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rev.10/10

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TMB, HIGH KINETICS

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

ALTERNATE NAME: 3,3',5,5'-Tetramethylbenzidine; TMBHK

CATALOG #: 1216-100

AMOUNT: 100 ml

STORAGE CONDITIONS: 2-8°C

SHELF LIFE: Stable for up to 12 months at -20°C

TMBHK SOLUTION: Contains 2.5 mMol L^{-1} TMB and Hydrogen Peroxide in a proprietary buffer at pH 3.6. The substrate also contains non-toxic proprietary stabilizers. *Warm to assay temperature before use.*

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. TMBHK, available from BioVision 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.

ASSAY DESCRIPTION:

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.

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STOP SOLUTION:

A. Yellow Stop Solution: 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. *(Not provided).*

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

B. Blue Stop Solution: 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. *(Not provided).*

PROTOCOL:

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. BioVision Inc. welcomes inquiries regarding dilution of TMBHK with special BioVision diluents.
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.

NOTE: Protect from direct sunlight. Discard if solution is blue or turbid.

UK & Rest of World
184 Milton Park, Abingdon
OX14 4SE, Oxon, UK
Tel: +44 (0) 1235 828 200
Fax: +44 (0) 1235 820 482

Switzerland
Centro Nord-Sud 2E
CH-6934 Bioggio-Lugano
Tel: +41 (0) 91 604 55 22
Fax: +41 (0) 91 605 17 85

Deutschland
Bockenheimer Landstr. 17/19
60325 Frankfurt/Main
Tel: +49 (0) 69 779099
Fax: +49 (0) 69 13376880

United States
23591 El Toro Rd, Suite #167
Lake Forest, CA 92630
Tel: + 1 800 987 0985
Fax: + 1 949 265 7703

amsbio
info@amsbio.com