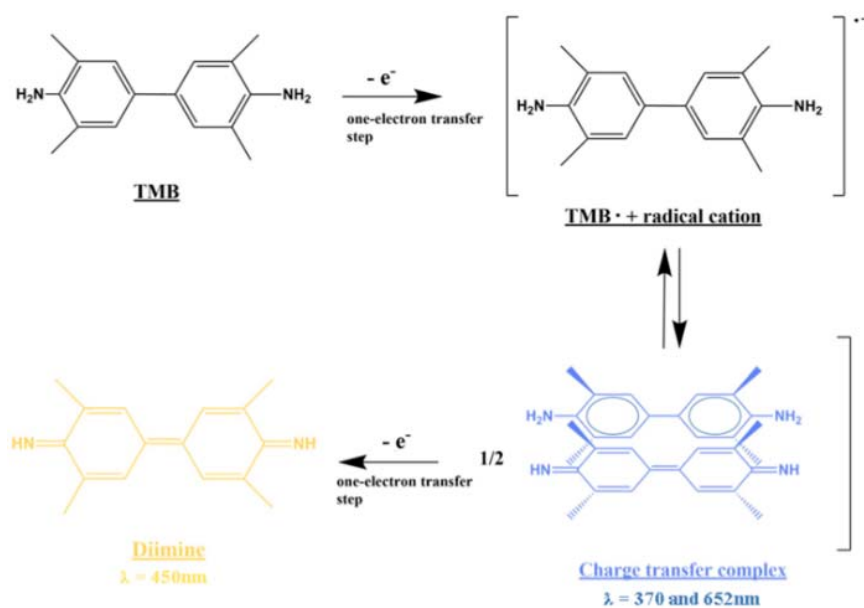


## Targeted Control of TMB Coloring

### 1. TMB Elisa Protocol

TMB substrate mainly consists of 3,3',5,5'-Tetramethylbenzidine(non-carcinogenic HRP substrate) and becomes the charge transfer complex(CTC) upon oxidation due to loss of electrons. The max absorption peak is 652nm(blue). The added acid substance results in the protonation which forces the separation of the CTC and also generates TMB(colourless) and TMB<sup>+</sup> (yellow, max absorption peak: 450nm). Meanwhile, HRP enzyme is inactive in acetic condition and the visualization stops.



## 2. Analyzing Data between OD652 and OD450

Seen from tables below, OD652 data is positively correlated to OD450 data. (The relative error between OD620 and OD652 is 10%.)

**Table 1.** Comparison between monitored OD620(blue) and OD450 after stopping the reaction.

	OD620	OD450		OD620	OD450		OD620	OD450
intensity 1	0.0648	0.1235	intensity 2	0.0573	0.1055	intensity 3	0.0670	0.1206
	0.0857	0.1889		0.0938	0.2081		0.0882	0.2067
	0.1182	0.2634		0.1356	0.3279		0.1170	0.3030
	0.1732	0.3996		0.1980	0.4991		0.1963	0.5374
	0.2793	0.3471		0.3065	0.8282		0.2993	0.8109
	0.4422	1.0680		0.4717	1.3234		0.4497	1.2408
	0.6420	1.6302		0.7296	1.9896		0.7349	1.9502
	0.9350	2.7292	0.9135	2.4595	0.8402	2.2595		
intensity 4	0.0688	0.1117						
	0.0673	0.1383						
	0.0844	0.1810						
	0.1206	0.2703						
	0.1829	0.4092						
	0.2772	0.6553						
	0.4488	1.1440						
	0.6255	1.7981						

**Table 2.** Visualization control of some items.

	R. ICG		H. S100B		H. MMP-8				H. tnfa	
620	0.0648	0.0688	0.0609	0.0507	0.0573	0.0670	0.0618	0.0538	0.0733	duplicated well 4
	0.0857	0.0673	0.0643	0.0521	0.0938	0.0882	0.0841	0.0654	0.0518	
	0.1182	0.0844	0.0830	0.0555	0.1356	0.1170	0.1171	0.0875	0.1359	duplicated well 3
	0.1732	0.1206	0.1219	0.0665	0.1980	0.1963	0.1793	0.1233	0.1320	
	0.2793	0.1829	0.1802	0.0680	0.3065	0.2993	0.2651	0.1969	0.3904	duplicated well 2
	0.4422	0.2772	0.2901	0.0722	0.4717	0.4497	0.4033	0.2866	0.3690	
	0.6420	0.4488	0.4764	0.0816	0.7296	0.7349	0.6345	0.4583	0.9640	duplicated well 1
	0.9350	0.6255	0.6385	0.0884	0.9135	0.8402	0.7861	0.5805	0.9131	
450	0.1235	0.1117	0.1032	0.0854	0.1055	0.1206	0.1080	0.0923	0.1252	duplicated well 4
	0.1889	0.1383	0.1379	0.1236	0.2081	0.2067	0.1908	0.1420	0.0961	
	0.2634	0.1810	0.1931	0.1115	0.3279	0.3030	0.2706	0.2108	0.3232	duplicated well 3
	0.3996	0.2703	0.2865	0.1359	0.4991	0.5374	0.4600	0.3283	0.3160	
	0.6471	0.4092	0.4317	0.1428	0.8282	0.8109	0.7261	0.4924	0.9789	duplicated well 2
	1.2680	0.6553	0.6714	0.1542	1.3234	1.2408	1.0877	0.7326	0.9225	
	1.6302	1.1440	1.1865	0.1712	1.9896	1.9502	1.7025	1.1755	2.4194	duplicated well 1
	2.7292	1.7981	1.8103	0.1812	2.4595	2.2595	2.1295	1.5146	2.3819	
	intensity 1 intensity 2		intensity 1 intensity 2		intensity 1 intensity 2	intensity 3 intensity 4		intensity 1		

### 3. Notes for Visualization Control and Accurate Calculation

1. Read TMB+ at 450nm in a microplate reader with resolution ratio of 3.0. The linearity is quite well under resolution ratio of 2.2(2X relation). For the quantitative detection, the visualization shouldn't be too dark. Please refer to data provided by Thermo FC microplate reader.

**Notes: Sensitivity of microplate readers among different vendors is also varied.**

2. Use professional curve fitting software. Recommended software is available on FineTest [e-resource center](#). MMF four-parameter function is recommended for the parabola.

3. The concentration of TMB+ is higher if the visualization is too dark( $OD_{450} > 3.5$ ). The solubility is much less than TMB. Gradually, TMB+ forms crystals and precipitates at the bottom of the well(black needle-like particle). Read the O.D. absorbance immediately otherwise the yellow will fade. A number of black crystals will appear at the bottom of the well after 12h due to the dark visualization. The yellow is unavailable as a result.