1	
2	
3	
4	
5	
6	
7	
8	
9	DRAFT ICCVAM RECOMMENDATIONS
10	In Vitro Acute Toxicity Test Methods
11	
12	
13	
14	March 17, 2006
15	
16	
17	

17	
18	
19	
20	
21	
22	
23	
24	
25	
26	
27	
28	
29	[This Page Intentionally Left Blank]
30	

30			TABLE OF CONTENTS
31			
32			
33			Page Number
34	Section 1.0	Draft ICCVA	M Recommendations for <i>In Vitro</i> Acute
35		Toxicity Test I	Methods5
36			
37	Appendix A	Draft Perform	ance Standards for <i>In Vitro</i> Acute
38		Toxicity Meth	ods
39	Appendix B	Draft Recomn	nended Test Method ProtocolsB-1
40		Appendix B1	Test Method Protocol for the BALB/c 3T3
41			Neutral Red Uptake (NRU) Cytotoxicity Test B1-1
42		Appendix B2	Test Method Protocol for the Normal
43			Human Epidermal Keratinocyte (NHK)
44			Neutral Red Uptake (NRU) Cytotoxicity Test B2-1
45			

45	
46	
47	
48	
49	
50	
51	
52	
53	
54	
55	
56	
57	[This Page Intentionally Left Blank]
58	

59	1.0	Draft ICCVAM Recommendations for In Vitro Acute Toxicity Test Methods
60		
61	1.1	Draft Recommended Test Method Uses
62		
63	This ir	dependent validation study evaluated two in vitro neutral red uptake (NRU) basal
64	cytoto	xicity assays: the BALB/c 3T3 (clone A31) mouse fibroblast NRU (hereafter referred
65	to as th	ne 3T3 NRU) test method and the normal human keratinocyte NRU (hereafter referred
66	to as N	THK NRU) test method. The objective of the study was to determine their ability to
67	estima	te rodent acute oral toxicity LD_{50} values to be used in a weight-of-evidence approach
68	to set t	he starting dose for <i>in vivo</i> acute oral toxicity tests. Based on the results of this
69	validat	ion study, ICCVAM proposes the following draft recommendations:
70	1.	The 3T3 and NHK NRU test methods are not sufficiently accurate to predict the acute
71		oral toxicity of substances for the purposes of hazard classification (see Section 6 of
72		the In Vitro Acute Toxicity Test Methods Background Review Document).
73	2.	For the purposes of acute oral toxicity testing, the 3T3 and NHK NRU test methods
74		may be used in a weight-of-evidence approach to determine the starting dose for the
75		current acute oral in vivo toxicity protocols (i.e., the Up-and-Down Procedure [UDP]
76		and Acute Toxic Class [ATC])
77	3.	Consistent with the U.S. Government Principles on the Use of Animals in Research,
78		Testing, and Education (National Research Council 1996), and the U.S. Public Health
79		Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002) ¹ , in vitro
80		basal cytotoxicity test methods as part of a weight-of-evidence approach to estimate
81		the starting dose for acute oral <i>in vivo</i> toxicity test methods should be considered and
82		used where appropriate before testing is conducted using animals. For some types of
83		substances, this approach will reduce the number of animals needed. In some testing
84		situations, the approach may also reduce the numbers of animals that die or need to be
85		humanely killed.

¹ National Research Council. 1996. Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academy Press.

PHS. 2002. Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals.

