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5.0 3T3 AND NHK NRU TEST METHOD DATA AND RESULTS

This section summarizes the IC_{50} results generated by testing 72 coded reference substances (see **Section 3**) in the 3T3 and NHK NRU test method protocols. These IC_{50} values were used to evaluate the accuracy (also known as concordance - see **Section 6**) of the two *in vitro* cytotoxicity test methods for predicting *in vivo* GHS acute oral toxicity categories and their reliability (intra- and inter-laboratory reproducibility - see **Section 7**). The individual test data for the passing and failing tests are provided in **Appendix I** for the reference substances and the PC. The raw data for each test (in EXCEL® and PRISM® files) are available upon request from NICEATM on compact disk(s), as are the laboratory reports. Requests can be made by mail, fax, or e-mail to Dr. William S. Stokes, NICEATM, NIEHS, P. O. Box 12233, MD EC-17, Research Triangle Park, NC, 27709, (phone) 919-541-2384, (fax) 919-541-0947, (e-mail) niceatm@niehs.nih.gov.

Section 5.1 discusses the timeline for the validation study, the study participants, and their roles in the study. **Section 5.2** documents the use of coded reference substances and the GLP compliance by the participating laboratories. **Section 5.3** discusses the protocol revisions that were made during the study and the effect the revisions had on the results. **Section 5.4** presents the IC_{50} data collected during each phase to assess the reliability and accuracy (relevance) of the NRU methods. **Section 5.5** presents the statistical analyses performed. **Section 5.6** summarizes the results of IC_{50} comparisons of the 3T3 and NHK methods. **Section 5.7** offers information about the availability of all the data (e.g., raw OD data from all tests, laboratory reports), and **Section 5.8** presents the solubility test results for the reference substances from all laboratories.

5.1 Study Timeline and Participating Laboratories

5.1.1 Statements of Work (SOW) and Protocols

The SMT provided the laboratories with SOWs for each test method prior to initiation of testing (see **Appendix G**), and proposed dates for completion of the various aspects of the study (e.g., transfer of data, provision of reports). The SOWs defined the following:

- Project objectives
- Management and key personnel
- Required facilities, equipment, and supplies
- Quality assurance requirements
- Test phases and schedules
- Products (e.g., reports) required
- Report preparation

The SOW for BioReliance contained all of the above requirements, and also included requirements for:

- Reference substance acquisition, coding, preparation, and distribution
- Solubility testing

The SMT, in consultation with the laboratories, prepared Test Method Protocols for each phase of the study. Cytotoxicity testing in each phase of the validation study was initiated in each laboratory when the SMT received a signed protocol specific for that phase from the

Study Director. Solubility testing for the Phases I and II substances was performed prior to cytotoxicity testing for those substances; most of the solubility testing for the Phase III substances was performed toward the end of Phase II and during the early part of Phase III.

5.1.2 Study Timeline

The actual timeline of the study is shown in **Table 5-1**. The SMT modified the original timeline presented in the SOWs because of a number of factors, such as, protocol revisions, side studies, difficulties with acquisition of medium, etc.

Table 5-1 Validation Study Timetable

Event	BioReliance	ECBC	FAL	IIVS
Receipt of SOW from SMT	Jun 2002	Jun 2002	Jun 2002	Jun 2002
Procurement of Test Substances	Jul 2002 - Jan 2003	NA	NA	NA
Solubility Testing Completed	Jul 2002 - Jan 2003	Dec 2003	Dec 2003	Jan 2004
Distribution of Reference Substances Phase Ia Phase Ib Phase II Phase III	 Jul 2002 Sep 2002 Nov 2002 Feb - Mar 2003	NA	NA	NA
Initiation of Phase Ia	NA	Aug 2002	Aug 2002	Aug 2002
Completion of Phase Ia	NA	Nov 2002	Nov 2002	Oct 2002
Initiation of Phase Ib	NA	Dec 2002	Dec 2002	Dec 2002
Completion of Phase Ib	NA	May 2003	May 2003	May 2003
Initiation of Phase II	NA	Jun 2003	Jun 2003	Jun 2003
Completion of Phase II	NA	Nov 2003	Nov 2003	Nov 2003
Initiation of Phase III	NA	Dec 2003	Dec 2003	Dec 2003
Completion of Phase III	NA	Dec 2004	Dec 2004	Jan 2005

Abbreviations: ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; SOW=Statement of Work; SMT=Study Management Team; NA=Not applicable.
Note: BioReliance distributed the reference substances and performed solubility testing. ECBC, FAL, and IIVS tested the reference substances for solubility and *in vitro* cytotoxicity.

5.1.3 Participating Laboratories

- BioReliance Corporation
  14920 Broschart Road
  Rockville, Maryland 20850-3349
  Study Director: Dr. Martin Wenk
- U.S. Army Edgewood Chemical Biological Center (ECBC)
  Molecular Engineering Team
  Aberdeen Proving Ground, MD 21010
  Study Director: Dr. Cheng Cao

- Institute for *In Vitro* Sciences (IIVS)
  21 Firstfield Road Suite 220
  Gaithersburg, MD 20878
  Study Director: Mr. Hans Raabe
- Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory (FAL)
  Queens Medical Centre, University of Nottingham
  Nottingham NG7 2UH
  United Kingdom
  Study Director: Dr. Richard Clothier

5.2 Coded Reference Substances and GLP Guidelines

5.2.1 <u>Coded Reference Substances</u>

BioReliance acquired 73 substances (72 reference substances and one PC substance) from reputable commercial sources (see **Appendix F1**). All but eight of the reference substances were >99% pure (see **Section 8.1.2.1**). BioReliance coded each substance with a unique, random identification number when repackaging them into smaller units for distribution to the laboratories. These units were given an additional code unique to the respective cytotoxicity laboratories, so that they could be provided in a blinded fashion (see **Section 3.4** for distribution procedures). The coded substance units were packaged and shipped such that their identities were concealed; however, all laboratories knew the identity of the positive control. The SMT revealed the codes for each phase after all laboratories had submitted their data and reports for that phase. The laboratories periodically required additional aliquots of reference substance, and BioReliance provided these aliquots from the original stock of reference substance in the same manner that the original aliquots were provided.

5.2.2 <u>Lot-to-Lot Consistency of Reference Substances</u>

Each substance was purchased as a single lot, and each laboratory received aliquots from this same lot throughout the validation study. The reference substance suppliers provided certificates of analysis for each lot, along with the MSDS documents containing substance, physical, and safety and handling information.

5.2.3 <u>Adherence to GLP Guidelines</u>

BioReliance, ECBC, and IIVS, followed GLP procedures for all testing, with the exception of tests designed to resolve technical challenges (e.g., formation of NR crystals; use of film plate sealers for volatile substances; slow growth of cells). The laboratories submitted all data to their respective quality assurance units (as per GLP requirements) and copies of the data were submitted to NICEATM. FAL followed most of the GLP guidelines, but did not employ independent quality assurance reviews of laboratory procedures or documentation. The Study Director for FAL performed all data reviews and provided copies to NICEATM. Hard copy printouts and electronic versions of all data are available at NICEATM.

5.3 3T3 and NHK NRU Test Method Protocols

The protocols for the 3T3 and NHK NRU test methods used during Phase III laboratory testing were the result of modifications and revisions to the *Guidance Document* (ICCVAM 2001b) protocols, the optimization of the protocols used in the laboratory evaluation Phases Ia and Ib, and the laboratory qualification phase (Phase II) (see **Section 2.6**). **Figure 1-2** provides an outline of the study phases, and identifies where repeated observations were carried out to permit protocol evaluation and comparison. **Sections 2.2** and **2.3** address the similarities and differences between the 3T3 and NHK protocols. The remaining subsections in **Section 5.3** address the modifications to the protocols used in each phase, and how those modifications affected each data set.

5.3.1 Phase Ia: Laboratory Evaluation Phase

During Phase Ia, each laboratory established an historical database for the PC substance, SLS. No reference substances were tested in this phase. Ten concentration-response tests were performed using SLS and no more than two tests were performed/day. The resulting data were used to calculate the acceptable response limits for the SLS IC_{50} for use during Phase Ib testing.

Section 2.6.1 summarizes issues that occurred during Phase I and addresses protocol changes made after the initiation of Phase Ia. The specific changes to the protocols for both cell systems are summarized below, along with the impact these changes had on the test data. Changes made in the protocols during Phase Ia were incorporated into the Phase Ib protocols.

5.3.1.1 *Protocol Changes and the Effect on the Data*

- *NR Dye Crystals:* Reduced the NR dye concentration for both cell types. No subsequent tests failed because of NR crystal formation. The background OD values decreased and this was not interpreted as a negative effect on the data.
- *3T3 Cell Growth*: Modified cell culture conditions for 3T3 cells to improve cell growth characteristics. No apparent effect on the data was detected.
- *NHK Cell Growth (96-well plates):* Removed the cell culture refeeding step performed prior to reference substance addition. Although the OD values for the vehicle controls became higher, the SLS IC_{50} results were similar whether or not the cells were re-fed.
- *NHK Cell Growth (in culture flasks)*: FAL coated their culture flasks with fibronectin-collagen prior to seeding thawed cells. This may have affected the SLS data from FAL because it had the highest SLS IC_{50} values of the three laboratories (7.45 μg/mL vs. 4.03 μg/mL for ECBC and 3.68 μg/mL for IIVS). The fibronectin-collagen coating procedure was eliminated, and subsequent SLS data and IC_{50} results from FAL were comparable to the data from the other two laboratories.
- *OD Limits*: Eliminated the VC OD range as a test acceptance criterion. The SMT decided to accept tests that had VC ODs outside the originally preset range if all other test acceptance criteria were met. Test data were not adversely affected by relaxing this criterion.
- *Dilution Factor*: The SMT accepted data generated using dilution factors other than the recommended 1.47 for definitive tests if all other test acceptance criteria were met. The use of smaller dilution factors generally increased the number of data points between 10 - 90% viability, and the precision of the IC_{50} calculation was improved.

5.3.2 Phase Ib: Laboratory Evaluation Phase

Phase Ib was designed to determine whether the protocol revisions following Phase Ia were effective in improving intra- and inter-laboratory reproducibility, and to determine whether the laboratories could obtain reproducible results when testing coded reference substances of various toxicities. Three coded reference substances representing the full range of toxicity were tested: arsenic trioxide (high toxicity: $5< LD_{50} \leq 50$ mg/kg), propranolol HCl (medium toxicity: $300< LD_{50} \leq 2000$ mg/kg), and ethylene glycol (low toxicity: $LD_{50} >5000$ mg/kg) (see **Section 3.3.5** for the selection of substances to be tested in Phases Ib and II). Because Phase Ib was part of the laboratory evaluation phase, the SMT decided that three substances would be sufficient, and that it was not necessary to represent all GHS acute oral toxicity categories. Each substance was tested in all laboratories at least once in a range finding experiment, and then in three, acceptable definitive tests performed on three different days. **Section 2.6.2** summarizes the technical challenges that arose during this phase and addresses protocol changes made after initiation of Phase Ib. The specific changes made in the 3T3 and NHK protocols, along with the effect the changes had on the test data, are summarized below.

5.3.2.1 *Protocol Changes and the Effect on the Data*

- *NR Dye Crystals*: Reduced the concentration of NR in the 3T3 method. The OD values and SLS IC_{50} results were similar in four exploratory experiments regardless of the NR concentration or NRU incubation time. The elimination of NR crystals reduced the background OD values without affecting the sensitivity of the procedure.
- *VC OD Range*: Used new VC OD ranges for guidance (e.g., as target values to assess cell growth), rather than as a test acceptance criterion, for the remainder of the study. This increased the number of tests that met the acceptance criteria. Relative toxicities did not change. The test data were not adversely affected by the removal of this criterion.

5.3.3 Phase II: Laboratory Qualification Phase

The results from Phase II were used to determine whether the protocol revisions from Phase Ib were effective in improving intra- and inter-laboratory reproducibility, and whether the laboratories could obtain reproducible results when testing a larger set of substances covering a wider range of physical/substance characteristics and toxicities. Nine coded reference substances were tested: aminopterin, cadmium chloride, chloramphenicol, colchicine, lithium carbonate, potassium chloride, 2-propanol, sodium fluoride, and sodium selenate. These substances (with the exception of sodium selenate) are included in the RC, and were selected because they fit the RC millimole regression line (i.e., they were within the acceptance intervals established by Halle [1998, 2003]). The RC is a database of acute oral LD_{50} values for rats and mice obtained from RTECS® and IC_{50} values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints for substances with known molecular weights (Halle 1998, 2003). Sodium selenate was selected because of its high toxicity, despite the fact that it was not in the RC, because there were no other substances in the highest GHS acute oral toxicity category, other than aminopterin, that were within the RC millimole regression acceptance intervals. Each laboratory tested each substance at least once in a range finding experiment, and then in three acceptable definitive tests performed on different days.

Section 2.6.2 summarizes the technical issues that arose during this phase and the protocol changes made prior to Phase II. The specific changes made in the 3T3 and NHK NRU protocols, along with the effect the changes had on the test data, are summarized below.

