

## **Appendix E**

### **Neutral Red Dye Experiments**

<b>E1</b>	<b>Institute for <i>In Vitro</i> Sciences (IIVS) Assessment of Protocol Variables in the NICEATM/ECVAM Evaluation of Cytotoxicity Assays.....</b>	<b>E-5</b>
<b>E2</b>	<b>Neutral Red (NR) Dye Experiments – 3T3 Cells – IIVS.....</b>	<b>E-13</b>
<b>E3</b>	<b>Neutral Red (NR) Dye Experiments – NHK Cells – IIVS .....</b>	<b>E-19</b>
<b>E4</b>	<b>Neutral Red (NR) Dye Experiments – 3T3 Cells – ECBC.....</b>	<b>E-25</b>

***[This Page Intentionally Left Blank]***

## APPENDIX E

### Neutral Red Dye Experiments

#### **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

µg NR dye/mL	NRU Incubation Time (hours)	Mean Vehicle Control OD <sub>540</sub> Value
25	1	0.255
25	3	0.508
50	1	0.330
50	3	0.457