Measurement Assurance in a Nanocytotoxicity Assay

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How do we improve confidence in alternative model measurements?

• Cellular measurements are complicated
  – Cell culture, extended periods, manual
  – Manual steps in setting up experiments
  – Multiple reagents
  – Instrumentation

• How do you prove measurement quality?
What can we do to increase confidence in the measurement

• Treat the assay as a measurement process
• Add process controls as evidence that the measurement process is proceeding as expected
• Adapt the “seven basic tools for quality” to cell assays
  – Cause and effect diagram
  – Check sheet
  – Control charts
  – Histogram
  – Pareto chart
  – Scatter diagram
  – Flow chart
The importance and challenge of nanotechnology risk assessment

• Nanotechnology is expected to have a massive commercial impact

• However, measuring their potential toxicological effects is challenging
  – Many of the standard methods for dissolved chemicals require nanoparticle-specific modifications
  – Nanoparticles may cause artifacts with many assays
  – There is a huge range of nanoparticles (different sizes, coatings, chemical compositions, etc.) to test
  – Prioritization is needed for screening the potential effects and in vitro methods have been suggested for this purpose
  – But, there are disagreements among laboratories on the cytotoxic effects of many nanoparticles
NIST Role in Nano-Environmental Health & Safety

National Nanotechnology Initiative 2011 Environmental Health and Safety Research Strategy
Identification and Avoidance of Potential Artifacts and Misinterpretations in Nanomaterial Ecotoxicity Measurements

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Use of Cause-and-Effect Analysis to Design a High-Quality Nanocytotoxicology Assay

- Seed cells - 24 h
- Treatment with nanoparticles and chemical control - 24 h
- Remove supernatant
- Treatment with MTS reagents - 1 h
- Absorption measurement at 490 nm with plate reader
Find sources of variability in assay

1. Cell-specific issues
   - Time before Assay
   - Handling
   - Passage Seed Density
   - Media
   - Culture Conditions
     - Temperature
     - Humidity
   - CO2 Incubator
   - Cell ID
   - Cell Growth
   - Plates
   - Manufacturer

2. Pipetting
   - Between Row
   - Between Columns
   - User-Technique
   - Gradients during Pipetting
   - Calibration
   - Tips

3. Instrument-specific issues
   - Curve fitting
   - Non Linearity
   - Stray light
   - Background
   - Signal

4. Chemical Control
   - Dosing
   - Solvent control
   - Solubility
   - Purity
   - Chemical Compound

5. Assay-specific issues
   - Prep to Prep
     - Freeze/Thaw Cycle
     - Reagent
     - Kit to Kit
     - Washing Step
     - Nano Particle Interference
     - Blank

6. Nanoparticle-specific issues
   - Dosing
     - Contaminants
     - Surface react.
     - Agglo/Aggre. exper. conditions
     - Chemical Composition
     - Particle Size
     - Morphology
     - Specific Surface
     - Surface "Chemistry"

Sources of Variability

Cause and effect diagram for MTS assay
Design a new plate format with process control measurements

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- **6 (8)-channel pipette**
- **Positive Chemical Ctrl**
- **ENM test**

**ENM concentration**
- BG indicates best guess of ED$_{50}$ value
- 0
- 0.05 (BG)
- 0.5 (BG)
- BG
- 2 (BG)
- 4 (BG)
Results

![Graph showing relative absorbance at 490 nm vs. concentration for PS-NP First Round and PS-NP Second Round.](image-url)
Results

![Bar chart showing Coefficient of Variation (CV) for different feature numbers in Figure 3. Features 1 and 2 show a higher coefficient of variation compared to other features. Features 3, 4, 6, and 7 have a lower coefficient of variation. The chart compares the 1st Round and 2nd Round results.](image-url)
Interlaboratory comparison

