Detection, Detection, Detection: Considerations for Enzyme/Substrate Selection in Immunoassay Development

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Introduction

Detection limit, dynamic range, and reproducibility are cornerstones in the development of a successful immunoassay application. During the optimization process, antibody/antigen systems are carefully chosen to provide the specificity and sensitivity of the desired analyte measurement. However, the choice of enzyme/substrate can also have a substantial effect on achieving the above-mentioned parameters and require equal attention for optimal selection. Often the most "sensitive" substrate is chosen without consideration of necessity or tradeoff with dynamic range and reproducibility. In order to give assay developers some guidance in the selection of detection methods, we have used horseradish peroxidase (HRP) and alkaline phosphatase (AP) conjugates of streptavidin to compare colorimetric (e.g. tetramethylbenzidine (TMB), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), para-Nitrophenylphosphate (pNPP)) and chemiluminescent (e.g. luminol and dioxetane) substrates in a simplified capture antibody assay. Using this model system, we have examined both the choice of detection enzyme as well as substrate to measure detection limit, dynamic range, and kinetics. The results indicate that the "fastest" substrate (e.g. "high sensitivity TMB") did not consistently have the best detection limit and was lacking in dynamic range. Chemiluminescent substrates did not show significantly better detection limits and dynamic range, as is often suggested by current literature. Surprisingly, substrates such as ABTS and our new developmental TMB (TMBX) gave both good detection limits as well as large dynamic ranges. Several substrates had linear kinetics over the assay development time, allowing for further optimization to get either better detection limits or dynamic range. Understanding the impact of these detection systems on the immunoassay application is essential and should always be a primary consideration for design.

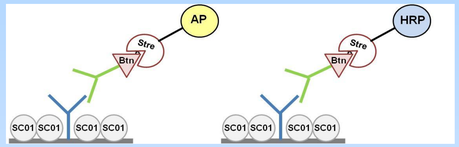
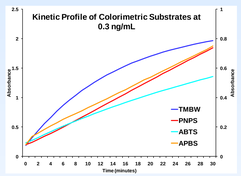


Table 1: SurModics BioFX® Substrates	Enzyme
TMB Super Sensitive HRP One Component (TMBS)	HRP
TMB HRP One Component (TMBW)	HRP
TMB Slow Kinetic HRP One Component (TMSK)	HRP
TMB Super Slow Kinetic HRP One Component (TTMB)	HRP
New TMB – In Development (TMBX)	HRP
2,2'-Axino-bis[3-ethylbenzothiazoline-6-sulfonic acid] HRP One Component (ABTS)	HRP
AP-Yellow One Component (PNPS)	AP
AP-Blue SpectraFX (APBS)	AP
Chemiluminescent Ultra Sensitive HRP (CHMI)	HRP
Chemiluminescent Sensitive Plus HRP (LERI)	HRP
Chemiluminescent Ultra Sensitive AP – 450 nm (APU4)	AP
Chemiluminescent Ultra Sensitive AP – 540 nm (APU5)	AP
Chemiluminescent Super Sensitive AP – 450 nm (APS4)	AP
Chemiluminescent Super Sensitive AP – 540 nm (APS5)	AP

Figure 1: Model Immunoassay System

Rabbit anti-mouse IgG (Jackson ImmunoResearch) was coated at 0.1µg/well on a 96-well ELISA plate (Nunc) and stabilized with StabilCoat® Stabilizer (SurModics, Inc.). Biotinylated mouse IgG (Jackson ImmunoResearch) was titrated in StabilZyme *SELECT*® Stabilizer (SurModics, Inc.) and incubated for 2 hours at room temperature. The plates were washed (3X PBS/Tween) and either streptavidin-peroxidase or streptavidin-alkaline phosphatase (Jackson ImmunoResearch) were diluted to 1 µg/mL in StabilZyme® AP Stabilizer or StabilZyme® HRP Stabilizer (SurModics, Inc.) respectively and incubated for 20 minutes at room temperature. After a final wash (6X PBS/Tween), the substrate was added and developed according to recommended protocols.

SUBSTRATE KINETICS



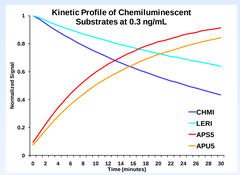


Figure 2: Kinetic Profile of Substrates

The signal generated by both colorimetric and chemiluminescent substrates for each enzyme was monitored for 30 minutes. The concentration chosen, 0.3 ng/mL (MsIgG) was within the dynamic range for all substrates. The most linear kinetic response was observed for the AP colorimetric substrates, PNPS and APBS (Table 1). When choosing a substrate, consideration to assay development timing and linearity should be made to determine the best substrate for your particular assay. Sometimes "faster" substrates are less desirable because linearity of response is lost for the assay's detection range.

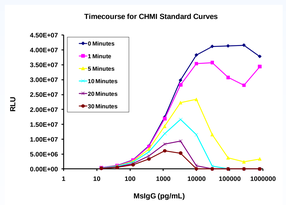


Figure #3: Kinetics of Chemiluminescent HRP Substrates

Standard curves were calculated at different time points for CHMI (Table 1) substrate. The rapid degradation of signal for the chemiluminescent HRP substrates (Fig. 2), especially at high HRP concentrations, caused oversaturation, limiting the measurable dynamic range. If it is possible to use these substrates as an immediate flash reading, saturation is less and allows for a larger dynamic range (see 0 time point). However, it is important to consider substrate addition time and plate read time (instrument specific) when optimizing an assay using chemiluminescent HRP substrates, especially if a large number of plates will be analyzed.

