

# **Stabilized TMB Substrate for Horseradish Peroxidase vs. 4-chloro-1-naphthol: A comparison on Western blots**

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*TMB Stabilized Substrate for Horseradish Peroxidase was compared to 4-chloro-1-naphthol on Western blots. TMB Stabilized Substrate gave at least 3-fold greater immunodetection sensitivity. In addition, the color produced by this new substrate developed faster and was less susceptible to fading.*

## **Introduction**

Researchers rely upon immunological methods for the detection of specific proteins or other antigens in complex mixtures. Two popular immunodetection methods are "dot" blots, in which antigen-containing solutions are spotted directly onto a membrane, and Western blots (1,2), in which proteins are transferred to a membrane following SDS-polyacrylamide gel electrophoresis (3).

A common method for the detection of antigens on blots is based on the enzyme-linked immunodetection of antibodies using anti-IgG secondary antibodies conjugated with an enzyme, typically alkaline phosphatase (AP) or horseradish peroxidase (HRP). After transfer of the antigen-containing sample to a membrane, unoccupied protein binding sites on the membrane are blocked. The blot is then incubated with the primary antibody directed against the antigen in question and then with the appropriate secondary antibody-enzyme conjugate. An enzyme substrate is then applied and the resulting colored precipitate localizes the antigen as a colored band or spot on the membrane.

Conventional AP and HRP substrates are not stable at working dilutions and therefore need to be prepared just before each use. Promega has introduced a stabilized substrate for alkaline phosphatase (Western Blue<sup>TM</sup> Substrate) (4) and, more recently, has introduced a stabilized substrate for horseradish peroxidase, TMB Stabilized Substrate. These substrates come ready to use and are stable at room temperature.

## **Advantages of TMB Stabilized Substrate**

Two substrates commonly used to detect horseradish peroxidase conjugated antibodies are 4-chloro-1-naphthol (CN) and TMB (3, 3', 5, 5'-tetramethylbenzidine). TMB Stabilized Substrate has several advantages over 4-chloro-1-naphthol.

TMB Stabilized Substrate allows for more sensitive detection than CN. TMB Stabilized Substrate is supplied premixed, fully diluted and ready for use in a proprietary buffer containing less than 0.5% organic solvent, and is stable at room temperature for up to one year. CN must be dissolved and H<sub>2</sub>O<sub>2</sub> added immediately before use.

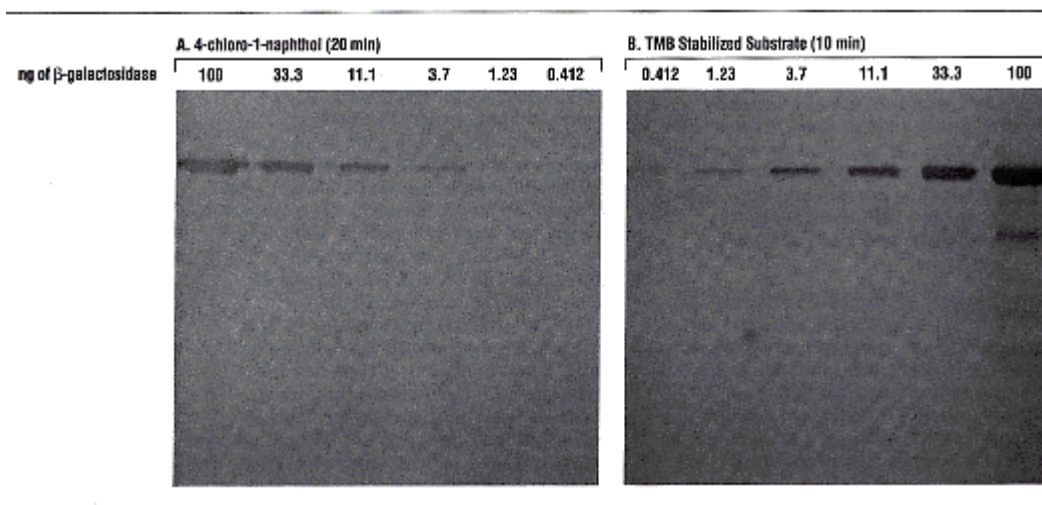
The stable, dark blue reaction product produced by TMB Stabilized Substrate develops rapidly and does not fade as quickly as the CN reaction product. TMB blots have remained stable in lab notebooks for several months, while CN blots tend to fade. When exposed to light, TMB blots also do not fade as quickly as CN blots.

