# Stability of 2 Component TMB Substrate System After Mixing

**Purpose:**

To evaluate the stability of the 2 Component TMB Substrate System after the TMB Solution and the Peroxidase Solution B have been mixed together. Stability will be evaluated by measuring background color over time, and by measuring performance in an ELISA.

**Reagents:**

| Background Test: | | Performance Test: | |
|---|---|---|---|
| TMB Substrate Solution | Lot UA001 | TMB Substrate Solution | Lot UA001 |
| Peroxidase Solution B | Lot TM002 | Peroxidase Solution B | Lot TF020 |

**Procedure:**

Samples of 2 Component TMB were mixed together in glass tubes and stored in the dark at room temperature and 4°C. Background color at 650 nm and 405 nm was measured for each sample at 0, 30 min., 1 hour, 2 hours, 4 hours, 7 hours, and 24 hours after mixing. Sample performance was also tested. After mixing, samples were stored for 1 hour, 4 hours, 24 hours, 48 hours and were compared in an ELISA to a freshly mixed sample of the same lot.

**Background Test Procedure:**

Background absorbance was measured at 405 and 650 nm in the Shimadzu UV-1601 spectrophotometer. Duplicate readings were obtained for each sample.

**Background Test Results:**

The O.D.s increase over time, but at the end of the 24 hour testing period, none of the samples had any visible blue color. (See absorbance at 650, Figure 1.) The room temperature samples appear to turn yellow over time; the 4°C samples do not. This is represented by the increase in absorbance seen at 405 nm (Figure 1).

*Figure 1: Background absorbance over time*

| Time After Mixing | Storage Condition | Mean $A_{650}$ | Mean $A_{405}$ |
|---|---|---|---|
| 0 | RT | 0.004 | 0.005 |
| | 4°C | 0.022 | 0.021 |
| 30 min. | RT | 0.010 | 0.013 |
| | 4°C | 0.006 | 0.005 |
| 1 hr. | RT | 0.005 | 0.007 |
| | 4°C | 0.009 | 0.008 |
| 2 hrs. | RT | 0.009 | 0.012 |
| | 4°C | 0.015 | 0.017 |
| 4 hrs. | RT | 0.011 | 0.021 |
| | 4°C | 0.029 | 0.032 |
| 7 hrs. | RT | 0.021 | 0.037 |
| | 4°C | 0.024 | 0.025 |
| 24 hrs. | RT | 0.025 | 0.068 |
| | 4°C | 0.034 | 0.052 |

**ELISA Test Procedure:**

A separate plate was used to assay samples from each different storage condition.

1. Coat plate with 100 µl/well Rabbit IgG (Cappel, lot 35000) diluted to 0.5, 0.25, 0.125, 0.063, 0.031, 0.015 µg/ml in PBS. Add dilutions down the plate from row A to row G; row H filled with PBS only. Incubate for 1 hour at room temperature.
2. Block plate for 15 minutes at room temperature with 300 µl/well BSA Diluent/Blocking Solution Concentrate (lot TF022) diluted 1:10 with reagent quality water.
3. Add 100 µl/well HRP Goat Anti-Rabbit IgG(H+L) (lot NF49) diluted to 0.1 µg/ml in diluted BSA solution. Incubate for 1 hour at room temperature.
4. Wash 3 X 5 minutes with Wash Solution Concentrate (lot RJ45) diluted 1:20 with reagent quality water.
5. Add 100 µl/well of each mixed TMB solution, in duplicate, to the appropriate wells Incubate 10 minutes, stop reaction with 100 µl/well of 1M $H_3PO_4$.
6. Determine the O.D. using an ELISA microplate reader at 450 nm.

**ELISA Test Results:**

Performance was very good for all samples tested, even 48 hours after mixing. As described previously, mixed substrate stored at room temperature developed a yellow color over time, while samples stored at 4°C remained colorless. Figures 2 & 3 show that there may be a nominal decrease in performance over time.

***Figure 2: ELISA Performance, samples stored at room temperature***

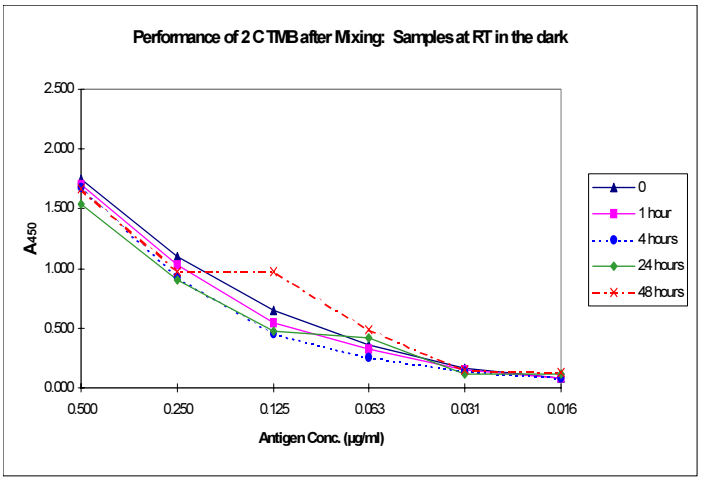
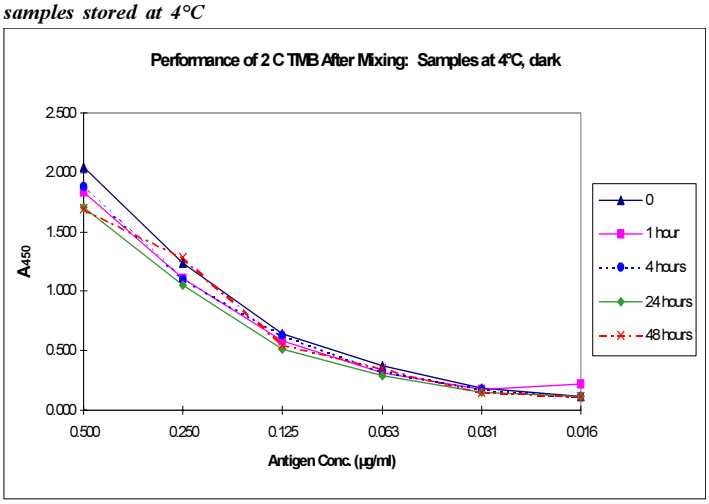



***Figure 3: ELISA Performance, samples stored at 4°C***




**Conclusion**:

It appears that the 2 Component TMB can be mixed together and used for at least one day. Best results will be obtained from substrate stored at 4°C in the dark. The mixed substrate may also be kept at room temperature, but it may develop a yellow color over time. After more than 48 hours, it is recommended that fresh substrate be mixed together before use.

Note: While it is appropriate to compare relative changes in performance over time on individual plates, absolute absorbance values may vary somewhat from plate to plate due to plate variations.

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