The lack of predictive value of absolute measures in the design and control of lot release potency assays

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Controlling the Bioassay

- Potency assays require a high degree of control within and between runs for multiple reasons:
  1. They can exhibit a high degree of variability
  2. The comparison of a qualified reference standard to an internal control sample
  3. Complex statistical analyses must be performed to generate a reportable potency result
  4. Multiple critical reagents are often necessary
  5. The use of advanced equipment (robotics, plate readers, automated cell counters)

- Therefore, knowledge of what components or parameters of a potency assay to control is of critical importance.
Common Potency Assay Controls

- Parallelism (similarity)
- Response linearity – for parallel line models
- Internal control potency
- Goodness-of-fit (full curve) / Linear regression (parallel line)
- Signal-to-noise/Background response
- Replicate variability
- Cell viability, count, passage number
- Minimum data requirements (# of plates or consecutive concentration groups)
- Absolute reference standard responses ($IC_{50}$/$ED_{50}$, response slope, asymptote location)
Guidance for Reference Standard Controls

- There is no shortage of published literature on potency assays
  - Assay and cell line development, validation, determining parallelism

- Surprisingly, there is relatively little that directly addresses the control of the reference standard response

- Given the paucity of available guidance, a pressing question for the assay developer should be:
  How does one determine the aspects of the reference standard response to the active substance that must be controlled between runs?
Approaches to Reference Standard Control

Determining which of these parameters to use, alone or in combination, can be a daunting task.
Amgen has performed a series of studies to explore the utility of certain absolute reference standard controls in potency assays.

- These studies were designed to empirically determine the impact of variability in the reference standard on the precision and accuracy of multiple potency assay platforms.
  - Half maximal response
    - IC$_{50}$ in a Homogeneous Time Resolved Fluorescence (HTRF) ligand binding assay
    - ED$_{50}$ in a cell proliferation assay
  - Steepness (slope) of the dose-response curve (referred to as fold stimulation in following slides)
    - Reporter gene assay
    - Cell proliferation assay
  - Distance between the asymptotes
    - Ratio of the maximum to minimum (Max/Min) in the HTRF method
Impact of IC$_{50}$ on Assay Accuracy/Precision

- **Purpose:** To determine the impact of variability in the IC$_{50}$ response on assay accuracy and precision in a HTRF ligand binding potency assay.

- **Methods:**
  - The starting concentration of an 11-point full dose response curve was varied in the reference standard so that it was 3X, 2X, 1X, 0.5X, and 0.33X the highest concentration.
  - Reference standard was compared against itself at simulated potencies that spanned the validated range of the method (70%, 100%, 130%) in duplicate in 30 assays.
Experimental IC$_{50}$ values were obtained that greatly exceeded those seen in routine assays.

<table>
<thead>
<tr>
<th></th>
<th>Routine</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>205</td>
<td>30</td>
</tr>
<tr>
<td>Mean (ng/mL)</td>
<td>35</td>
<td>56</td>
</tr>
<tr>
<td>Range (ng/mL)</td>
<td>22 - 50</td>
<td>11 - 136</td>
</tr>
</tbody>
</table>
Dramatic shifts in the reference standard curve do not impact the potency results

<table>
<thead>
<tr>
<th>Starting Ref. Std. Concentration</th>
<th>Std. IC50 (ng/mL)</th>
<th>Potency Result – expected = 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.33X</td>
<td>123</td>
<td>96%</td>
</tr>
<tr>
<td>1X</td>
<td>33</td>
<td>99%</td>
</tr>
<tr>
<td>3X</td>
<td>12</td>
<td>99%</td>
</tr>
</tbody>
</table>
The accuracy and precision of an HTRF potency assay is not impacted by variability in the reference standard IC$_{50}$ value.

A regression analysis showed that the value of the relative potency result does not depend on the reference standard IC$_{50}$ ($p = 0.1885$).
Long-term trending of ED$_{50}$ in a cell proliferation bioassay – comparing the variability of absolute and relative measures of potency

- The ED$_{50}$ of the reference standard was trended over a 33 month period.
- Assay data was then used to generate relative measurements of the biological activity and results were compared to the absolute values.

Reference Standard ED$_{50}$ = 12.0 ng/mL

Relative Potency = Group 1 ED$_{50}$ / Group 2 ED$_{50}$

$= 12.0$ ng/mL / 11.9 ng/mL = 101%
The variability of the ED$_{50}$ response over time is much greater than a relative estimate of potency.

- The ED$_{50}$ results observed over the course of the 33 month trending period cover two thirds of the linear range of the method (1.56 – 25 ng/mL). Limits established using a 95% tolerance interval encompass the entire range (1.1 – 34.8 ng/mL).

<table>
<thead>
<tr>
<th></th>
<th>ED$_{50}$ (ng/mL)</th>
<th>Rel. Pot. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.82</td>
<td>1.05</td>
</tr>
<tr>
<td>Std. Dev.</td>
<td>5.09</td>
<td>0.09</td>
</tr>
<tr>
<td>% CV</td>
<td>65</td>
<td>9</td>
</tr>
</tbody>
</table>
There is no correlation between ED$_{50}$ and relative potency results. Relative potency plotted against ED$_{50}$ fails to show a strong correlation ($r = 0.3096$), suggesting that ED$_{50}$ is not a consistent indicator of reference standard activity.
Impact of Slope on Assay Accuracy and Precision

The graph depicts the reference standard response observed in three consecutive cell proliferation bioassays.

Two important questions are raised by these assays:

1. Should the lab be concerned about the difference in the absolute response of the reference standard?
2. If so, how does one determine the point at which assay capability is negatively affected?
Impact of Slope in a Reporter Gene Bioassay

- **Purpose:** To determine the impact of variability in the slope of the reference standard on assay accuracy and precision in a reporter gene bioassay.

