPL offers TMB 2-Component, SureBlue, or SureBlue Reserve for colorimetric ELISA detection. For chemiluminescent detection, LumiGLO or LumiGLO Reserve are available.

   See RELATED PRODUCTS on page 22 for more information on HRP substrates for ELISA.

4. Stop reaction by adding 100 µl of ABTS Peroxidase Stop Solution (See RELATED PRODUCTS) or 1% Sodium Dodecyl Sulfate (SDS) to each well. ABTS substrate will remain blue-green after addition of stop solution.
5. Read plate using a plate reader at the appropriate wavelength (405-410 nm for ABTS) or a luminometer (chemiluminescent detection).

AP ELISA DETECTION

Protocol is for a standard 96-well plate. Perform all steps at room temperature.

1. Dilute HisDetector™ Nickel-AP 1/1,000 to 1/10,000 in 1X Detector Block. Add 100 µL diluted Nickel-AP per well. Incubate for 30 minutes.
2. Wash plate by adding 300 µL 1X TBST Wash Solution per well. Let plate stand for 3 minutes with wash solution in the wells. Remove wash solution from wells and repeat two additional times.

   Other wash solutions may be used, however, the solution must not contain chelators of divalent metal cations, i.e., no EDTA.

3. Add 100 µL/well of BluePhos® Substrate for detection. As the color develops, tap gently to mix. Incubation times will vary depending on the assay.

   Other substrates are available for AP detection in ELISA. KPL also offers pNPP for colorimetric ELISA detection. For chemiluminescent detection, PhosphaGLO™ or PhosphaGLO™ Reserve are available.

   See RELATED PRODUCTS on page 22 for more information on AP substrates for ELISA.

4. Stop reaction by adding 100 µl of BluePhos Stop Solution (See RELATED PRODUCTS) or 2.5% EDTA to each well.
5. Read plate using a plate reader at the appropriate wavelength (630 nm for BluePhos) or a luminometer (chemiluminescent detection).

TROUBLESHOOTING GUIDE
HisDetector™ Western Blot Protocols

Use the dot blot protocol on page 13 to troubleshoot Western Blot protocols. The dot blot protocol saves time by omitting electrophoresis and transfer steps.

Problem 1: No Signal

Possible Cause	Corrective Measure
• The membrane was blocked in an incompatible block solution, such as those containing milk-based or phosphorylated proteins.	Block membrane in 1% BSA/TBST for Nickel-HRP or 1X Detector Block Solution for Nickel-AP.
• Inhibition of nickel-NTA binding to His-tagged protein.	Ensure buffers or reagents do not contain chelators such as EDTA, imidazole, or other divalent metal cations.
• Presence of an inhibitor of enzymatic activity.	Ensure buffers do not contain inhibitors such as sodium azide or phosphatase inhibitors. Sodium azide is a potent inhibitor of HRP activity.
• Insufficient sample loaded in lane or low level of expression of His-tagged protein.	Determine total protein concentration. Using protein molecular markers, estimate level of protein expression from a Coomassie stain.
• Lack of His-tag fusion with recombinant protein.	Ensure the recombinant protein is His-tagged.
• Incomplete transfer of proteins to membrane.	Use a pre-stained protein ladder to establish level of transfer.
• Incorrect orientation of membrane during exposure to film.	Check that the correct orientation of the membrane was maintained during the assay, that it was not placed “wrong” side down on film.
• Reagent omitted or improperly prepared.	Check that all procedures were carried out correctly.

Problem 2: Weak Signal

Possible Cause	Corrective Measure
• The membrane was blocked in an incompatible block solution, such as those containing milk-based or phosphorylated proteins.	Block membrane in 1% BSA/TBST for Nickel-HRP or 1X Detector Block Solution for Nickel-AP.

• Insufficient sample loaded or low expression of His-tagged protein.	Determine total protein concentration. Using protein molecular markers, estimate level of protein expression from a Coomassie stain.
• Short exposure of membrane to film	Expose the film to the membrane for extended time, such as 10 minutes.
• His-tag region of protein is not exposed.	Add ß-mercaptoethanol to denature protein. Heat sample prior to loading on gel. After transfer, use chaotropic agents (6M urea or 3M potassium thiocyanate[1]) to increase accessibility of the nickel binding region.
• Assay system may require greater quantities of Nickel-HRP or AP conjugate or longer exposure to conjugate.	Titrate nickel conjugate concentration. Increase the incubation time of nickel conjugate. Increase the time of film exposure.
• Incomplete transfer of proteins to membrane.	Use a pre-stained protein ladder to establish level of transfer.

Problem 3: Excessive Signal

Possible Cause	Corrective Measure
• Bands "blasting" their signal or bands not visible due to excessive signal.	Titrate total amount of protein loaded on the gel. Reduce concentration of Nickel-NTA conjugate. Reduce substrate incubation time or film exposure.
• Film exposure time is too long.	Decrease film exposure time.

