

Then prepare a 1x TBS solution by adding 200 ml of 10x buffer to 1.8 L of dd H₂O.

B. HRP Color Development Solution

1. Dissolve 60 mg of 4CN into 20 ml of methanol. Dissolve immediately before use and protect solution from light.
2. Immediately prior to use, add 60 µl of **ice cold** 30% H₂O₂ (or 600 µl of 3% H₂O₂) to 100 ml of TBS. Mix with solution (A) at room temperature. Use immediately.

2. Color Development

A. Immerse the membrane in the HRP color development solution. Protein concentrations greater than 100 ng will immediately become visible as purple bands or dots. Lower concentrations of protein will take longer, but should be visible within 30 minutes. Avoid development periods longer than 45 min-

utes, as bleaching or fading of color in positive reactions will occur. If a heavy precipitate forms on the color development solution, a fresh solution should be prepared and used immediately.

- B. Stop the development by immersing the membrane in dd H₂O for 10 minutes with gentle agitation. Change the water at least once during the 10 minute period to remove residual color development solution.



**Instructions for Preparing the
HRP Color Development Solution,
4CN (4-chloro-1-naphthol)
for Use with the Immun-Blot[®]
Horseradish Peroxidase Assay Kit**

Catalog 170-6534



The HRP Color Development Reagent, 4CN (4-chloro-1-naphthol), is used for detecting antigens bound to nitrocellulose or other blotting membranes. This substrate will develop an insoluble, purple product on the membrane surface after exposure to horseradish peroxidase conjugated antibodies.

Note: Do not use sodium azide in any solutions, as NaN_3 is an inhibitor of horseradish peroxidase. If a bacteriostat is necessary, use Merthiolate[®] (thimerosal) at a concentration of 0.01%. Do not use Tween-20 in developer solution, as this will cause a precipitate to form.

Storage and Stability

Reagent Storage	Temperature	Shelf Life
HRP Color Development Reagent (4CN)	-20 °C (desiccated)	1 year

Reagents Available from Bio-Rad

Catalog Number	Product Description	Quantity
170-6534	HRP Chloro Development Reagent (4-chloro-1-naphthol)	5 g
170-6430	Premixed Tris-Buffered Saline (10x)	1 L
161-0715	Tris	100 g
161-0716	Tris	500 g
170-6515	Affinity Purified Goat Anti-Rabbit IgG (H+L) - HRP	2 ml
170-6516	Affinity Purified Goat Anti-Mouse IgG (H+L) - HRP	2 ml
172-1050	Affinity Purified Goat Anti-Human IgG (H+L) - HRP	2 ml
170-6522	Protein A - HRP	1 ml
170-6425	Protein G - HRP	1 ml
161-0307	Biotinylated LMW Standards Kit , contains Biotinylated LMW Standards, 250 µl, and Avidin-HRP, 2 ml	
161-0312	Biotinylated HMW Standards Kit , contains Biotinylated HMW Standards, 250 µl, and Avidin-HRP, 2 ml	

Other Reagents Required to Generate the Color Development Solution

1. Sodium Chloride - ACS reagent grade.
2. Methanol - ACS reagent grade.
3. Hydrochloric Acid - ACS reagent grade.
4. Hydrogen Peroxide - 30% H_2O_2 aqueous solution.

Procedure

1. Prepare the following solutions:
 - A. Tris-Buffered Saline, 2 L (20 mM Tris, 500 mM NaCl, pH 7.5): Add 4.84 g Tris to 58.48 g NaCl and bring to 2 liters with distilled, deionized H_2O (dd H_2O). Adjust to pH 7.5 with HCl.

Note: Premixed TBS (catalog number 170-6430) may be substituted. Follow the directions to prepare the 10x stock solution.