KPL APPLICATION NOTE

Comparison of HisDetector™ Nickel-NTA Enzyme Conjugates with Other Methods for the Detection of His-Tagged Fusion Proteins in Western Blots

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INTRODUCTION

With the sequencing of the human genome, the need to clone and express large numbers of proteins has greatly expanded. Consequently, over-expression of proteins in various host systems (bacterial, yeast, insect and mammalian cells) has become an invaluable biological tool. The wide usage of protein fusion tags has simplified the expression, isolation, purification and detection of recombinant proteins. Commonly used fusion tags include polyhistidine, glutathione-S-transferase (GST) and maltose binding protein tags. Advantages and disadvantages of each system have been extensively described (1-3).

Polyhistidine tags have emerged as the most popular recombinant protein fusion tag for a number of reasons. The small size of the His-tag, *i.e.*, 4 to 6 amino acids, renders it less likely to alter the activity or structure of a protein of interest. Contributing to the popularity of His-tags is the commercial availability of various vectors and hosts for protein expression. The vectors enable the attachment of a polyhistidine sequence at either the N- or C- terminus of the protein. The simplicity and efficiency of purification protocols has also contributed to their wide usage.

Accompanying the growth in the use of His-tagged proteins has been the development of several specific detection reagents (Figure 1). The main approach in most detection applications has been the use of antibodies. Although popular, the detection of His-tagged proteins with antibodies has several limitations. Most detection protocols are based on a two-step method using unconjugated anti-His antibodies along with a corresponding secondary antibody-enzyme conjugate (Figure 1c). This approach, although effective, increases assay complexity and time to results, factors that may limit the utility of this approach in high-volume situations. Protocols require 4-5 hours to complete and the hands-on time is long because several washing steps are required. Moreover, Western blotting protocols requiring multiple antibody incubations are prone to higher levels of background.

An alternative approach based on the use of labeled anti-His antibodies (Figure 1b) reduces assay complexity and time to results. Both approaches require raising an universal antibody against polyhistidine motifs in various sequence and structural contexts, an inherently difficult task. As a result, a variety of anti-His antibodies are offered for various sized His-tags in different structural contexts. The choice of anti-His antibodies makes assay development more complex.

Direct detection of His-tagged proteins without the use of antibodies is enabled by technology developed by Peterson and colleagues at Virginia Commonwealth University (USPTO # 5,674,677) (4, 5). This approach is based on the use of transition metals such as nickel, cobalt, copper and zinc that have high affinity for the imidazole ring of histidine. Nickel ions are bound to the metal chelator, nitrilotriacetic acid (NTA), via four coordination sites (Figure 2). The two unoccupied coordination sites that are available on the nickel ion bind to the His-tag. Reporter molecules such as enzymes or fluorophores, covalently conjugated to the nickel-NTA complex, enable detection (Figure1a). For purification applications (IMAC), the nickel-NTA complex is covalently linked to a solid matrix, thereby exploiting the same affinity of transition metals for histidine residues. Matrices covalently linked to nickel-NTA are widely used affinity resins.

Incorporating the Peterson technique and novel cross-linking technology (6), KPL, Inc. has developed nickel-NTA enzyme

