SLAS2019 Tutorial: Coupling Assay Design And Process Optimization Toward Minimizing Variability

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Acknowledgment: Becky Kitchener, PhD
Tutorial Agenda

- Assay variability
- Liquid handler optimization: current practices
- Case study: model assay & findings
- Conclusions
OVERVIEW: ASSAY VARIABILITY
Parameters that Effect Assay Variability

- Temperature, humidity, light, vibration
- Viscosity, density, surface tension, volatility
- Mixing, incubation time, centrifugation, # liquid transfers, wash steps, dilution steps, serial dilutions
- Hardware / Labware
- ALH Settings
- Sample
- Detector
- Assay
- Plate type, tip type, tubing, automation
- Off-set volume, single/multi dispense, aspirate/dispense rate, aspirate/dispense height, liquid class, pre / post air gaps, etc.
- PMT, wavelength, x-y-z position
Assay Validation in a High-Throughput World

- Plate Uniformity
- Reagent Stability
- Signal Variability
- DMSO Compatibility
- Reaction Stability
So, What Does A “Good” Assay Look Like?

Let’s define a couple of parameters:

- First, let’s consider the assay variability
- Second, let’s consider the assay window

$$Z = 1 - \left( \frac{3\sigma_{\text{max}} + 3\sigma_{\text{min}}}{\mu_{\text{max}} - \mu_{\text{min}}} \right)$$

<table>
<thead>
<tr>
<th>Z-Factor Value</th>
<th>Assay Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ideal assay</td>
</tr>
<tr>
<td>1 &gt; Z ≥ 0.5</td>
<td>Excellent assay</td>
</tr>
<tr>
<td>0.5 &gt; Z &gt; 0</td>
<td>Marginal assay</td>
</tr>
<tr>
<td>0</td>
<td>Yes/no assay</td>
</tr>
<tr>
<td>&lt;0</td>
<td>Assay not useful</td>
</tr>
</tbody>
</table>
The Result

Z' = 0.74

Z' = 0.94
The Result

Useful assay descriptors:
- Hill slope
- Assay span
- Upper asymptote
- Lower asymptote
- Variability
OVERVIEW:
LIQUID HANDLER OPTIMIZATION
## Liquid Dispense Technologies

<table>
<thead>
<tr>
<th>Dispense Technology</th>
<th>Attributes</th>
</tr>
</thead>
</table>
| Air displacement        | • Problematic for volatile liquids  
                          | • Possible cross contamination  
                          | • Wide range of volumes     |
| Positive displacement   | • Useful for volatile solvents  
                          | • Cross contamination possible  
                          | • Wide range of volumes     |
| Droplet (acoustic)      | • Non-contact  
                          | • Useful for small volumes (pL – nL)                                     |
| Droplet (solenoid/inkjet)| • Useful for small volumes (nL)  
                          | • Sensitive to fluid types                                             |
| Capillary (pintool)     | • Useful for small volumes  
                          | • Direct contact with sample (contamination)  
                          | • Sensitive to fluid type                                             |
| Peristaltic             | • Useful for bulk dispense;  
                          | • More frequent calibrations needed                                     |
Parameters that Effect Volume Transfer

- **Hardware/labware** – plate format, tip types, tubing type
  - **ALH settings** – target (or off-set) volume, single/multi dispense, aspirate/dispense rate, aspirate/dispense height, liquid class, pre and post air gaps, accuracy/precision of volume transfer, transfer speed/time delays, on-board mixing
- **Assay** – reagent mixing, incubation, centrifugation, number of liquid transfers, wash steps, dilution steps, serial dilutions
- **Sample** – viscosity, density, surface tension, temperature, volatility
- **Environment** – temperature, humidity, light, vibration
Typical Liquid Handler Optimization

- Usually performed as a stand-alone activity
  - Precision is always checked, but accuracy is not as easy
- Several methods for volume verification
  - In-house (e.g., fluorescence, gravimetric, absorbance, etc.)
  - Commercial (e.g., dual-dye spectrophotometry)
- Volume verification is typically performed with ideal solutions
- Liquid handler is certified, calibrated, or repaired (if necessary)
- Then….someone programs ALH for assay use
  - Default method
  - Specific to basic assay requirements
Artel MVS: A Useful Tool

- Employs a dual-dye, dual-wavelength, ratiometric absorbance-based measurement method for calculating the dispense volume.

- How it works: dyes of known concentration are dispensed into well-characterized microtiter plate. The plate is mixed on a plate shaker to ensure solution homogeneity. Absorbance readings are taken at 520 nm and 730 nm.

\[
V_S = V_T \left( \frac{a_b}{a_r} \right) \left( \frac{A_{520}}{A_{730}} \right)
\]
ADOPTING A PROCESS OPTIMIZATION APPROACH
Sources of Assay Variability

**Biology**
- Diffusion
- Binding equilibrium
- Steric hindrance
- Cell population diversity
- Protein activity

**Environment**
- Temperature
- Light
- Humidity
- People

**Instrumentation**
- Liquid handlers
- Detectors
- Mixers
- Incubators
- Centrifuges

**Consumables**
- Reagents
- Tips
- Plate type
- Plate seals

What can we control or optimize?
Artel MVS is More Than a Calibration Tool

- Mixing
- Plate Evaluation
- Plate Washing
- Serial Dilutions
- Tip Evaluation
- Trouble-shooting

Calibrations
CASE STUDY
Assessing Effects of Liquid Handling on the Assay

- **Phase I**: Using a well-characterized model assay, develop and optimize the assay platform at bench scale.

- **Phase II**: Perform method transfer to ALH: examine effects of automated liquid handling parameters on the same assay.

