State of the Art High-throughput Approaches to Genotoxicity: Flow Micronucleus, Ames II, GreenScreen and Comet
June 28, 2012 EPA Computational Toxicology Communities of Practice

Dr. Marilyn J. Aardema Chief Scientific Advisor, Toxicology
Dr. Leon Stankowski Principal Scientist/Program Consultant
Ms. Kamala Pant Principal Scientist

Helping to bring your products from discovery to market
1. Introduction  
   *Marilyn Aardema 5 min*

2. In Vitro Flow Micronucleus Assay - 96 well  
   *Leon Stankowski, 10 min*

3. Ames II Assay  
   *Kamala Pant 10 min*

4. GreenScreen Assay  
   *Kamala Pant 10 min*

5. In Vitro Comet Assay - 96 well TK6 assay  
   *Kamala Pant 10 min*

6. Questions/Discussion  
   *15 min*
Genetic Toxicology Testing in Product Development

Discovery/Prioritization

Structure activity relationship analyses useful in very early lead identification

High throughput early screening assays

Lead Optimization

Screening versions of standard assays to predict results of GLP assays

GLP

Perform assays for regulatory submission according to regulatory guidelines

Gate

Additional supplemental tests to investigate mechanism and to help characterize human risk

GLP Gene Tox Battery

Follow-up assays to solve problems
High Throughput Genotoxicity Assays

- Faster
- Cheaper
- Uses Less Chemical/Drug
- Non-GLP
- Predictive of GLP assay/endpoint
- Mechanistic Studies (large number of conc./replicates)
- Automation
Example of Use of Genotoxicity Screening Assays: EPA ToxCast™

**Problem:** Tens of thousands of poorly characterized environmental chemicals

**Solution:** ToxCast™ – US EPA program intended to use:
- High throughput screening
- Genomics
- Computational chemistry and computational toxicology

To permit:
- Prediction of potential human toxicity
- Prioritization of limited testing resources

[www.epa.gov/ncct/toxcast](http://www.epa.gov/ncct/toxcast)
BioReliance EPA ToxCast Award July 15, 2011

• Assays
  – In vitro flow MN
  – In vitro Comet
  – Ames II
  – GreenScreen

• 25 chemicals of known genotoxicity to evaluate the process/assays (April-June 2012)
  – In vitro flow MN
  – In vitro Comet
  – Ames II
1. Introduction  
   *Marilyn Aardema 5 min*

2. In Vitro Flow Micronucleus Assay - 96 well  
   *Leon Stankowski, 10 min*

3. Ames II Assay  
   *Kamala Pant 10 min*

4. GreenScreen Assay  
   *Kamala Pant 10 min*

5. In Vitro Comet Assay - 96 well TK6 assay  
   *Kamala Pant 10 min*

6. Questions/Discussion  
   *15 min*
In Vitro Micronucleus Assay
OECD TG 487 (approved July 2010)

- Alternative to chromosome aberration assay
- Comparatively easy to evaluate
- Experimental design nearly same, but typically use cytochalasin B (cytokinesis inhibitor)
- Nuclei divide in presence of cytochalasin B, but not cytoplasm (ensuring cell division)
Mechanisms of Micronucleus Formation

Double Strand Breaks

Mitotic Cells

i.e. Clastogens

Daughter Cells

Spindle Fiber Dysfunction

i.e. Aneugens
Cytotoxicity: Cytokinesis-Block Proliferation Index (CBPI) method

\[
CBPI = 1 \times \text{No. of Mononucleated cells} + 2 \times \text{No. of Binucleated cells} + 3 \times \text{No. of Multinucleated cells} \quad \text{Total number of Cells}
\]

At least 1000 cells/dose level analyzed for CBPI, and 2000 binucleated cells/dose level for MN induction
In Vitro Micronucleus Assay Manual Scoring
ToxCast Experimental Design

- 96-well format
- no cytochalasin B
  - test article @ 9 dose levels
  - 3 positive controls @ 2 dose levels
  - 1 vehicle control
  - four test articles per plate with or without metabolic activation (S9)
  - limit dose (~200 µM) or 40 – 60% relative survival
  - 5000 cells analyzed per well
  - all in duplicate cultures
Experimental Design (ToxCast)

Treatment similar to typical but harvesting/scoring is different

- Remove media
- Place culture dish on ice (20 min)
- EMA (ethidium monoazide bromide) photoactivate on ice
- Lysis solution 1 (SYTOX green + detergent + RNase)
- Lysis solution 2 (SYTOX green + latex counting beads)
- Analyze (within 3 – 7 days)
Flow Cytometric Scoring