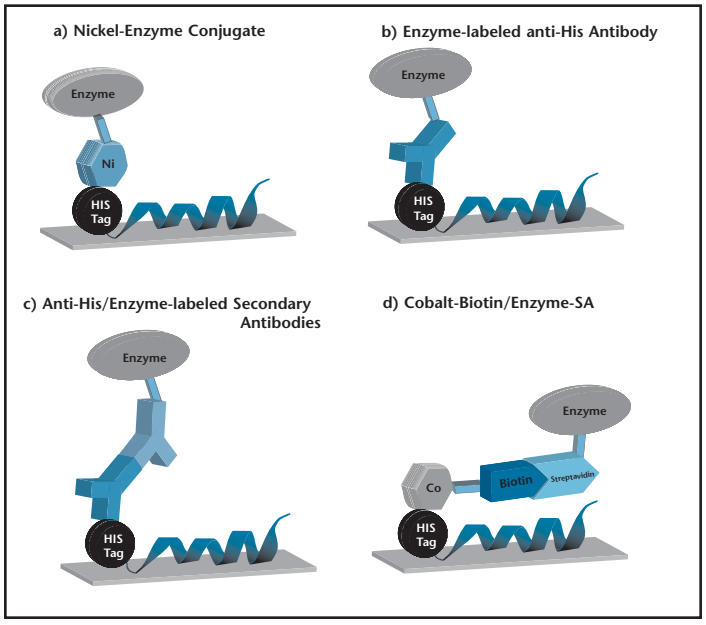

Figure 1. Four schematics representing different Western blot His-tagged protein detection methodologies.

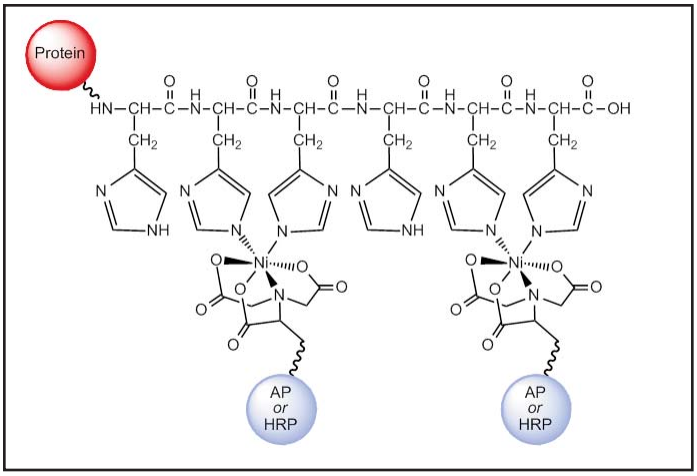

Figure 2. Interaction of polyhistidine sequence with Ni-NTA conjugated to HRP or AP.

conjugates that enable the direct detection of His-tagged proteins. In this report, we describe the detection of His-tagged protein using HisDetector™ Nickel-HRP and Nickel-AP conjugates. Data showing the sensitivity, specificity, and background levels of the KPL HisDetector Nickel conjugates compared to other commercially available methods are presented.

Abbreviations-Polyhistidine sequence fusion tag (4-10 consecutive histidine residues) is denoted as His-tag. Other abbreviations are: HRP, horseradish peroxidase; AP, alkaline phosphatase; SA, streptavidin; Ab, antibody; NTA, nitrilotriacetic acid; Ni, nickel; Co, cobalt; ELISA, Enzyme-linked Immunosorbent Assay.

MATERIAL AND METHODS

HRP and AP were purchased from Biozyme, Inc. LumiGLO® Chemiluminescent Peroxidase Substrate, TMB Membrane Peroxidase Substrate, PhosphaGLO™ AP Substrate and BCIP/NBT Phosphatase Substrate were from KPL, Inc. Methanol, sodium periodate ($NaPIO_3$), β-mercaptoethanol, culture media components and antibiotics were all purchased from Sigma-Aldrich Co. Pre-cast SDS-PAGE gels (Tris-HCl, 4-20% gradient), nitrocellulose membrane (0.45 mm) and Western blotting apparatus were obtained from Bio-Rad Laboratories Inc. Protein sample buffer (Laemmli) and other buffers used for the separation and transfer steps were also obtained from Bio-Rad Laboratories Inc. His-tagged protein ladder containing five His-tagged recombinant proteins ranging from 15 to 100 kDa was obtained from Qiagen, Inc. The *Escherichia coli* strain BL21-DE3 pLysS containing the over-expression clone of His-tagged β-galactosidase was obtained from Novagen, Inc.

E. coli BL21 cells were grown in the presence of antibiotics, and the expression of β-galactosidase was induced by the addition of IPTG as described in the instruction provided by the vendor. The cells were disrupted by sonication and the resulting preparation was centrifuged to remove cellular debris. A sample of the supernatant was diluted into Laemmli sample buffer containing 5% β-mercaptoethanol and heated for 3 minutes at 95°C. Five concentrations of the His-tagged protein ladder and three concentrations of BL21 cell lysate were prepared.