5.3.3.1 *Protocol Changes and the Effect on the Data*

- *Blank Wells*: Added reference substance to blank wells of the test plate to determine if reference substance affected (i.e., increased OD values) compared to medium-filled blank wells. There was no apparent effect on the test data as there were no noticeable differences in OD values between blanks with culture medium or culture medium and reference substance.
- *VC OD Range*: Eliminated the VC OD range as an acceptance criterion. There was no apparent effect on test data from not restricting the OD values to a pre-set range.
- *Harmonization of Laboratory Techniques*: Made revisions to the Phase II protocols as a result of the harmonization training by the testing laboratories (see **Section 2.6.2.6**). There was no apparent effect on the test data from IIVS and ECBC, but there was an improvement in the FAL data quality (e.g., fewer lost OD values due to cell seeding errors, more uniform OD values for six replicate wells per reference substance).
- *3T3 Cell Seeding Density*: Added a range of cell seeding densities to be used by the laboratories. This optimized the cell confluence at the end of chemical exposure and no apparent effects on the data were detected because of this modification.
- *NHK Cell Growth from Cryopreserved Stock Cells*: Eliminated the use of fibronectin-collagen coating of 80-cm^2 flasks for the initial propagation of NHK cells. By doing this, FAL achieved better cell growth, lower IC_{50} values for the PC, and better agreement of the mean SLS IC_{50} values with those of the other laboratories.
- *Volatile Substances*: Added the use of a CO_2 permeable plate sealer to control volatility (as identified by cross contamination of the control wells). The use of plate sealers for volatile substances was incorporated into the Phase III protocols.
- *R^2 Acceptance Criterion*: Relaxed the R^2 criterion for the fit of the dose-response data to the Hill function. Some tests that did not meet the original criterion were accepted by the SMT after determining that even though the curve fit was not optimum, it adequately conveyed the toxicity of the substance (i.e., an IC_{50} could be calculated with an adequate number of toxicity points between 0 and 100% viability).
- *Unusual Concentration-Response*: Revised the Hill function calculation to address substances that produced a concentration-response in which toxicity plateaued before reaching 0% viability. This modification allowed for a curve fit to the Hill function for such substances, and thus a better estimation of their IC_{50} values.
- *PC IC_{50} Range*: Expanded the SLS IC_{50} acceptable range, which resulted in additional tests in Phase II being acceptable. Expanding the PC range reduced the number of reference substance retests, and thereby qualified additional

definitive tests as acceptable because they would not fail simply because the PC was out of the pre-set range.

5.3.4 Phase III: Main Validation Phase

The purpose of Phase III was to generate high quality *in vitro* cytotoxicity data using the 3T3 and NHK NRU test methods with protocols that were optimized based on the experience and results in Phases I and II. Sixty coded reference substances were tested; 46 of these were RC substances that covered a broad range of toxicity. The reference substances in Phase III spanned all five GHS toxicity categories and unclassified substances. Each substance was tested in each laboratory at least once in a range finding experiment, and then in three acceptable definitive tests performed on different days.

Section 2.6.4 addresses protocol changes made before the initiation of Phase III. The specific changes made in the 3T3 and NHK protocols, along with the effect the changes had on the test data, are summarized below.

5.3.4.1 *Protocol Changes and the Effect on the Data*

- *Prequalification of NHK Culture Medium*: Included a protocol for prequalifying NHK culture medium and supplements. This prevented the participating laboratories from using medium and supplements that did not support adequate growth of the cells.
- *Stopping Rule for Testing*: Added this rule for reference substances that were insoluble (i.e., <200 μg/mL) and/or did not produce sufficient cytotoxicity for the calculation of an IC_{50}. This rule allowed testing to end for substances that produced no IC_{50} data after three definitive tests. Substances for which an IC_{50} was not produced by one or more laboratories are presented in **Table 5-2**. Carbon tetrachloride did not produce an IC_{50} in any of the laboratories in either the 3T3 or the NHK NRU test methods, and methanol did not produce an IC_{50} in the 3T3 NRU test method.
- *Acceptable Range for Dose-Response Data Points:* Modified the test acceptance criterion for the number of data points required on the toxicity curve. The criterion was changed from requiring a minimum of two points (at least one >0% and ≤50% viability, and at least one >50% and <100% viability) to one point >0% and <100% viability, if the smallest practical dilution factor (i.e., 1.21) was used, and all other test acceptance criteria were met. This reduced the number of failed experiments for substances with very steep concentration-response curves, without reducing the quality of the IC_{50} data. For the 3T3 NRU test method, diquat dibromide (1/9 definitive tests), epinephrine bitartrate (2/9 definitive tests), and 1,1,1-trichloroethane (2/8 definitive tests) had such steep dose-responses that some acceptable tests met these revised criteria. None of the NHK NRU tests needed the revised criteria.
- *R^2 Acceptance Criterion*: Rescinded the R^2 criterion for the fit of the Hill function. The SMT determined that the R^2 criterion was best used to characterize the shape of the concentration-response curve rather than to establish a criterion for test acceptability. This reduced the number of failed experiments without affecting the calculation of the IC_{50} values as long as an

adequate number of toxicity points between 0 and 100% viability were obtained.

- *PC Acceptance Criteria*: Modified the PC acceptance criterion for Hill function fit.
- *Hill Function Analysis*: Altered the PRISM® template for the Hill function analysis to perform calculations for IC_x values in two ways: (1) constraining Bottom parameter to zero, and (2) fitting the Bottom parameter. As a result of the changes and efforts by the laboratories to use dilution schemes that captured the entire concentration-response range, very few tests in Phase III had $R^2 < 0.9$.
- *Biphasic Dose-Response in Range Finder Test*: Provided guidance for proceeding with definitive testing when a biphasic dose-response was obtained in the range-finder test. The definitive test was to focus on the lowest concentrations that produced responses around 50% viability (See **Section 2.6.3.2**).

Table 5-2 Reference Substances Affected by Stopping Rule[1]

Reference Substance	Testing Stopped -- No IC_{50} Data					
	3T3 NRU Test Method			NHK NRU Test Method		
	ECBC	FAL	IIVS	ECBC	FAL	IIVS
Carbon tetrachloride	X	X	X	X	X	X
Disulfoton		X				
Gibberellic acid		X				
Methanol	X	X	X	X		
1,1,1-Trichloroethane	X				X	X
Valproic acid			X			
Xylene	X	X		X	X	

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

[1]Substances that did not provide sufficient cytotoxicity for the calculation of an IC_{50} in one or more laboratories (identified by X).

5.4 Data Used to Evaluate Test Method Accuracy and Reliability

This section first presents the acceptable PC data and IC_{50} results from each laboratory for each phase of the validation study, and then presents the reference substance IC_{50} results and Hill Slopes from each phase. The individual test data for both passing and failing tests are provided in **Appendix I** for the PC and reference substances. Accuracy (concordance for the prediction of GHS acute oral toxicity category) and reliability assessments are provided in **Sections 6** and **7**, respectively.

5.4.1 <u>PC Data</u>

A summary of the acceptable SLS data IC_{50} results used to calculate quality control acceptance limits for each test method in each laboratory are provided in **Table 5-3**. The SLS IC_{50} results were used to calculate acceptable limits for each laboratory to use in subsequent study phases. One of the test acceptance criteria for each reference substance test was that the associated SLS IC_{50} must be within the acceptance limits. The individual test data for both passing and failing PC tests are provided in **Appendix I3** for the 3T3 and in **Appendix I4** for the NHK methods.

Table 5-3 Positive Control (PC)[1] IC_{50} Results by Study Phase

Study Phase	ECBC				FAL				IIVS			
	Mean IC_{50} (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N	Mean IC_{50} (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N	Mean IC_{50} (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N
3T3 NRU												
Ia[2]	38.3	4.71	28.8 – 47.7	15	42.3	8.56	25.2 – 59.5	25	40.9	3.19	34.5 – 47.3	12
Ib[3]	41.3	5.99	26.4 – 56.3	12	43.2	4.68	31.5 – 54.9	17	42.1	3.40	33.6 – 50.6	13
II[4]	41.2	4.20	30.8 – 51.6	29	45.9	7.50	27.2 – 64.7	36	40.6	3.50	31.8 – 49.3	21
III[5]	41.6	3.41	NA	65	41.1	6.23	NA	26	41.5	3.74	NA	22
NHK NRU												
Ia[2]	4.03	1.32	1.40 – 6.67	15	7.45	3.07	1.34 – 13.6	18	3.68	0.555	2.57 – 4.79	30
Ib[3]	3.65	0.98	1.22 – 6.10	11	5.35	2.32	0[6] – 11.1	15	3.57	0.59	2.10 – 5.04	17
II[4]	3.59	1.41	0.07 – 7.11	22	3.20	1.05	0.57 – 5.82	15	3.78	0.73	1.94 – 5.61	26
III[5]	3.03	0.75	NA	57	3.45	0.90	NA	35	3.12	0.53	NA	20

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; N=Number of acceptable tests; NA=Not applicable

[1]PC was sodium lauryl sulfate (SLS).
[2]Values generated from Phase Ia data were used as acceptance criteria for Phase Ib tests; Acceptance limits = Mean ±2 X standard deviation.
[3]Values generated from Phases Ia and Ib data were used as acceptance criteria for Phase II tests; Acceptance limits = Mean ±2.5 X standard deviation.
[4]Values generated from Phases Ia, Ib, and II data were used as acceptance criteria for Phase III tests; Acceptance limits = Mean ±2.5 X standard deviation.
[5]Values generated from Phase III test data.
[6]Calculation of lower limits yielded a negative value, so that lower limit was set at 0 and later revised to 0.1 µg/mL.

5.4.1.1 *Phase Ib PC Data Acceptance Limits*

The SLS IC_{50} acceptance limits for Phase Ib testing were calculated using the Phase Ia data. The data sets from each laboratory were examined for outliers using the method of Dixon and Massey (1981), but none were identified. The acceptance limits for the SLS IC_{50} values for each laboratory and test method were the mean ±2 SD.

5.4.1.2 *Phase II PC Data Acceptance Limits*

The IC_{50} values from the Phase Ia and Ib SLS tests were used to calculate laboratory-specific and test method-specific quality control acceptance limits for Phase II. Phase Ib tests that had SLS IC_{50} values outside of the acceptance limits were considered acceptable if they met all other test acceptance criteria. For any day during which there was more than one SLS test (for any one method and laboratory), the IC_{50} values were averaged to better reflect day-to-day variation and avoid overweighting the overall mean with multiple values from a single day. Outliers at the 99% level were removed and the remaining values were used to calculate the mean ±2.5 SD acceptance limits. The acceptance limits were expanded from 2 SD in Phase Ib to 2.5 SD for Phase II to allow for the fact that the SDs decrease as more data are collected.

5.4.1.3 *Phase III PC Data Acceptance Limits*

The IC_{50} values from the Phase I and II SLS tests were used to calculate laboratory-specific and method-specific quality control acceptance limits for Phase III data. The SLS IC_{50} values outside the acceptance limits were considered acceptable if the tests met all other acceptance criteria. For any day for which there was more than one SLS test (for any one method and laboratory), the IC_{50} values were averaged to better reflect day-to-day variation and avoid overweighting the overall mean with multiples values from a single day. ANOVA was used to compare the Phase Ia, Ib, and II data within each laboratory to determine whether the SLS IC_{50} for each method and laboratory was changing over the course of the study. For PC data that were not significantly different from phase to phase at $p < 0.05$, the IC_{50} values were used to calculate the mean ±2.5 SD as the acceptance limits for Phase III. The only significant differences in SLS values seen between study phases ($p < 0.0002$) were the FAL results for NHK. This difference was attributed to the changes in cell culture practices between Phases Ib and II (see **Section 5.3.3**). Thus, only the Phase II SLS IC_{50} values were used to calculate the acceptance limits for Phase III NHK data at FAL.

5.4.2 Reference Substance Data

Reference substance data and results from the individual 3T3 and NHK tests (both acceptable and unacceptable) from each laboratory are presented in **Appendices I1** and **I2**. **Tables 5-4** and **5-5** summarize the IC_{50} and Hill Slope data from the acceptable 3T3 and NHK tests, respectively, for each reference substance and laboratory. The Hill Slope data are provided for supplemental information on the concentration-response characteristics for each reference substance, but were not used for reliability or accuracy analyses. These tables are organized alphabetically by substance name and provide substance class (based on the NLM Medical Subject Heading [MeSH index]), arithmetic mean IC_{50} and SD for each laboratory, arithmetic mean Hill Slope and SD for each laboratory, and the number of tests used to produce the mean values. **Figure 5-1** graphically presents the 3T3 IC_{50} data from **Table 5-4**, and **Figure 5-2** presents the NHK IC_{50} data from **Table 5-5**. The reference substances in **Figures 5-1** and **5-2** are ordered by ascending IC_{50} (lowest value [most toxic] to highest value [least toxic]) using the 3T3 IC_{50} values from IIVS (the lead laboratory for the study). This allows a simple comparison of each reference substance value from each laboratory. **Table 5-6** provides the numerical key to the reference substances in **Figures 5-1** and **5-2**.

Because of their low toxicity and/or low solubility, some substances were not sufficiently toxic for calculation of an IC_{50} value. For the 3T3 NRU test method, no IC_{50} values were obtained for carbon tetrachloride or methanol in any laboratory (see **Table 5-4**). ECBC was the only laboratory that obtained IC_{50} values for lithium carbonate, and IIVS was the only laboratory that obtained IC_{50} values for xylene. Only one acceptable test (and IC_{50} value) was obtained for disulfoton at FAL, for 1,1,1-trichloroethane at ECBC, and for valproic acid at IIVS. FAL did not achieve sufficient toxicity for the calculation of an IC_{50} for gibberellic acid in any 3T3 NRU tests performed. For the NHK NRU test method (see **Table 5-5**), there was insufficient toxicity in all tests in all laboratories for a calculation of an IC_{50} for carbon tetrachloride. Only one laboratory achieved sufficient toxicity for the calculation of an IC_{50} for 1,1,1-trichloroethane (ECBC) and xylene (IIVS). One laboratory, ECBC, failed to achieve sufficient toxicity for the calculation of an IC_{50} for methanol. All of these substances, with the exception of methanol, produced precipitate in the cell culture medium. The solvent used for methanol was DMSO, and because the amount of DMSO that could be used in the cell culture was limited to 0.5%, the amount of DMSO that could be used to dissolve methanol was also limited. The differences among laboratories regarding their ability to attain a high enough concentration to achieve an IC_{50} for some substances may be due to the differing perceptions of the laboratory personnel regarding whether or not the substance was sufficiently dissolved, or differences in the techniques used to dissolve the substances.

