Introduction

• Considerations for Substrate Choice
  – Kinetics
    • Colorimetric vs. Chemiluminescence
    • Optimization of Timing
    • HRP vs. AP
  – Dynamic Range
    • Role of Kinetics (Chemi.)
    • Limitations?
  – Sensitivity vs. Detectability
    • Definitions
    • Analytical sensitivity
    • Precision Profiles (upper and lower limits of quantitaion)
Model System

- Rabbit anti-mouse IgG coated at 0.1ug/well was used as the “capture antibody” and biotinylated mouse IgG as the “antigen”. Incubation with either streptavidin-peroxidase or streptavidin-alkaline phosphatase allowed each enzyme to be used interchangeably and with different substrates.
Kinetics – Colorimetric vs Chemiluminescent

- All Substrates are at a concentration within their dynamic range and are somewhat linear. 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.
Kinetics – Colorimetric

- **TMBW Kinetics**
  - Absorbance at 650nm
  - Time (minutes)
  - Graphs show absorbance over time for different concentrations.

- **ABTS Kinetics**
  - Absorbance at 405nm
  - Time (minutes)
  - Graphs show absorbance over time for different concentrations.

- **PNPS Kinetics**
  - Absorbance at 405nm
  - Graphs show absorbance over time for different concentrations.

© 2011 SurModics, Inc.
An example of a saturation curve for both TMBW and TMBX with their corresponding four parameter fits was shown. Dynamic range is an important consideration when measurement of a broad range of sample values is needed. While TMBX was a slower substrate, it provided the ability to quantify into the upper range of the assay while maintaining similar detection levels. TMBS was 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!
Kinetics - Chemiluminescence

LERI Signal Decay

CHMM Signal Decay

APS4

APU4

© 2011 SurModics, Inc.
Dynamic Range - Chemiluminescence

CHMM

LERI Response

APS4

APU4
Sensitivity vs. Detectability

Fig. 1.1. The different concepts of sensitivity (= $dR/dC$) and detectability (detection limit) are represented in frame 1. System (1) has a higher detectability (ability to detect small amounts) than system (2), whereas the latter is more sensitive (more responsive to slight changes in the concentration). The different concepts of precision (i.e., with its relative scatter; Section 15.1) and accuracy (i.e., conformity with theoretical results) are illustrated in frames 2–5 in which the broken lines show the theoretical relation and the dots with the solid lines represent the experimental results. Frame 2 shows high precision (small standard deviation) but low accuracy, 3 high precision and high accuracy, 4 has low precision and low accuracy, whereas 5 shows results having low precision but high accuracy.
Analytical Sensitivity

Linear graph of signal versus concentration (MslgG) 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 sample values close to the detection limit may benefit from a substrate choice with better analytical sensitivity.
Precision profiles were calculated assuming a perfect fit. The standard deviations in the absorbance measurements at each data point were used to calculate the resulting variation in the back calculated 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) was illustrated at 20% CV for TMBX, PNPS, and ABTS (ULQ off scale). The combination of analytical sensitivity and standard error impact the quantitation limits for each substrate.
## Detection Limit

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