SDS-PAGE was performed using Bio-Rad Tris-HCl 4 - 20% Criterion gels (1.0 mm, 26 wells) at the running condition of a constant 200V for 45 minutes in Tris-glycine-SDS running buffer (Bio-Rad). Proteins on the gels were transferred to 0.45 µm nitrocellulose membranes at a constant 100 volt, for 33 minutes. Membranes were blocked and washed according to manufacturer's recommendations; steps are summarized in Table 1 for each detection method. HisDetector™ Nickel-HRP and AP conjugates were used with their respective chemiluminescent or colorimetric substrate. Detection procedures were as described in the instruction manuals provided by the vendors. LumiGLO® and TMB HRP substrates were used for all protocols, except for the BD™ Universal HIS Western Blot system where the chemiluminescent substrate provided in the kit was used.

RESULTS AND DISCUSSION

Results of comparative measurements are presented using chemiluminescent and colorimetric detections for HisDetector™ Nickel-HRP (KPL, Inc.), HisProbe™-HRP (Pierce, Inc.), Penta-His™ HRP Conjugate (Qiagen, Inc.), and BD™ Universal HIS Western Blot kit (BD Biosciences, Inc.).

KPL HisDetector™ *2 Hours 30 minutes*	Pierce HisProbe™ *3 Hours 15 minutes*	Qiagen Penta-His HRP™ *3 Hours 35 minutes*	BD™ Universal HIS *3 Hours 45 minutes*
Block (1 hour)	Block (1 hour)	Wash 2x (20 minutes)	Block (1 hour)
↓	↓	↓	↓
HisDetector Ni-HRP binding (1 hour)	Wash 2x (20 minutes)	Block (1 hour)	**HIS Detection Reagent binding (1 hour)**
↓	↓	↓	↓
Wash 3x (15 minutes)	**His-Probe-HRP binding (1 hour)**	Wash 2x (20 minutes)	Wash 2x (10 minutes)
↓	↓	↓	↓
Substrate detection (15 minutes)	Wash 4x (40 minutes)	Wash 1x (10 minutes)	Wash 4x (20 minutes)
	↓	↓	↓
	Substrate detection (15 minutes)	**Penta-His HRP binding (1 hour)**	**Streptavidin-HRP Complex binding (30 minutes)**
		↓	↓
		Wash 2x (20 minutes)	Wash 6x (30 minutes)
		↓	↓
		Wash 1x (10 minutes)	Substrate detection (15 minutes)
		↓	
		Substrate detection (15 minutes)	

Table 1. Four Western blot protocols for detection of His-tagged proteins.

COMPARISON OF METAL CONJUGATES WITH ANTI-HIS ANTIBODY CONJUGATES

Comparison of HisDetector™ Nickel-HRP with HRP-labeled Penta-His™ antibody for His-tagged protein detection on Western blots was performed with either a colorimetric or chemiluminescent substrate (Figure 3). The Penta-His™ HRP antibody is directed against a 5X His motif and when conjugated to HRP is the basis for a single-step detection method for His-tagged proteins.