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Acetaminophen	Amide	III	40.8	9.12	3	-1.53	0.354	66.2	23.0	3	-1.23	0.503	43.4	11.4	3	-1.55	0.165
Acetonitrile	Nitrile	III	6433	129	3	-2.29	0.648	9690	5634	3	-1.55	0.196	9330	1217	3	-2.63	0.245
Acetylsalicylic acid	Carboxylic Acid; Phenol	III	646	61.5	3	-1.75	0.473	1234	298	3	-1.99	0.393	401	62.0	3	-1.31	0.167
Aminopterin	Heterocyclic	II	0.005	0.001	3	-2.00	0.395	0.012	0.005	3	-3.36	1.59	0.005	0.001	3	-1.46	0.198
5-Aminosalicylic acid	Carboxylic Acid; Phenol	III	1467	203	3	-1.82	0.267	2070	334	3	-2.33	0.809	1557	179	3	-1.64	0.326
Amitriptyline HCl	Polycyclic	III	6.03	1.38	3	-2.47	0.668	7.86	2.20	3	-2.98	0.446	7.81	1.38	3	-4.48	0.916
Arsenic III Trioxide	Arsenical	Ib	2.41	0.782	4	-1.94	0.204	1.04	0.070	4	-3.02	2.09	4.09	2.23	3	-1.62	0.285
Atropine sulfate	Heterocyclic	III	54.1	29.6	3	-1.32	0.480	133	41.1	3	-2.20	0.695	70.0	5.7	3	-1.27	0.165
Boric acid	Boron compound; Acid	III	1497	484	3	-1.14	0.039	3987	693	3	-1.86	0.654	1202	581	3	-1.71	0.677
Busulfan	Alcohol; Sulfur compound; Acyclic hydrocarbon	III	40.4	19.3	3	-0.515	0.003	321	180	3	-1.14	0.802	43.7	1.77	3	-0.627	0.164
Cadmium II chloride	Cadmium compound; Chlorine compound	II	0.480	0.066	3	-1.85	0.529	0.400	0.129	3	-3.05	0.743	0.817	0.427	3	-2.45	0.449
Caffeine	Heterocyclic	III	133	13.3	3	-1.11	0.097	157	81.7	3	-0.866	0.250	191	14.4	3	-1.27	0.077
Carbamazepine	Heterocyclic	III	83.0	12.0	3	-1.94	0.539	152	56.9	3	-3.50	1.27	91.8	11.0	3	-2.34	0.307
Carbon tetrachloride	Halogenated hydrocarbon	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	NA	NA	-	NA	NA
Chloral hydrate	Alcohol	III	151	15.6	3	-1.73	0.172	241	25.1	3	-2.16	0.597	170	19.9	3	-1.68	0.084
Chloramphenicol	Alcohol; Nitro compound; Cyclic hydrocarbon	II	55.3	12.4	4	-0.779	0.057	273	82.2	4	-1.16	0.249	156	27.9	3	-0.952	0.036
Citric acid	Carboxylic acid	III	473	138	3	-1.89	0.423	1148	143	4	-3.68	0.407	865	160	3	-2.51	0.530

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Colchicine	Polycyclic	II	0.021	0.002	4	-1.69	0.049	0.093	0.042	3	-1.61	1.80	0.028	0.0003	3	-1.69	0.255
Cupric sulfate pentahydrate	Sulfur compound; Metal	III	82.7	3.18	3	-4.85	0.700	123	54.0	4	-17.7	15.5	5.72	1.75	3	-5.71	1.14
Cycloheximide	Heterocyclic	III	0.125	0.057	3	-1.19	0.167	0.647	0.451	3	-1.53	0.128	0.109	0.025	3	-0.937	0.158
Dibutyl phthalate	Carboxylic acid	III	23.5	3.98	3	-3.37	1.27	191	94.5	4	-0.965	0.140	20.7	1.37	3	-2.62	0.283
Dichlorvos	Organophos-phorous	III	9.83	3.42	3	-1.32	0.297	32.8	2.07	3	-3.42	1.00	18.3	2.09	3	-2.13	0.439
Diethyl phthalate	Carboxylic acid	III	85.5	29.0	3	-1.11	0.340	147	37.8	3	-2.03	0.422	106	25.3	3	-2.35	0.824
Digoxin	Polycyclic; Carbohydrate	III	351	137	3	-2.11	2.05	892	319	3	-3.26	2.21	317	67.9	2	-3.04	1.52
Dimethyl-formamide	Amide; Carboxylic acid	III	5343	515	3	-1.96	0.087	5483	517	3	-1.80	0.143	4900	183	3	-1.87	0.102
Diquat dibromide monohydrate	Heterocyclic	III	3.87	0.887	3	-1.59	0.197	36.1	35.5	3	-11.5	10.1	5.39	1.36	3	-3.00	0.784
Disulfoton	Organophos-phorous; Sulfur compound	III	137	74.9	3	-2.06	1.88	11200	NA	1	-1.22	NA	60.4	52.5	3	-2.23	1.08
Endosulfan	Heterocyclic Sulfur compound	III	5.27	3.01	3	-0.669	0.243	15.2	11.9	4	-0.762	0.221	3.61	1.53	3	-0.871	0.636
Epinephrine bitartrate	Alcohol; Amine	III	51.5	6.16	3	-5.99	3.08	63.4	6.63	3	-45.1	32.0	63.4	1.91	3	-4.74	1.51
Ethanol	Alcohol	III	5360	1754	3	-1.33	0.104	8420	1205	3	-1.88	0.128	6413	345	3	-1.99	0.372
Ethylene glycol	Alcohol	Ib	18325	1658	4	-3.79	4.08	31650	7453	4	-1.70	0.166	25900	3081	3	-1.67	0.079
Fenpropathrin	Nitrile; Ester; Ether	III	22.6	2.41	3	-2.54	0.350	42.4	26.8	4	-1.44	0.645	16.7	2.03	3	-2.53	0.495
Gibberellic acid	Polycyclic	III	8027	908	3	-1.95	0.678	NA	NA	-	NA	NA	7657	745	3	-1.66	0.087
Glutethimide	Heterocyclic	III	167	7.00	3	-1.3	0.045	284	20.7	3	-1.47	0.131	125	9.25	4	-1.20	0.163
Glycerol	Alcohol	III	20000	2987	3	-2.02	0.273	38878	28238	4	-2.27	1.29	27833	10882	3	-1.87	0.306
Haloperidol	Ketone	III	5.32	0.649	3	-2.34	0.445	7.99	0.655	3	-4.99	0.378	5.47	0.654	3	-1.86	0.048

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Hexachlorophene	Cyclic hydrocarbon Phenol	III	5.02	2.41	3	-1.62	0.189	5.35	1.75	3	-1.17	0.322	3.06	0.289	3	-1.66	0.217
Lactic acid	Carboxylic acid	III	2943	315	3	-4.13	1.54	3487	561	3	-6.62	3.23	2790	259	3	-3.64	1.09
Lindane	Halogenated hydrocarbon	III	125	119	3	-0.737	0.231	266	94.8	4	-1.26	1.283	90.4	111	5	-1.46	0.262
Lithium I carbonate	Alkalies; Inorganic carbon; Lithium compound	II	564	67.6	3	-1.59	0.313	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Meprobamate	Carboxylic acid	III	353	49.7	3	-1.16	0.438	877	128	4	-1.32	0.270	386	9.02	3	-1.12	0.133
Mercury II chloride	Mercury compound; Chlorine compound	III	3.45	0.177	3	-4.18	0.988	5.99	1.87	3	-4.34	1.11	3.51	0.120	3	-4.16	1.31
Methanol	Alcohol	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	NA	NA	-	NA	NA
Nicotine	Heterocyclic	III	272	65.3	3	-1.58	0.357	412	136	3	-12.0	6.99	450	54.7	3	-49.6	70.9
Paraquat	Heterocyclic	III	21.3	7.29	3	-1.32	0.341	24.9	16.5	3-	-4.10	3.13	23.7	15.2	3	-1.92	0.581
Parathion	Organophos-phorous; Sulfur compound	III	22.7	12.1	3	-1.89	1.33	141	98.7	4	-1.62	0.520	22.0	4.94	3	-1.55	0.562
Phenobarbital	Heterocyclic	III	634	134	3	-1.43	0.177	726	255	3	-1.84	0.851	476	111	4	-1.67	0.418
Phenol	Phenol	III	50.2	10.9	3	-1.46	0.318	104	24.8	3	-1.55	0.205	58.1	6.78	3	-1.41	0.259
Phenylthiourea	Sulfur compound; Urea	III	30.1	19.8	3	-0.781	0.218	239	65.8	3	-0.890	0.206	89.0	21.9	3	-1.40	0.127
Physostigmine	Carboxylic acid; Heterocyclic	III	28.2	14.9	3	-1.51	0.595	37.8	1.93	3	-7.22	1.04	20.4	6.71	4	-1.70	0.157
Potassium I chloride	Potassium compound; Chlorine compound	II	3352	468	4	-3.32	1.17	3842	1198	5	-4.31	2.27	3710	417	3	-2.87	0.147

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Potassium cyanide	Potassium compound; Nitrogen compound	III	15.3	3.76	3	-1.48	0.677	159	81.9	3	-1.03	0.152	18.9	0.950	3	-3.43	0.488
Procainamide HCl	Carboxylic acid; Amide	III	400	15.3	3	-12.4	1.91	431	4.73	3	-45.6	18.4	497	39.3	3	-19.9	13.1
2-Propanol	Alcohol	II	2610	240	2	-1.80	0.001	3970	139	3	-1.65	0.241	4110	161	3	-1.93	0.160
Propranolol HCl	Alcohol	Ib	13.6	4.37	4	-2.54	0.627	13.5	6.85	4	-3.31	2.53	17.6	3.78	3	-3.45	1.44
Propylparaben	Carboxylic acid; Phenol	III	20.9	3.33	3	-1.23	0.259	51.8	14.8	3	-1.45	0.442	17.1	2.10	3	-1.24	0.245
Sodium arsenite	Sodium compound; Arsenical	III	0.496	0.028	3	-1.43	0.087	1.44	0.819	3	-3.79	1.22	0.683	0.117	3	-1.90	0.535
Sodium chloride	Sodium compound; Chlorine compound	III	4790	233	3	-1.55	0.182	4625	611	4	-2.67	0.620	4877	457	3	-2.03	0.366
Sodium dichromate dihydrate	Sodium compound; Chromium compound	III	0.603	0.087	3	-1.64	0.136	0.657	0.244	3	-5.01	1.51	0.547	0.092	3	-1.93	0.194
Sodium I fluoride	Sodium compound; Fluorine compound	II	61.3	5.55	3	-5.06	1.50	96.1	17.7	3	-4.40	0.971	82.0	5.81	3	-2.73	0.850
Sodium hypochlorite	Sodium compound Oxygen compound; Chlorine compound	III	823	108	3	-2.57	1.12	805	367	3	-4.13	3.05	2005	872	4	-3.20	0.279
Sodium oxalate	Sodium compound; Carboxylic acid	III	42.0	17.3	3	-1.83	0.380	31.0	8.66	3	-3.11	0.367	49.5	26.3	4	-2.32	0.592
Sodium selenate	Sodium compound; Selenium compound	II	12.7	1.62	3	-1.59	0.217	54.2	10.4	3	-3.76	0.968	36.5	5.23	3	-1.65	0.112

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Strychnine	Heterocyclic	III	389	80.9	3	-2.51	0.728	124	20.3	3	-5.85	0.922	83.5	5.35	3	-6.49	2.12
Thallium I sulfate	Sulfur compound; Metal	III	2.81	0.671	3	-1.02	0.201	13.4	10.4	4	-0.714	0.302	6.27	1.75	3	-0.752	0.081
Trichloroacetic acid	Carboxylic acid	III	762	99.1	3	-1.66	0.118	1220	72.1	3	-2.22	0.089	801	114	3	-1.77	0.130
1,1,1-Trichloro-ethane	Halogenated hydrocarbon	III	41100	NA	1	-2.38	NA	21250	2357	3	-31.5	32.1	9827	180	3	-21.8	8.47
Triethylene-melamine	Heterocyclic	III	0.086	0.009	3	-0.567	0.018	1.45	0.265	3	-1.88	1.04	0.169	0.049	3	-0.615	0.138
Triphenyltin hydroxide	Organo-metallic compound	III	0.026	0.004	3	-1.66	0.257	0.026	0.021	3	-4.78	3.37	0.015	0.008	3	-1.46	0.149
Valproic acid	Carboxylic acid; Lipids	III	547	67.1	3	-2.24	0.742	1807	175	3	-4.07	0.766	574	NA	1	-1.24	NA
Verapamil HCl	Amine	III	32.2	5.82	3	-4.43	1.362	34.6	1.72	3	-29.1	18.6	38.9	4.20	3	-5.00	0.935
Xylene	Cyclic hydrocarbon	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	724	87.1	3	-1.91	0.473

Abbreviations: ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; SD=Standard deviation; N=Number of data points; NA=Not available (i.e., IC_{50} values or Hill Slope values could not be generated [see notes in **Appendix I** for more information])

[1]Arithmetic mean.

[2]Standard deviation of IC_{50}.

[3]Arithmetic Mean of Hill Slope values.

[4]Standard deviation of Hill Slope values.

[5]Chemical class assigned is based on the classification of the National Library of Medicine's Medical Subject Heading (MeSH), http://www.nlm.nih.gov/mesh/meshhome.html.

