MOS #TMBE5005
#TMBE1000

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_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 ($E_{653\,nm} = 3.9 \times 10^4$ mol^{-1} cm^{-1}) and diimine ($E_{450\,nm} = 5.9 \times 10^4$ mol^{-1} 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^{-1} and Hydrogen Peroxide, 2.21 mMol L^{-1} and less than **1 %** dimethyl sulfoxide in a 0.08 Mol L^{-1} 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 $MolL^{-1}$ sulfuric acid or 0.25 Mol L^{-1} 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^{-7} Mol L^{-1} 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.

STABILITY DATA for TMBE

TMBE STABILITY DATA AVERAGED FOR LOT NOS.07211931,12348931, 04095941,08227941, 11327942

DEG C	DAYS	CONC R	CONC P	DEG C	DAYS	CONC R	CONC P	DEG C	DAYS	CONC R	CONC P
4	1	0.00221	0	25	1	0.00221	0	37	1	0.00221	0
4	14	0.0022	0.00001	25	14	0.0022	0.00001	37	14	0.00218	0.00003
4	28	0.002195	0.000015	25	28	0.00219	0.00002	37	28	0.00216	0.00005
4	56	0.00218	0.00003	25	56	0.00217	0.00004	37	56	0.00208	0.00013
4	120	0.00217	0.00004	25	120	0.00216	0.00005	37	120	0.00195	0.00026
4	180	0.002155	0.000055	25	180	0.00213	0.00008	37	180	0.00183	0.00038
4	365	0.002145	0.000065	25	365	0.00211	0.0001	37	365	0.00142	0.00079
4	455	0.00214	0.00007	25	455	0.00208	0.00013	37	455	0.00113	0.00108
4	540	0.00212	0.00009	25	540	0.00206	0.00015	37	540	0.00092	0.00129

DEG C	k (mol/DAY)	HALF LIFE, DAYS	DAYS TO LOSE 10% R
4	1.81E-08	10629.42	1063
25	3.33E-08	5784.96	578
37	2.81E-07	685.45	89

4 DEG C			25 DEG C		37 DEG C		
DAY	$A_{283\ nm}$	$dA_{653\ nm}$	A_{283nm}	$dA_{653\ nm}$	$A_{283\ nm}$	$dA_{653\ nm}$	
1	0.282	0.456	0.283	0.456	0.283	0.456	THE pH VALUES AND
14	0.281	0.453	0.283	0.451	0.28	0.453	650, 450, AND 370 nm
28	0.282	0.454	0.281	0.451	0.282	0.448	READINGS WERE ALL
56	0.284	0.452	0.28	0.452	0.28	0.447	IN SPECIFICATIONS
120	0.283	0.451	0.283	0.45	0.284	0.44	
180	0.286	0.449	0.279	0.451	0.281	0.43	
365	0.281	0.446	0.281	0.449	0.279	0.398	
455	0.282	0.445	0.283	0.447	0.281	0.372	
540	0.281	0.44	0.28	0.44	0.283	0.314	

3,3'5,5'-TETRAMETHYLBENZIDINE SOLUTION

TMB

PRODUCT NO. TMBE-S

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

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
Microtiter Plates

1. After addition of an HRP labeled probe and incubation for pre-determined 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/ 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

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

STABILITY DATA FOR TMBE-S

TMBE-S KINETIC DATA

DEG C	HOURS	CONC R	CONC P	DEG C	HOURS	CONC R	CONC P	DEG C	HOURS	CONC R	CONC P
4	1	0.00221	0	25	1	0.00221	0	37	1	0.00221	0
4	168	0.0022	0.00001	25	168	0.0022	0.00001	37	168	0.00218	0.00003
4	336	0.00219	0.00002	25	336	0.00219	0.00002	37	336	0.00214	0.00007
4	720	0.00218	0.00003	25	720	0.00217	0.00004	37	720	0.00203	0.00018
4	1440	0.00217	0.00004	25	1440	0.00215	0.00006	37	1440	0.00188	0.00033
4	2880	0.00216	0.00005	25	2880	0.00214	0.00007	37	2880	0.00171	0.0005
4	4320	0.00216	0.00005	25	4320	0.00212	0.00009	37	4320	0.00136	0.00085
4	10000	0.00213	0.00007	25	10000	0.00208	0.00013	37	10000	0.00087	0.00134

R = HYDROGEN PEROXIDE

DEG C	k(Mol/s)	t 1/2 (h)	t (10%)
4	7.64E-10	252040	2.9 YEARS
25	1.57E-09	122716.4	1.6 YEARS
37	2.62E-08	7346.74	30 DAYS

k = rate constant describing loss of peroxide as Mols/second

t 1/2 = hours required for a 50% loss of peroxide

t (10%) = time required for a 10% loss of peroxide. This is used for setting expiration dates.