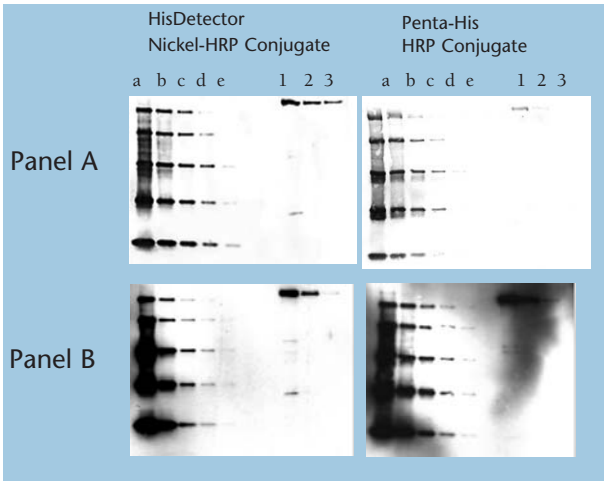

Figure 3. Western blots prepared using HisDetector™ Nickel-HRP and Penta-His™ HRP conjugates for the detection of His-Tagged proteins with colorimetric (Panel A) and chemiluminescent substrates (Panel B). A 10-minute film exposure was used for Panel B results. Lanes a-e: His-tagged protein ladder loaded at 50-75 ng (a), 17-25 ng (b), 6-8 ng (c), 2-3 ng (d), and 620-925 pg (e). Lanes 1-3: Bacterial lysate containing β-galactosidase loaded at 200 ng (1), 67 ng (2), and 22 ng (3).

Using HisDetector™ Nickel-HRP or HRP-labeled Penta-His™ antibody with a colorimetric detection system, each of the five protein bands present in a ladder composed of His-tagged proteins was detected. This suggests that detection is not dependent on the amino acid composition or size of the protein (Figure 3, Panel A, Lanes a-e). Picogram levels of the His-tagged proteins in the ladder were detectable with the Nickel-HRP conjugate whereas the sensitivity of the Penta-His™ HRP was no lower than 2-3 ng. With either type of conjugate, background using colorimetric detection methods was low.

When HisDetector™ Nickel-HRP was used with a colorimetric detection system to detect His-tagged β-galactosidase in a bacterial lysate, the enzyme was detected at <22 ng (Panel A, Lanes 1-3) implying a strong affinity of Ni-NTA for His-tag moieties. In contrast, the enzyme was not detected at levels lower than 200 ng using Penta-His™ HRP.

The insignificant level of detection of other host proteins in the *E. coli* crude lysate using either type of conjugate is indicative of the high specificity of both conjugates.

The level of protein detection using chemiluminescent detection was somewhat better with HRP-labeled Penta-His™ antibody than that observed with Nickel-HRP (Figure 3, Panel B). However, background levels observed when using the Penta-His™ HRP conjugate for detection were significantly higher even though the technique requires more washing steps to reduce the high background sometimes associated with antibody-based detection methods.

The relative sensitivity of the HisDetector™ Nickel-HRP conjugate as compared to the Penta-His™ HRP conjugate makes it the preferred method when using a colorimetric detection system. Moreover, HisDetector™ Nickel-HRP conjugates are a better choice when low background levels are desired with a chemiluminescent system. The high background observed with the Penta-His™ HRP method, combined with the length of the protocol, makes it impractical for the detection of proteins that are expressed at low levels. Because most HRP substrates decay over the course of data collection, methods that inherently generate high background levels provide less flexibility with regard to film exposure times.

COMPARISON OF METAL CONJUGATE DETECTION METHODOLOGIES

The HisDetector™ Nickel-HRP conjugate was compared to two other commercially available products (HisProbe™-HRP, Pierce Inc.; and BD™ Universal HIS Western Blot kit; BD Biosciences,