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Acetaminophen	Amide	III	558	80.7	3	-1.09	0.108	447	83.7	3	-1.09	0.646	571	79.0	3	-1.20	0.154
Acetonitrile	Nitrile	III	10868	7824	4	-2.61	0.424	10153	1960	4	-5.95	3.34	9290	413	3	-2.79	0.306
Acetylsalicylic acid	Carboxylic Acid; Phenol	III	631	19.9	3	-1.94	0.367	694	98.3	3	-1.85	0.324	514	79.1	3	-1.97	0.083
Aminopterin	Heterocyclic	II	889	182	3	-2.03	0.375	545	42.2	3	-1.27	0.225	611	70.7	2	-1.72	0.547
5-Aminosalicylic acid	Carboxylic Acid; Phenol	III	29.9	6.52	3	-3.45	0.806	78.2	42.3	3	-7.96	6.90	48.8	7.90	3	-3.66	0.629
Amitriptyline HCl	Polycyclic	III	10.8	3.34	3	-1.79	0.236	7.57	5.43	3	-1.43	0.479	10.9	1.04	3	-2.27	0.278
Arsenic III Trioxide	Arsenical	Ib	7.77	2.54	4	-2.67	0.470	2.55	1.92	6	-1.78	1.14	20.9	6.4	3	-2.02	0.338
Atropine sulfate	Heterocyclic	III	85.4	10.5	3	-1.26	0.307	104	88.2	3	-2.90	3.48	83.2	21.0	3	-1.21	0.101
Boric acid	Boron compound; Acid	III	440	138	3	-1.19	0.233	517	378	3	-0.752	0.117	464	11	3	-1.33	0.194
Busulfan	Alcohol; Sulfur compound; Acyclic hydrocarbon	III	253	68.2	3	-0.783	0.323	268	193	3	-1.50	0.357	313	37.2	3	-1.66	0.459
Cadmium II chloride	Cadmium compound; Chlorine compound	II	2.20	0.823	5	-4.01	1.25	1.88	1.22	3	-3.36	3.14	1.86	0.151	3	-4.65	1.38
Caffeine	Heterocyclic	III	817	256	3	-1.44	0.504	591	186	3	-1.06	0.499	574	7.81	3	-1.28	0.117
Carbamazepine	Heterocyclic	III	66.1	8.4	3	-1.15	0.307	253	325	3	-2.57	2.53	63.9	5.27	3	-1.34	0.444
Carbon tetrachloride	Halogenated hydrocarbon	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	NA	NA	-	NA	NA
Chloral hydrate	Alcohol	III	140	34.2	3	-1.55	0.378	159	50.1	3	-1.33	0.105	112	1.73	3	-1.42	0.123
Chloramphenicol	Alcohol; Nitro compound; Cyclic hydrocarbon	II	318	142	3	-1.51	0.794	414	182	4	-1.16	0.091	367	79.7	3	-0.917	0.249
Citric acid	Carboxylic acid	III	526	82.4	3	-1.62	0.158	312	51.6	4	-1.25	0.249	433	22.3	3	-1.62	0.080

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Colchicine	Polycyclic	II	0.005	0.002	3	-2.15	1.39	0.008	0.001	3	-3.16	1.96	0.008	0.002	3	-13. 8	11.0
Cupric sulfate pentahydrate	Sulfur compound; Metal	III	190	19.6	3	-6.16	3.16	195	12.5	3	-3.85	0.328	207	7.09	3	-5.69	0.871
Cycloheximide	Heterocyclic	III	0.053	0.012	3	-1.24	0.152	0.120	0.094	3	-0.850	0.388	0.071	0.013	3	-1.54	0.178
Dibutyl phthalate	Carboxylic acid	III	28.3	7.64	3	-1.40	0.295	47.4	34.3	3	-1.02	0.352	22.0	1.32	3	-1.33	0.197
Dichlorvos	Organophos-phorous	III	8.56	2.28	3	-1.17	0.147	12.4	3.74	3	-2.29	2.33	12.2	0.416	3	-1.50	0.214
Diethyl phthalate	Carboxylic acid	III	174	14.4	3	-2.21	0.358	71.5	67.3	3	-1.67	0.637	189	33.1	3	-1.97	0.242
Digoxin	Polycyclic; Carbohydrate	III	0.0054	0.0007	3	-2.00	0.127	0.0001	0.00002	3	-1.38	0.684	0.004	0.0003	3	-4.59	1.73
Dimethyl-formamide	Amide; Carboxylic acid	III	9353	155	3	-3.67	0.273	7817	100	3	-2.85	0.590	6397	202	3	-3.00	0.161
Diquat dibromide monohydrate	Heterocyclic	III	3.59	0.825	3	-1.44	0.051	6.77	3.73	4	-1.38	0.488	3.84	0.313	3	-1.10	0.139
Disulfoton	Organophos-phorous; Sulfur compound	III	140	27.0	3	-1.65	1.15	808	213	3	-0.841	0.452	186	59.2	3	-0.836	0.209
Endosulfan	Heterocyclic Sulfur compound	III	3.44	0.573	3	-1.68	0.438	1.42	0.701	4	-1.19	0.369	2.19	0.437	3	-2.20	0.242
Epinephrine bitartrate	Alcohol; Amine	III	115	10.8	3	-7.37	2.10	81.7	28.4	3	-8.39	5.81	75.0	12.2	3	-4.90	2.81
Ethanol	Alcohol	III	8290	390	3	-2.13	0.035	12013	2286	3	-1.82	0.635	10250	867	3	-2.29	0.185
Ethylene glycol	Alcohol	Ib	38000	4681	3	-3.22	0.650	49800	4371	3	-3.02	0.188	40000	5341	4	-2.56	0.444
Fenpropathrin	Nitrile; Ester; Ether	III	3.73	1.01	3	-1.42	0.486	2.23	0.616	3	-4.37	4.45	1.82	0.310	3	-1.78	0.617
Gibberellic acid	Polycyclic	III	2850	402	3	-2.45	0.372	2940	276	3	-5.90	2.69	2807	121	3	-3.30	1.104
Glutethimide	Heterocyclic	III	187	64.3	3	-1.47	0.616	170	24.1	3	-1.29	0.145	176	27.5	3	-1.54	0.237
Glycerol	Alcohol	III	34267	15399	3	-3.32	1.97	18023	8334	3	-1.62	0.521	29033	4596	3	-2.69	0.511
Haloperidol	Ketone	III	3.69	1.01	3	-0.964	0.206	3.72	1.81	3	-0.732	0.097	3.29	1.15	3	-0.840	0.100

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Hexachlorophene	Cyclic hydrocarbon Phenol	III	0.027	0.004	3	-2.21	0.301	0.046	0.020	3	-2.91	0.662	0.021	0.002	3	-2.36	0.059
Lactic acid	Carboxylic acid	III	1290	52.9	3	-2.36	0.306	1320	60.8	3	-3.25	0.328	1313	138	3	-3.23	0.408
Lindane	Halogenated hydrocarbon	III	19.1	3.14	3	-3.02	0.969	23.2	7.09	3	-2.24	0.315	15.6	2.4	3	-2.61	0.265
Lithium I carbonate	Alkalies; Inorganic carbon; Lithium compound	II	411	119	3	-1.95	0.456	486	95.7	3	-1.78	1.31	535	31.6	3	-2.64	0.164
Meprobamate	Carboxylic acid	III	761	116	3	-1.90	0.695	163	189	3	-0.806	0.206	624	84.2	3	-2.04	0.170
Mercury II chloride	Mercury compound; Chlorine compound	III	6.87	1.04	3	-16.3	4.95	5.4	1.02	3	-17.8	13.1	5.35	0.09	3	-17.8	3.31
Methanol	Alcohol	III	NA	NA	-	NA	NA	1133	213	3	-1.79	0.874	2100	226	3	-1.86	0.297
Nicotine	Heterocyclic	III	94.3	24.7	3	-0.654	0.092	134	78.4	3	-0.668	0.077	112	27.7	3	-0.733	0.047
Paraquat	Heterocyclic	III	48.3	6.03	3	-1.04	0.158	96.6	37.2	3	-1.34	0.326	53.4	5.52	3	-1.47	0.034
Parathion	Organophos-phorous; Sulfur compound	III	34.0	10.0	3	-1.60	0.640	31.2	11.9	3	-1.18	0.200	29.0	8.34	3	-1.85	0.956
Phenobarbital	Heterocyclic	III	693	180	3	-1.10	0.214	360	95.5	3	-0.976	0.229	381	69.9	3	-1.68	0.353
Phenol	Phenol	III	59.1	21.4	3	-0.919	0.084	93.2	5.97	3	-1.15	0.209	80.8	5.12	3	-0.915	0.029
Phenylthiourea	Sulfur compound; Urea	III	363	58	3	-1.55	0.726	401	83.6	3	-3.49	1.91	272	71.7	3	-1.00	0.053
Physostigmine	Carboxylic acid; Heterocyclic	III	164	5.51	3	-3.05	0.552	212	238	3	-3.81	2.44	139	8.74	3	-2.97	0.135
Potassium I chloride	Potassium compound; Chlorine compound	II	2560	432	3	-2.23	0.383	2287	631	3	-1.09	0.163	1990	161	3	-2.05	0.165

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Potassium cyanide	Potassium compound; Nitrogen compound	III	29.3	6.9	3	-1.21	0.241	89.0	100	3	-1.10	0.319	16.9	2.21	3	-1.37	0.154
Procainamide HCl	Carboxylic acid; Amide	III	1480	200	3	-3.56	0.813	1787	221	3	-4.22	1.57	2027	229	3	-4.42	0.459
2-Propanol	Alcohol	II	5263	583	3	-2.01	0.173	4273	1139	3	-2.31	0.211	7087	480	3	-3.01	0.406
Propranolol HCl	Alcohol	Ib	38.3	4.54	3	-3.44	0.559	43.8	2.52	3	-2.72	1.461	28.6	3.28	4	-2.09	0.413
Propylparaben	Carboxylic acid; Phenol	III	18.1	2.42	3	-1.18	0.122	18.6	2.84	3	-1.58	0.399	13.8	1.21	3	-1.20	0.065
Sodium arsenite	Sodium compound; Arsenical	III	0.79	0.248	3	-1.69	0.222	0.336	0.187	3	-1.54	0.317	0.470	0.066	3	-1.96	0.197
Sodium chloride	Sodium compound; Chlorine compound	III	3583	263	3	-2.43	0.153	1118	1388	3	-1.96	0.371	3470	300	3	-2.47	0.208
Sodium dichromate dihydrate	Sodium compound; Chromium compound	III	0.784	0.113	3	-2.35	0.282	0.851	0.302	4	-3.52	1.49	0.576	0.100	3	-2.32	0.199
Sodium I fluoride	Sodium compound; Fluorine compound	II	48.7	6.92	3	-2.50	0.263	39.7	9.61	3	-2.60	1.04	53.7	6.82	4	-2.71	0.150
Sodium hypochlorite	Sodium compound Oxygen compound; Chlorine compound	III	1863	581	3	-5.19	1.14	1243	576	3	-2.78	1.27	1633	180	3	-3.86	0.211
Sodium oxalate	Sodium compound; Carboxylic acid	III	355	54.9	3	-4.00	1.99	350	147	4	-6.10	6.40	360	94.6	3	-3.13	0.555
Sodium selenate	Sodium compound; Selenium compound	II	7.47	0.861	3	-1.78	0.529	16.1	9.55	3	-3.07	0.456	10.0	1.33	3	-1.75	0.226

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Strychnine	Heterocyclic	III	100	76.6	4	-1.30	0.729	52.5	28.0	3	-1.60	0.260	55.1	3.43	3	-1.47	0.466
Thallium I sulfate	Sulfur compound; Metal	III	0.198	0.100	3	-2.08	1.01	0.153	0.031	3	-2.64	0.639	0.127	0.020	3	-2.90	0.338
Trichloroacetic acid	Carboxylic acid	III	348	63.5	3	-1.36	0.241	541	150	3	-1.34	0.411	394	50.8	3	-1.48	0.103
1,1,1-Trichloro-ethane	Halogenated hydrocarbon	III	8137	591	3	-14.0	6.08	NA	NA	-	NA	NA	NA	NA	-	NA	NA
Triethylene-melamine	Heterocyclic	III	1.69	0.950	3	-0.838	0.076	2.03	0.471	3	-1.37	0.471	2.13	0.480	3	-1.95	0.369
Triphenyltin hydroxide	Organo-metallic compound	III	0.021	0.007	3	-2.46	0.698	0.007	0.007	3	-3.55	1.68	0.011	0.003	3	-3.34	0.396
Valproic acid	Carboxylic acid; Lipids	III	468	116	3	-1.31	0.252	702	160	3	-1.83	0.455	430	71.5	3	-1.24	0.115
Verapamil HCl	Amine	III	60.5	13.6	3	-1.72	0.238	79.4	33.9	3	-1.88	0.915	66.2	5.57	3	-2.53	0.221
Xylene	Cyclic hydrocarbon	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	486	185	3	-2.88	1.99

Abbreviations: ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; SD=Standard deviation; N=Number of data points; NA=Not available (i.e., IC_{50} values or Hill Slope values could not be generated [see notes in **Appendix I** for more information])

[1]Arithmetic mean.

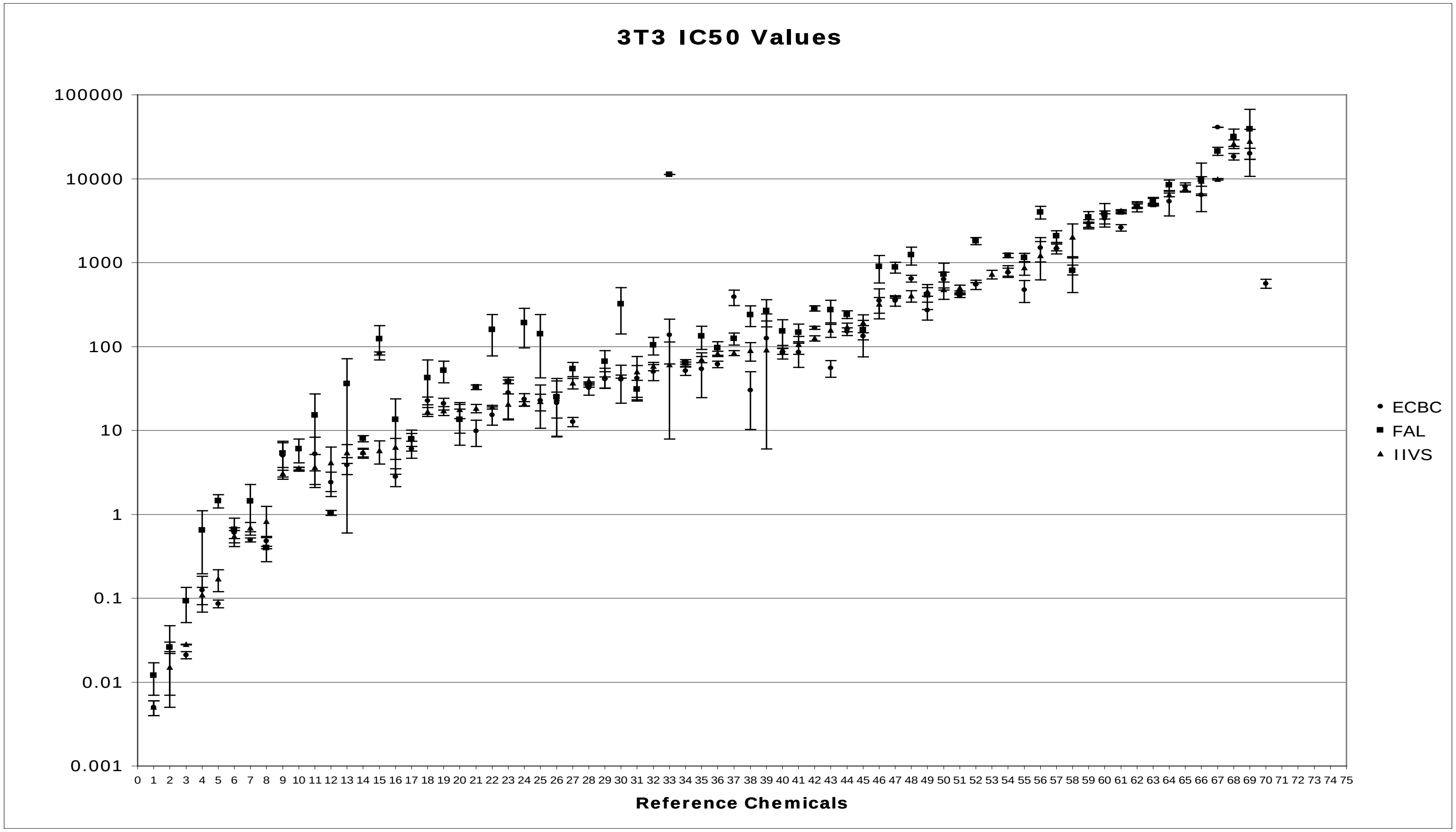
[2]Standard deviation of IC_{50}.