86	4.	Substances with specific toxic mechanisms that are not expected to be active in 3T3
87		or NHK cells (e.g., those that are neurotoxic, cardiotoxic, interfere with energy
88		utilization, or alkylate proteins and other macromolecules) will likely be
89		underpredicted by these in vitro basal cytotoxicity test methods. Therefore, until such
90		time as a more predictive testing approach is developed, the results from basal
91		cytoxicity testing with such substances may not be appropriate.
92	5.	The regression formula used to determine starting doses should be the revised
93		Registry of Cytotoxicity (RC) regression line [with IC_{50} values in $\mu g/mL$ and LD_{50}
94		values in mg/kg] developed with the RC chemicals using rat LD50 data only and
95		excluding chemicals with mechanisms of action that are not expected to be active in
96		in vitro basal cytotoxicity test methods.
97	6.	The performance of other in vitro basal cytotoxicity test methods that are based on
98		similar scientific principles and that measure or predict the same biological response
99		(i.e., basal cytotoxicity and the rat acute oral LD_{50} value, respectively) should be
100		demonstrated to meet or exceed the accuracy and reliability of the 3T3 and NHK
101		NRU test methods.
102	7.	Compared to the NHK NRU test method, the 3T3 NRU test method appears to be less
103		labor intensive and less expensive to conduct; therefore, the 3T3 NRU cytotoxicity
104		test method is recommended for general use.
105		
106		
107	1.2	Draft Recommended Future Studies
108		
109	1.	Additional data should be collected using the 3T3 and/or the NHK NRU test methods
110		to evaluate their usefulness for predicting the in vivo acute oral toxicity of chemical
111		mixtures.
112	2.	Additional high quality comparative in vitro basal cytotoxicity data should be
113		collected in tandem with in vivo rat acute oral toxicity test results to supplement the
114		high quality validation database started by this study. Periodic evaluations of the
115		expanded database should be conducted to further characterize the usefulness and

6

116		limitations of using in vitro cytotoxicity data as part of a weight-of-evidence approach	
117		to estimate starting doses.	
118	3.	Additional efforts should be conducted to identify additional in vitro tests and other	
119		methods necessary to achieve accurate acute oral hazard classification; specifically,	
120		studies should be conducted to investigate the potential use of in vitro cell-based test	
121		methods that incorporate mechanisms of action and evaluations of ADME	
122		(absorption, distribution, metabolism, excretion) to provide improved estimates of	
123		acute toxicity hazard categories.	
124	4.	The in vivo database of reference substances used in this validation study should be	
125		used to evaluate the utility of other nonanimal approaches to estimate starting doses	
126		for acute oral systemic toxicity tests (e.g., widely available software that uses	
127		quantitative structure-activity relationships [QSAR]).	
128	5.	Standardized procedures to collect information pertinent to an understanding of the	
129		mechanisms of lethality should be included in future in vivo rat acute oral toxicity	
130		studies. Such information will likely be necessary to support the further development	
131		of predictive mechanism-based in vitro methods.	
132	6.	An expanded list of reference substances with estimated rat LD_{50} values substantiated	
133		by high quality in vivo data should be developed for use in future in vitro test method	
134		development and validation studies.	
135			
136	Appe	ndix A provides Draft Performance Standards for In Vitro Acute Toxicity Methods that	
137	are ba	sed on ICCVAM guidelines (ICCVAM 2003 ²). Appendix B provides two draft	
138	recom	mended in vitro NRU basal cytotoxicity protocols that are revised versions of the	
139	Phase	III protocols used in the validation study. Appendix B-1 is the <i>Test Method Protocol</i>	
140	for the	e BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test. Appendix B-2 is the Test	
141	Method Protocol for the Normal Human Epidermal Keratinocyte (NHK) Neutral Red Uptake		
142	(NRU)) Cytotoxicity Test.	

² ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508. National Institute for Environmental Health Sciences, Research Triangle Park, NC. Available: <u>http://iccvam.niehs.nih.gov/</u>. [accessed 2 June 2005].

143	
144	
145	
146	
147	
148	
149	
150	
151	Appendix A
152	
153	
154	Draft Performance Standards for In Vitro
155	Acute Toxicity Methods
156	

156	
157	
158	
159	
160	
161	
162	
163	
164	
165	
166	
167	
168	
169	
170	[This Page Intentionally Left Blank]

