MOS TMBZ-100 TMBZ
TMBK-100

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TETRAMETHYLBENZIDINE REAGENT FOR KINETIC OR ENDPOINT ASSAY OF HORSERADISH PEROXIDASE LABELED PROBES

TMBM 500,

PRODUCT NO. TMBEKS

INTRODUCTION

Benzidine and its derivatives are well documented electron donors for the horseradish peroxidase (HRP) /H_2O_2 oxidation/reduction system. These amines are known to be potent carcinogens(1). The synthesis of 3,3',5,5'-tetramethylbenzidine (TMB) by Holland *et al* provided a reagent more sensitive than benzidine (2) and much less hazardous due to ortho methylation (3). Using H_2O_2 as substrate, the reaction of TMB with HRP proceeds through 2 phases. First, a charge transfer complex, the result of a 1-electron oxidation is formed(4). This is a blue product displaying an absorption maximum at 650 nm. Further oxidation proceeds through a green color shift to the fully oxidized yellow diimine with an absorption maximum at 450 nm. The diimine is quantitatively formed only if the molar concentration of H_2O_2 is twice that of TMB(4). The molar extinction coefficient of the charge transfer complex is reported to be 3.9×10^4 M^{-1} cm^{-1} and that of the diimine to be 5.9×10^4 M^{-1} cm^{-1}(4). The blue reaction product can be converted to the diimine non-enzymatically by the addition of acid providing an increase in sensitivity of at least 1.5. The product supplied by **MOSS, INC.** is designed to measure low levels of HRP preventing the formation of the diimine allowing the user to determine enzyme activity or analyte concentration kinetically at 650 nm or as an end point at 450 nm after the addition of acid.

REAGENT PROVIDED

TETRAMETHYLBENZIDINE SOLUTION: A single component ready to use reagent containing TMB (1.58 mMol/L) and Hydrogen Peroxide (2.21 mMol/L) in an 80 mMol/L acetate buffer with stabilizers.

The TMB solution is stable at room temperature or at 2-8 °C. **PROTECT FROM EXPOSURE TO SUNLIGHT!** Discard if turbidity develops or a definite blue color is present.

REAGENT REQUIRED BUT NOT PROVIDED

HYDROCHLORIC ACID, 0.5 Mol/L: Prepare this solution as follows from low-iron Analytical Reagent(AR) 6 Mol/L HCl: To 917 mL of reverse osmosis (RO) water or equivalent, add 83 mL of 6 Mol/L AR HCl. Mix well and store tightly stoppered at room temperature.

RECOMMENDED METHOD, TMBEKS
Microtiter Plates

1. After addition of ad HRP labeled probe and incubation for a predetermined time, discard probe and wash wells thoroughly (at least 4 times) with phosphate buffered saline containing 0.1% Tween 20.

2. Add 100 microliters of TMB Solution to each well, mix thoroughly, and incubate at a constant temperature until the required intensity of the blue reaction product is obtained.

3. Read results at 650nm or add 100 microliters of 0.5 Mol/L HCl, mix thoroughly, and read results at 450 nm. The yellow color is stable for at least 30 minutes after the addition of acid.

RECOMMENDED METHOD
Kinetic Assay Using Cuvettes

NOTE: This procedure describes use of 1 cm lightpath cuvettes.

1. To 3 mL of TMB Solution add 100 microliters of an appropriately diluted HRP mixture. Mix well and place cuvette in spectrophotometer.

2. Record the absorbance change at 650 nm for 5 minutes. NOTE: If dA_{650} is greater than 0.2/minute, dilute HRP solution to obtain this rate.

3. Record the absorbency increase per minute using the linear portion of the curve.

CALCULATIONS: $\frac{dA_{650\ nm}/min \times 1000 \times dilution}{39000 \times 3.1} = Mol/L/min$

EXAMPLE: Where $dA_{650} = 0.2$ and dilution = 1/500

$$\frac{0.2 \times 1000 \times 500}{39000 \times 3.1} = 0.827\ Mol/L/min$$

This procedure is useful for determining the activity of various HRP probes and determining the dilution factor necessary for use with microtiter plate assays employing TMB.

INTERFERING SUBSTANCES AND LIMITING CONCENTRATIONS

SUBSTANCE	HIGHEST ACCEPTABLE LEVEL
Fe^{+3}	6.5×10^{-6} mol/L
Fe^{+2}	6.5×10^{-6} mol/L
Cu^{+2}	5.3×10^{-6} mol/L
Hypochlorite (bleach)	7.8×10^{-6} mol/L
Thimerosal	Not allowed
Sunlight	Not allowed

Iron, copper and bleach contamination in levels higher than listed will turn the TMB solution blue. Thimerosal reacts with TMB inhibiting the reaction and sunlight rapidly oxidizes TMB.

REFERENCES

1. Haley TJ: Clin Toxicol 8: 13, 1975

2. Holland VR, Saunders BC et al: Tetrahedron 30: 3299, 1974

3. Garner RC, Walpole AL et al: Cancer Lett 1: 39, 1975

4. Josephy PD, Eling T, Mason RP: J Biol Chem 257: 3669, 1982

TMBM-500

TMB
(3,3',5,5'-tetramethylbenzidine)

An early hypothesis for the carcinogenicity of aromatic amines such as benzidine involved ortho hydroxylation[1]. Since this mechanism is negated with TMB, it appeared as a logical and safe substitute for benzidine[2]. TMB was developed primarily as a substitute for benzidine to detect occult blood and for studying retrograde axonal transport[3,4]. In recent years, variations of original procedures have been developed that allows use of TMB as an ELISA substrate and as a substrate for blotting procedures to detect horseradish peroxidase labeled probes. The latter are usually modifications of a technique developed by Scopsi and Larrson[5] while the former employ copious quantities of organic solvent to prevent precipitate formation. MOSS Inc. now has available to the researcher 2 proprietary single component TMB products for use in ELISA techniques and blotting methods. Both are stable at room temperature and contain less than 0.5 % organic solvent. The membrane reagent produces a vivid stable blue reaction product. The ELISA system also provides a blue soluble end product that may be quantitated at either 370nm or 655nm. Addition of acid converts the blue color to yellow that can be read at 450 nm. The molar extinction of the yellow oxidation material is greater than the blue thus affording greater sensitivity if required.

TMBE
(for ELISA)

TMBE-100	100 mL
TMBE-500	500 mL
TMBE-1000	1000 mL

TMBM
(for blotting)

TMBM-100	100 mL
TMBM-500	500 mL
TMBM-1000	1000 mL

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4. Mesulam M-M, Rosene DL: Neuro Sci Lett 5: 7, 1977
5. Scopsi L, Larrson L-I: Histochemistry 84: 221, 1986