## Immunodetection on Western blots

Immunodetection on Western blots using TMB Stabilized Substrate and CN was performed to compare the detection sensitivities obtainable with these color development substrates. Samples of serially diluted beta-galactosidase (Worthington) were run on an 8% SDS-polyacrylamide minigel and electroblotted to nitrocellulose (1). Blocking was performed for 30 minutes in TBS (20mM Tris-HCl, pH 7.4, 150mM NaCl) containing 1% BSA and 0.05% Tween<sup>®</sup> 20 (blocking buffer). The blot was incubated with the primary antibody, monoclonal anti-beta-galactosidase (Promega) diluted 1:5000 in blocking buffer, for one hour and washed three times for 5 minutes each in TBS containing 0.1% BSA. Goat anti-mouse IgG HRP conjugate (Promega), diluted 1:5000 in blocking buffer, was then applied for 30 minutes. Three 5-minute washes were performed with TBS containing 0.5% Tween 20, and the blots were rinsed briefly with water. The blot was bisected with a razor blade and developed with 4-chloro-1-naphthol (Promega) (Panel A) or with TMB Stabilized Substrate (Panel B), according to the instructions provided with the substrates. The color was allowed to develop on the CN blot for 20 minutes, and on the stabilized TMB blot for 10 minutes. Color development was stopped by soaking the blots in water for 5 minutes.

### 3-fold better detection sensitivity

**Figure 1** shows the sensitivity of detection obtained on the CN and TMB Stabilized Substrate Western blots. On the CN blot, 1.23ng of beta-galactosidase was detected, while the lower limit of detection on the stabilized TMB blot was 412pg. With longer incubation times, we have been able to detect as little as 100pg of beta-galactosidase on a Western blot using TMB Stabilized Substrate. A further advantage of TMB Stabilized Substrate is that the signal produced is easier to see when photographed than the signal produced with CN.



**Figure 1. Immunodetection of beta-galactosidase using 4-chloro-1-naphthol and TMB Stabilized Substrate.** The indicated amounts of serially diluted beta-galactosidase were resolved by SDS gel electrophoresis and detected by Western blotting. Color development

was performed for 20 minutes with 4-chloro-1-naphthol (Panel A) and for 10 minutes with TMB Stabilized Substrate (Panel B).

## Summary

TMB Stabilized Substrate for HRP provides greater sensitivity and a longer lasting, more rapidly developed signal on Western and dot blots than 4-chloro-1-naphthol. These benefits and its ease of use should make TMB Stabilized Substrate the substrate of choice for HRP Western blots.

## References

1. Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350.
2. Burnette, W.N. (1981) *Anal. Biochem.* **112**, 195.
3. Laemmli, U.K. (1979) *Nature* **277**, 680.
4. Larson, G. (1992) *Promega Notes* **35**, 18.

## Ordering Information

Product	Size	Cat.#
TMB Stabilized Substrate for Horseradish Peroxidase	200ml	W4121
Western Blue™ Stabilized Substrate for Alkaline Phosphatase	100ml	S3841
Anti-Beta-Galactosidase, Purified Mouse Monoclonal Antibody	2mg	Z3783
Anti-Mouse IgG (H + L) HRP Conjugate	300µl	W4021
4-Chloro-1-Naphthol Color Development Substrate	1g	W4111

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