Cells at Time of Harvest

<table>
<thead>
<tr>
<th>Healthy</th>
<th>Micronucleated</th>
<th>Necrotic</th>
<th>Apoptotic</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Healthy Cell" /></td>
<td><img src="image2" alt="Micronucleated Cell" /></td>
<td><img src="image3" alt="Necrotic Cell" /></td>
<td><img src="image4" alt="Apoptotic Cell" /></td>
</tr>
</tbody>
</table>

**Add Nucleic Acid Dye A (stains red)**

Identifies Dead/Dying Cells

- Dead and Dying Cells

**Add Nucleic Acid Dye B (stains green)**

Labels Nuclei and Micronuclei

- Double Positives are Gated Out

Courtesy of Litron Laboratories
Flow Cytometric Scoring

BD FACS Canto II

Flow Cell
Results

Mitomycin C (Clastogen)

Vehicle control

Vinblastine (aneugen)
96-Well Flow MN Validation

“OECD 10” from TG 487

• Clastogens not requiring S9
  – araC
  – MMC

• Clastogens requiring S9
  – B(a)P
  – CP

• Aneugens
  – COL
  – VB

• Negative substances
  – DEHP
  – NAL
  – PYR
  – NaCl
### 96-Well Flow MN Validation

<table>
<thead>
<tr>
<th>Substance</th>
<th>Condition</th>
<th>DEHP</th>
<th>NaCl</th>
<th>CP</th>
<th>MMC</th>
<th>NaCl</th>
<th>DEHP</th>
<th>CP</th>
<th>MMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC</td>
<td>-S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>-S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEHP</td>
<td>-S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>-S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMC</td>
<td>+S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>+S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEHP</td>
<td>+S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>+S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: The table above shows the results of 96-well flow MN validation for different substances (DEHP, NaCl, CP, MMC) under different conditions (with and without S9). The data is presented in a grid format.*

*BioReliance*
96-Well Flow MN Validation

<table>
<thead>
<tr>
<th>Test</th>
<th>Article</th>
<th>S9</th>
<th>Dose Level</th>
<th>Trial</th>
<th>fold-increase MN</th>
<th>fold-increase Hypodiploid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>+</td>
<td>0.00</td>
<td></td>
<td></td>
<td>0.77</td>
<td>0.94</td>
</tr>
<tr>
<td>Vehicle</td>
<td>+</td>
<td>0.00</td>
<td></td>
<td></td>
<td>0.81</td>
<td>1.12</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>0.156</td>
<td></td>
<td></td>
<td>1.02</td>
<td>1.12</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>0.313</td>
<td></td>
<td></td>
<td>0.92</td>
<td>1.62</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>0.625</td>
<td></td>
<td></td>
<td>1.31</td>
<td>1.91</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>1.25</td>
<td></td>
<td></td>
<td>1.98</td>
<td>3.24</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>2.50</td>
<td></td>
<td></td>
<td>3.06</td>
<td>5.48</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>5.00</td>
<td></td>
<td></td>
<td>3.62</td>
<td>6.67</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>10.0</td>
<td></td>
<td></td>
<td>3.71</td>
<td>6.95</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>20.0</td>
<td></td>
<td></td>
<td>2.98</td>
<td>4.79</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>40.0</td>
<td></td>
<td></td>
<td>1.40</td>
<td>2.20</td>
</tr>
<tr>
<td>CP</td>
<td>+</td>
<td>80.0</td>
<td></td>
<td></td>
<td>1.02</td>
<td>1.02</td>
</tr>
</tbody>
</table>

- Positive response (≥3-fold increase in MN, or ≥10-fold increase in hypodiploidy)
- Excessive cytotoxicity (<40% relative survival)
### 96-Well Flow MN Validation

