

Substrate Selection in Immunoassay Development

Assay development requires that many components such as antibody/antigen systems, blocking and stabilization, and enzyme detection substrates be evaluated to produce the most robust assay possible. While the choice of antibody or antigen is often the primary consideration, the choice of substrate also has a substantial effect on performance and deserves equal attention for optimal selection. This article examines several key areas that can influence your choice of colorimetric substrate including substrate kinetics, stop reagent, dynamic range, and assay timing. The terms “sensitivity” and “detection limit” are defined here and used to illustrate how these key parameters affect assay performance. Our goal is to provide the assay developer with the tools necessary to make an informed selection of tetramethylbenzidine (TMB) substrate by understanding its impact on assay performance.



Sensitivity versus Detection Limit

The term “sensitivity” has been defined several different ways in the current literature and is the topic of much controversy in the immunoassay field.¹ Assay developers will often ask for “the most sensitive substrate” when what they really need is a substrate with a low detection limit. For the purpose of this paper two separate terms are defined: sensitivity and detection limit. Defining sensitivity and detection limit separately allows for a clear distinction in understanding how each may affect immunoassay design differently.

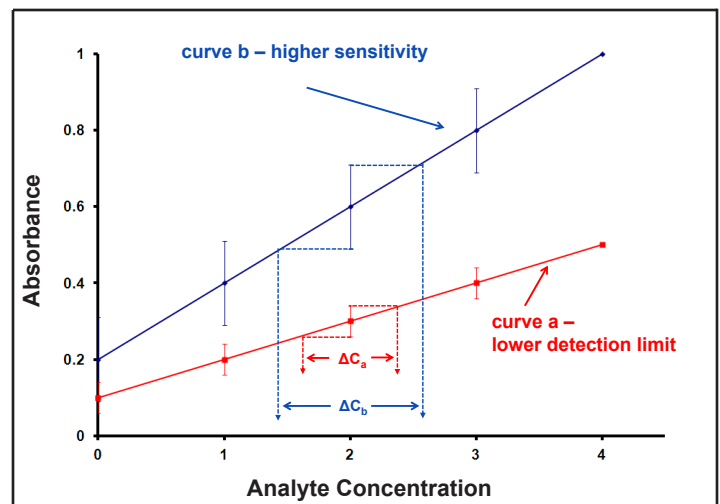


Figure 1. Illustration of the difference between sensitivity and detection limit. Curve a represents an assay with a lower detection limit due to lower standard deviation. Curve b represents an assay with higher sensitivity but poorer detection limit due to a higher standard deviation.

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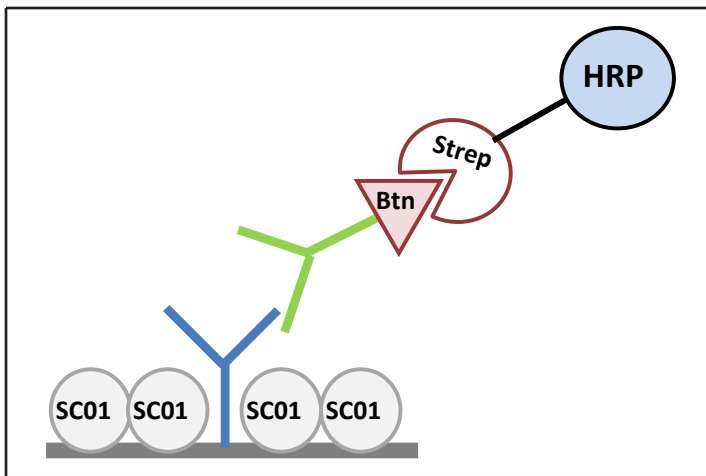


Figure 2. Model Assay System. Rabbit anti-mouse IgG shown in blue, biotinylated (Btn) mIgG shown in green, streptavidin-HRP (Strep) and horseradish peroxidase (HRP).

SurModics IVD Products	Product Code
TMB Super Sensitive One Component HRP	TMBS
TMB One Component HRP	TMBW
TMB Slow Kinetic One Component HRP	TMSK
TMB Super Slow One Component HRP	TTMB
TMB Extended Range HRP (One Component)	TMBX
450 nm Liquid Stop Solution for TMB	LSTP
650 nm Liquid Stop Solution for TMB	LBSP
StabilCoat® Immunoassay Stabilizer	SC01
StabilZyme® SELECT Stabilizer	SZ03
StabilZyme® HRP Conjugate Stabilizer	SZ02

Table 1. SurModics IVD Products and Product Codes.