171				TABLE OF CONTENTS
172				Page Number
173	LIST	OF TA	ABLES.	
174	LIST	OF A	CRONY	MS AND ABBREVIATIONS
175 176 177	ALT	ERNA	ΓIVE M	OORDINATING COMMITTEE ON THE VALIDATION OF ETHODS (ICCVAM) - DESIGNATED AGENCY ES
178 179	ALT	ERNA	ΓIVE M	OORDINATING COMMITTEE ON THE VALIDATION OF ETHODS
180				TOXICITY WORKING GROUP (ATWG) A-9
181 182 183	THE	EVAL	UATIO	COLOGY PROGRAM (NTP) INTERAGENCY CENTER FOR N OF ALTERNATIVE TOXICOLOGICAL METHODS
184			·	
185				/IARY
186	1.0	PUR	POSE A	ND BACKGROUND OF PERFORMANCE STANDARDS A-15
187		1.1	Intro	luctionA-15
188		1.2	Eleme	ents of ICCVAM Performance StandardsA-16
189		1.3	ICCV	AM Process for the Development of Performance StandardsA-17
190 191		1.4		AM Development of Recommended Performance Standards <i>Vitro</i> Acute Toxicity Test MethodsA-18
192 193			1.4.1	Current Regulatory Testing Requirements for Acute Systemic Toxicity
194			1.4.2	Test Methods for Assessing Acute Systemic ToxicityA-19
195 196			1.4.3	Intended Regulatory Uses for <i>In Vitro</i> Cytotoxicity Test Methods
197 198 199			1.4.4	Similarities and Differences in the Endpoints of <i>In Vitro</i> Cytotoxicity Test Methods and <i>In Vivo</i> Acute Oral Toxicity Test MethodsA-21
200	2.0	IN V.	ITRO A	CUTE TOXICITY TEST METHODS
201		2.1	Backg	ground
202 203		2.2		iples of <i>In Vitro</i> Basal Cytotoxicity Assays to Predict Starting for Acute Oral Toxicity TestsA-23
204 205 206		2.3	Assay	tial Test Method Components for <i>In Vitro</i> Basal Cytotoxicity s to Predict Starting Doses for Acute Oral Toxicity (Lethality)

207		2.3.1	In Vitro Cell Culture Conditions	A-25
208		2.3.2	Application of the Test Substances	A-25
209		2.3.3	Control Substances	A-27
210		2.3.4	Viability Measurements	A-28
211		2.3.5	Interpretation of Results	A-29
212		2.3.6	Test Report	A-30
213 214	2.4		ence Substances for <i>In Vitro</i> Basal Cytotoxicity Ass ing Doses for Acute Oral Toxicity Tests	•
215	2.5	Accur	racy and Reliability	A-35
216		2.5.1	Accuracy	A-35
217		2.5.2	Reliability	A-37
218			'ES	

219 220 221		LIST OF TABLES
222 223	Table 1-1	Summary of Current U.S. Legislation for Using Acute Systemic Toxicity Data for Product Labeling
224	Table 1-2	Regulatory Classification Systems for Acute Oral ToxicityA-20
225 226 227	Table 2-1	Recommended Reference Substances for Evaluation of <i>In Vitro</i> Basal Cytotoxicity Methods for Predicting Starting Dose for Acute Systemic Toxicity Tests
228	Table 2-2	Linear Regression Analyses of In Vitro and In Vivo Results
229 230	Table 2-3	Prediction of GHS Toxicity Category by RC Rat-Only Weight Regression Excluding Chemicals with Specific Mechanisms of ToxicityA-38
231 232 233 234 235 236 237	Table 2-4	Summary of CV Results for the 3T3 and NHK NRU Test MethodsA-40

237 LIST OF ACRONYMS AND ABBREVIATIONS 238 239 240 ANOVA Analysis of Variance 241 ATC Acute Toxicity Class 242 BRD Background review document 243 CASRN Chemical Abstracts Service Registry Number 244 **CPSC** U.S. Consumer Product Safety Commission 245 CS Calf serum 246 CV Coefficient of Variation °C 247 **Degrees** Celsius 248 DMSO Dimethyl sulfoxide U.S. Department of Transportation 249 DOT 250 DPBS Dulbecco's Phosphate Buffered Saline 251 **ECVAM** European Center for the Validation of Alternative Methods 252 EPA U.S. Environmental Protection Agency 253 ETOH Ethanol 254 FDA U.S. Food and Drug Administration 255 FDP Fixed Dose Procedure 256 FL Fluorescein leakage 257 FR Federal Register 258 GHS Globally Harmonized System of Classification and Labelling of 259 Chemicals (UN 2005). 260 IC_{50} Test substance concentration producing 50% inhibition of the endpoint 261 measured 262 **ICCVAM** Interagency Coordinating Committee on the Validation of Alternative Methods 263 264 LD_{50} Lethal dose that produces lethality in 50% of test animals 265 LDH Lactate dehydrogenase