<table>
<thead>
<tr>
<th>Test</th>
<th>S9</th>
<th>Dose Level</th>
<th>fold-increase MN</th>
<th>fold-increase Hypodiploid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1    2    3    4    5    6    7    8</td>
<td>1    2    3    4    5    6    7    8</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>0.00</td>
<td>0.86 0.85 1.10 0.99 0.99 0.96 0.98 0.94</td>
<td>1.06 0.36 1.09 0.78 1.19 0.87 0.37 0.69</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>0.00</td>
<td>0.96 1.04 0.91 0.99 1.05 0.98 0.95 0.84</td>
<td>1.51 0.45 1.03 1.04 0.98 0.94 0.35 0.90</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.00195</td>
<td>0.80 1.20 1.02 1.12 1.07 1.01 1.04 1.35</td>
<td>1.11 0.35 0.60 0.72 1.06 0.80 0.34 0.99</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.00391</td>
<td>0.88 1.33 1.24 1.28 1.07 1.22 0.98 1.26</td>
<td>1.00 0.31 1.14 0.97 1.04 1.02 0.31 0.76</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.00781</td>
<td>1.17 1.50 1.61 1.41 1.28 1.09 1.01 1.48</td>
<td>0.93 0.31 1.04 0.71 1.24 0.89 0.35 0.73</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.0156</td>
<td>1.29 1.66 1.61 1.49 1.25 1.33 1.45 1.61</td>
<td>0.94 0.25 0.94 1.03 1.02 0.90 0.29 0.73</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.0313</td>
<td>1.68 1.98 1.94 1.91 1.57 1.78 1.30 2.45</td>
<td>0.95 0.33 0.89 1.03 0.94 0.97 0.31 0.98</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.0625</td>
<td>2.32 2.41 2.85 2.53 2.04 1.99 2.04 2.74</td>
<td>1.06 0.31 1.00 1.12 1.29 1.28 0.32 1.07</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.125</td>
<td>2.52 3.35 3.43 3.77 2.85 2.98 2.90 3.74</td>
<td>1.01 0.33 1.31 1.25 1.17 1.16 0.41 1.10</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.250</td>
<td>2.91 4.26 4.38 4.85 3.84 3.08 3.61 4.42</td>
<td>1.17 0.45 1.78 1.67 1.41 1.63 0.52 1.28</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>0.500</td>
<td>3.04 4.68 4.02 4.68 3.84 3.93 3.97 5.16</td>
<td>1.34 0.54 2.42 2.22 1.66 1.85 0.58 1.96</td>
</tr>
<tr>
<td>MMC</td>
<td>-</td>
<td>1.00</td>
<td>5.20 4.97 5.31</td>
<td>4.56 4.97</td>
</tr>
</tbody>
</table>

- Positive response (≥3-fold increase in MN, or ≥10-fold increase in hypodiploidy)
- Excessive cytotoxicity (<40% relative survival)
## 96-Well Flow MN Validation

<table>
<thead>
<tr>
<th></th>
<th>%MN</th>
<th>%Hypo</th>
</tr>
</thead>
<tbody>
<tr>
<td>-S9:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>795</td>
<td>795</td>
</tr>
<tr>
<td>average</td>
<td>1.76</td>
<td>0.73</td>
</tr>
<tr>
<td>SD</td>
<td>0.54</td>
<td>0.28</td>
</tr>
<tr>
<td>min</td>
<td>0.40</td>
<td>0.12</td>
</tr>
<tr>
<td>max</td>
<td>4.20</td>
<td>2.10</td>
</tr>
<tr>
<td>95% UCL</td>
<td>2.85</td>
<td>1.28</td>
</tr>
<tr>
<td>+S9:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>801</td>
<td>801</td>
</tr>
<tr>
<td>average</td>
<td>1.46</td>
<td>0.31</td>
</tr>
<tr>
<td>SD</td>
<td>0.49</td>
<td>0.15</td>
</tr>
<tr>
<td>min</td>
<td>0.50</td>
<td>0.08</td>
</tr>
<tr>
<td>max</td>
<td>5.60</td>
<td>2.96</td>
</tr>
<tr>
<td>95% UCL</td>
<td>2.44</td>
<td>0.61</td>
</tr>
</tbody>
</table>
## Results

<table>
<thead>
<tr>
<th>Wells</th>
<th>Conc. (µM)</th>
<th>Nucleated #Events</th>
<th>Beads and Nuclei to MN #Events</th>
<th>MN #Events</th>
<th>%Parent</th>
<th>Hypo #Events</th>
<th>%Parent</th>
<th>Relative Survival (%)</th>
<th>MN fold increase</th>
<th>Hypo fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.00</td>
<td>5000</td>
<td>230</td>
<td>21.9</td>
<td>44</td>
<td>0.85</td>
<td>14</td>
<td>0.27</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>A3 B3</td>
<td>0.781</td>
<td>5000</td>
<td>207</td>
<td>24.2</td>
<td>40</td>
<td>0.80</td>
<td>16</td>
<td>0.32</td>
<td>111</td>
<td>0.94</td>
</tr>
<tr>
<td>A4 B4</td>
<td>1.56</td>
<td>5000</td>
<td>223</td>
<td>22.5</td>
<td>41</td>
<td>0.80</td>
<td>15</td>
<td>0.29</td>
<td>103</td>
<td>0.94</td>
</tr>
<tr>
<td>A5 B5</td>
<td>3.13</td>
<td>5000</td>
<td>172</td>
<td>29.1</td>
<td>47</td>
<td>0.90</td>
<td>15</td>
<td>0.29</td>
<td>133</td>
<td>1.06</td>
</tr>
<tr>
<td>A6 B6</td>
<td>6.25</td>
<td>5000</td>
<td>193</td>
<td>26.0</td>
<td>44</td>
<td>0.85</td>
<td>17</td>
<td>0.34</td>
<td>119</td>
<td>1.00</td>
</tr>
<tr>
<td>A7 B7</td>
<td>12.5</td>
<td>5000</td>
<td>233</td>
<td>21.5</td>
<td>54</td>
<td>1.05</td>
<td>17</td>
<td>0.33</td>
<td>98</td>
<td>1.24</td>
</tr>
<tr>
<td>A8 B8</td>
<td>25.0</td>
<td>5000</td>
<td>199</td>
<td>25.2</td>
<td>46</td>
<td>0.90</td>
<td>14</td>
<td>0.27</td>
<td>115</td>
<td>1.06</td>
</tr>
<tr>
<td>A9 B9</td>
<td>50.0</td>
<td>5000</td>
<td>260</td>
<td>19.2</td>
<td>47</td>
<td>0.90</td>
<td>14</td>
<td>0.28</td>
<td>88</td>
<td>1.06</td>
</tr>
<tr>
<td>A10 B10</td>
<td>100</td>
<td>5000</td>
<td>265</td>
<td>18.9</td>
<td>43</td>
<td>0.85</td>
<td>18</td>
<td>0.35</td>
<td>86</td>
<td>1.00</td>
</tr>
<tr>
<td>A11 B11</td>
<td>200</td>
<td>5000</td>
<td>269</td>
<td>18.6</td>
<td>62</td>
<td>1.20</td>
<td>24</td>
<td>0.47</td>
<td>85</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Non-toxic and negative