Sensitivity is the change in signal per unit of concentration, i.e. the slope of the dose/response curve. Ideally if an immunoassay produces a larger signal for a given concentration, then it may be possible to detect lower concentrations above background. **Figure 1** illustrates two assays with different sensitivity levels. *Curve b* has a larger slope than *Curve a*, and therefore higher sensitivity. While overall assay signal can impact detectability, it neglects the importance of error associated with each measurement.

The error associated with signal generation relates to the definition of detection limit. Detection limit is defined as the lowest concentration that gives statistically different signal from the background signal where no analyte is present. The term detection limit is often what assay designers really want when they ask for more sensitivity. Detection limit is governed by the standard deviation of your signal measurement, i.e. the less error, the more able you are to distinguish a low concentration from background.

An assay with high sensitivity does not always give the lowest level of analyte detection. **Figure 1** graphically illustrates the difference between sensitivity and detection limit where *Curve a* represents an assay that has a lower detection limit while *Curve b* is a more sensitive assay (steeper slope).

In summary, the detection limit is defined by the error in signal measurement while sensitivity is determined by the overall signal magnitude. Both concepts are important to consider when choosing the optimal TMB substrate for your assay.

Methods

In order to illustrate the differences between various TMB substrates, a model assay system was developed (**Figure 2**). Rabbit anti-mouse IgG was coated at 0.1 µg/well on a 96-well ELISA plate and stabilized with StabilCoat® Immunoassay Stabilizer (SC01). Biotinylated mouse IgG (mIgG) was titrated in StabilZyme® Select Conjugate Stabilizer (SZ03) and incubated for two hours at room temperature. The plates were washed with PBS/0.05% Tween-20®. Streptavidin-peroxidase was diluted to 1 µg/mL in StabilZyme® HRP Conjugate Stabilizer (SZ02) and incubated for 20 minutes at room temperature. After a final wash in PBS/0.05% Tween-20® the substrate was added and developed. **Table 1** provides a list of SurModics IVD products used for this study.

TMB Substrate Choice - Kinetic Rate and Dynamic Range

A common detection enzyme used in the development of immunoassays for diagnostic applications is horseradish peroxidase (HRP), which is a small, stable enzyme, ~40 kDa, that can be easily conjugated to an antibody, hapten, or peptide. Several substrates are available for use with HRP, but the most common is TMB. SurModics IVD offers several choices for one component TMB substrates with varying kinetic rates for optimization of your assays (**Table 1**). Further examples are provided, illustrating the use of these substrates and offering guidance in selecting the best substrate depending on your assay requirements.

As discussed above, the concepts of sensitivity and detection limit are important considerations when choosing a substrate during assay design. TMB substrate kinetics has been thought to dictate the sensitivity level of an assay, where “faster” substrates give higher signal for a given concentration, thereby increasing its sensitivity. However, a “faster” substrate does not always provide a lower detection limit than a “slower” substrate. Substrates with

