

# 3,3',5,5'-TETRAMETHYLBENZIDINE SOLUTION

## TMB

### PRODUCT NO. TMBE

#### A Single Component-Soluble Substrate for Kinetic and Endpoint Assays of Horseradish Peroxidase

##### INTRODUCTION

3,3',5,5'-Tetramethylbenzidine (TMB) has been shown to be a safe-sensitive substrate for the assay of horseradish peroxidase (HRP). Initially, in the presence of HRP and hydrogen peroxide, a one-electron oxidation product is formed. This compound, a cation free radical, is blue in color with an adsorption maximum at 653 nm. Further reaction with HRP/H<sub>2</sub>O<sub>2</sub> or acidification of the radical with acid yields the diimine terminal oxidation product adsorbing light at 450 nm. The extinction coefficient of the radical ( $E_{653\text{ nm}} = 3.9 \times 10^4 \text{ mol}^{-1} \text{ cm}^{-1}$ ) and diimine ( $E_{450\text{ nm}} = 5.9 \times 10^4 \text{ mol}^{-1} \text{ cm}^{-1}$ ) provide an remarkably sensitive system for the assay of HRP and HRP labeled probes. Product No. TMBE, available from **MOSS, INC.**, is a single component reagent stable at room temperature and not sensitive to normal laboratory light. It is optimized with respect to TMB and hydrogen peroxide concentrations and yields a linear response with the concentrations of HRP usually employed in immunologic assays.

##### METHOD SYNOPSIS

After completion of analyte binding to a solid phase and reaction with a HRP labeled probe, TMBE solution is added. Oxidation of TMB produces a blue reaction product that is measured at 650 nm. The color formation as a function of time can be recorded or the reaction stopped with sodium fluoride after a fixed interval. Increased sensitivity can be achieved by converting the blue radical to the diimine by addition of acid. The resulting yellow chromogen is measured immediately at 450 nm.

##### REAGENT PROVIDED

TMBE SOLUTION: Contains TMB, 1.25 mMol L<sup>-1</sup> and Hydrogen Peroxide, 2.21 mMol L<sup>-1</sup> and less than 1 % dimethyl sulfoxide in a 0.08 Mol L<sup>-1</sup> acetate buffer at pH 4.9. Also contains non-toxic proprietary stabilizers.

Store at room temperature, 15-28 °C. Refrigerator temperatures, 2-8 °C, will not harm the product. Warm to assay temperature prior to use.

**Protect from exposure to direct sunlight.**

Discard if solution is blue or turbid.

**REAGENTS REQUIRED FOR STOPPING THE REACTION, BUT NOT  
PROVIDED BY MOSS, INC.**

- A. Sodium fluoride, 0.1%, for stopping reaction and preserving blue chromogen.

Prepare by dissolving 1 g of sodium fluoride in 1 liter of reagent grade water.  
Observe all precautions on label for use of sodium fluoride.

- B. Acid for stopping the reaction and converting the blue radical to the yellow diimine. Either 0.5 Mol L<sup>-1</sup> sulfuric acid or 0.25 Mol L<sup>-1</sup> hydrochloric acid may be used. THE ABSORBANCE MUST BE OBTAINED WITHIN 5 MINUTES AFTER ADDING ACID. If longevity of the yellow reaction product is required, order **MOSS, INC.** Product No. TMBE-S.

NOTE: Reagent grade water must contain less than 10<sup>-7</sup> Mol L<sup>-1</sup> of iron or copper salts otherwise unreacted TMB will be converted non-enzymatically to the diimine.

**PROCEDURE**

1. Complete all required incubations with antibodies, probes and HRP labeled reagents.
2. Wash plate wells at least 4 times with phosphate buffered saline or tris buffered saline containing 0.1% Tween-20.
3. After the final wash, shake and blot all residual buffer from plate wells.
4. Add 0.1 mL of TMBE Solution to appropriate wells and incubate 5-30 minutes.

NOTE: The reaction time will depend upon the activity of the HRP probe. If color develops too briskly, zero order kinetics will not prevail. Dilution of a probe, antibody or HRP labeled reagent may be required.

5. The reaction can be monitored as a function of time for kinetic assays or stopped with 0.1 mL of 0.1% sodium fluoride and read at 650 nm.
6. If the procedure demands conversion to the yellow diimine, add 0.1 mL of either acid described in the reagent section and record the absorbance within 5 minutes.

The method described is similar to that employed by **MOSS, INC.** Variations of time, reagent volume and temperature require standardization by the user.

MOSS DOCUMENT NO. TMBE, MARCH 1996 FOR TECHNICAL ASSISTANCE OR INFORMATION ABOUT OTHER  
MOSS, INC. PRODUCTS, CALL 800-932-6677. OUTSIDE THE USA CALL 410-768-3442 OR FAX 410-768-3971.