*For Illustration Purposes*
## Results

**TX011587 -S9**

<table>
<thead>
<tr>
<th>Wells</th>
<th>Conc. (µM)</th>
<th>Nucleated Beads and Nuclei to MN #Events</th>
<th>MN Conc. %Parent</th>
<th>Hypo-Conc. %Parent</th>
<th>Relative Survival (%)</th>
<th>MN fold increase</th>
<th>Hypo fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.00</td>
<td>5000 230 21.9 44 0.85 14 0.27</td>
<td>100</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3 H3</td>
<td>0.797</td>
<td>5000 214 23.4 52 1.00 15 0.29</td>
<td>107</td>
<td>1.18</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4 H4</td>
<td>1.59</td>
<td>5000 184 27.2 55 1.05 14 0.27</td>
<td>125</td>
<td>1.24</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G5 H5</td>
<td>3.19</td>
<td>5000 212 23.6 50 1.00 16 0.31</td>
<td>108</td>
<td>1.18</td>
<td>1.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G6 H6</td>
<td>6.38</td>
<td>5000 207 24.2 82 1.55 19 0.36</td>
<td>111</td>
<td>1.82</td>
<td>1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G7 H7</td>
<td>12.8</td>
<td>5000 213 23.5 77 1.50 24 0.46</td>
<td>108</td>
<td>1.76</td>
<td>1.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G8 H8</td>
<td>25.5</td>
<td>5000 302 16.6 60 1.20 27 0.53</td>
<td>76</td>
<td>1.41</td>
<td>1.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G9 H9</td>
<td>51.0</td>
<td>5000 324 15.5 52 1.00 25 0.49</td>
<td>71</td>
<td>1.18</td>
<td>1.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G10 H10</td>
<td>102</td>
<td>5000 526 9.5 51 1.00 19 0.37</td>
<td>44</td>
<td>1.18</td>
<td>1.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G11 H11</td>
<td>204</td>
<td>65 478 0.1 1 0.85 1 0.76</td>
<td>1</td>
<td>1.18</td>
<td>1.38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cytotoxic and negative
## Results