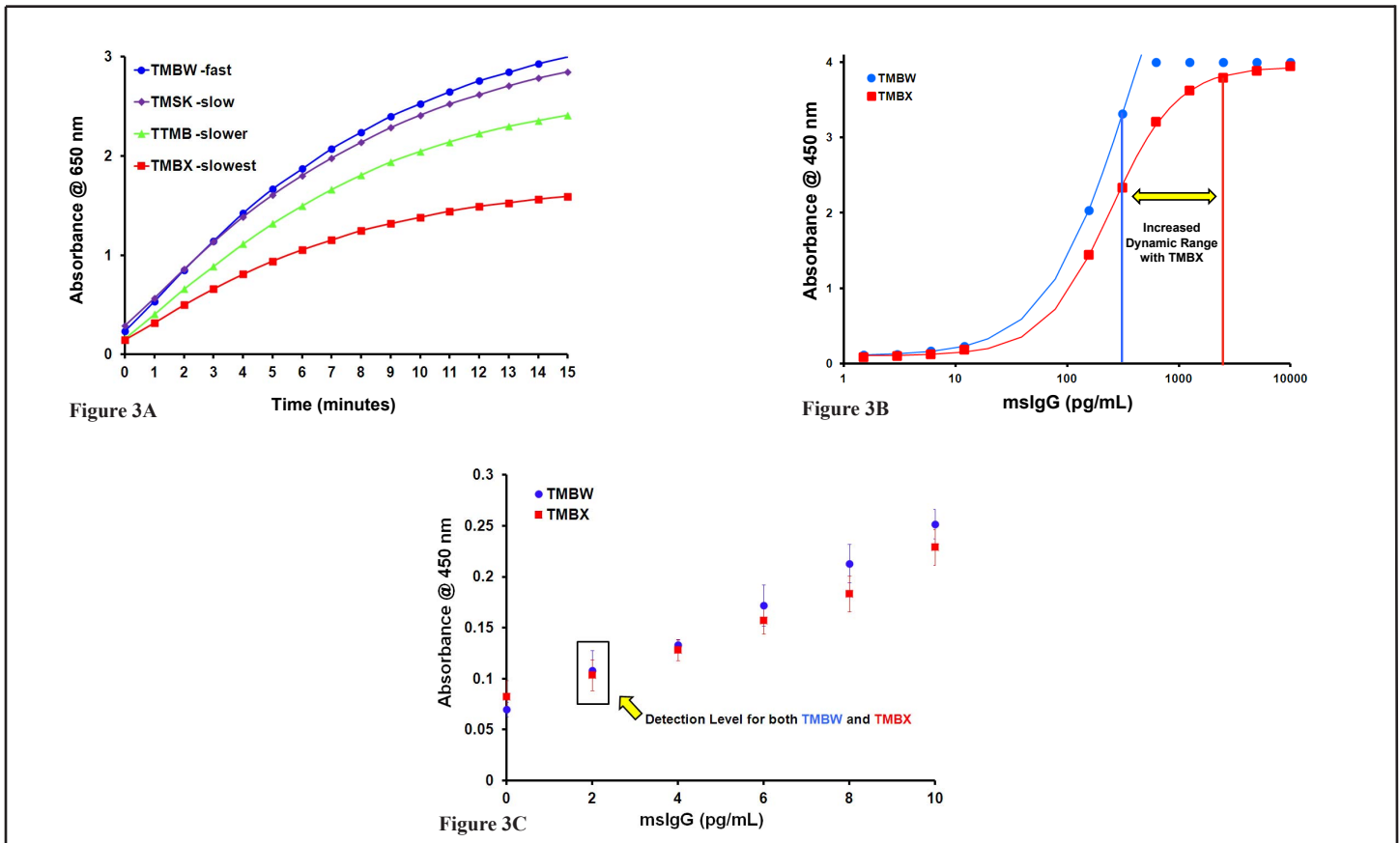


Figure 3. TMB Microwell Substrates. A. Comparison of the kinetic curves of several TMB substrates at 500 pg/mL msIgG in the model assay system monitored over time at 650 nm. B. Standard curve comparison for TMBW and TMBX stopped and read at 450 nm using the model assay system. Results show an extended dynamic range when TMBX is used. C. Expanded graph at the lower concentrations in the model assay using TMBW and TMBX. Both TMBW and TMBX have the same detection limit.

increased kinetics are often limited at the higher concentrations because they “top out” in OD and therefore have a limited dynamic range.

SurModics offers several choices of TMB substrates that vary in kinetic rate, a few of which are compared in **Figure 3A**. Our new TMBX substrate has the slowest kinetic rate of our TMB family but offers the advantage of combining a low level of detection with an extended dynamic range. This means that a broader range of concentrations can be measured using the same substrate. In **Figure 3B** TMBW and TMBX are compared using our model assay when stopped and read at 450 nm. Typically TMBW will saturate at an OD of 4 at higher concentrations after approximately 15 minutes, while TMBX never has an OD (@450 nm) of above 4.0, even after 30 minutes. **Figure 3C** indicates that TMBX maintains the same low limit of detection as TMBW.

