Product Description

<table>
<thead>
<tr>
<th>Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>15164</td>
<td>INDIA™ HRP, 2 mg</td>
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</tbody>
</table>

INDIA™ HRP is supplied lyophilized from 0.1 M MES, pH 5.5. Reconstitute with 0.4 ml of high quality distilled water for a final concentration of 5 mg/ml. See specification sheet for additional information. Product may be stored at 4° C after reconstitution, or may be aliquoted and frozen for long-term storage.

Introduction

Horseradish peroxidase (HRP, donor: hydrogen-peroxide oxidoreductase, E.C. 1.11.1.7), isolated from horseradish root, is a high activity enzyme used as a label to detect small amounts of antibodies, protein, peptides, DNA and RNA. HRP substrates are available for ELISA, immunohistochemistry, and Western Blotting applications. Direct probes can be created by cross-linking HRP to a probe specific for the target molecule. Indirect HRP probes are often commercially available. Although indirect probes are convenient, these systems require additional processing steps.

INDIA™ HRP is a metal-chelating derivative of horseradish peroxidase. The active chelator is a tridentate chelator which allows metals to be bound in an active form for subsequent interaction and detection of target molecules. The active chelator has similar binding capabilities as that reported for iminodiacetic acid, which has been long used for immobilized metal affinity chromatography (IMAC). Active metal chelates of HRP can be generated by simply mixing a metal solution with INDIA™ HRP. No desalting of free metal is required as long as the metal binding capacity of the INDIA™ HRP is not exceeded (approximately 25 fold molar excess).

A variety of metals have been used for IMAC chromatography and may also be used with INDIA™ HRP. These include Fe³⁺, Ni²⁺, Co²⁺, Zn²⁺, Ce³⁺, La³⁺, Lu³⁺, Sc³⁺, Ti³⁺, Yb³⁺, and Th⁴⁺.¹ The majority of applications for IMAC chromatography have focused on iron, nickel, cobalt, zinc, and copper. Iron can be used for binding interactions with phosphoproteins and organophosphates.²⁻⁴ Special blocking conditions are employed when using ferric iron activated INDIA™ HRP for phosphorylated protein/peptide detection.⁵ A carboxyl-containing non-phosphate binding motif on some proteins and peptides must be blocked to prevent interactions with the ferric iron activated INDIA™ HRP. Nickel (Ni²⁺) can be used for detection of recombinant histidine-tagged fusion proteins.⁶⁻⁸

Zinc (Zn²⁺) has been used for binding of histidine-tagged protein A.⁹ Human α-fetoprotein is bound by copper and nickel.¹⁰ Zinc (Zn²⁺) has been used for purification of serum albumin and cadmium has been used for purification of α₂-macroglobulin.¹¹ Copper, nickel, and zinc interactions were studied for 66 peptides in one study and for 30 peptides in another study.¹²,¹³ One study, involving model peptides retention behavior, was focused on nickel.¹⁴ Copper interactions were examined for an additional 19 peptides.¹⁵

Copper, nickel, cobalt and zinc are some common metals used for binding interactions with histidines. Copper requires one histidine for binding while nickel requires two histidines. Cobalt and zinc require that two histidines are in the same vicinity, either sequential or conformational, for binding.¹⁶ In addition to histidines, other electron rich ligands such as cysteine and tryptophan interact with these metals.¹⁷⁻¹⁹ Nickel and cobalt contain six coordination sites for
interaction with the enzyme-chelate and histidines. The nickel-affinity constant for polyhistidine is $K_a = 10^{13}$. Binding can occur even in chaotropic agents such as 8 M urea or 6 M guanidine HCl so purification and detection of even insoluble proteins is possible. Occasionally, folding may prevent detection of his-tag proteins. 3 M thiocyanate treatment may expose buried histidines.\(^6\)

Most antibodies contain a histidine repeat in the Fc region. Both nickel and cobalt have been shown to bind antibodies through the Fc region. Peroxide treatment of antibodies on cobalt-IMAC support leads to irreversible linkage of antibodies to a support.\(^20\) We have shown that antibodies may be immobilized through their Fc region to cobalt-INDIA™ HRP using this same technique.\(^21\)

**Suggestions for Use**

INDIA™ HRP may be loaded with a metal of choice for use as a detection probe for a target molecule of interest. Possible formats include the use of ELISA plates where the target molecule is passively coated onto polystyrene or nitrocellulose where the target molecule is immobilized through typical transfer procedures. The choice of blocking agent will be dependent on the metal used for interaction; a variety of blockers may be screened. Biotinylated target molecules can be tightly bound in a plate format by using streptavidin, avidin, or neutravidin coated microtiter plates (Pierce). Non-metal activated INDIA™ HRP may be useful as a negative control for nonspecific binding interactions.