### Aneugenic signature

<table>
<thead>
<tr>
<th>Wells</th>
<th>Conc. (µM)</th>
<th>Nucleated Beads</th>
<th>Beads and Nuclei to MN</th>
<th>MN %Parent</th>
<th>Fold Increase</th>
<th>Relative Survival (%)</th>
<th>MN</th>
<th>Hypo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.00</td>
<td>5000</td>
<td>230</td>
<td>21.9</td>
<td>44</td>
<td>0.85</td>
<td>14</td>
<td>0.27</td>
</tr>
<tr>
<td>C3 D3</td>
<td>0.916</td>
<td>5000</td>
<td>205</td>
<td>24.4</td>
<td>53</td>
<td>1.05</td>
<td>20</td>
<td>0.38</td>
</tr>
<tr>
<td>C4 D4</td>
<td>1.83</td>
<td>5000</td>
<td>174</td>
<td>28.8</td>
<td>44</td>
<td>0.85</td>
<td>19</td>
<td>0.38</td>
</tr>
<tr>
<td>C5 D5</td>
<td>3.66</td>
<td>5000</td>
<td>196</td>
<td>25.5</td>
<td>47</td>
<td>0.95</td>
<td>20</td>
<td>0.39</td>
</tr>
<tr>
<td>C6 D6</td>
<td>7.33</td>
<td>5000</td>
<td>156</td>
<td>32.2</td>
<td>52</td>
<td>1.05</td>
<td>27</td>
<td>0.53</td>
</tr>
<tr>
<td>C7 D7</td>
<td>14.7</td>
<td>5000</td>
<td>212</td>
<td>23.6</td>
<td>84</td>
<td>1.65</td>
<td>42</td>
<td>0.82</td>
</tr>
<tr>
<td>C8 D8</td>
<td>29.3</td>
<td>5000</td>
<td>251</td>
<td>19.9</td>
<td>127</td>
<td>2.35</td>
<td>150</td>
<td>2.84</td>
</tr>
<tr>
<td>C9 D9</td>
<td>58.6</td>
<td>5000</td>
<td>302</td>
<td>16.6</td>
<td>369</td>
<td>5.70</td>
<td>651</td>
<td>10.81</td>
</tr>
<tr>
<td>C10 D10</td>
<td>117</td>
<td>5000</td>
<td>539</td>
<td>9.3</td>
<td>101</td>
<td>1.95</td>
<td>32</td>
<td>0.62</td>
</tr>
<tr>
<td>C11 D11</td>
<td>234</td>
<td>29</td>
<td>497</td>
<td>0.1</td>
<td>20</td>
<td>36.75</td>
<td>2</td>
<td>3.00</td>
</tr>
</tbody>
</table>

For Illustration Purposes
# Results

**TX003211**  +S9

<table>
<thead>
<tr>
<th>Wells</th>
<th>Conc. #Events</th>
<th>Beads and Nuclei to MN</th>
<th>Nucleated Beads and Nuclei</th>
<th>MN #Events</th>
<th>MN %Parent</th>
<th>Hypo #Events</th>
<th>Hypo %Parent</th>
<th>Relative Survival (%)</th>
<th>fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.00</td>
<td>4766</td>
<td>538</td>
<td>8.9</td>
<td>92</td>
<td>1.88</td>
<td>18</td>
<td>0.37</td>
<td>100</td>
</tr>
<tr>
<td>A3 B3</td>
<td>0.781</td>
<td>5000</td>
<td>532</td>
<td>9.4</td>
<td>113</td>
<td>2.15</td>
<td>19</td>
<td>0.36</td>
<td>106</td>
</tr>
<tr>
<td>A4 B4</td>
<td>1.56</td>
<td>5000</td>
<td>492</td>
<td>10.2</td>
<td>101</td>
<td>1.95</td>
<td>22</td>
<td>0.43</td>
<td>114</td>
</tr>
<tr>
<td>A5 B5</td>
<td>3.13</td>
<td>5000</td>
<td>471</td>
<td>10.6</td>
<td>120</td>
<td>2.30</td>
<td>20</td>
<td>0.39</td>
<td>119</td>
</tr>
<tr>
<td>A6 B6</td>
<td>6.25</td>
<td>5000</td>
<td>492</td>
<td>10.2</td>
<td>129</td>
<td>2.50</td>
<td>16</td>
<td>0.30</td>
<td>114</td>
</tr>
<tr>
<td>A7 B7</td>
<td>12.5</td>
<td>5000</td>
<td>578</td>
<td>8.7</td>
<td>170</td>
<td>3.25</td>
<td>20</td>
<td>0.39</td>
<td>97</td>
</tr>
<tr>
<td>A8 B8</td>
<td>25.0</td>
<td>5000</td>
<td>568</td>
<td>8.8</td>
<td>227</td>
<td>4.25</td>
<td>27</td>
<td>0.51</td>
<td>99</td>
</tr>
<tr>
<td>A9 B9</td>
<td>50.0</td>
<td>5000</td>
<td>577</td>
<td>8.7</td>
<td>412</td>
<td>7.45</td>
<td>39</td>
<td>0.71</td>
<td>97</td>
</tr>
<tr>
<td>A10 B10</td>
<td>100</td>
<td>5000</td>
<td>682</td>
<td>7.3</td>
<td>504</td>
<td>8.90</td>
<td>43</td>
<td>0.78</td>
<td>82</td>
</tr>
<tr>
<td>A11 B11</td>
<td>200</td>
<td>3561</td>
<td>919</td>
<td>3.9</td>
<td>402</td>
<td>9.85</td>
<td>49</td>
<td>1.21</td>
<td>43</td>
</tr>
</tbody>
</table>