Effects of Stop Reagent Choice on Assay Detection

The choice of stop reagent can also be used to optimize the dynamic range of an assay. The TMB reaction is routinely stopped by using an acid to

convert the blue reaction product ($\lambda_{\max} = 650 \text{ nm}$) to a yellow product ($\lambda_{\max} = 450 \text{ nm}$). The yellow product has a higher extinction coefficient that yields about 2.6 times the absorbance signal for a given concentration of analyte. There is an ongoing perception that converting the TMB to the yellow product increases the ability to detect the analyte. This section will demonstrate, both theoretically and empirically, that the detection limit is not improved by this process. This knowledge can lead to useful insights into better assay designs.

Assume theoretically that the addition of yellow stop reagent produces a 2-fold increase in the absorbance measurement. Consider the sample standard deviation (S) of a set of replicate wells read in the blue (650 nm). This standard deviation is calculated as shown here:

$$S_{\text{blue}} = \sqrt{\frac{\sum_{i=1}^N (x_i - \mu)^2}{(N-1)}} \quad \begin{array}{l} \mu = \text{sample mean} \\ x_i = \text{individual measurements} \\ N = \text{number of measurements} \end{array}$$

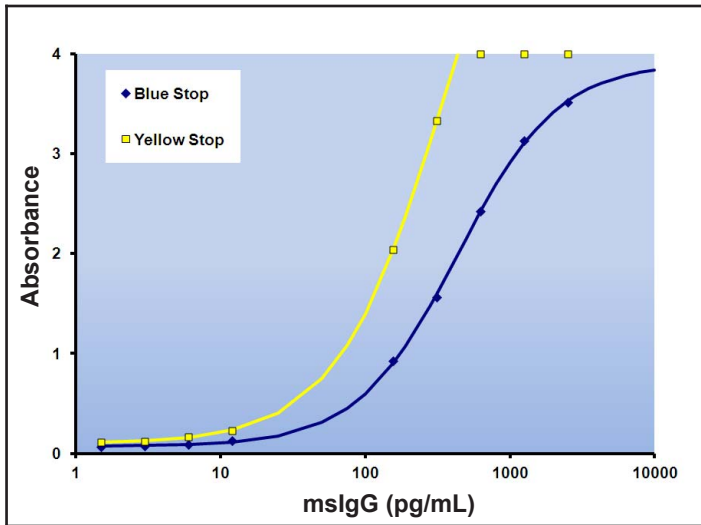


Figure 4. Standard Curves for Blue Stop (650 nm) versus Yellow Stop (450 nm) Standard curves for the model assay system using TMBW either read at 650 nm (blue) or 450 nm (yellow). Reading at 650 nm allows for an extended dynamic range without losing detectability.

If the addition of the stop reagent exactly doubles the absorbance signals, this would have the effect of doubling every well signal and the overall mean. This results in the calculation of standard deviation as shown here:

$$S_{\text{yellow}} = \sqrt{\frac{\sum_{i=1}^N (2x_i - 2\mu)^2}{(N-1)}} = \sqrt{\frac{4 \sum_{i=1}^N (x_i - \mu)^2}{(N-1)}} = 2 \times \sqrt{\frac{\sum_{i=1}^N (x_i - \mu)^2}{(N-1)}} = 2 \times S_{\text{blue}}$$

If the signal is doubled for each measurement, the error is doubled as well. The end result is that there is no improvement in detection limit. This can be easily demonstrated by calculating the standard deviations of a set of data and its doubled cohort (**Table 2**).

