コスモ・バイオ株式会社

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

HIGH KINETICS TMB

PRODUCT NO. TMBHK

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_2O_2 or acidification of the radical with acid yields the diimine terminal oxidation product adsorbing light at 450 nm. The extinction coefficient of the radical ($E_{653\ nm}$ = 3.9 x 10^4 mol^{-1} cm^{-1}) and diimine ($E_{450\ nm}$ = 5.9 x 10^4 mol^{-1} cm^{-1}) provide a remarkably sensitive system for the assay of HRP and HRP labeled probes. Product No. TMBHK, available from **Moss Inc.**, is a highly sensitive single component reagent that is ready to use for the quantitative detection of HRP bound to a solid phase or in free solution. TMBHK is stable at room temperature for six months and is 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 commonly employed in immunologic assays.

METHOD SYNOPSIS

After completion of analyte binding to a solid phase and reaction with HRP labeled probe, TMBHK solution is added. Alternatively, TMBHK can be spiked with a small volume of buffered HRP. Oxidation of TMB by HRP produces a blue reaction product that is measured at 650 nm. Color formation can be recorded as a function of time or the reaction can be stopped using an equal volume of 0.3 M sulfuric acid after a fixed interval. Increased sensitivity is achieved by converting the blue radical to the yellow diimine by addition of acid. The resulting yellow chromogen is measured immediately at 450 nm.

REAGENT PROVIDED

TMBHK SOLUTION: Contains 2.5 mM TMB and Hydrogen Peroxide in a proprietary buffer at pH 3.6. The substrate also contains non-toxic proprietary stabilizers.

Store at refrigerator temperatures, 2-8^{o}C. TMBHK may be stored at controlled room temperature for up to six months, and can also be frozen. Warm to assay temperature prior to use.

Protect from exposure to sunlight.

Discard if solution is blue or turbid.

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

A. **Yellow Stop**: 0.3 M sulfuric acid is added in a volume **equal to substrate volume** to stop the reaction and to convert the blue chromogen to the more highly absorbing 450 nm yellow chromogen.

NOTE: Sulfuric acid should be diluted with reagent grade water that contains less than 10^{-7} M of iron or copper salts to prevent non-enzymatic conversion of unreacted TMB to chromogen.

B. **Blue Stop**: 0.1% sodium fluoride or 0.15% sodium dodecyl sulfate is added in a volume **equal to substrate volume** to stop the reaction and to preserve the 650 nm blue chromogen.

PROCEDURE FOR 96 WELL MICROTITER PLATES

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 TMBHK 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 probe, antibody, or HRP-labeled reagent may be required. Moss Inc. welcomes inquiries regarding dilution of TMBHK with special Moss diluent.

5. Highest sensitivity of detection is achieved by stopping the TMBHK reaction with an **equal volume,** 0.1 mL of 0.3 M sulfuric acid. Read the yellow chromogen in the stopped reaction at 450 nm.

6. Assay kinetics may be monitored at 650 nm as a function of time. The reaction may be stopped to preserve the blue chromogen using an equal volume, 0.1 mL of 0.1% sodium fluoride or 0.15% sodium dodecyl sulfate. Measure the blue chromogen in the stopped reaction at 650 nm.

The method described is comparable to that used at **Moss Inc.** Variations of time, reagent volume and temperature require standardization by the user.