266	MTT	[3-(4,5,dimethylthiazol-2yl)2,5-diphenyl tetrazolium bromide]
267	NCS	Newborn calf serum
268	NHK	Normal human epidermal keratinocytes
269	NICEATM	National Toxicology Program Center for the Evaluation of Alternative
270		Toxicological Methods
271	NIEHS	National Institute of Environmental Health Sciences
272	NIOSH	U.S. National Institute for Occupational Safety and Health
273	NR	Neutral red
274	NRR	Neutral red release
275	NRU	Neutral red uptake
276	NTP	U.S. National Toxicology Program
277	OD	Optical density
278	OECD	Organisation for Economic Cooperation and Development
279	OSHA	U.S. Occupational Safety & Hazards Administration
280	RC	Registry of Cytotoxicity
281	RTECS	Registry of Toxic Effects for Chemical Substances
282	3T3	BALB/c mouse fibroblasts, clone A31
283	UDP	Up and Down Procedure
284	UN	United Nations
285	XTT	[Sodium 3,3,-[(Phenylamino)carbonyl]-3,4-Tetrazolium-Bis(4-
286		methoxy-6-nitro)benzenesulfonic acid hydrate]
287	ZEBET	German Center for Documentation and Evaluation of Alternative
288 289 290		Methods to Animal Experiments

INTERAGENCY COORDINATING COMMITTEE ON THE VALIDATION OF ALTERNATIVE METHODS (ICCVAM) Designated Agency Representatives

Agency for Toxic Substances and Disease Registry • Moiz Mumtaz, Ph.D.

Consumer Product Safety Commission

- Marilyn L. Wind, Ph.D. (Vice-Chair)
- * Kailash C. Gupta, D.V.M., Ph.D.
- * Patricia Bittner, M.S.

290

291

292 293

* Kristina Hatelid, Ph.D.

Department of Agriculture

- Jodie Kulpa-Eddy, D.V.M.
- ◊ Elizabeth Goldentyer, D.V.M.

Department of Defense

- Robert E. Foster, Ph.D.
- ◊ Patty Decot
- * Harry Salem, Ph.D.
- * John M. Frazier, Ph.D.

Department of Energy

• Marvin Stodolsky, Ph.D.

Department of the Interior

• Barnett A. Rattner, Ph.D.

◊ Sarah Gerould, Ph.D.

Department of Transportation

- George Cushmac, Ph.D.
- ◊ Steve Hwang, Ph.D.

Environmental Protection Agency

Office of Science Coordination and Policy • Karen Hamernik, Ph.D.

Office of Research and Development

◊ Julian Preston, Ph.D.

* Suzanne McMaster, Ph.D.

- **OECD** Test Guidelines Program
- * Jerry Smrchek, Ph.D.
- **Office of Pesticides Programs**
- * Amy Rispin, Ph.D.
- * Deborah McCall

Food and Drug Administration

- Leonard M. Schechtman, Ph.D. (Chair)
- Principal Agency Representative
- ◊ Alternate Principal Agency Representative
- * Other Designated Agency Representatives

Office of Science and Health Coordination ◊ Richard Canady, Ph.D., D.A.B.T. Center for Drug Evaluation and Research * Abigail C. Jacobs, Ph.D. Center for Devices and Radiological Health * Raju Kammula., D.V.M., Ph.D., D.A.B.T. * Melvin E. Stratmeyer, Ph.D. Center for Biologics Evaluation and Research * Richard McFarland, Ph.D., M.D. * Ying Huang, Ph.D. Center for Food Safety and Nutrition * David G. Hattan, Ph.D. * Robert L. Bronaugh, Ph.D. **Center for Veterinary Medicine** * Devaraya Jagannath, Ph.D. * M. Cecilia Aguila, D.V.M. National Center for Toxicological Research * William T. Allaben, Ph.D. **Office of Regulatory Affairs** * Lawrence A. D'Hoostelaere, Ph.D.