The theoretical result was tested empirically using

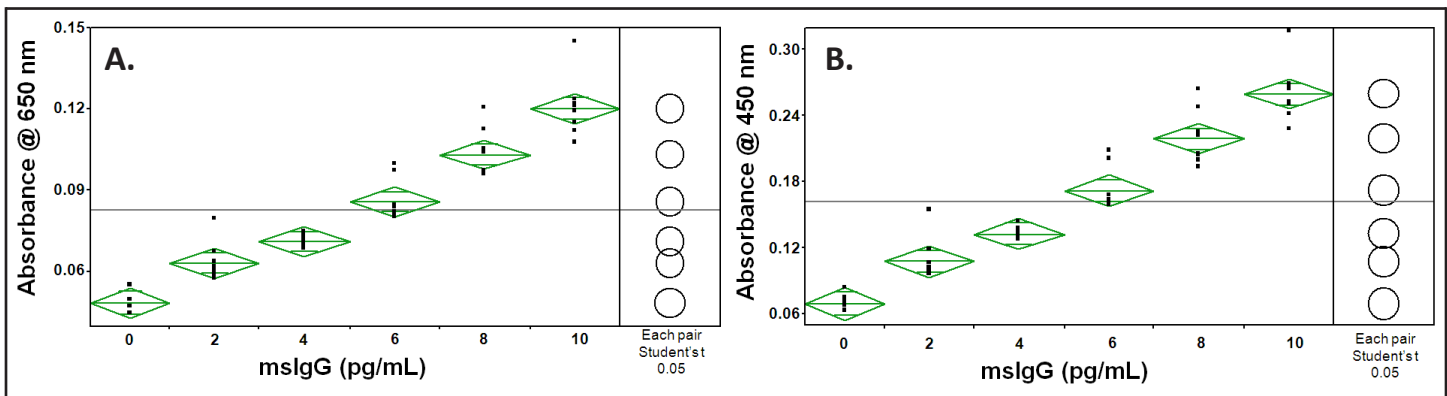


Figure 5. A. ANOVA representation for A650, blue stop reagent. B. ANOVA representation for A450, yellow stop reagent. Means diamonds represent the mean (the center line) and 95% confidence levels (tips of the diamonds). Circles not connected in the Student's t test are significantly different.

mslgG (pg/mL)	N	Average Absorbance		Standard Deviation		SD Ratio
		650 nm	450 nm	S ₆₅₀	S ₄₅₀	S ₄₅₀ /S ₆₅₀
10	8	0.120	0.260	0.011	0.026	2.33
8	8	0.103	0.219	0.009	0.025	2.76
6	8	0.086	0.172	0.008	0.020	2.66
4	8	0.071	0.133	0.002	0.006	2.61
2	8	0.063	0.108	0.007	0.020	2.74
0	8	0.049	0.070	0.003	0.007	2.21

Table 2. Values from lowest concentrations in model assay. Experimental standard deviations for 450 nm measurements are almost 2.6 times greater than 650 nm measurements.

the mslgG model assay described previously. After substrate incubation, the assay was read at 650 nm and then stopped by adding BioF_x® yellow stop reagent (LSTP). The plate was then read at 450 nm. The overall standard curves are shown in **Figure 4**. Addition of the yellow stop clearly produced higher signals at each analyte concentration thereby showing an increased sensitivity; however, some points were saturated at an OD ≥ 4.0. A benefit of reading at 650 nm is that these points now fall in the quantifiable range.

The experiment was further analyzed to compare detection limits. The absorbance readings and standard deviations for the lowest concentrations are displayed in **Table 2**. The last column in the table is the ratio of the standard deviations S_{yellow}/S_{blue}. The increase in standard deviation for each point is about 2.6-fold higher for the 450 nm reading versus the 650 nm reading. This is equivalent to the signal increase achieved by stopping and reading at 450 nm. So, the signal and the error have been increased by exactly the same factor. This results in no net change in the detection limit. The data from this experiment

was analyzed in a statistical program (JMP) using a Student's t test. **Figure 5A and 5B** show a visual representation of this comparison. There was no difference in the detection limit or the ability to distinguish between mslgG concentrations across this experiment. Stopping the TMB reaction in the blue and reading at 650 nm is a valuable strategy to increase the dynamic range of an assay without sacrificing the detection.

Considerations for Substrate Choice

During assay development it is important to consider your substrate selection carefully and to have an understanding of your assay's requirements. Several key elements can be important such as dynamic range, kinetic rate, and assay timing.

1. Dynamic Range

Dynamic range is especially important when developing an assay that requires quantitation of a large range of analyte concentrations. Drawing on information from previous sections, two potential options emerge as ways to extend your assay's dynamic range when using TMB substrates.

If an important design criteria for your assay is that it must be stopped and read at 450 nm, then TMBX may be the optimal solution. Due to its slower kinetic rate, TMBX maintains a lower OD at 450 nm than a faster kinetic substrate like TMBW. Often TMBW will become saturated at an $OD \geq 4$ and limit your quantitation range at higher concentrations. (See **Figure 3B** as an example.) Choosing TMBX allows for the development of an assay consistent with traditional formats, which may be important for certain IVD applications.