For Illustration Purposes

**Clastogenic signature**
## Results

<table>
<thead>
<tr>
<th>Wells</th>
<th>Conc. (µM)</th>
<th>Nucleated #Events</th>
<th>Beads and P4 #Events</th>
<th>Nuclei to MN #Events</th>
<th>MN %Parent</th>
<th>Hypo #Events</th>
<th>Hypo %Parent</th>
<th>Relative Survival (%)</th>
<th>MN Fold Increase</th>
<th>Hypo Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.00</td>
<td>5000</td>
<td>152</td>
<td>33.2</td>
<td>123</td>
<td>2.36</td>
<td>31</td>
<td>0.61</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>C3 D3</td>
<td>0.781</td>
<td>5000</td>
<td>161</td>
<td>31.2</td>
<td>107</td>
<td>2.05</td>
<td>32</td>
<td>0.61</td>
<td>94</td>
<td>0.87</td>
</tr>
<tr>
<td>C4 D4</td>
<td>1.56</td>
<td>5000</td>
<td>147</td>
<td>34.0</td>
<td>115</td>
<td>2.20</td>
<td>40</td>
<td>0.77</td>
<td>102</td>
<td>0.93</td>
</tr>
<tr>
<td>C5 D5</td>
<td>3.13</td>
<td>5000</td>
<td>167</td>
<td>30.0</td>
<td>105</td>
<td>2.00</td>
<td>32</td>
<td>0.61</td>
<td>90</td>
<td>0.85</td>
</tr>
<tr>
<td>C6 D6</td>
<td>6.25</td>
<td>5000</td>
<td>143</td>
<td>35.1</td>
<td>124</td>
<td>2.40</td>
<td>36</td>
<td>0.70</td>
<td>106</td>
<td>1.02</td>
</tr>
<tr>
<td>C7 D7</td>
<td>12.5</td>
<td>5000</td>
<td>162</td>
<td>30.9</td>
<td>131</td>
<td>2.50</td>
<td>30</td>
<td>0.58</td>
<td>93</td>
<td>1.06</td>
</tr>
<tr>
<td>C8 D8</td>
<td>25.0</td>
<td>5000</td>
<td>182</td>
<td>27.5</td>
<td>195</td>
<td>3.70</td>
<td>41</td>
<td>0.78</td>
<td>83</td>
<td>1.57</td>
</tr>
<tr>
<td>C9 D9</td>
<td>50</td>
<td>1744</td>
<td>304</td>
<td>5.7</td>
<td>1227</td>
<td>36.80</td>
<td>142</td>
<td>4.56</td>
<td>17</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>C10 D10</td>
<td>100</td>
<td>10</td>
<td>300</td>
<td>0.0</td>
<td>4</td>
<td>25.45</td>
<td>1</td>
<td>3.70</td>
<td>0</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>C11 D11</td>
<td>200</td>
<td>1</td>
<td>328</td>
<td>0.0</td>
<td>1</td>
<td>50.00</td>
<td>1</td>
<td>33.33</td>
<td>0</td>
<td>Cytotoxic</td>
</tr>
</tbody>
</table>

Inconclusive
1. Introduction  
   *Marilyn Aardema 5 min*

2. In Vitro Flow Micronucleus Assay - 96 well  
   *Leon Stankowski, 10 min*

3. Genetic Toxicology Screening Assays  
   a) Ames II Assay  
      *Kamala Pant 10 min*
   b) GreenScreen Assay  
      *Kamala Pant 10 min*
   c) In Vitro Comet Assay - 96 well TK6 assay  
      *Kamala Pant 10 min*

6. Questions/Discussion  
   *15 min*
Rationale for using these Screening Assays

Ames II assay is liquid based Ames assay and can be automated.

- Treatment in 24-well plates and dispense and growth in 384-well plates, well suited for high throughput screening.

GreenScreen Assay is performed in 96-well plates and depending on the number of concentrations tested either 4 or 12 test articles per 96-well plate can be tested. Thus making this also a high throughput assay.

In Vitro Comet Assay – although the assay can be performed in the 96-well plate format, it is not really a high throughput screening assay since a huge number of slides have to be scored.
Non-GLP Assays can be used at early stages in drug discovery to select chemical candidates for further development.

Early screening assay advantages include:
- Low cost
- Rapid turn-around time
- Require minimal amounts of test articles
- Can be highly predictive

Customized design based on available sample
- Caution: should mimic GLP study as closely as possible to provide as good a correlation with the GLP study as possible
Screening Genetic Toxicology Assays

- Miniaturized “standard assays”
  - Bacterial Mutation Assays
    - Ames II
  - “New” assays using engineered cells or “upstream” signals

In-Vitro COMET Assay

- GreenScreen Assay
Topics for Discussion

• Screening assays
  – Ames II
  – GreenScreen HC assay with and without activation
  – In-Vitro Comet assay

• Review protocols
  – Design and methodology

• Advantages and Limitations of each assay (if any)
1. Introduction
   *Marilyn Aardema 5 min*