National Cancer Institute

- Alan Poland, M.D.
- ◊ Vacant

National Institute of Environmental Health Sciences

- William S. Stokes, D.V.M., D.A.C.L.A.M.
- ◊ John R. Bucher, Ph.D., D.A.B.T.
- * Rajendra S. Chhabra, Ph.D., D.A.B.T
- * Jerrold J. Heindel, Ph.D.

National Institute for Occupational Safety and Health

- Paul Nicolaysen, V.M.D.
- ◊ K. Murali Rao, MD, Ph.D.

National Institutes of Health

• Margaret D. Snyder, Ph.D.

National Library of Medicine

- Vera Hudson, M.S.
- ◊ Jeanne Goshorn, M.S.

Occupational Safety and Health Administration

• Surender Ahir, Ph.D.

294 INTERAGENCY COORDINATING COMMITTEE ON THE 295 VALIDATION OF ALTERNATIVE METHODS (ICCVAM) ACUTE 296 TOXICITY WORKING GROUP (ATWG)

297

298 299

- 300 Consumer Product Safety Commission301 (CPSC)
- 302
- 303 Patricia Brundage, Ph.D.
- 304 Kailash Gupta, Ph.D.
- 305 Cassandra Prioleau, Ph.D.
- 306 Marilyn Wind, Ph.D. (ICCVAM Co-
- 307 Chair)
- 308

309 Department of Energy (DOE)

- 310
- 311 Po-Yung Lu, Ph.D.
- 312
- 313 Environmental Protection Agency
- 314 (EPA)
- 315
- 316 Karen Hamernik, Ph.D.
- 317 Masih Hashim, Ph.D.
- 318 Marianne Lewis, Ph.D.
- 319 Elizabeth Margosches, Ph.D.
- 320 Debbie McCall, Ph.D.
- 321 John Redden, Ph.D.
- 322 Amy Rispin, Ph.D.
- 323
- 324
- 349

325

326 Food and Drug Administration (FDA)

- 327
- 328 Leonard Schechtman, Ph.D. (ICCVAM Chair)
- 329 Kenneth Hastings, Ph.D.
- 330 Abigale Jacobs, Ph.D.
- 331 Suzanne Morris, Ph.D.
- 332 David Morse, Ph.D.
- 333 Thomas Umbreit, Ph.D.
- 334
- 335 National Institute for Occupational
- 336 Safety & Health (NIOSH)
- 337
- 338 Stephen Reynolds, Ph.D.
- 339
- 340 National Institute of Environmental
- 341 Health Sciences (NIEHS)
- 342
- 343 Rajendra Chhabra, Ph.D.
- 34William Stokes, D.V.M., D.A.C.L.A.M.
- 345 (Executive Director, ICCVAM)
- 346 Raymond Tice, Ph.D., (Deputy Director,
- 347 NICEATM)
- 348

349			GRAM (NTP) INTERAGENCY
350	CENTER FOR THE E	VALUA	TION OF ALTERNATIVE
351	TOXICOLOGIC	CAL MET	ГНОDS (NICEATM)
352			
353			
354	Bradley Blackard, M.S.P.H.	374	Michael Paris
355	ILS, Inc.	375	ILS, Inc.
356		376	
357	Sue Brenzel	377	William Stokes, D.V.M., Diplomate,
358	ILS, Inc.	378	ACLAM (Director)
359		379	NIEHS
360	Thomas Burns, M.S.	380	
361	ILS, Inc.	381	Judy Strickland, Ph.D., D.A.B.T.
362		382	ILS, Inc.
363	Jeffery Charles, Ph.D., M.B.A.,	383	
364	D.A.B.T.	384	Raymond Tice, Ph.D. (Deputy Director)
365	ILS, Inc.	385	NIEHS
366		386	
367	Linda Litchfield	387	
368	ILS, Inc.		
369			
370	Deborah McCarley		
371	NIEHS		
372			
373			
388			