- 5 national metrology institutes were involved in the interlaboratory comparison
- Experimental design:
  - Share two A549 cell lines from ATCC and EMPA
  - Serum from local provider
  - Reagents from local provider
  - Serum and serum-free tests
  - Multiple replicates
  - Share nanoparticles (+ve PS) and chemical control (CdCl₂)
Dose Response Curves NP

A549 cell-1

Laboratory:
- Consensus
- A
- B
- C
- D

Serum free

Relative absorbance at 490 nm

0 25 50 75 100

concentration of NH₂-PS NP (μg/mL)

outlier

A

A549 cell-2

Laboratory:
- Consensus
- A
- B
- C
- D

Serum free

Relative absorbance at 490 nm

0 25 50 75 100

concentration of NH₂-PS NP (μg/mL)

B

C

D

Serum

Relative absorbance at 490 nm

0 25 50 75 100

concentration of NH₂-PS NP (μg/mL)
NP EC50 values

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td>Serum Free</td>
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<tr>
<td>Serum</td>
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Cell Line:
- A549 - A
- A549 - B

- Looks like harmonization between the laboratories
- No cell line differences
- The serum conditions increases variability
Let's look at the controls

- Chemical Process Control tests overall measurement system

Serum free conditions, variability less than with NP
Differences between cell lines
Cell line differences?

<table>
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<th>Medium volume (µm³)</th>
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<tr>
<td>2327±94</td>
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<tr>
<td>2047±90</td>
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Cell ID

A549-A  22.6±2.23  STR

A549-B  22.5±2.43  STR

• a. FAM dye
• b. NED dye
• c. PET dye
• d. VIC dye
How sensitive are we to cell seeding variability

- Correlation between no-treatment cells and NP EC50
- If outliers are removed, no strong correlation
- Suggests that within this range of cell seeding variability (OD=1.5-2.5) no big effect on EC50
Pipetting volumes and cells

Within pipette volume control

Within pipette cell control

Variability in pipetting volumes< variability in pipetting cells
**Specification of process controls:**

<table>
<thead>
<tr>
<th>Control</th>
<th>Serum free: target value</th>
<th>Serum free: range</th>
<th>Serum free: variability</th>
<th>Serum: target value</th>
<th>Serum: range</th>
<th>Serum: variability</th>
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</thead>
<tbody>
<tr>
<td>Control 1 (within) B6 – G6</td>
<td>1.8 OD</td>
<td>1.5-2.0 OD</td>
<td>&lt;10%</td>
<td>2.0 OD</td>
<td>1.8-2.3</td>
<td>&lt;7%</td>
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<td>Control 2 (between) B3-B6 B8-B10</td>
<td>1.5 OD</td>
<td>1.3-1.8 OD</td>
<td>&lt;12%</td>
<td>2.2 OD</td>
<td>1.8-2.8</td>
<td>&lt;7%</td>
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<tr>
<td>Control 3A Background B7-G7</td>
<td>0.06 OD</td>
<td>0.05-0.09 OD</td>
<td>&lt;6%</td>
<td>0.06 OD</td>
<td>0.05-0.09 OD</td>
<td>&lt;6%</td>
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<tr>
<td>Control 3B 1) Background Chemical Control B2-G2</td>
<td>0.06</td>
<td>0.05-0.09</td>
<td>&lt;6%</td>
<td>0.06</td>
<td>0.05-0.09</td>
<td>&lt;6%</td>
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<td>Control 3C 2) Background NP B11-G11</td>
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<td>Control 4 3) Chemical reaction control</td>
<td>49.9</td>
<td>47.5-51.5</td>
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<td>77.2</td>
<td>54.3-99.4</td>
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Conclusions:

• Interlab data with process controls presents a powerful view of a biological assay
• The findings regarding the sources of variability in this assay may be relevant for other cytotoxicity assays
• Check cell line ID. May affect controls and not test result
• The process used to quantify the sources of variability and generate test specifications can be used with other assays
• Meeting specifications provides evidence that the test procedure is as expected. “Accept test result”
• Adds Measurement Assurance to a Cell Assay
Collaborators

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