[3]Arithmetic Mean of Hill Slope values.

[4]Standard deviation of Hill Slope values.

[5]Chemical class assigned is based on the classification of the National Library of Medicine's Medical Subject Heading (MeSH), http://www.nlm.nih.gov/mesh/meshhome.html.

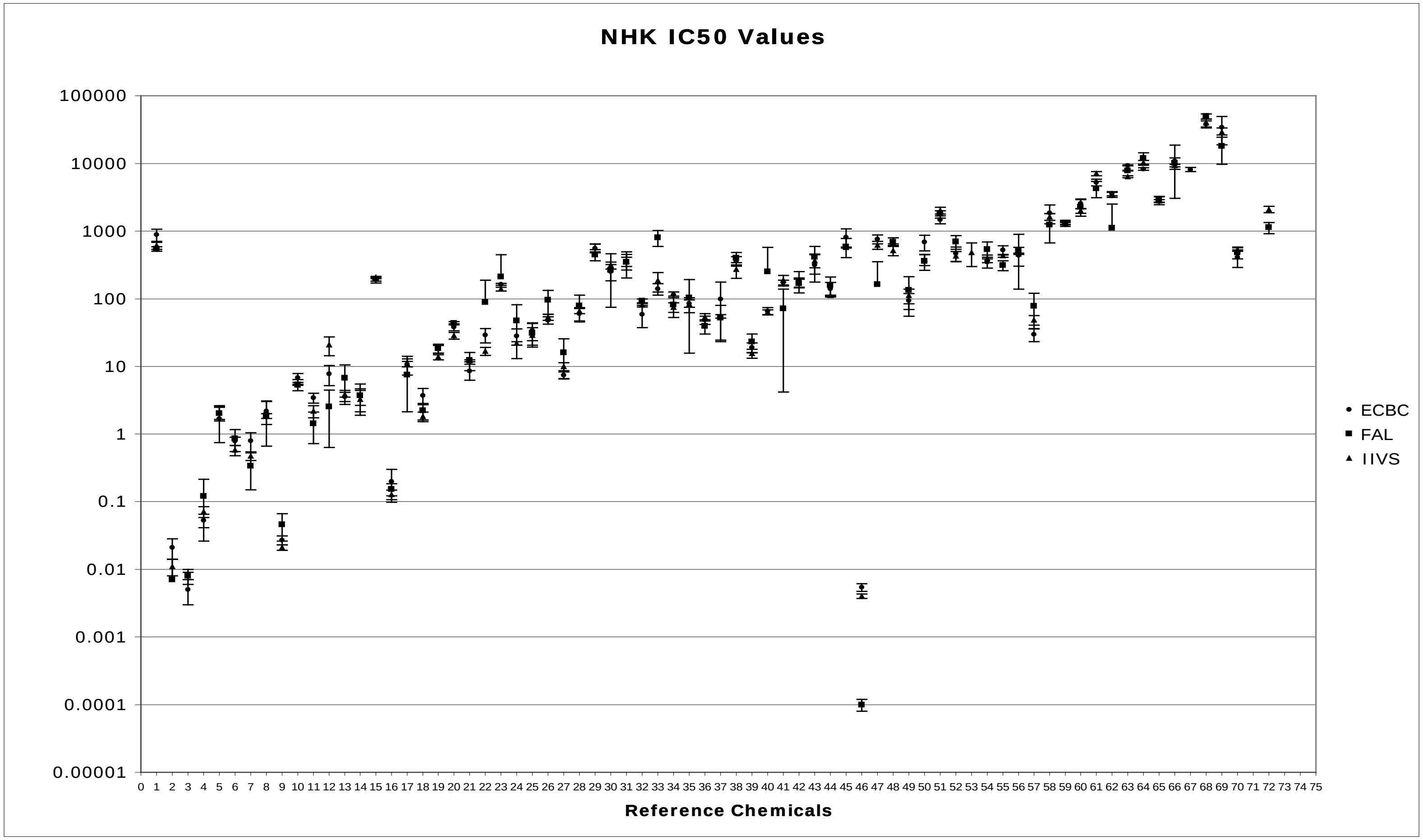
Figure 5-1 Reference Substance IC_{50} Results for the 3T3 NRU Test Method by Laboratory



Abbreviations: ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

Points show the mean arithmetic IC_{50} (µg/mL) for each reference substance from each laboratory. Error bars show the standard deviation. Data were sorted in ascending order of 3T3 IC_{50} values from IIVS (lead laboratory in the validation study). **Table 5-6** provides the numerical key for reference substance identification.

Figure 5-2 Reference Substance IC_{50} Results for the NHK NRU Test Method by Laboratory



Abbreviations: ECBC=Edgewood Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.
Points show the mean arithmetic IC_{50} (µg/mL) for each reference substance from each laboratory. Error bars show the standard deviation. Data were sorted in ascending order of 3T3 IC_{50} values from IIVS (lead laboratory in the validation study). **Table 5-6** provides the numerical key for reference substance identification.

Table 5-6 Key to Validation Study Reference Substances[1]

No	Reference Substance	No	Reference Substance	No	Reference Substance	No	Reference Substance
1	Aminopterin	19	Propylparaben	37	Strychnine	55	Citric acid
2	Triphenyltin hydroxide	20	Propranolol HCl	38	Phenylthiourea	56	Boric acid
3	Colchicine	21	Dichlorvos	39	Lindane	57	5-Aminosalicylic acid
4	Cycloheximide	22	Potassium cyanide	40	Carbamazepine	58	Sodium hypochlorite
5	Triethylenemelamine	23	Physostigmine	41	Diethyl phthalate	59	Lactic acid
6	Sodium dichromate dihydrate	24	Dibutyl phthalate	42	Glutethimide	60	Potassium I chloride
7	Sodium arsenite	25	Parathion	43	Chloramphenicol	61	2-Propanol
8	Cadmium II chloride	26	Paraquat	44	Chloral hydrate	62	Sodium chloride
9	Hexachlorophene	27	Sodium selenate	45	Caffeine	63	Dimethylformamide
10	Mercury II chloride	28	Verapamil HCl	46	Digoxin	64	Ethanol
11	Endosulfan	29	Acetaminophen	47	Meprobamate	65	Gibberellic acid
12	Arsenic III trioxide	30	Busulfan	48	Acetylsalicylic acid	66	Acetonitrile
13	Diquat dibromide monohydrate	31	Sodium oxalate	49	Nicotine	67	1,1,1-Trichloroethane
14	Haloperidol	32	Phenol	50	Phenobarbital	68	Ethylene glycol
15	Cupric sulfate pentahydrate	33	Disulfoton	51	Procainamide HCl	69	Glycerol
16	Thallium I sulfate	34	Epinephrine bitartrate	52	Valproic acid	70	Lithium I carbonate
17	Amitriptyline HCl	35	Atropine sulfate	53	Xylene	71	Carbon tetrachloride
18	Fenpropathrin	36	Sodium I fluoride	54	Trichloroacetic acid	72	Methanol

Abbreviations: No=Number.
[1]As used in **Figures 5-1** and **5-2**.

5.5 Statistical Approaches to the Evaluation of 3T3 and NHK Data

The statistical approaches used for data evaluation are reviewed in the following sections for each phase of the validation study. **Section 2.2.3** discussed the endpoint measurements for the 3T3 and NHK test methods. The OD values of each of six replicate wells ([minimum of four] in the 96-well plate) per test concentration (eight concentrations/reference substance or PC) were used to determine relative cell viability in relation to the mean VC OD on the same plate. The cell viability values calculated for the replicate wells for each concentration were used to determine the concentration-response curve (percent viability vs. log concentration) for each test. The IC_{50} value was determined from fitting the curve to a Hill function.

5.5.1 Statistical Analyses for Phase Ia Data

The laboratories reported the IC_{50} results for SLS in µg/mL. The SMT used the results from the acceptable tests to calculate means and SDs for each method at each laboratory.

5.5.1.1 *Outlier Determination for Replicate Well Concentration Data*

A test for outliers at the 99% level (Dixon and Massey 1981) was used to determine the presence of outlier OD values among the six replicate wells for each reference substance concentration. The SMT applied the outlier test to the Phase Ia data when extreme values were noted. Outliers were excluded from the data set, and the IC_{50} was recalculated. The raw data files include all data provided by the laboratories, including the excluded outlier OD values. Because the protocol required a minimum of four acceptable test wells per reference substance concentration, no more than two wells of the six replicates could be excluded.

5.5.1.2 *Curve Fit Criteria*

After the completion of Phase Ia testing, a curve fit criterion was implemented for test acceptance following a visual review of the fit of the OD data to the Hill function curve. The SMT considered the fit of the concentration-response curve to the Hill function to be acceptable when $R^2 > 0.9$. A fit of $R^2 < 0.8$ was considered unacceptable and the data from that test were rejected. Curves with a fit of $0.8 < R^2 < 0.9$ were evaluated visually for goodness of fit and accepted if the SMT concluded that there were sufficient data points between 0 and 100% cytotoxicity, and a reasonable shape to the curve, to calculate a reasonably accurate IC_{50} value. Each test with a curve fit in this range was analyzed on a case-by-case basis, and no standard pass/fail criterion was developed. [Note: The use of a curve fit criterion was reevaluated in Phases Ib and II, and was eliminated as a test acceptance criterion for Phase III test results. An R^2 value ≥ 0.85 was maintained as a test acceptance criterion for the PC because its fit to the Hill function was well characterized.]

5.5.1.3 *Reproducibility Analyses for PC IC_{50} Values*

To evaluate reproducibility of the IC_{50} values for the PC for each test method, within and between the laboratories, the SMT considered the American Society of Testing and Materials (ASTM) Standard E691-99, *Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method* (ASTM 1999). This method uses two statistics, *h* and *k*, to judge the consistency of means and variances between laboratories. However, a minimum of six laboratories is required for this type of analysis and the SMT decided that it could not be appropriately applied to three laboratories. The variability of the PC IC_{50} results obtained from each test and laboratory was assessed using CV analysis and one-way ANOVA. Dividing the SD by the arithmetic mean IC_{50} value, and multiplying by 100 produced the CV. CV values were calculated for the acceptable tests within each laboratory to determine intralaboratory reproducibility. To compare the variation among laboratories, the CV was calculated using the arithmetic mean IC_{50} values from each of the three laboratories. Although no criterion for an acceptable CV was determined for this study, ECVAM recently used CV <30% as an acceptable range for both intra- and inter-laboratory reproducibility (Zuang et al. 2002; Fentem et al. 2001). Although CV <30% was intended to reflect an acceptable maximum for normal biological variability, the range was not supported by data.

For the ANOVA, IC_{50} values were first converted to mM units and then log-transformed to obtain normal distributions. One-way ANOVA was performed with SAS PROC GLM software (SAS Institute 1999; see **Appendix D1** for example SAS code). A significance level of $p < 0.01$ was used to test results between the laboratories in order to be conservative with respect to identifying laboratory differences.

5.5.2 Statistical Analyses of Phase Ib Data

5.5.2.1 *Outlier Determination for Replicate Well Concentration Data*

For consistency of replicate well concentration data, the SMT applied the same outlier test used for the Phase Ia data (Dixon and Massey 1981) when extreme OD values were noted. If the extreme value was an outlier at the 99% level, it was excluded from the data set, and the IC_{50} was recalculated. All data are available in the data files provided by the laboratories, including the excluded outlier OD values.

5.5.2.2 *Reproducibility Analyses of the Reference Substance IC_{50} Values*

One-way ANOVA and CV analyses were used to assess method reproducibility within and among laboratories. For the ANOVA, the IC_{50} values were first converted to mM units and then log-transformed to obtain normal distributions. One-way ANOVA was performed with SAS PROC GLM (SAS Institute 1999; see **Appendix D1** for example SAS code). A significance level of $p < 0.01$ was used to test results between the laboratories in order to be conservative with respect to identifying laboratory differences. When the ANOVA detected significant differences among the laboratories, contrast analyses were performed to determine which laboratory was different from the others. These analyses compared the results of each laboratory with those of the other two laboratories. A significant difference in response among the laboratories was indicated by $p < 0.01$.

CV values were calculated for each reference substance by dividing the SD by the arithmetic mean IC_{50} value and multiplying by 100. CV values were calculated for the acceptable tests in each laboratory to determine intralaboratory reproducibility. To compare the variation among laboratories, the CV was calculated using the arithmetic mean IC_{50} values from each of the three laboratories.

As an additional approach to the assessment of interlaboratory reproducibility for each test substance, the maximum:minimum IC_{50} ratios (i.e., the maximum arithmetic mean laboratory IC_{50} value compared to the minimum arithmetic mean laboratory IC_{50} value) were calculated. This approach is similar to the calculation of maximum:minimum LD_{50} ratios for examining reproducibility of reference LD_{50} values (see **Section 4.4.1**).