389

390

PREFACE

391 The National Toxicology Program (NTP) Interagency Center for the Evaluation of 392 Alternative Toxicological Methods (NICEATM) collaborated with the European Centre for 393 the Validation of Alternative Methods (ECVAM), a component of the European 394 Commission's Joint Research Centre, to further characterize the usefulness of *in vitro* basal 395 cytotoxicity test methods as predictors of starting doses for acute oral systemic toxicity (i.e., 396 lethality) assays. NICEATM and ECVAM designed a multi-laboratory validation study to 397 evaluate the performance of two standardized *in vitro* basal cytotoxicity test methods using 398 72 reference substances with the ZEBET approach of using the Registry of Cytotoxicity (RC) 399 regression model to estimate starting doses for acute oral systemic toxicity tests. Based on 400 the procedures described in the Guidance Document on Using In Vitro Data to Estimate In 401 Vivo Starting Doses for Acute Toxicity (hereafter referred to as Guidance Document (ICCVAM 2001), the validation study used two mammalian cell types (i.e., BALB/c 3T3 402 403 mouse fibroblasts [3T3] and primary normal human epidermal keratinocytes [NHK]) with a 404 neutral red uptake (NRU) cell viability endpoint.

405

406 NICEATM developed draft performance standards that could be used to evaluate the 407 acceptability of test methods that are based on similar scientific principles and that measure 408 or predict the same biological or toxic effect as the *in vitro* basal cytotoxicity test methods. 409 The Interagency Coordinating Committee on the Validation of Alternative Methods 410 (ICCVAM) and its Acute Toxicity Working Group (ATWG) reviewed the Background 411 Review Document (BRD) for the NICEATM/ECVAM validation study and the draft 412 performance standards. After commenting and recommending revisions, ICCVAM and the 413 ATWG recommended that these standards be presented to and reviewed by an Expert Peer 414 Panel convened for evaluation of the validation study. ICCVAM, in collaboration with 415 NICEATM, subsequently proposed and sought public comment on draft performance 416 standards for these types of test methods. Following consideration of public and advisory 417 committee comments, ICCVAM will finalize recommended performance standards for in 418 vitro basal cytotoxicity test methods.

419

420	Performance standards provide the basis by which a validated and accepted proprietary (i.e.,
421	copyrighted, trademarked, registered) or non-proprietary test method has been determined to
422	have sufficient accuracy and reliability for a specific testing purpose. In addition,
423	performance standards should assist other test developers in the validation of test methods
424	that are similar in structure and function, and facilitate acceptance of test methods that adhere
425	to the applicable performance standards.
426	
427	This document is available online at <u>http://iccvam.niehs.nih.gov</u> ; printed copies are available
428	on request from the NICEATM (NIEHS, P.O. Box 12233, MD EC-17, Research Triangle
429	Park, NC 27709; telephone: 919-541-3398, fax: 919-541-0947, e-mail:
430	<u>iccvam@niehs.nih.gov)</u> .
431	
432	We gratefully acknowledge the significant contributions of the ICCVAM agency
433	representatives and members of the ICCVAM Acute Toxicity Working Group (ATWG) in
434	the preparation of this document, and the NICEATM staff that assisted throughout the
435	process. We also appreciate the constructive suggestions from interested stakeholders in
436	response to the Federal Register notice.
437	
438	Leonard M. Schechtman, Ph.D.
439	Chair, ICCVAM
440	
441	William S. Stokes, D.V.M., Diplomate, A.C.L.A.M.
442	Director, NICEATM
443	Executive Director, ICCVAM
444	

444	
-----	--

EXECUTIVE SUMMARY

445

446 The purpose of performance standards is to communicate the basis by which validated new 447 proprietary (e.g., copyrighted, trademarked, registered) and nonproprietary test methods have 448 been determined to have sufficient accuracy and reliability for specific testing purposes. 449 Performance standards can then be used to evaluate the accuracy and reliability of other test 450 methods that are based on similar scientific principles and that measure or predict the same 451 biological or toxic effect. The three elements of performance standards are essential test 452 method components (i.e., structural, functional, and procedural elements of a validated test 453 method that a proposed, mechanistically and functionally similar test method should adhere 454 to), a minimum list of reference chemicals for assessing the accuracy and reliability of the 455 proposed test method, and the accuracy and reliability values that should be achieved by the 456 proposed test method using the minimum list of reference chemicals.