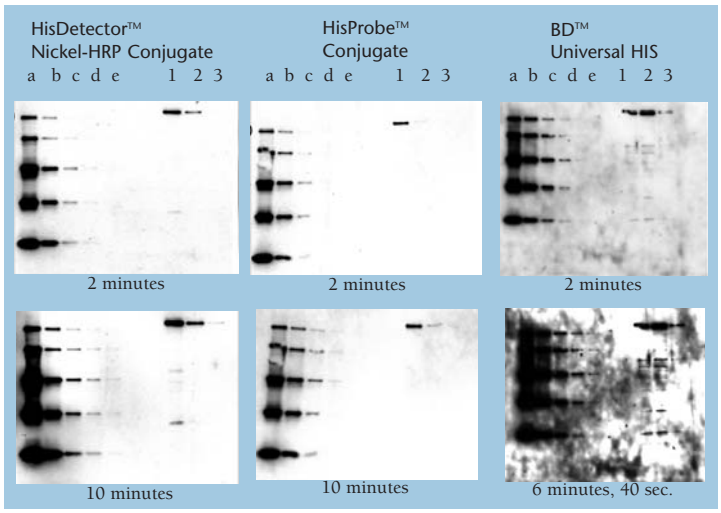

Figure 4. Western blots prepared using HisDetector™ Nickel-HRP, HisProbe™-HRP, and BD™ Universal HIS for the detection of His-Tagged proteins with chemiluminescent substrates. Lanes a-e: His-tagged protein ladder loaded at 50-75 ng (a), 17-25 ng (b), 6-8 ng (c), 2-3 ng (d), and 620-925 pg (e). Lanes 1-3: Bacterial lysate containing β-galactosidase loaded at 200 ng (1), 67 ng (2), and 22 ng (3). Film exposure times were as noted.

Inc.) designed to detect His-tagged proteins using transition metal conjugates. The HisDetector™ and HisProbe™ products are based on Ni-NTA technology. The BD™ Universal HIS Western Blot kit is based on the binding of a Cobalt-biotin complex to the His-tag and detection of the complex with a HRP-streptavidin (HRP-SA) conjugate (Figure 1d). The protocols used for detection were those recommended by the vendor of each kit. Chemiluminescent and colorimetric detection methods were used to evaluate product performance except in the case of the BD™ Universal HIS Western Blot system for which no protocol was available for colorimetric detection.

Figure 4 shows Western blots of His-tagged proteins developed with a chemiluminescent substrate at two substrate exposure times. When HisDetector™ Nickel-HRP was used for detection, picogram levels of the His-tagged proteins in a ladder were detectable (lanes a-e). His-tagged β-galactosidase in a cell lysate was detected at low nanogram levels (lanes 1-3). In contrast, detection of the proteins in the ladder with the HisProbe™-HRP conjugate was no lower than 1 ng; the lowest level of detection of β-galactosidase was 67 nanograms. Background levels observed when using either product were low.

Picogram levels of His-tagged protein were detectable with the BD™ Universal HIS Western kit. However, in the bacterial lysate five host protein bands in addition to the β-galactosidase band were detectable after only two minutes of film exposure when β-galactosidase was present at 60 ng (lanes 1-3). Under identical conditions, only the β-galactosidase band was visible using the HisDetector™ Nickel-HRP conjugate (lanes 1-3). The differences in specificity appear to be due to the selectivity of the Co-biotin/HRP-SA method as compared to the nickel-HRP method. Background levels with the Co-biotin/HRP-SA system were also significantly higher than those observed with either of the other two systems based on nickel-NTA. For chemiluminescent detection, the Pierce and KPL systems provide superior results in a simpler assay protocol.

Figure 5 depicts Western blot results comparing the HisDetector™ Nickel-HRP and HisProbe™-HRP conjugates using the colorimetric detection substrate TMB. Use of HisDetector™ Nickel-HRP conjugate enabled a significantly lower level of detection as compared to the HisProbe-HRP conjugate. HisDetector™ Ni-HRP detected the His-tagged ladder at high picogram levels (lanes a-e) and the His-tagged β-galactosidase at 22 nanograms (lanes 1-3).

The detection level of the ladder observed with the HisProbe product was negligible even at a protein concentration of 75 ng (lanes a-e). Comparable results were obtained for detection of His-tagged β-galactosidase in a bacterial lysate (lanes 1-3).

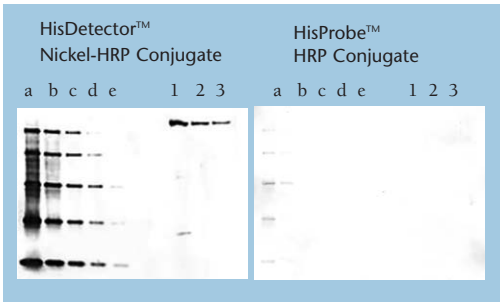

Figure 5. Western blots prepared using HisDetector™ Nickel-HRP and HisProbe™-HRP for the detection of His-tagged proteins with colorimetric TMB substrate. Lanes a-e: His-tagged protein ladder loaded at 50-75 ng (a), 17-25 ng (b), 6-8 ng (c), 2-3 ng (d), and 620-925 pg (e). Lanes 1-3: Bacterial lysate containing β-galactosidase loaded at 200 ng (1), 67 ng (2), and 22 ng (3).