- **Methods:**
  - Assay incubation time was varied from 1 to 5 hours after addition of all components to assay plates.
  - Reference standard was compared against itself at simulated potencies that spanned the validated range of the method in a full curve format.
  - Each time course experiment was performed 10 times for a total of 50 assays.
Assay incubation time is an effective means of modifying the dose-response slope in a reporter gene bioassay.

Fold stimulation is used as a surrogate for slope. It is calculated by dividing the average reference standard response at the highest point in the linear portion of the response by the average reference standard response at the lowest point in the linear portion of the response.
Response slope must be forced to a minimum before there is an impact to the accuracy and precision of the assay.

### Assay Accuracy and Precision as a Function of Fold Stimulation in the Reporter Gene Bioassay

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>1 Hour</th>
<th>2 Hours</th>
<th>3 Hours</th>
<th>4 Hours</th>
<th>5 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope/Fold Stimulation</td>
<td>1.1</td>
<td>2.0</td>
<td>3.3</td>
<td>4.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Theoretical Potency (%)</td>
<td>70 100 130</td>
<td>70 100 130</td>
<td>70 100 130</td>
<td>70 100 130</td>
<td>70 100 130</td>
</tr>
<tr>
<td>Actual Potency Average (%)</td>
<td>2999 170 248</td>
<td>67 97 128</td>
<td>69 101 133</td>
<td>71 100 130</td>
<td>70 99 136</td>
</tr>
<tr>
<td>% CV</td>
<td>300 145 118</td>
<td>12 20 13</td>
<td>8 13 7</td>
<td>10 9 7</td>
<td>8 10 8</td>
</tr>
</tbody>
</table>

![Graph showing Relative Potency vs Fold Stimulation](image)

- ▲ 1.30 Simulated Potency
- □ 1.00 Simulated Potency
- ○ 0.70 Simulated Potency
We further investigated the impact of Slope in an alamar Blue™ Cell Proliferation Assay

- **Purpose:** To determine the impact of variability in the slope of the reference standard on assay accuracy and precision in a cell proliferation bioassay.

- **Methods:** Cells were synchronized in different stages of the cell cycle with paclitaxel or aphidicolin pretreatment. Reference standard was run against itself at potencies that spanned the validated range of the method (65%, 100%, 135%) in 30 total assays.
Impact of Cell Synchronization on Reference Standard Response

- Aphidicolin pretreatment effectively decreased the slope of the biological response while paclitaxel pretreatment increased the slope.
- The untreated cell response was inconsistent and overlapped with the two treatment groups.
The proliferation assay is accurate and precise at all response slopes observed.

All individual potency results were within 15% of the expected value, regardless of the corresponding fold stimulation.

The same results are obtained with both a 4PL or parallel line analysis (mean potency difference = 0.24%).
Impact of Reference Standard Max/Min on Assay Accuracy and precision

- **Purpose:** To investigate the impact of variability in the reference standard maximum: minimum ratio on the accuracy and precision of a HTRF ligand binding potency assay.

- **Methods:**
  - The amount of a critical reagent necessary for fluorescence was added to the reaction at 1, 0.5, 0.2, 0.15, 0.1, 0.075, or 0.05 times the concentration qualified for the method. As a result, fluorescence output is limited and the max/min response decreases.
  - Reference standard was compared against itself at simulated potencies that spanned the validated range of the method (70%, 100%, 130%) in duplicate in 30 assays.
  - Relative potency was determined with a 4PL model and the relationship of max/min to assay accuracy/precision at each potency level was investigated.
Altering the concentration of a critical reagent was an effective means of intentionally varying the reference standard max/min response.

<table>
<thead>
<tr>
<th>Critical Reagent Concentration</th>
<th>Reference Standard Max/Min</th>
<th>Potency Result – expected = 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X</td>
<td>6.3</td>
<td>97%</td>
</tr>
<tr>
<td>0.2X</td>
<td>3.7</td>
<td>101%</td>
</tr>
<tr>
<td>0.15X</td>
<td>2.9</td>
<td>118%</td>
</tr>
</tbody>
</table>
The HTRF assay is accurate and precise when the Max/Min ratio exceeds 3.0

![Graph showing accuracy of relative potency](image)

### Experimental Results in Assays with Reference Standard Max/Min ≥ 3.0

<table>
<thead>
<tr>
<th>Expected Potency (%)</th>
<th>Mean</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>74.2</td>
<td>7.6</td>
</tr>
<tr>
<td>100</td>
<td>104.1</td>
<td>8.0</td>
</tr>
<tr>
<td>130</td>
<td>132.3</td>
<td>6.4</td>
</tr>
</tbody>
</table>
Results Summary

- IC50 is not predictive of assay capability in a HTRF potency method. Across a ten-fold range of IC50 results, assay accuracy and precision are maintained through the validated range of the method.

- ED50 exhibited much greater variability than a relative measurement of potency in a cell proliferation bioassay and is of no predictive value in monitoring activity in a product reference standard.

- Loss of assay accuracy and precision are observed in one of two potency assay platforms below a certain slope threshold.

- Assay capability is also lost below a minimum reference standard max/min response in the HTRF method.

- In the HTRF, reporter gene, and proliferation assay platforms, assay capability are maintained as the value of the absolute parameter of interest increased.
Conclusions

- In a full-curve relative potency assay, correlating potency to an absolute response of the reference standard is of no value due to the inherent variability when compared to relative measurements.

- Before including an absolute response as an acceptance criterion, one must test the limits of the assay, first to determine if it is predictive of assay capability and then to determine where to set the limit.

- Failure to systematically establish these limits will result in unnecessarily invalidating assays or releasing poor data.
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