BIOMEDICAL RESEARCH SERVICE CENTER
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TMB Substrate Kit, HRP-based ELISA (Cat #: A-121)

COMPONENTS: TMB Substrate- 2 x 4 ml; store at room temperature in small aliquots and shield from light
TMB Peroxide- 100 ml (1,000 wells); store at room temperature

PRODUCT DESCRIPTION: One of the most widely used enzymes for chromogenic detection in immunoassays is horse-radish peroxidase (HRP), a highly stable enzyme (Clin. Chim. Acta 81:1,1977). The compound 3,3',5,5'-tetramethylbenzidine (TMB) has emerged as the safest colorigenic substrate of HRP exhibiting excellent sensitivity (Tetrahedron 30:3299,1974). HRP in the presence of peroxide converts TMB to a bluish reaction product that can be read spectrophotometrically at 370 or 655 nm. The blue end product upon acidification by HCl or H_2SO_4 generates a bright yellowish color enabling sensitive HRP detection and quantification by absorbance at 450 nm. This application has become widely used in enzyme-linked immunosorbent assay (ELISA) because of its lowest detection limit compared to other reporter enzymes and substrate systems. TMB working solution needs to be prepared fresh by mixing TMB Substrate and TMB Peroxide (75 µl TMB Substrate per ml of TMB Peroxide).

PROTOCOL:

* **Storage of TMB Substrate:** Each vial of the TMB Substrate solution should be aliquoted in the dark and dispensed into small amber tubes during the first use. The solution should be shielded from light and free from water contamination during storage.

Note: TMB Substrate solution is freshly prepared prior to delivery and is good for 10 – 12 months depending on use and storage conditions. Discard the substrate solution if it turns bluish or turbid upon mixing with TMB peroxide.

** **Preparation of working substrate solution:** Calculate the amount of working substrate solution required for each assay. Each well of a 96-well ELISA plate receives 0.1 ml of working substrate solution. The working substrate solution is prepared in the dark by adding 75 µl of TMB Substrate to each ml of TMB Peroxide. The freshly prepared working substrate solution should be shielded from light and used IMMEDIATELY. DO NOT prepare working substrate solution until the final ELISA wash/aspiration step.

1. Perform ELISA protocol as directed by the ELISA kit. Estimate the amount of working substrate solution required, which is 0.1 ml multiplied by the number of wells. Transfer a calculated amount of TMB Peroxide to a 15-ml culture tube.
2. Finish the final wash/aspiration step. Quickly add a calculated amount of TMB Substrate to TMB Peroxide in the 15-ml culture tube. Gently invert tube several times to mix. Immediately add 0.1 ml of the working substrate solution to each well, cover plate, and incubate for 40 min with gentle agitation at room temperature. Shield the plate from light during incubation. A blue color should begin to appear in wells during incubation.
3. Stop color reaction by adding 50 µl of 1N HCl to each well. Gently agitate the plate to ensure thorough mixing. The solution should turn yellowish instantly upon HCl addition.
4. Measure absorbance at 450 nm and 540 (or 570) nm with a microplate reader. Subtract readings at 540 or 570 nm from the readings at 450 nm, and plot the result. See the representative SDF1 standard curve of an SDF1 ELISA.

NOTE:

- TMB Substrate is formulated in methanol. The compound is highly unstable in aqueous solution and is sensitive to photo-degradation. The solution may turn bluish or precipitate during storage if contaminated with water.
- Prepare 1N hydrochloric acid (HCl) for termination of ELISA reaction. Preparation of 1N HCl should be performed in a fume hood. HCl causes severe skin burns and eye damage, and may cause damage to organs. Avoid skin contact.
- Please contact us or visit the product webpage for MSDS information on HCl, TMB, methanol and hydrogen peroxide.