Introduction
Detection limit, dynamic range, and reproducibility are components in the development of a successful immunoassay application. During the optimization process, antibody/reagent systems are carefully chosen to provide the specificity and sensitivity of the desired assay. However, the choice of enzyme/substrate can also have a substantial effect on achieving the above-mentioned parameters and requires 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), 3,3′,5,5′-tetramethylbenzidine (TMB), p-nitrophenol phosphate (PNPS), and chemiluminescent (e.g., luminol and dioxetanes) substrates in a simplified capture antibody assay. Using this model system, we have examined both the choice of detection enzymes as well as substrate to measure detection limit, dynamic range, and kinetics. The results indicate that the “fastest” substrates (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 or our new developmental TMB (TMBW) give both good detection limits as well as large dynamic ranges. Several substrates had lower kinetics over the assay development time, allowing for further optimization to get either better detection limits or dynamic range. Understanding the impact of these selection decisions on the immunoassay application is essential and should always be a primary consideration for design.

Figure 1: Model Immunoassay System
Rabbi antibodies (Jackson ImmunoResearch) were coated at 0.1 μg/mL on a 96-well ELISA plate (Nunc) and stabilized with StabilCoat Stable (SurModics, Inc.). Blutser antibodies (Jackson ImmunoResearch) were diluted in StabilCoat SELECT™ Stabilizer (SurModics, Inc.) and incubated for 2 hours at room temperature. The plates were washed (3X PBS/Tween) and either streptavidin-peroxidase or alkaline phosphatase antibodies were diluted to 1 μg/mL in StabilDyn AP Stabilizer or StabilDyn HRP Stabilizer (SurModics, Inc.) and incubated for 20 minutes at room temperature. After a final wash (3X PBS/Tween), the substrate was added and developed according to recommended protocols.

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, PAPS and APBS (Table 1). When choosing a substrate, consider 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 CHM (Table 1) substrates. The rapid degradation of signal for the chemiluminescent HRP substrates (Fig. 2), especially at high HRP concentrations, can be 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 min 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.

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 TMB 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 2 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.

Table 2: Analytical Sensitivity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Component Type</th>
<th>TMB</th>
<th>TMBW</th>
<th>TMBX</th>
<th>PNPS</th>
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<tr>
<td>APs</td>
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<td>TMBX (HRP)</td>
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<td>PAPS (AP)</td>
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<td>0.989</td>
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</tbody>
</table>

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 titration range of values is needed. With TMBW as a slower substrate, it provided the ability to quantitate the upper range of the assay while maintaining similar detection levels. TMBX is faster but “crops 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!

Table 3: Experimentally Determined 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 > 30% 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 high detection limit is the only requirement, the TMBW reagents would provide this in a minimum amount of time. For larger dynamic range with good detection limits is needed, PNPS might be an excellent substrate.

Figure 6: Theoretical Precision Profiles
Precision profiles were calculated assuming a perfect fit. The standard deviation in the absorbance measurement at each data point were used to back calculate the resulting relative in concentration. This deviation was then used to calculate a coefficient of variation based on the theoretical concentration. LOQ (lower limit of quantitation) and LLOQ (upper limit of quantitation) were illustrated of 20% CV for TMBX, PAPS, and ABTS (LLOQ off scale). The combination of analytical sensitivity (Fig. 4) and standard error impact the quantitation limits for each 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.