5.5.3 Statistical Analyses of Phase II Data

5.5.3.1 *Outlier Determination for Replicate Well Concentration Data*

The Dixon and Massey (1981) outlier test was incorporated into the EXCEL® templates to assess the consistency of replicate well data for each reference substance concentration. Outliers at the 99% level were highlighted and the Study Director was offered the option of removing the value from subsequent calculations (e.g., mean OD of the six replicates; % viability; IC_{50}).

5.5.3.2 *Reproducibility Analyses of the Reference Substance IC_{50} Values*

The intra- and inter-laboratory reproducibility of the IC_{50} values were assessed using the acceptable tests to calculate the mean IC_{50}, SD, and CV for each substance, method, and laboratory, as described in **Section 5.5.2.2**. One-way ANOVAs and calculations of maximum:minimum IC_{50} ratios were performed as described in **Section 5.5.2.2**.

5.5.3.3 *Comparison of 3T3 and NHK Test Results with the RC Millimole Regression*
To compare the 3T3 and NHK test results for the reference substances to those of the RC millimole regression, each IC_{50} value was transformed to mM units for the calculation of geometric mean IC_{50} values. The use of geometric means corresponded with the approach used to obtain single IC_{50} values from multiple IC_{50} values for the RC millimole regression (Halle 1998, 2003). The log geometric mean IC_{50} values (in mM) of the 11 RC substances tested during Phases Ib and II (see **Table 3-8**) were used with the log RC LD_{50} values, after transformation to log mmol/kg units (see **Appendices J1** and **J2**), to calculate least squares linear regressions for the data from each test method and laboratory. Each of these method/laboratory regressions was compared to the RC millimole regression using an F test with SAS PROC REG (SAS Institute 1999; see **Appendix D2** for example SAS code). An F test with a significance level of $p < 0.01$ was used to determine whether the joint comparison of slope and intercept indicated that the method/laboratory regressions were significantly different from the RC millimole regression.

As an alternate analysis, a least squares linear regression using IC_{50} and LD_{50} values from the RC was constructed for the 11 RC substances (*the RC-11 regression*) tested in Phases Ib and II. Each of these method/laboratory regressions was compared to the RC-11 regression using an F test with SAS PROC GLM (SAS Institute 1999; see **Appendix D2** for example SAS code) at a significance level of $p < 0.01$. This was used to determine whether the comparisons of slope and intercept indicated that the laboratory regressions were significantly different from the RC-11 regression.

5.5.4 <u>Statistical Analyses of Phase III Data</u>

5.5.4.1 *Outlier Determination for Replicate Well Concentration Data*
The laboratories used the Dixon and Massey (1981) outlier test at the 99% level that was incorporated into the EXCEL® templates to test for outlier values among replicate well data at the different reference substance concentrations. The Study Director had the option of excluding the outliers from the data set, which were highlighted by the template, and subsequent calculations. All data are available in the data files provided by the laboratories, including the outlier OD values.

5.5.4.2 *Reproducibility Analyses of the PC IC_{50} Data*
A number of analyses were performed to determine whether the SLS IC_{50} values were reproducible across study phases. The SLS IC_{50} values used to access variability were different from those shown in **Table 5-3**. To get an assessment of the true variation of SLS IC_{50} values, the reproducibility analyses included additional IC_{50} values from SLS tests that did not meet the IC_{50} acceptance limits (see **Table 5-3**) for each laboratory and study phase if they passed all other test acceptance criteria. If more than one SLS test was performed on a single day (for any test method and laboratory), the IC_{50} values were averaged to determine a single IC_{50} for the day. This prevented multiple data values from a single day from overly influencing the mean for each phase. CV analyses were performed as described in **Section 5.5.1** using the arithmetic mean SLS IC_{50} values for each method, laboratory, and study phase.

For the remaining analyses of reproducibility, the IC_{50} values were first log-transformed to obtain normal distributions. One-way ANOVAs were performed with SAS PROC GLM (SAS Institute 1999; see **Appendix D1** for example SAS code) for each method using study phase and laboratory as individual variables. A significance level of $p < 0.01$ was used to test for a statistical difference among the laboratory and/or phase results.

To determine whether there was a linear time trend for the SLS IC_{50} data, linear regression analyses using a least squares method were performed for each laboratory and method using SAS PROC REG (SAS Institute 1999). Time was expressed as an index for each test. The index number of each SLS test reflected its order of testing without respect to the time lapsing between tests. For example, the first SLS test was assigned a time index of 1 and the second SLS test was assigned a time index of 2 whether it occurred the day after the first test or one week after the first test. The slopes of the linear regressions were judged to be statistically significant at $p < 0.05$, which indicated that the IC_{50} had changed significantly over time.

5.5.4.3 *Reproducibility Analyses of the Reference Substance IC_{50} Values*

CV, one-way ANOVA analyses, and maximum:minimum IC_{50} ratios were performed to assess the intra- and/or inter-laboratory reproducibility of the Phase III reference substance data, as described in **Section 5.5.2.2**. An additional evaluation to determine whether normalizing the reference substance IC_{50} to the SLS IC_{50} would reduce interlaboratory variability was performed using five substances (for each test method) for which the ANOVAs indicated significant interlaboratory differences. The reference substance IC_{50} values were normalized to the SLS IC_{50} by calculating the reference substance IC_{50}:SLS IC_{50} ratio. CVs were calculated for each substance using the mean ratios from each laboratory. To determine whether this normalization reduced variability among the laboratories, the CVs for the substance IC_{50}:SLS IC_{50} ratios were compared to the CVs for the substance IC_{50}.
In addition, the geometric mean IC_{50} values were used to calculate least squares linear regression models after log transforming the data. Linear regressions were fit for each method and laboratory using the log-transformed reference LD_{50} values from **Table 4-2 (**in mmol/kg), with log IC_{50} in mM. To detect differences among the linear regressions in each laboratory, two models were fit for each method. The first was a full model that included effects for laboratory and interactions, and generated a regression line for each substance in each laboratory, by test method. The second model, which was considered to be a reduced model, assumed that one model fit all the laboratories. A goodness of fit F test was performed to compare the full and reduced models for each method. A significance level of $p < 0.01$ was used to test whether the regressions among laboratories were significantly different from one another. The following criteria were established for selection of data for use in the regression analyses for each test method:

- The substance was included in the RC
- All three laboratories reported IC_{50} values
- There was an associated rat oral reference LD_{50} value (see **Table 4-2**)

There were 47 reference substances that fit these criteria for the 3T3 and 51 test substances that fit the criteria for the NHK test methods.

5.5.4.4 *Comparison of 3T3 and NHK Results with the RC Millimole Regression*

To determine whether the IC_{50} values determined in the validation study were significantly different from the RC values, the laboratory-specific regression values for each method were combined using the geometric means of the laboratory-specific geometric mean IC_{50} values in mM and the reference LD_{50} in mmol/kg. Thus, there was one regression analysis with pooled laboratory data for the 3T3 NRU test method and another regression analysis (also with pooled data) for the NHK NRU test method. A third linear regression was calculated using the IC_{50} and LD_{50} values from the RC. The IC_{50} values and LD_{50} values were log-transformed for the regression calculations. The following criteria were established for the selection of substances to be used for the regression analyses:

- The substance was included in the RC
- All three laboratories reported IC_{50} values for both the 3T3 and NHK NRU test methods
- There was an associated rat oral reference LD_{50} value (see **Table 4-2**)

Forty-seven substances met these criteria. Two models were fit for each test method to detect differences between the NRU regression and the 47 RC substance regression. The first regression model was a full model that included effects for the RC and the NRU regression, and generated one regression line each for the RC and the NRU test method. The second (reduced) model assumed that a single model fit the combined RC and NRU IC_{50} data. The RC regression for the 47 reference substances was compared to the combined laboratory regression for each NRU test method using an F test to simultaneously compare slopes and intercepts. The NRU regressions were statistically different from the RC regressions if $p < 0.01$.

To assess the accuracy of the NRU methods and the associated IC_{50}-LD_{50} regressions, a predicted LD_{50} was calculated for each reference substance using its laboratory geometric mean IC_{50} in two analyses:

- The RC rat-only millimole regression calculated from the 282 RC substances with rat LD_{50} values, using units of mM for the IC_{50} and mmol/kg for the LD_{50} (see **Section 6.4.2**)
- The RC rat-only weight regression calculated from the 282 RC substances with rat LD_{50} values, using units of μg/mL for the IC_{50} and mg/kg for the LD_{50} (see **Section 6.4.3**)

The LD_{50} values predicted from the regression analyses were used to predict GHS acute oral toxicity categories (see **Section 6.4**). The accuracy of the predictions was determined by calculating the proportion of substances for which the predicted GHS toxicity category matched the GHS toxicity category. The LD_{50} predictions from these regression models were also used to determine starting doses for acute systemic toxicity test simulations for the purpose calculating animal use and savings that would be achieved using the NRU test methods. The simulation modeling methods, and results from the UDP and ATC methods, are described in **Section 10**.

5.5.5 Summary of the Data Used for Statistical Analyses

Table 5-7 summarizes the number of substances that were tested and the number of substances used for the various analyses performed to determine the accuracy and reliability of the *in vitro* NRU test methods.

Table 5-7 Datasets Used for Validation Study Analyses[1]

Use	3T3 NRU Test Method[1]	NHK NRU Test Method[1]	Characteristics of Dataset
Testing	72	72	Substances tested
Comparison of laboratory IC_{50}-LD_{50} regressions to one another	47	51	RC substances with IC_{50} values from all laboratories and reference rat oral LD_{50} values
Comparison of combined-laboratory IC_{50}-LD_{50} regressions to a regression calculated with RC data	47	47	RC substances with IC_{50} values for both test methods from all laboratories and rat oral reference LD_{50} values
Prediction of GHS accuracy using IC_{50} values in IC_{50}-LD_{50} regressions; prediction of starting doses for acute oral toxicity test (UDP and ATC) simulations	67	68	Substances with IC_{50} values from at least one laboratory
Reproducibility of acceptable rat oral LD_{50} values	NA	NA	62 substances with more than one acceptable rat oral LD_{50} value
Reproducibility of IC_{50} values	64	68	Substances with IC_{50} values from all laboratories
Comparison of reproducibility of IC_{50} values with reproducibility of LD_{50} values	53	57	Substances with IC_{50} values from all laboratories and more than one acceptable rat oral LD_{50} value

Abbreviations: RC=Registry of Cytotoxicity; 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; NA=Not applicable.
[1]Number of substances.

5.6 Summary of NRU Test Results

Table 5-8 shows the 3T3 and NHK IC_{50} values as geometric means of the geometric mean laboratory values, as a basis to compare the 3T3 and NHK NRU IC_{50} values for each reference substance. The substances in **Table 5-8** are organized by ascending 3T3 NRU IC_{50} values (as was done for **Figures 5-1** and **5-2**). For each method, the table provides the geometric mean IC_{50} (combined across laboratories) in µg/mL, the ratio of the geometric mean IC_{50} to the SLS IC_{50}, and the 3T3 IC_{50}:NHK IC_{50} ratios. Geometric means were used for this comparison because they were used for both the IC_{50} and LD_{50} regression analyses (see **Sections 5.5.3.3**, **5.5.4.3,** and **5.5.4.4**). The 3T3 and NHK NRU IC_{50} values were compared using the ratios of their geometric means. The IC_{50} values for each reference substance were also compared to the IC_{50} for SLS using the ratio of reference substance geometric mean IC_{50} to SLS geometric mean IC_{50}.

Table 5-8 Comparison of 3T3 and NHK NRU IC_{50} Geometric Means

Reference Substance	3T3 NRU Geometric Mean[1] IC_{50} (µg/mL)	3T3 NRU Ratio Geometric Mean IC_{50} to SLS IC_{50}	NHK NRU Geometric Mean[1] IC_{50} (µg/mL)	NHK NRU Ratio Geometric Mean IC_{50} to SLS IC_{50}	IC_{50} Ratios 3T3:NHK
Carbon tetrachloride	NA	NA	NA	NA	NA
Methanol	NA	NA	1529[3]	383.2	NA
Aminopterin	0.006	0.0001	669	167.7	0.00001
Triphenyltin hydroxide	0.017	0.0004	0.01	0.003	1.7
Colchicine	0.034	0.001	0.007	0.002	4.9
Cycloheximide	0.187	0.004	0.073	0.02	2.6
Triethylenemelamine	0.272	0.007	1.85	0.5	0.1
Cadmium II chloride	0.518	0.01	1.84	0.5	0.3
Sodium dichromate dihydrate	0.587	0.01	0.721	0.2	0.8
Sodium arsenite	0.759	0.02	0.477	0.1	1.6
Arsenic trioxide	1.96	0.05	5.26	1.3	0.4
Mercury II chloride	4.12	0.1	5.8	1.5	0.7
Hexachlorophene	4.19	0.1	0.029	0.01	144.5
Thallium I sulfate	5.74	0.1	0.152	0.04	37.8
Haloperidol	6.13	0.1	3.36	0.8	1.8
Endosulfan	6.35	0.2	2.13	0.5	3.0
Amitriptyline HCl	7.05	0.2	8.96	2.2	0.8
Diquat dibromide monohydrate	8.04	0.2	4.48	1.1	1.8
Propranolol	13.9	0.3	35.3	8.8	0.4
Dichlorvos	17.7	0.4	10.7	2.7	1.7
Paraquat	20.1	0.5	61.6	15.4	0.3
Fenpropathrin	24.2	0.6	2.43	0.6	10.0
Physostigmine	25.8	0.6	88.5	22.2	0.3
Propylparaben	26.1	0.6	16.6	4.2	1.6
Sodium selenate	29	0.7	10.2	2.6	2.8
Potassium cyanide	34.6	0.8	29	7.3	1.2
Verapamil HCl	34.9	0.8	66.5	16.7	0.5
Parathion	37.4	0.9	30.3	7.6	1.2
Sodium oxalate	37.7	0.9	337	84.5	0.1
Sodium lauryl sulfate (SLS)*	***41.7***	***1.0***	***3.99***	***1.0***	***10.5***
Cupric sulfate pentahydrate	42.1	1.0	197	49.4	0.2
Acetaminophen	47.7	1.1	518	129.8	0.1
Dibutyl phthalate	49.7	1.2	28.7	7.2	1.7
Epinephrine bitartrate	59	1.4	87.4	21.9	0.7
Phenol	66.3	1.6	75	18.8	0.9
Atropine sulfate	76	1.8	81.8	20.5	0.9
Busulfan	77.7	1.9	260	65.2	0.3
Sodium I fluoride	78	1.9	49.8	12.5	1.6
Phenylthiourea	79	1.9	336	84.2	0.2
Carbamazepine	103	2.5	83.2	20.9	1.2