- **Phase III**: “Deconstruct” the assay: decide which parameters, when altered, significantly vary assay outcome.
Streptavidin: Biotin-Fl Assay Principle

Streptavidin (SA) is a tetravalent biotin-binding protein that is isolated from *Streptomyces avidinii* and has a mass of 60.0 kDa. SA has a very high affinity for biotin ($K_d = 10^{-14}$ to $10^{-15}$ M).

**Assay Set-up**

- PBS, PBS+0.1%BSA, and PBS+0.1% glycerol
- Add 25 µL of biotin-4-fluorescein (Fl) to black 96w plate
- Add 25 µL of inhibitor
- Add 25 µL of streptavidin (SA)
- Incubate for 30 min at room temperature
- Read fluorescence: Ex = 485 nm and Em = 515 nm

## Assay Development Parameters Evaluated

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Manual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution stability</td>
<td>X</td>
</tr>
<tr>
<td>Plate type</td>
<td>X</td>
</tr>
<tr>
<td>Background buffer</td>
<td>X</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>X</td>
</tr>
<tr>
<td>Inhibitor conc.</td>
<td>X</td>
</tr>
<tr>
<td>Incubation time</td>
<td>X</td>
</tr>
<tr>
<td>Mixing</td>
<td>X</td>
</tr>
<tr>
<td>Pipette calibration</td>
<td></td>
</tr>
<tr>
<td>Asp/Disp rates</td>
<td></td>
</tr>
<tr>
<td>Fluid exit rate</td>
<td></td>
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<tr>
<td>Air gaps</td>
<td></td>
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</table>
Effect of Mixing on Assay Variability

Mixing was conducted by aspirating and dispensing 3 cycles after the third reagent was added. The plates were then incubated for 30 minutes at room temperature.

Key Takeaway
Buffer has an impact on mixing; Mixing improved BSA and glycerol buffers

PBS = phosphate buffered saline / BSA = PBS + 0.1% bovine serum albumin / GL = PBS + 0.1% glycerol
Effect of Buffer Type on Assay Performance

Key Takeaway
Manual mixing can behave differently than automated mixing, especially depending on assay buffer ingredients.
Effect of Source Plates on Automated Liquid Handler Verification

Artel MVS plates containing reagent from non-binding (A) and untreated (B) 96w black source plates.

MVS precision: 6.3% for non-binding and 1.2% for untreated

Key Takeaway
Source plate affected the destination plate – observed by visual inspection
Effect of Plate Type on Assay Variability

Black, 96-well plates used:
• Corning #3650 (Non-binding)
• Corning #3915 (Untreated)

**Key Takeaway**
When subjected to the assay, the uncoated plate yielded slightly lower variability.

PBS = phosphate buffered saline / BSA = PBS + 0.1% bovine serum albumin / GL = PBS + 0.1% glycerol
Effect of Reagent Temperature on Assay Variability

Key Takeaway
Cold reagent dispense yielded slightly lower potency

“Cold reagents” were stored at 4°C until use. The reagents were not maintained at 4°C during pipetting.

IC50 (µM)
- Cold Reagent Dispense: 0.311
- Room Temp Dispense: 0.235
Effect of Aspirate (ASP) Speed on Assay Performance

Key Takeaway
Aspirate speed affected potency for glycerol and BSA buffers

IC50 (µM)
- Aspirate 1 PBS: 0.157
- Aspirate 5 PBS: 0.163
- Aspirate 1 GL: 0.151
- Aspirate 5 GL: 0.225
- Aspirate 1 BSA: 0.145
- Aspirate 5 BSA: 0.207

PBS = phosphate buffered saline / BSA = PBS + 0.1% bovine serum albumin / GL = PBS + 0.1% glycerol
Effect of Dispense (DISP) Speed on Assay Performance

Key Takeaway
Dispense speed affected potency for glycerol but not PBS or BSA

**IC50 (µM)**
- Dispense 1 BSA: 0.268
- Dispense 5 BSA: 0.266
- Dispense 1 GL: 0.136
- Dispense 5 GL: 0.197
- Dispense 1 PBS: 0.164
- Dispense 5 PBS: 0.167

PBS = phosphate buffered saline / BSA = PBS + 0.1% bovine serum albumin / GL = PBS + 0.1% glycerol
Effect of Air Gap on Inhibitor Potency

Key Takeaway
Air gap affects the assay containing PBS and BSA buffers with respect to potency and variability

**IC50 (µM)**
- AirGap0BSA: 0.243
- AirGap2BSA: 0.269
- AirGap5BSA: 0.171
- AirGap0GL: 0.179
- AirGap2GL: 0.155
- AirGap5GL: 0.163
- AirGap0PBS: 0.172
- AirGap2PBS: 0.175
- AirGap5PBS: 0.205

PBS = phosphate buffered saline / BSA = PBS + 0.1% bovine serum albumin / GL = PBS + 0.1% glycerol
Effect of Air Gap on Assay Variability

PBS = phosphate buffered saline / BSA = PBS + 0.1% bovine serum albumin / GL = PBS + 0.1% glycerol
Study Summary

- Four assay parameters were identified which required optimization both during development at bench-scale and on the ALH.
  - Assay buffer selection
  - Mixing
  - Reagent stability
  - Plate type
- Certain ALH parameters were dependent on buffer type. Not all ALH parameters will effect an assay.
Conclusions

- Performing assay optimization AND liquid handler optimization together as a whole process reduces potential for error introduction and prolonged/difficult method transfer.

- Evaluate critical liquid handling parameters and potential sources of variability at bench scale and on the ALH for each new assay.

Assay optimization or LH qualification alone isn’t enough.
Putting It Together…

Assays depend on reagent concentrations

Reagent concentrations are volume-dependent

THEREFORE:
Assay integrity is dependent on accurate volume delivery

Assay results are impacted by liquid handling variability