Based on these results, the practical use of HisProbe™-HRP conjugate is limited to chemiluminescent detection systems.

COMPARISON OF NICKEL-HRP WITH NICKEL-AP CONJUGATES

HisDetector™ products are also available as AP conjugates, providing an alternative enzyme reporter system for the detection of His-tagged proteins. The performance of the Nickel-AP conjugate as compared to Nickel-HRP conjugate is presented in Figure 6.

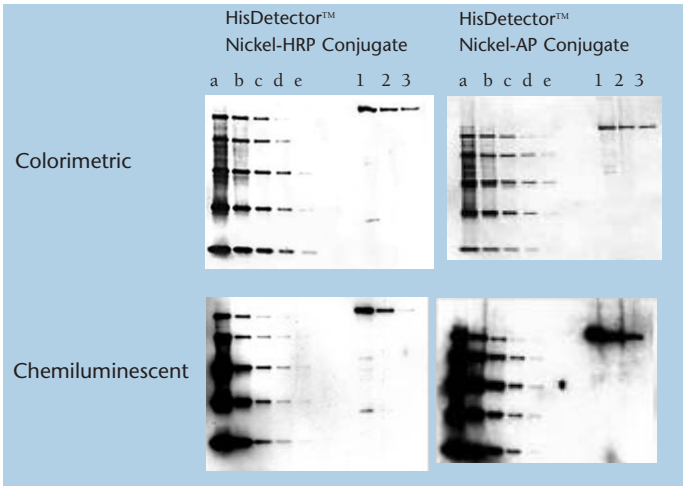

Figure 6. Western blots prepared using HisDetector™ Nickel-HRP and Nickel-AP conjugates for the detection of His-Tagged proteins with colorimetric and chemiluminescent substrates. In the colorimetric assays, TMB and BCIP/NBT were used to detect Nickel-HRP and Nickel-AP, respectively. In the chemiluminescent assays, LumiGLO® and PhosphaGLO™ were used to detect Nickel-HRP and Nickel-AP, respectively. A 10-minute film exposure was used for chemiluminescent results. Lanes a-e: His-tagged protein ladder loaded at 50-75 ng (a), 17-25 ng (b), 6-8 ng (c), 2-3 ng (d), and 620-925 pg (e). Lanes 1-3: Bacterial lysate containing β-galactosidase loaded at 200 ng (1), 67 ng (2), and 22 ng (3).

The results demonstrate that regardless of the detection system used, the nickel-AP conjugate can be used effectively to detect His-tagged proteins. Sensitivity and background were similar to those observed using the nickel-HRP conjugate.

TIME TO RESULTS AND NUMBER OF STEPS

The total time and number of steps for each of the methods tested herein is summarized in Table 2. The antibody-based method, Penta-His™ HRP, is the most complex. It required eight steps and more than 3.5 hours to complete. The BD Universal HIS method required one fewer step but the time for completion was even longer, 3.75 hours versus 3.58 hours. For simplicity and time savings, the methods based on nickel-NTA are clearly superior. Furthermore, results were obtained with the KPL HisDetector™ products in 2.5 hours with only four steps whereas the HisProbe™ product required one more step and about 45 minutes more processing time.

Product	Technology	Detection Time (hrs)	Number of Steps
KPL HisDetector	Nickel-NTA	2.5	4
Pierce HisProbe	Nickel-NTA	3.25	5
BD Universal HIS	Cobalt-Biotin/ Streptavidin	3.75	7
Qiagen Penta-His	Antibody	3.58	8

Table 2. Time and steps required for Western blot detection of His-tagged proteins using different products.

