Appendix E

Neutral Red Dye Experiments

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APPENDIX E
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Appendix E1: Institute for In Vitro Sciences (IIVS) Assessment of Protocol Variables in the NICEATM/ECVAM Evaluation of Cytotoxicity Assays

IIVS performed experiments using the 3T3 cells and the NRU test methods before the NICEATM/ECVAM validation study was initiated. The laboratory examined: optimal solvent concentrations (DMSO and ETOH), cell seeding densities, doubling times, and exposure duration of a test chemical (24, 48, and 72-hour exposures). Data are presented in the appendix.

Appendix E2: Neutral Red (NR) Dye Experiments – 3T3 Cells

IIVS performed three sets of experiments to compare the optical density (OD) readings obtained in an NRU assay using various concentrations of NR dye and different incubation periods.

- **Experiment 1**: NR Stain Time Course in 3T3 Cells; NRU incubation times: 0.25, 0.50, 1.0, 2.0, and 3.0 hour.

- **Experiment 2**: Neutral Red Stain Prepared in DMEM/5%NCS; Test of NR Preparation 1 Day Prior to Use; Tested in 90-100% Confluent 3T3 Cultures

- **Experiment 3**: Neutral Red Stain Prepared in DMEM/5%NCS; Filtered Immediately before Use; Tested in 90-100% Confluent 3T3 Cultures

Appendix E3: Neutral Red (NR) Dye Experiments – NHK Cells

IIVS performed three sets of experiments to compare the optical density (OD) readings obtained in an NRU assay using various concentrations of NR dye and different incubation periods.

- **Experiment 1**: NR Stain Time Course in NHK Cells; NRU incubation times: 0.25, 0.50, 1.0, 2.0, and 3.0 hour.

- **Experiment 2**: Neutral Red Stain Prepared in KGM; Test of NR Preparation 1 Day Prior to Use; Tested in 90-100% Confluent NHK Cultures

- **Experiment 3**: Neutral Red Stain Prepared in KGM; Filtered Immediately before Use; Tested in 90-100% Confluent NHK Cultures
Appendix E4: Neutral Red (NR) Dye Experiments – Concentration vs Time – 3T3 Cells

ECBC performed experiments using the 3T3 cells and the NRU test methods.
- *in vitro* cytotoxicity NRU tests (3T3 cells) using SLS (range = 100 µg/mL to 6.7 µg/mL)
- NR dye mixed with DMEM culture medium with 10% NCS; final concentrations = 25 µg/mL and 50 µg/mL
- Tests performed with two NRU incubation times: 1 hour and 3 hours

<table>
<thead>
<tr>
<th>µg NR dye/mL</th>
<th>NRU Incubation Time (hours)</th>
<th>Mean Vehicle Control OD₅₄₀ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1</td>
<td>0.255</td>
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<tr>
<td>25</td>
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