A variety of solutes have been observed to be inert with respect to metal interactions on macromolecules, including nonionic detergents, urea, ethylene glycol, dimethylsulfoxide.\(^2\) Sodium chloride may enhance or decrease metal affinity interactions. Phosphate buffers should be avoided when using ferric iron activated INDIA™ HRP. Imidazole is known to be an eluent for interactions involving nickel, cobalt, zinc, and copper. EDTA and other chelators should be avoided. Reducing agents such as mercaptoethanol should also be avoided. Metal binding to the target molecule may be related to topology, microenvironment, reversible reactions, pH, ionic strength, and other factors.\(^18\)

**Metal Activation Protocol**

The following protocol illustrates the activation procedure for INDIA™ HRP. This can be used as a general guideline for metal activation. A variety of molar excesses of metal can be used. It has been our general experience that an 8 fold molar excess is well suited for most applications. Detection sensitivity increases with higher metal loadings, but background interferences can be observed at high metal loadings. Desalting should be used when attempting to saturate the metal binding sites of INDIA™ HRP (molar loadings in excess of 25 moles/mole enzyme).

**Materials**

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
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<tbody>
<tr>
<td>MES Buffer</td>
<td>Composition is 0.1 M MES, pH 5.0 (Prod. No. 28390)</td>
</tr>
<tr>
<td>Metal Solution</td>
<td>Dissolve metal at 4 mM in MES Buffer.</td>
</tr>
<tr>
<td>INDIA™ HRP stock</td>
<td>Reconstitute to 5 mg/ml by dissolving 2 mg in 0.4 ml high quality water.</td>
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**Method**

1. Add 100 µl Metal Solution to a test tube. Add 400 µl INDIA™ HRP stock. Mix briefly.
2. Incubate at room temperature for 30 minutes. Final concentration of metal-activated INDIA™ HRP will be 4 mg/ml.

**Microtiter Plate Protocol**

**Materials**

<table>
<thead>
<tr>
<th>Buffer</th>
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<tbody>
<tr>
<td>Coating Buffer</td>
<td>0.1 M sodium carbonate, pH 9.4</td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>1% BSA or other</td>
</tr>
<tr>
<td>Wash Buffer</td>
<td>PBS/0.05% Tween 20, pH 7.4</td>
</tr>
</tbody>
</table>
Metal-activated INDIA™ HRP
1 N H₂SO₄. Used as a stop solution for the colorimetric assay.

Method

1. Coat 100 µl of target molecule by adding 5-20 µg/ml in coating buffer to plate wells. Incubate overnight at room temperature or for two hours at 37° C.
2. Block wells with 200 µl of Blocking Buffer for 30 minutes at 37° C.
3. Wash plates with 3 X 200 µl of Wash Buffer.
4. Dilute metal-activated INDIA™ HRP to approximately 1 µg/ml in Wash Buffer. Add 100 µl of metal-activated INDIA™ HRP to each well and incubate for 15 minutes at room temperature.
5. Wash plates with 3 X 200 µl of Wash Buffer.
6. Add 100 µl of Turbo-TMB. Stop with 50 µl of 1 N sulfuric acid. Read A₄₅₀.

Immunoblotting Protocol

Materials

Proteins transferred onto nitrocellulose
Tris Buffer: 0.1 M Tris, 0.15 M NaCl, pH 7.2
Blocking Buffer: 3% BSA in Tris Buffer
Wash Buffer: Tris buffer with 0.3% BSA
Metal-activated INDIA™ HRP, 1 µg/ml in Tris Buffer with 0.3% BSA

Method

1. Block the membrane containing transferred proteins with Blocking Buffer for 1 hour at room temperature.
2. Wash the membrane 3 X 10 minutes with Wash Buffer.
3. Add metal-activated INDIA™ HRP and incubate for 1 hour at room temperature with shaking.
4. Wash the membrane 3 X 10 minutes with Wash Buffer.
5. Develop with appropriate substrate.

References


**Compatible Products**

Please consult your Pierce catalog for a complete line of compatible products including preformulated buffers, transfer membranes and activated micro-plates.

A variety of soluble HRP substrates for ELISA applications are available from Pierce with varying sensitivities:
(Products are listed in order of sensitivity.)

<table>
<thead>
<tr>
<th>Product</th>
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<tbody>
<tr>
<td>TMB Substrate Kit</td>
<td>34021</td>
</tr>
<tr>
<td>Turbo TMB, 1-Step™</td>
<td>34022</td>
</tr>
<tr>
<td>OPD Tablets</td>
<td>34006</td>
</tr>
<tr>
<td>Slow TMB, 1-Step™</td>
<td>34024</td>
</tr>
<tr>
<td>ABTS, 1-Step™</td>
<td>37615</td>
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**Peroxidase substrates for blotting and histochemistry include:**

<table>
<thead>
<tr>
<th>Product</th>
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<tbody>
<tr>
<td>DAB Metal Enhanced Substrate Kit</td>
<td>34065</td>
</tr>
<tr>
<td>CN/DAB Substrate Kit</td>
<td>34000</td>
</tr>
<tr>
<td>TMB-Blotting, 1-Step™</td>
<td>34018</td>
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**Pierce offers several chemiluminescent HRP substrates for blotting applications:**

<table>
<thead>
<tr>
<th>Product</th>
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<tbody>
<tr>
<td>SuperSignal™ Ultra</td>
<td>34075</td>
</tr>
<tr>
<td>SuperSignal™ Nucleic Acid</td>
<td>17015 or 17017 (kit)</td>
</tr>
<tr>
<td>SuperSignal™ Substrate, Western Blotting</td>
<td>34080</td>
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</tbody>
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