Problem 4: Non-Specific Binding of Nickel Conjugate

Possible Cause	Corrective Measure
• Lane is overloaded with protein.	Load less amount of protein sample. Decrease film exposure time.
• Nickel-HRP or AP is too concentrated.	Optimize Nickel-HRP or AP concentration using serial dilutions.

Problem 5: High Background

Possible Cause	Corrective Measure
• Insufficient washing.	Increase wash times if necessary. Ensure blot is covered with 1X TBST
• Insufficient blocking.	Use blocking solutions recommended in appropriate western blot protocol. Ensure blot is covered with blocking solution.
• Endogenous phosphatase or peroxidase activity in blocking solution.	Use blocking solutions recommended in appropriate western blot protocol
• Excess substrate on the membrane during film exposure.	Remove excess substrate from membrane before exposure to the film by touching membrane to a piece of filter paper.

Problem 6: Detection of Multiple Protein Bands Containing His-tags

Possible Cause	Corrective Measure
• Insufficient amount of reducing agent.	Titrate concentration of reducing agent.
• Degradation of protein of interest due to protease activity.	Consider use of protease inhibitors at the lysis step.

TROUBLESHOOTING GUIDE:
HisDetector™ ELISA Protocols

Problem 1: No Signal

Possible Cause	Corrective Measure
• The ELISA plate was blocked in an incompatible block solution, such as those containing milk-based or phosphorylated proteins.	Block the ELISA plate in 1% BSA/TBST for Nickel-HRP or 1X Detector Block Solution for Nickel-AP.
• Inhibition of nickel-NTA binding to His-tagged protein.	Ensure buffers or reagents do not contain EDTA, imidazole, or other metal chelators.
• Presence of an inhibitor of HRP or AP in assay.	Use buffers without chelators such as EDTA, imidazole, or other divalent metal cations.
• Reagent omitted or improperly prepared.	Check that all procedures were carried out correctly.
• Insufficient amount of sample plated in each well	Optimize the protein concentration needed via several titrations.
• Lack of His-tag fusion on the recombinant protein.	Ensure the recombinant protein contains a His-tagged portion.

Problem 2: Weak Signal

Possible Cause	Corrective Measure
• Insufficient quantity of target protein plated or low level of expression of His-tagged protein.	Increase amount of protein coated on plate.
• His-tag region of protein is not well exposed.	Add ß-mercaptoethanol or DTT to denature protein. Heat sample prior to coating on plate.
• Assay system may require higher levels of Nickel conjugate.	Titrate nickel conjugate concentration. Increase nickel conjugate incubation time.

Problem 3: High Background

Possible Cause	Corrective Measure
• Excess protein coated on ELISA plate.	Reduce concentration of His-tagged protein coated on plate.
• Nickel-HRP or AP conjugate is too concentrated.	Optimize Nickel-HRP or AP concentration using serial dilutions.
• Insufficient washing.	Increase soak time for each wash step or add additional wash steps.
• Insufficient blocking.	Use the recommended blocking solution. Increase time of blocking step.

REFERENCES

Jin, L. et al. (1995) *Analytical Biochem*. 229, 54-60.
Peterson, D. (1996) *Australian Biotechnology*. 6, 103-106.
Jin, L. & Peterson, D. (1995) *Arch. Biochem. Biophys*. 323, 47-53.

RELATED PRODUCTS

Western Blotting Products	***Catalog No***	***Size***
Detector™ Block Solution (5X)	71-83-00	240 mL
TMB Membrane Peroxidase Substrate	50-77-18	100 mL
LumiGLO Chemiluminescent Substrate	54-61-00	240 mL
LumiGLO Reserve Chemiluminescent Substrate	54-71-00	2400 cm^2
PhosphaGLO AP Substrate	55-60-04	100 mL
PhosphaGLO Reserve AP Substrate	55-60-02	100 mL
BCIP/NBT Substrate	50-81-18	100 mL
ELISA Products		
Coating Solution Concentrate	50-84-00	50 mL
ABTS Microwell Peroxidase Substrate	50-66-18	100 mL
ABTS Microwell Peroxidase Substrate	50-62-00	600 mL
ABTS Peroxidase Stop Solution	50-85-01	200 mL
SureBlue TMB Microwell Peroxidase Substrate	52-00-01	100 mL
SureBlue Reserve TMB Microwell Substrate	53-00-01	100 mL
TMB Stop Solution	50-85-05	400 mL
pNPP Microwell Substrate System	50-80-00	500 mL
pNPP Microwell Substrate (100 tablets)	50-80-01	5 mg tabs
BluePhos Microwell Substrate Kit	50-88-00	600 mL
BluePhos Stop Solution (10X Concentrate)	50-89-00	200 mL

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