Table 5-8 Comparison of 3T3 and NHK NRU IC_{50} Geometric Means

Reference Substance	3T3 NRU		NHK NRU		
	Geometric Mean[1] IC_{50} (µg/mL)	Ratio Geometric Mean IC_{50} to SLS IC_{50}	Geometric Mean[1] IC_{50} (µg/mL)	Ratio Geometric Mean IC_{50} to SLS IC_{50}	IC_{50} Ratios 3T3:NHK
Diethyl phthalate	107	2.6	120	30.1	0.9
Lindane	108	2.6	18.7	4.7	5.8
Chloramphenicol	128	3.1	348	87.2	0.4
Disulfoton	133	3.2	270	67.7	0.5
Caffeine	153	3.7	638	159.9	0.2
Strychnine	158	3.8	62.5	15.7	2.5
Glutethimide	174	4.2	174	43.6	1.0
Chloral hydrate	183	4.4	133	33.3	1.4
Nicotine	361	8.7	107	26.8	3.4
Procainamide HCl	441	10.6	1741	436.3	0.3
Digoxin	466	11.2	0.001	0.0003	466000.0
Meprobamate	519	12.4	357	89.5	1.5
Lithium I carbonate	562[2]	13.5	468	117.3	1.2
Phenobarbital	573	13.7	448	112.3	1.3
Acetylsalicylic acid	676	16.2	605	151.6	1.1
Xylene	721[2]	17.3	466[2]	116.8	1.5
Citric acid	796	19.1	400	100.3	2.0
Trichloroacetic acid	902	21.6	413	103.5	2.2
Valproic acid	916	22.0	512	128.3	1.8
Sodium hypochlorite	1103	26.5	1502	376.4	0.7
5-Aminosalicylic acid	1667	40.0	46.7	11.7	35.7
Boric acid	1850	44.4	421	105.5	4.4
Lactic acid	3044	73.0	1304	326.8	2.3
Potassium I chloride	3551	85.2	2237	560.7	1.6
2-Propanol	3618	86.8	5364	1344.4	0.7
Sodium chloride	4730	113.4	1997	500.5	2.4
Dimethylformamide	5224	125.3	7760	1944.9	0.7
Ethanol	6523	156.4	10018	2510.8	0.7
Gibberellic acid	7810[3]	187.3	2856	715.8	2.7
Acetonitrile	7951	190.7	9528	2388.0	0.8
1,1,1-Trichloroethane	17248	413.6	8122[2]	2035.6	2.1
Ethylene glycol	24317	583.1	41852	10489.2	0.6
Glycerol	24655	591.2	24730	6198.0	1.0

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; SLS=Sodium lauryl sulfate; NA=Not available.
Reference substances are ordered by 3T3 NRU IC_{50} values.
[1]Geometric mean IC_{50} of the laboratory geometric mean values.
[2]Data available from only one laboratory.
[3]Data available from only two laboratories.
*Acceptable positive control (SLS) values from all study phases: N=293 for the 3T3 NRU and N=281 for the NHK NRU.

Table 5-8 shows that there are nine reference substances for which the 3T3 and NHK NRU IC_{50} values differ by at least one order of magnitude (i.e., 3T3 IC_{50}:NHK IC_{50} $\leq$0.1 or $\geq$10): aminopterin, triethylenemelamine, hexachlorophene, thallium sulfate, fenpropathrin, sodium oxalate, acetaminophen, digoxin, and 5-aminosalicylic acid. The IC_{50} values for SLS, also differed by slightly more than one order of magnitude in the two NRU test methods (41.7 µg/mL for 3T3 and 3.99 µg/mL for NHK). One test method was not more consistently sensitive (i.e., produced lower IC_{50} values) than the other for these nine reference substances. The 3T3 NRU test method was more sensitive than the NHK NRU test method for four of the nine substances: aminopterin, triethylenemelamine, sodium oxalate, and acetaminophen. The NHK NRU test method was more sensitive than the 3T3 NRU test method for five substances: hexachlorophene, thallium sulfate, fenpropathrin, digoxin, and 5-aminosalicylic acid. Despite the normalization procedure, the reference substance IC_{50}:SLS IC_{50} ratios for the two methods were still greater by at least one order of magnitude for six of the nine substances (aminopterin, triethylenemelamine, hexachlorophene, sodium oxalate, acetaminophen, and digoxin) and the order of magnitude difference increased for all six substances. A number of factors could potentially be responsible for these differences between the 3T3 and NHK NRU IC_{50} values:

- Cell culture conditions (i. e., the 3T3 treatment medium contains serum while the NHK treatment medium does not; differences in cell density in the treatment medium)
- Differences in sensitivity between the fibroblast cell line and primary keratinocytes
- Differences in sensitivity between human and mouse cells
- Differences in metabolic activity between the cell types

These factors may affect the results for some substances more than others. For example, a substance that binds to serum proteins would be less available to the 3T3 cells (which have serum in their growth medium) than to NHK cells (which are grown without serum). No additional testing was performed to investigate the differences between the 3T3 and NHK NRU IC_{50} values.

Two substances, digoxin and aminopterin, have IC_{50} values that differ by five orders of magnitude between the two NRU test methods. Digoxin was much more toxic to the NHK cells and aminopterin was more toxic to the 3T3 cells. Both substances are known substrates for organic anionic transporters (OAT) (ICCVAM 2006). Such transporters are important for *in vivo* toxicity responses in terms of the ability of challenge substances to be absorbed, reach target tissues, accumulate, or be excreted. The differential susceptibilities of the 3T3 and NHK cells may be explained by differential functioning of OAT between the cell types. Although species and tissue differences in OAT have been reported (Sekine et al. 2000; Miyazaki et al. 2004), the reason for these differential sensitivities is not known.

The 3T3 IC_{50}:NHK IC_{50} ratios shown in **Table 5-8** were used to determine the frequency distributions shown in **Table 5-9.** These distributions indicate that the 3T3 and NHK NRU IC_{50} values were within one order of magnitude of each other for 85% of the reference substances (obtained by adding 38.9% and 45.8% for the 0.1 < IC_{50} ratio $\leq$1 and 1 < IC_{50} ratio <10 ranges). Ninety-three percent of the reference substances have 3T3 and NHK NRU

IC_{50} values within two orders of magnitude of each other (obtained by adding 4.2% each for the $10 \leq IC_{50}$ ratio ≤ 100 and $0 < IC_{50}$ ratio ≤ 0.1 ranges to the 85% above).

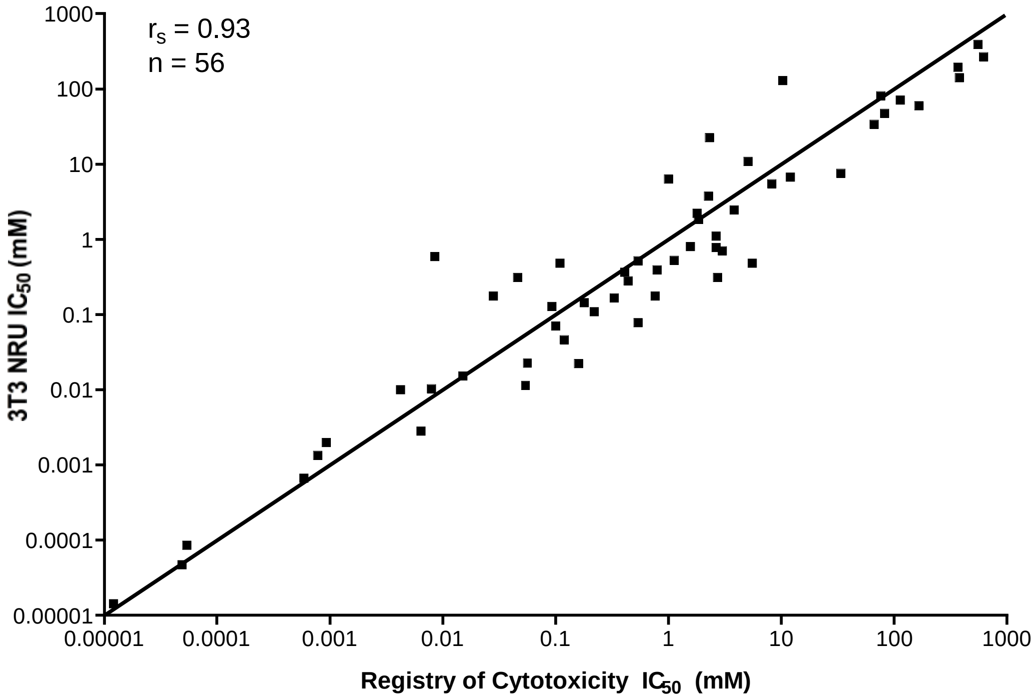
Table 5-9 Frequency of 3T3:NHK IC_{50} Ratios[1] for Reference Substances

3T3:NHK IC_{50} Ratio Range	Number of Substances	% of Substances
IC_{50} Ratio <0.00001	1	1.4
$0 < IC_{50}$ Ratio ≤ 0.1	3	4.2
$0.1 < IC_{50}$ Ratio ≤ 1	28	38.9
$1 < IC_{50}$ Ratio <10	33	45.8
$10 \leq IC_{50}$ Ratio <100	3	4.2
$100 \leq IC_{50}$ Ratio <1000	1	1.4
IC_{50} Ratio ≥ 1000	1	1.4
Not Available	2	2.8

Abbreviations: 3T3=Neutral red uptake using BALB/c 3T3 fibroblasts; NHK= Neutral red uptake using normal human epidermal keratinocytes.
Note: Compiled using reference substance data from **Table 5-7**.

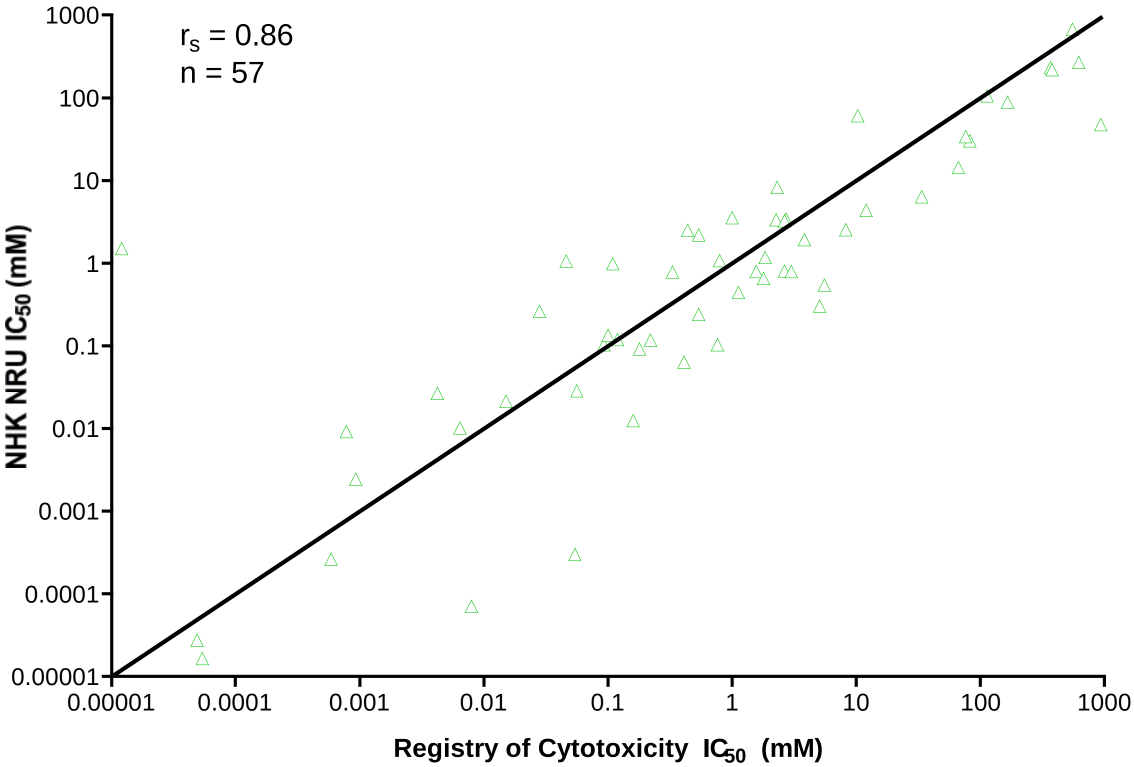
Correlations of the mean IC_{50} values for the reference substances common to the RC database with the IC_{50} values (i.e., geometric mean of IC_{50} values obtained from the literature for various basal cytotoxicity endpoints and cell types) from the RC (Halle 1998, 2003) are shown in **Figure 5-3** (3T3 values) and **Figure 5-4** (NHK values). Although the validation study tested 58 RC substances in common with the RC, IC_{50} values were obtained for 56 substances using the 3T3 NRU test method and 57 substances using the NHK NRU test method. Spearman correlation analyses of the log-transformed IC_{50} data (in mM) indicated that the NRU IC_{50} values were significantly correlated with the RC IC_{50x} values ($p<0.001$, for both the 3T3 and NHK NRU test methods). The Spearman correlation coefficient, r_s, was 0.93 for the 3T3 values and 0.86 for the NHK values.

Figure 5-3 RC IC_{50} Values vs 3T3 NRU IC_{50} Values for 56 Substances in Common



Abbreviations: RC=Registry of Cytotoxicity; 3T3=BALB/c 3T3 fibroblasts; NRU=Neutral red uptake; r_s=Spearman correlation coefficient; n=Number of substances; mM=Millimolar.
The diagonal line indicates the predicted values for a 1:1 correspondence. No IC_{50} values were obtained for carbon tetrachloride or methanol because of insufficient toxicity. The Registry of Cytotoxicity IC_{50} values are geometric means of IC_{50} values obtained from the literature for various basal cytotoxicity endpoints and cell types.