2. In Vitro Flow Micronucleus Assay - 96 well
   *Leon Stankowski, 10 min*

3. Genetic Toxicology Screening Assays
   a) *Ames II Assay*
      *Kamala Pant 10 min*
   b) *GreenScreen Assay*
      *Kamala Pant 10 min*
   c) *In Vitro Comet Assay - 96 well TK6 assay*
      *Kamala Pant 10 min*

6. Questions/Discussion
   *15 min*
Ames II™ Assay

• Ames II™ – Screening version of the Ames assay
• Strains engineered for base-pair mutagens (TA7001 to TA7006)
• Standard TA-98 strain for frameshift mutagens
• University of California Berkley/Xenometrix technology licensed to BioReliance
  – Exclusive license in US and Japan
• Automated plating system can be used
• 2 to 5 mg test article needed
Ames II™ Mutagenicity Assay

Ames II Assay

Ames II Salmonella unable to make histidine

DNA damaging agent

Salmonella regain ability to make histidine

Medium without histidine
Ames II™ Mutagenicity Assay

Genotype of the Ames II Tester Strains

- **transition** mutation
  - Gly 153 (GGT) mutated to Asp 153 (GAT)

- **transversion** mutation
  - Lys 217 (AAA) mutated to Ile 217 (ATA)
  - Gly 153 (GGT) mutated to Val 153 (GTT)
  - Asp 169 (GAG) mutated to Gly 169 (GGG)
  - Asp 169 (GAG) mutated to Ala 169 (GCG)
  - Gly 163 (GGA) mutated to Arg 163 (CGA)
Ames II Assay Method
Comparison of Ames & Ames II Tests

• Ames tests – testing using traditional Ames strains
  – GLP or ISO Ames test (testing for TA98,100,1535, 1537,102 or E.coli)

• Ames II tests – testing using both traditional and Ames II strains
  – Ames II individual tests: testing for TAMIX and TA98
  – Concordance with the regular Ames assay – 88%
  – Eight concentrations – highest ~ 200 µg/mL were tested in triplicate.
Ames II Assay
Evaluation Criteria

• At least a two fold increase in the number of positive wells in two test article concentrations.
• The increase must be higher than the vehicle control historical range and dose dependence.
• Positive control – at least 25 positive wells or more.
• Negative control – within historical range.
• Limitation – toxicity measurement
1. Introduction
   Marilyn Aardema 5 min

2. In Vitro Flow Micronucleus Assay - 96 well
   Leon Stankowski, 10 min

3. Genetic Toxicology Screening Assays
   a) Ames II Assay
      Kamala Pant 10 min
   b) GreenScreen Assay
      Kamala Pant 10 min
   c) In Vitro Comet Assay - 96 well TK6 assay
      Kamala Pant 10 min

6. Questions/Discussion
   15 min
GreenScreen Human Cell Assay

GreenScreen Assay in Human TK6 Cells

Cell signal associated with DNA damage processing

- Uses p53 proficient cells
- With and without S9
- Population-wide response to “upstream” signal of DNA damage processing
- GADD45 target gene and Green Fluorescent Protein report gene
- 1 to 4 mg test article with and without S9
Assay principle:
DNA damage increases cell fluorescence

Slide courtesy of Gentronix, LTD.
GreenScreen HC Cell Lines

- GreenScreen cells constructed using TK6 cell line
- GADD45a gene (Growth Arrest and DNA Damage)
- Two cell constructs used in study
- GenM-T01 – fully functional plasmid with GADD45a and GFP genes
  - Functional GFP expressed following DNA damage
- GenM-C01 – plasmid missing 4 base pairs from start on GFP gene
  - Nonfunctional GFP produced
  - Control for cytotoxicity and non-specific autoflorescence
- Cells also contain hygromycin B resistance gene
  - Plasmids maintained in TK6 by adding hygromycin B to media
GreenScreen HC Without S9 Assay Overview

- 4 compounds per plate
- 9 two-fold dilutions
- internal positive controls
- control & test strain
- plate set up in 20/30 minutes
- automated data collection
- results in 24/48 hours
- 1mg required to test up to 1000 µg/ml
- 10 µl of 10mg/ml stock

Slide courtesy of Gentronix, LTD.
4-Nitroquinoline N-oxide (4-NQO)

**Cytotoxicity Results**

![Cytotoxicity Graph](image)

**Cytotoxicity Control**

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>CELL DENSITY</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GenM-C01</td>
<td>35.3</td>
<td>PASS</td>
</tr>
<tr>
<td>GenM-T01</td>
<td>67.5</td>
<td>PASS</td>
</tr>
</tbody>
</table>

**Genotoxicity Evaluation**

![Genotoxicity Graph](image)

**Genotoxicity Control**

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>GFP INDUCTION</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GenM-T01</td>
<td>2.87</td>
<td>PASS</td>
</tr>
</tbody>
</table>