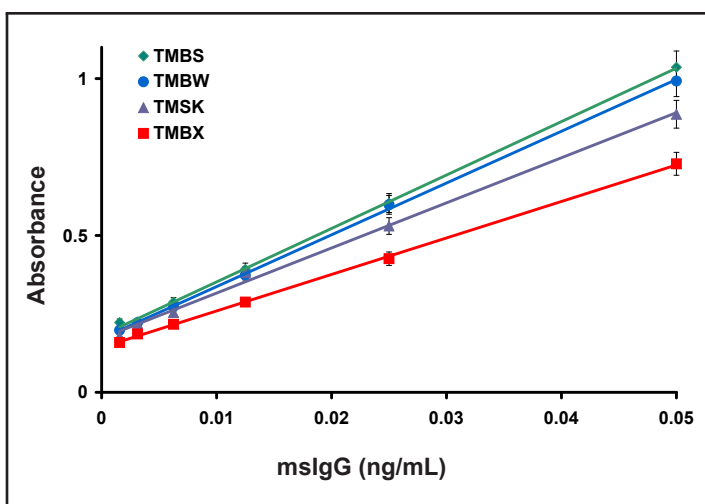


Figure 6. Sensitivity (slope) of SurModics TMB Family Using the Model Assay System

Substrate	Relative Kinetic Rate	Detection Limit (pg/mL)	Dynamic Range (pg/mL)
TMBS	Fastest	2-4	2-320
TMBW	Standard	2-4	2-320
TMSK	Slow	2-4	2-625
TTMB	Slower	3-6	4-625
TMBX	Slowest	2-4	2-2500

Table 3. Detection Limits and Dynamic Range for SurModics' TMB Family determined Using the Model Assay System.

A less traditional assay design, where TMB signal is monitored and stopped at 650 nm, can also allow for a larger dynamic range. An optimal choice for this method might be TMBW or TMSK. Here the reaction can be monitored real-time and/or stopped with a reagent that maintains the blue color at 650 nm (LBSP). If the OD is kept below 3.0, the assay should remain linear with concentration. However, many assay developers don't consider this option because it isn't widely used.

2. Kinetic Rate

The kinetic rate of the substrate has been thought to drive the detection limit of the assay, however this has not been generally observed with the TMB substrates. **Table 3** compares the detection limits and dynamic range of several of the TMB substrates in the SurModics TMB Family. Notice that the "fastest" substrate, TMBS, and the "slowest" substrate, TMBX, have the same detection limit. However, when TMBS and TMBX are compared using identical assay development times, TMBS and TMBW are more sensitive than TMBX, as observed by their slope shown in **Figure 6**. Again higher sensitivity does not always mean better detection limits as indicated in **Table 3** where all these substrates have similar levels of detection. Some assays that have all sample concentrations close to the low end of the dynamic range may benefit from a substrate choice with better sensitivity such as TMBS or TMBW.

3. Assay Timing

The timing of your assay is important when considering factors such as dynamic range, reproducibility and detection limit. An assay that has a slower substrate and a longer development time can exhibit less plate-to-plate variability than a faster substrate. For example, if you consider there could be a 15-30 second timing variation when adding stop reagent to all wells in a plate, an assay that is stopped

at 15 minutes could have more error than a similar assay that develops for 30-40 minutes. The slower development time could provide better within-plate and between-plate reproducibility. Also, an assay detecting a broad range of concentrations is best suited with a longer assay development time to ensure both ends develop signal.

However, sometimes process efficiency and fast timing are important design characteristics, and an assay that develops quickly is a necessity in order to process as many plates as possible. In this case an optimal choice might be TMB. Assay timing can be optimized by choosing a TMB substrate with the kinetic rate that allows your assay to be performed within the desired timeframe.

Summary

We have provided guidance for assay developers to aid in their choice of TMB substrate. Sensitivity and detection limit have been defined in this paper as a way to distinguish the important characteristics of each substrate. The range of TMB substrate choices will allow the developer to customize their assay based on its desired assay requirements. Key assay parameters, such as dynamic range, kinetic rate and assay timing, have been discussed as they relate to the different TMB substrate choices and can be used to make an informed decision. Each TMB substrate impacts these parameters differently. Choosing the best substrate for your assay will allow the developer to create a robust assay with superior performance.

References

1. Ekins, R. and Edwards, P. On the meaning of "sensitivity". *Clinical Chemistry* 1997; Vol. 43 Issue 10; pp 1824-1837.

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