Figure 5-4 RC IC_{50} Values vs NHK NRU IC_{50} Values for 57 Substances in Common



Abbreviations: RC=Registry of Cytotoxicity; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; r_s=Spearman correlation coefficient; n=Number of substances; mM=Millimolar.
The diagonal line indicates the predicted values for a 1:1 correspondence. No IC_{50} values were obtained for methanol because of insufficient toxicity. The Registry of Cytotoxicity IC_{50} values are geometric means of IC_{50} values obtained from the literature for various basal cytotoxicity endpoints and cell types.

5.7 Availability of Data

All data were provided to the SMT as electronic files and paper copies. The laboratories also maintained copies of all raw data and the electronic files. The individual test data and IC_{50} results for both passing and failing tests are provided in **Appendix I** for the reference substances and the PC.

5.8 Solubility Test Results

A solubility protocol (see **Section 2-8** and **Appendix B3**) designed to identify the solvent that would provide the highest concentration of a reference substance for *in vitro* testing was evaluated. Each laboratory performed solubility tests on all reference substances. However, to avoid the use of different solvents by the laboratories when testing the same substance, which might increase the variability of the IC_{50} results among the laboratories, the SMT assigned the solvents to be used (see **Table 5-10**). The objectives of the solubility testing were to evaluate the utility and appropriateness of the solubility protocol, and to evaluate the concordance among laboratories in selecting the solvents for each of the 72 reference substances.

Table 5-10 Solubility Test Results (mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Phase I																	
Arsenic III trioxide	0.25	0.05	<2	<2	Medium	***0.025***[6]	***0.025***[6]	***<0.2***	***<0.2***	***0.135***[6]	***0.135***[6]	***<0.2***	***<0.2***	***<0.02***[6]	***<0.02***[6]	***<0.2***	***<0.2***
Ethylene glycol	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Propranolol HCl	<2	10	200	20	DMSO	0.2	2	200	NT	20	20	200	NT	20	2	NT	NT
Phase II																	
Aminopterin	2	2	NT	NT	DMSO	2.0	<2	200	NT	<2	2	200	NT	0.2	0.2	200	NT
Cadmium II chloride	<2	<2	200	<200	DMSO	<2	<2	200	NT	<2	<2	200	NT	<0.2	<0.2	20	<20
Chloramphenicol	2	2	400	<200	DMSO	2.0	<2	200	NT	<2	<2	200	NT	0.2	0.2	20	20
Colchicine	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Lithium I carbonate	0.25	10	<2	NT	Medium	0.2	2.0	<20	<20	0.2	2	<200	<200	0.2	2	<2	<2
Potassium I chloride	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
2-Propanol	400	400	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium I fluoride	20	20	<200	<200	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium selenate	200	200	<200	<200	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Phase III																	
Acetaminophen	10	10	400	<200	DMSO	2	2	NT	NT	2	2	NT	NT	<2	<2	200	NT
Acetonitrile	400	400	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Acetylsalicylic acid	10	10	400	200	DMSO	2	2	NT	NT	<2	<2	200	NT	2	2	NT	NT
5-Aminosalicylic acid	2	2	<200	<200	Medium	2	2	NT	NT	2	2	NT	NT	2	2	NT	NT
Amitriptyline HCl	200	200	NT	NT	DMSO	<2	<2	200	NT	<2	<2	200	NT	0.2	0.2	200	NT

Table 5-10 Solubility Test Results (mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Atropine sulfate	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Boric aid	40	40	200	<200	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Busulfan	<2	<2	40	<200	DMSO	<2	<2	200	NT	<2	<2	50[6]	<200	<0.2	<0.2	20	<200
Caffeine	10	10	20	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Carbamazepine	<2	<2	40	<200	DMSO	0.2	0.2	20	20	<2	<2	200	NT	<0.2	<0.2	2	<20
Carbon tetrachloride	2	10	NT	NT	DMSO	20	20	NT	NT	<0.2	<0.2	2	NT	20	20	NT	NT
Chloral hydrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Citric acid	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Cupric sulfate pentahydrate	1	0.5	<2	2	Medium	2	0.2	<200	<200	2	2	NT	NT	0.2	0.2	<200	NT
Cycloheximide	20	20	400	<200	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Dibutyl phthalate	<2	<2	400	400	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Dichlorvos	10	10	NT	NT	DMSO	2	2	NT	NT	<2	<2	200	NT	2	2	NT	NT
Diethyl phthalate	<2	<2	400	400	DMSO	<2	<2	200	NT	0.2	<0.2	200	NT	<2	<2	200	NT
Digoxin	0.05	0.05	200	< 200	DMSO	<2	<2	200	NT	<0.2	<0.2	200	NT	<2	<2	200	NT
Dimethylformamide	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Diquat dibromide monohydrate	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Disulfoton	<2	<2	500	NT	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Endosulfan	<0.05	<0.05	40	NT	DMSO	<0.2	<0.2	20	<200	<0.2	<0.2	2	<200	<0.2	<0.2	20	<200
Epinephrine bitartrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Ethanol	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT

Table 5-10 Solubility Test Results (mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Fenpropathrin	<20	<20	500	NT	DMSO	<2	<2	200	NT	<0.2	<0.2	200	NT	<2	<2	200	NT
Gibberellic acid	10	10	NT	NT	Medium	2	2	NT	NT	2	2	NT	NT	2	2	NT	NT
Glutethimide	<2	<2	500	NT	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Glycerol	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Haloperidol	<20	<20	40	NT	DMSO	<0.2	<0.2	20	<20	<0.2	<0.2	20	<20	<2	<2	20	<20
Hexachlorophene	0.05	<0.05	400	400	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Lactic acid	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Lindane	<0.05	<0.05	400	<200	DMSO	<2	<2	200	NT	<2	<2	200	NT	<0.2	<0.2	20	<200
Meprobamate	1	1	200	NT	DMSO	2	2	200	NT	2	2	200	NT	<0.2	<0.2	200	NT
Mercury II chloride	0.125	0.125	400	<200	DMSO	<2	<2	200	NT	<2	<2	200	NT	<0.2	<0.2	200	NT
Methanol	40	40	400	400	DMSO	20	20	NT	NT	20	20	NT	NT	<2	<2	200	NT
Nicotine	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Paraquat	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Parathion	0.05	<0.05	400	400	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Phenobarbital	2	2	200	<200	DMSO	2	2	NT	NT	<2	<2	200	NT	<2	<2	200	NT
Phenol	40	40	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Phenylthiourea	2	2	400	<200	DMSO	2	<2	200	NT	20	20	NT	NT	<2	<2	200	NT
Physostigmine	2	2	400	200	DMSO	2	2	NT	NT	<2	<2	200	NT	<2	<2	200	NT
Potassium cyanide	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Procainamide HCl	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT

Table 5-10 Solubility Test Results (mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Propylparaben	0.25	0.25	400	400	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Sodium arsenite	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium chloride	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium dichromate dihydrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium hypochlorite	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium oxalate	<0.05	20	0.125	<0.05	Medium	<0.2	20	0.2	<2	20	20	NT	NT	***<0.2***	***<0.2***	***<0.2***	***<0.2***
Strychnine	< 2	<2	2	2	Medium	0.2	<0.2	2	2	0.2	0.2	<200	<200	***<0.2***	***<0.2***	***<0.2***	***<0.2***
Thallium I sulfate	1	0.5	<2	<2	Medium	0.2	0.2	<200	<200	***<0.2***	***<0.2***	***<0.2***	***<0.2***	0.2	0.2	<20	<200
Trichloroacetic acid	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
1,1,1-Trichloroethane	10	10	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Triethylenemelamine	<2	<2	2	<20	DMSO	0.2	0.2	<200	<200	<0.2	<0.2	2	<2	***<0.2***	***<0.2***	***<0.2***	***<0.2***
Triphenyltin hydroxide	<0.05	<0.05	10	<20	DMSO	<0.2	<0.2	2	<20	<0.2	<0.2	2	<200	<2	<2	2	<20
Valproic acid	10	2	NT	NT	DMSO	2	2	NT	NT	<2	<2	200	NT	2	<2	200	NT
Verapamil HCl	<0.05	0.25	200	NT	DMSO	<2	<2	200	NT	<2	<2	200	NT	<0.2	<0.2	20	NT
Xylene	1	1	500	NT	DMSO	<2	<2	200	NT	2	<2	200	NT	<2	<2	200	NT

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; SMT=Study Management Team; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; DMSO=Dimethyl sulfoxide; ETOH=ethanol; NT=Not tested.

Note: Table sorted by study phase and alphabetical by substance.

[1]The solubility protocol used was different from that used by the testing laboratories.

[2]Solvents selected by the SMT for cytotoxicity testing. The BioReliance results were used to determine solvents for Phases I and II. Results from all laboratories were used to determine solvents for Phase III. 3T3 and NHK media were treated as a single solvent. If a substance insoluble in one medium, and not the other, and soluble in DMSO, then DMSO was selected for use with both cell types.

[3]Used protocol in **Figure 2-7**.

[4]Dulbecco's Modification of Eagle's Medium.

[5]Keratinocyte Growth Medium (KGM® from CAMBREX Clonetics®).

[6]The results were obtained using a deviation from the standard protocol.

Laboratories agreed on solvent. Laboratories did not agree on solvent. **bold** Protocol did not provide enough guideline information to select a single solvent.

5.8.1 Solubility Data

BioReliance evaluated the solubility of the reference substances, first in media, then in DMSO, and then in ETOH, at 400 and 200 mg/mL. Based on their experience, a solubility protocol was developed for the testing laboratories. This revised protocol required testing at lower concentrations, and use of the various solvents at concentrations that would be equivalent when applied to the cell cultures (see **Table 2-5**). The solubility flow chart (**Figure 2-7**) illustrates the tests for solubility in 3T3 and NHK medium, DMSO, and ETOH. **Table 5-10** provides the solubility test results.

5.8.2 Solubility and Volatility Effects in the Cytotoxicity Tests

The laboratories reported solubility results for the stock solutions of reference substance for each 3T3 and NHK test. Prior to the addition of the NR dye medium, the laboratories visually observed the test cultures and documented noticeable precipitate. **Table 5-11** illustrates the existence of solubility issues (in both the 3T3 and NHK NRU test methods) as evidenced by the observation of precipitates with some reference substances. **Sections 3.2.6** and **5.4.2** provide additional information on ability of the laboratories to achieve sufficient toxicity for the calculation of an IC_{50} in the presence of limited solubility. **Table 5-11** also notes the presence of volatility, as indicated by the use of film plate sealers during incubation.

Table 5-11 Reference Substances with Precipitate (PPT) and Volatility Issues[1]

Reference Substances	3T3 NRU Test Method				NHK NRU Test Method			
	PPT in 2X Stock Dilutions	PPT in 1X Plate Dilutions	PPT in Stock and Plate Dilutions	Volatility	PPT in 2X Stock Dilutions	PPT in 1X Plate Dilutions	PPT in Stock and Plate Dilutions	Volatility
Acetonitrile				X				X
Aminopterin		X			X			
5-Aminosalicylic acid	X							
Arsenic III trioxide	X				X			
Cadmium II chloride		X					X	
Carbamazepine			X					
Carbon tetrachloride			X		X			
Citric acid						X		
Cupric sulfate pentahydrate						X		
Dibutyl phthalate		X					X	
Dichlorvos				X				X
Diethyl phthalate	X						X	
Digoxin			X					
Dimethylformamide						X		
Disulfoton			X				X	
Endosulfan	X			X				X
Ethanol				X				X
Fenpropathrin			X				X	
Gibberellic acid	X				X			
Glutethimide					X			
Lindane			X	X			X	
Lithium I carbonate	X				X			
Nicotine				X				X
Parathion	X						X	
Phenol				X				X
Potassium I chloride		X						
Potassium cyanide		X		X				X

Table 5-11 Reference Substances with Precipitate (PPT) and Volatility Issues[1]

Reference Substances	3T3 NRU Test Method				NHK NRU Test Method			
	PPT in 2X Stock Dilutions	PPT in 1X Plate Dilutions	PPT in Stock and Plate Dilutions	Volatility	PPT in 2X Stock Dilutions	PPT in 1X Plate Dilutions	PPT in Stock and Plate Dilutions	Volatility
2-Propanol				X				X
Sodium arsenite		X						X
Sodium chloride						X		
Sodium I fluoride		X				X		
Sodium hypochlorite				X				
Sodium oxalate			X			X		
Strychnine	X				X			
Trichloroacetic acid						X		
1,1,1-Trichloroethane	X						X	
Valproic acid	X							
Verapamil HCl					X			
Xylene	X				X			

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; PPT=Precipitate.
Note: Table sorted alphabetical by reference substance.
[1]Results are based on at least one laboratory having precipitate or volatility issues with a substance. Volatility was denoted by the use of plate sealers during testing. 2X stock dilutions are prepared for each of 8 test substance concentrations. 1X plate dilutions are the result of diluting the 2X stock solutions with medium in the 96-well plates.

5.9 Summary

- The BioReliance, ECBC, and IIVS laboratories performed the 3T3 and NHK NRU tests in compliance with GLP guidelines.
- The quality and consistency of the reference substances was maintained during the study by the central purchase and distribution of individual lots of reference substances to the testing laboratories.
- Modifications and revisions made to the protocols during Phases I and II contributed to the optimization of the final protocols used in Phase III of the study. As a general rule, the protocol changes enhanced the performance of the methods and allowed more tests to meet the acceptance criteria.
- FAL improved the quality of its NHK data prior to Phase II testing by modifying the methods used to propagate the cells. Positive control IC_{50} data in Phases II and III from FAL more closely resemble the data from the other laboratories.
- Summary test data and IC_{50} results are presented in tabular and graphic formats. Comparisons of 3T3 NRU IC_{50} values to NHK NRU IC_{50} values show that the values for 85% of the reference substances are within one order of magnitude of each other. Digoxin and aminopterin yielded differences of up to five orders of magnitude when the IC_{50} values of the 3T3 and NHK NRU test methods were compared.
- Although each laboratory followed the same solubility protocol, they sometimes obtained different results. This may have been due to the subjective judgment of whether or not solubility was achieved. Additionally, the laboratories may have used solubility procedures that were beyond the level of detail in the solubility protocol.