**Raw Data for Individual Strains**

![Graph](image)
GreenScreen Assay with Metabolic Activation

- GreenScreen assay is performed with S9 activation.
- Same type of setup as without activation in 96-well plates.
- Test article treatment performed with S9 for only three hours.
- Cells centrifuged, washed and refed with media and grown for 48 hours.
- Stained with Propidium Iodide.
- Sample from the microplate on the flow cytometer.
- Read with the GreenScreen HC S9 acquisition and analysis flow cytometry template, collecting 10,000 events per well.
GreenScreen + S9 Overview

4 compounds per plate
9 two-fold dilutions
internal positive controls
control & test strain

Treatment time – 3 hours
automated data collection
results in 48 hours

Data by Flow Cytometry.

1mg required to test up to 1000 µg/ml
10 µl of 10mg/ml stock
GreenScreen Assay +S9 Positive Control Data (CPA)

**GENOTOXICITY RESULTS**

**GENOTOXICITY EVALUATION**

![Graph showing fluorescence induction vs. test compound concentration for GenM-T01 with a threshold line.]

**GENOTOXICITY RESULT**

- **LEC:** 12.50 µg/ml

**GENOTOXIC CONTROLS RESULT**

- RESULT: PASS

**NOTES:**

- **POSITIVE DATA FOR INDIVIDUAL STRAINS:**
  - GenM-C01
  - GenM-T01

---

**RAW DATA FOR INDIVIDUAL STRAINS**

![Graph showing fluorescence induction vs. test compound concentration for GenM-C01 and GenM-T01.]

---

**Blank 0.20 0.39 0.78 1.56 3.13 6.25 12.50 25.00 50.00**

**Test Compound Concentration**

**Fluorescence Induction**

- GenM-C01
- GenM-T01

---

**BioReliance**
Evaluation Criteria

• With and Without S9

  – Assay should give a pass reading according to the software provided by Gentronix. Parameters checked – media contamination, optimum cell growth, auto-fluorescent or colorful test articles

  – Cytotoxicity – less than or equal to 80% relative cell survival (cytotoxic), 50% or less RCS (very cytotoxic)

  – Fold increase in GFP –
    – 1.5 or greater (genotoxic without metabolic activation),
    – 1.3 or greater (genotoxic with metabolic activation)

  – Limitation – solubility of test article, precipitating doses interfere with readings.
1. Introduction

   Marilyn Aardema 5 min

2. In Vitro Flow Micronucleus Assay - 96 well

   Leon Stankowski, 10 min

3. Genetic Toxicology Screening Assays
   a) Ames II Assay
      Kamala Pant 10 min
   b) GreenScreen Assay
      Kamala Pant 10 min
   c) In Vitro Comet Assay - 96 well TK6 assay
      Kamala Pant 10 min

6. Questions/Discussion
   15 min
In Vitro Comet Screening Assay

Single Cell Gel Electrophoresis to detect DNA damage

Why do in vitro Comet Assay?

1. Mechanism of action
2. Predictive of genotoxicity
3. Could be an alternative to in vitro clastogenicity assay.
4. Could be used as a test for early drug candidate selection.
5. Clinical trial (if a DNA damaging drug is going through clinical trial – patient blood/bone marrow monitoring)
In Vitro Comet Screening Assay

Test System (cell lines, human blood, primary cells etc.) for ToxCast –TK6 cells

• Five concentrations of test article (no pre-tox), stating at ~ 200 µM highest dose.
• Positive (2AA and MMS, with and without activation respectively) and vehicle controls
• 4-hour treatment, with and without metabolic activation
• 96-well plate format
• Toxicity measured by number of clouds on the slides. Have scored up to 90% clouds.
In Vitro Comet Assay Methodology

1. **Cell suspension**
   - 0.5% LMPA

2. **Lysis Solution**

3. **Unwinding & Electrophoresis**

4. **Viability**

5. **Neutralization Buffer (pH 7)**

6. **Dehydrate**

7. **Store**

8. **Stain**

9. **IMAGE ANALYZER**
Parameters of DNA Damage

**Tail Length**
DNA migration length from center of the head to smallest detectable fragment

**% Tail DNA (Intensity)**
Amount of DNA fragments in the tail

**Olive Tail Moment**
\[ (% \text{ Tail intensity}) \times (\text{tail length}) \]

**Tail Migration**
DNA migration length from the edge of the head to smallest detectable fragment
In Vitro Comet Assay Evaluation

• Positive control – significant increase in DNA damage
• Negative/vehicle control within historical vehicle control range
• Highest dose scored up to 90% clouds
• Statistical significance in doses to evaluate positive response
• Limitation – so far toxicity measurements and statistical analysis are not well defined.
Thank You

Any Questions?