SENSITIVITY, DYNAMIC RANGE, AND DETECTION LIMITS

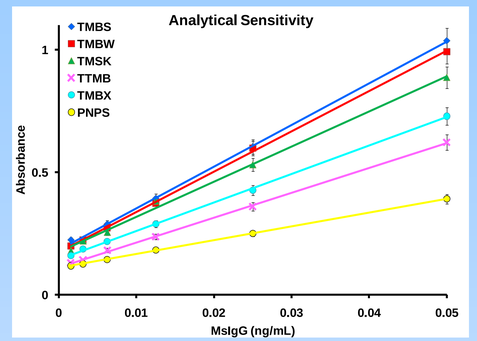


Table 2: Analytical Sensitivity		
Substrate	Slope (mOD/ng/mL)	r^2
TMBS (HRP)	17.0	0.9993
TMBW (HRP)	16.5	0.9995
TMSK (HRP)	14.4	0.9965
TTMB (HRP)	10.2	0.9995
TMBX (HRP)	11.6	0.9995
PNPS (AP)	5.7	0.9998

Figure 4: Lower Limit of Detection – Analytical Sensitivity

Linear graph of signal versus concentration (MsIgG) at the lower limits of detection for TMB and PNPS substrates. Analytical sensitivity is traditionally defined as the signal change per unit of concentration. Here TMBS and TMBW have the steepest slopes and therefore have the highest analytical sensitivity. Analytical sensitivity does not always mean better detection limits as observed in Table 3 where all these substrates have similar levels of detection. Some assays that have all samples close to the detection limit may benefit from a substrate choice with better analytical sensitivity.

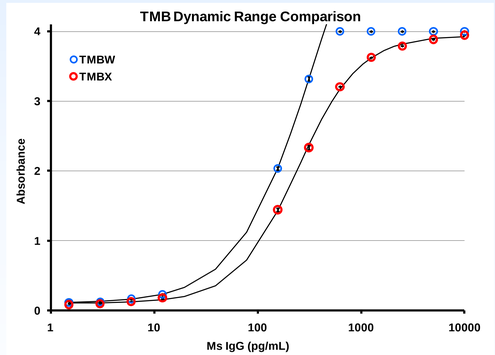


Figure 5: Dynamic Range Considerations

An example of a saturation curve for both TMBW and TMBX with their corresponding four parameter fits is shown. Dynamic range is an important consideration when measurement of a broad range of values is needed. While TMBX is a slower substrate, it provided the ability to quantify into the upper range of the assay while maintaining similar detection levels. TMBW is faster but "tops out" and the upper detection levels are lost. This was observed even at time points as short as 5 minutes. Depending on your assay's requirements, faster is not always better!

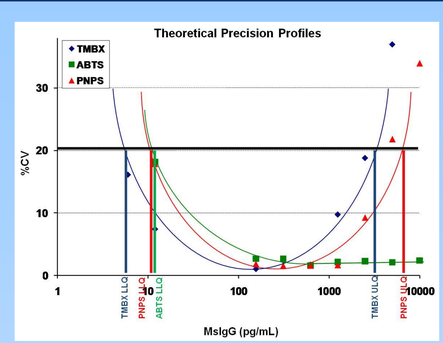


Figure 6: Theoretical Precision Profiles

Precision profiles were calculated assuming a perfect fit. The standard deviations in the absorbance measurements at each data point were used to back-calculate the resulting variation in concentration. This deviation was then used to calculate a coefficient of variation based on the theoretical concentration. LLQ (lower limit of quantitation) and ULQ (upper limit of quantitation) were illustrated at 20% CV for TMBX, PNPS, and ABTS (ULQ off scale). The combination of analytical sensitivity (Fig. 4) and standard error impact the quantitation limits for each substrate.

Substrate	Enzyme	Type	Detection Limit (pg/mL)	Time to reach detection limit (min.)	Dynamic Range (pg/mL)
TMBS	HRP	Colorimetric	3-6	5	3-320
TMBW	HRP	Colorimetric	3-6	15	3-320
TMSK	HRP	Colorimetric	3-6	30	6-625
TTMB	HRP	Colorimetric	3-6	30	6-625
TMBX	HRP	Colorimetric	3-6	30	6-2500
ABTS	HRP	Colorimetric	6-12	15	6->10,000
CHMI	HRP	Chemiluminescence	1-3	5	1-3000
LERI	HRP	Chemiluminescence	1-3	5	3-3000
PNPS	AP	Colorimetric	1-6	50-100	3-5000
APBS	AP	Colorimetric	1-3	15	3-625
APU4	AP	Chemiluminescence	3-6	20-30	3-10,000
APS4	AP	Chemiluminescence	3-6	20-30	3-10,000

Table 3: Experimentally Determine Detection Limits and Ranges for Substrates

The detection limit for each substrate was determined from experimental data using both Student's *t*-test and Tukey's multiple comparison. Values were determined from at least three separate experiments with n ≥ 4 for each substrate. Upper end of dynamic range was determined either by where the reading was saturated or where the precision profile indicated > 20% CV. Optimization of timing allows for selection of the dynamic range. The requirements of the assay should be considered when selecting the substrate. For example, if a low detection limit is the only requirement, the TMB reagents would provide this in a minimum amount of time. If larger dynamic range with good detection limits is needed, PNPS might be an excellent substrate.

Considerations When Choosing the Optimal Substrate

1. **Kinetics of both the enzyme and substrate – a faster enzyme/substrate does not always give better detection limits**
2. **Detection limit and analytical sensitivity are not always equivalent**
3. **Dynamic range is an important consideration – choosing a substrate with a large dynamic range does not always mean a significantly lower detection limit, e.g., TMBX, ABTS, and PNPS**
4. **Chemiluminescent substrates provide only slightly better detection limits and dynamic range than colorimetric substrates; the kinetics, especially of the HRP substrates, can cause difficulty with plate to plate variation and reproducibility.**

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