PERFORMANCE SUMMARY

The performance of the HisDetector™ Nickel-enzyme conjugates and other products used for the detection of His-tagged proteins is summarized in Table 3. HisDetector™ conjugates enable the detection of His-tagged proteins with the fewest number of steps and in the shortest amount of time. In contrast, other methods require more "hands-on" steps and longer time. HisDetector™ conjugates also enable high levels of sensitivity and specificity with low background in both colorimetric and chemiluminescent detection methods. With some alternatives, (Penta His™-HRP, BD™ Universal HIS) sensitivity is equivalent. However, increased background and reduced specificity in chemiluminescent assays is also observed. Other products (HisProbe™-HRP) demonstrate low background and high specificity, but lower sensitivity limits use to chemiluminescent assays with higher concentrations of His-tagged.

CONCLUSIONS

HisDetector™ technology is based on nickel-NTA interaction with His-tagged motifs, an interaction that has also been widely employed for purification of recombinant proteins. Unlike antibody-based methodologies, the universality of the interaction between nickel-enzyme conjugates and His-tagged sequences in any structural context, enables the rapid, specific detection of His-tagged proteins. Picogram levels of His-tagged protein can be detected, ensuring identification even at low expression levels. Negligible background, both in chemiluminescent and colorimetric Western blot assays is an added benefit of HisDetector™ Nickel conjugates.

HisDetector™ Nickel-HRP and Nickel-AP conjugates can be used with both chemiluminescent and colorimetric detection protocols. Research laboratories can choose the nickel conjugate and detection method that best meets their needs. The HisDetector™ protocols provide molecular biologists and protein researchers with shorter assay protocols and a more robust method of characterizing gene products making them well suited for proteomics research.

Parameter	HisDetector HRP	HisDetector AP	HisProbe HRP	BD Universal HIS HRP	Penta-His HRP
Technology	Nickel-NTA	Nickel-NTA	Nickel-NTA	Cobalt-Biotin/ SA-HRP	Antibody
Steps to Complete	4	4	5	7	8
Time to Complete (hr.)	2.5	2.5	3.25	3.75	3.6
Sensitivity - Colorimetric	++++	++++	+	Not available	++++
Background - Colorimetric	+	+	+	Not available	+
Specificity - Colorimetric	++++	++++	++++	Not available	++++
Sensitivity - Chemiluminescent	++++	++++	+++	+++++	++++
Background - Chemiluminescent	+	+	+	++++	+++
Specificity - Chemiluminescent	++++	++++	++++	++	++++

Table 3. Performance Summary of His-tagged Protein Detection Products + = low +++++ = high

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Related Products

Description	Size	Catalog No.
HisDetector™ Nickel Conjugates and Western Blotting Kits		
HisDetector Western Blot Kit, HRP Colorimetric	40 blots	24-00-01
HisDetector Western Blot Kit, HRP Chemiluminescent	40 blots	24-00-02
HisDetector Western Blot Kit, AP Colorimetric	40 blots	25-00-01
HisDetector Nickel-HRP Conjugate	1.0 mL	24-01-01
HisDetector Nickel-AP Conjugate	1.0 mL	25-01-01
Western Blotting Products		
Detector™ Block Solution	240 mL	71-83-00
TMB Membrane Peroxidase Substrate	100 mL	50-77-18
LumiGLO® Chemiluminescent Substrate	240 mL	54-61-00
LumiGLO Reserve™ Chemiluminesscent Substrate	2400 cm^2	54-71-00
PhosphaGLO AP™ Substrate	100 mL	55-60-04
PhosphaGLO Reserve™ AP Substrate	100 mL	55-60-02
BCIP/NBT Substrate	100 mL	55-81-18
ELISA Products		
Coating Solution Concentrate	50 mL	50-84-00
ABTS® Peroxidase Stop Solution	200 mL	50-85-01
ABTS® Microwell Peroxidase Substrate	600 mL	50-62-00
SureBlue™ TMB Microwell Peroxidase Substrate	100 mL	52-00-01
SureBlue Reserve™ TMB Microwell Substrate	100 mL	53-00-01
TMB Stop Solution	400 mL	50-85-05
*p*NPP Microwell Substrate System	500 mL	50-80-00
BluePhos® Microwell Substrate Kit	600 mL	50-88-00

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