457

458 The Interagency Coordinating Committee on the Validation of Alternative Methods 459 (ICCVAM) has reviewed and evaluated information presented in the Background Review 460 Document (BRD) for a validation study that evaluated the performance of two standardized 461 *in vitro* basal cytotoxicity test methods for predicting starting doses for acute oral systemic 462 toxicity tests. The study, a collaboration of the National Toxicology Program (NTP) 463 Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) 464 and the European Centre for the Validation of Alternative Methods (ECVAM), tested 72 465 reference substances using *in vitro* neutral red uptake (NRU) test methods with two 466 mammalian cell types (i.e., BALB/c 3T3 mouse fibroblasts [3T3] and primary normal human 467 epidermal keratinocytes [NHK]). The 3T3 and NHK NRU test methods are not sufficiently 468 accurate to predict the acute oral toxicity of substances for the purposes of hazard 469 classification. However, for the purposes of acute oral toxicity testing, the 3T3 and NHK 470 NRU test methods may be used in a weight-of-evidence approach to determine the starting 471 dose for the current acute oral in vivo acute systemic toxicity protocols for the Up-and-Down 472 Procedure (UDP; OECD 2001a; EPA 2002a) and Acute Toxic Class (ATC; OECD 2001b) 473 method. The performance of other in vitro basal cytotoxicity test methods that are based on 474 similar scientific principles and that measure or predict the same biological response (i.e.,

basal cytotoxicity and the rat acute oral LD_{50} [median lethal dose], respectively) should be demonstrated to meet or exceed the accuracy and reliability of the 3T3 and NHK NRU test methods.

478

479 ICCVAM, the ICCVAM Acute Toxicity Working Group (ATWG), and NICEATM have 480 drafted proposed performance standards for the 3T3 and NHK NRU test methods. This 481 document describes those proposed performance standards. The extent to which proposed *in* 482 *vitro* basal cytotoxicity test methods should demonstrate comparable performance to these 483 two *in vitro* NRU cytotoxicity test methods should be considered on a case-by-case basis. 484 While it would be desirable for proposed test methods to have reliability and accuracy values 485 at least as good as that of these two NRU test methods, some flexibility might be acceptable 486 to the extent that it would not compromise the ultimate protection of human and animal 487 health.

488

489 To demonstrate technical proficiency with the validated test method, ICCVAM recommends 490 that the user evaluate his/her ability to calculate IC_{50} values (i.e., the test concentration that 491 produces 50% inhibition of the endpoint measure) for at least 12 of the 25 reference 492 substances (minimum of two unclassified chemicals and two from each from the five GHS 493 hazard categories) listed in **Table 2-1**. The resulting IC_{50} values should be within 2.5 494 standard deviations of the reported RC IC₅₀ values in the table and a linear regression 495 calculated with the IC_{50} values from the proposed test method and LD_{50} values in the table 496 should not be different from a linear regression calculated using the IC_{50} and LD_{50} values 497 from the table. Intralaboratory Coefficient of Variation (CV) for the IC₅₀ of the reference test 498 substances should not exceed 129% for reference substance test chemicals and the mean for 499 the substances tested should not exceed 30%. The proposed *in vitro* basal cytotoxicity test 500 method should provide correct predictions of GHS acute oral toxicity category using the 501 recommended IC₅₀-LD₅₀ regression for at least 38% of the substances tested. 502

503

.

504 1.0 PURPOSE AND BACKGROUND OF PERFORMANCE STANDARDS

505

506 **1.1 Introduction**

507

508 Prior to the acceptance of a new test method for regulatory testing applications, validation 509 studies are conducted to assess its reliability (i.e., the extent of intra- and inter-laboratory reproducibility) and its relevance (i.e., the ability of the test method to correctly predict or 510 511 measure the biological effect of interest) (ICCVAM 1997, 2003; OECD 1996, 2002). The 512 purpose of performance standards is to communicate the basis by which new proprietary (i.e., 513 copyrighted, trademarked, registered) and nonproprietary test methods have been determined 514 to have sufficient relevance and reliability for specific testing purposes. These performance 515 standards, based on test methods accepted by regulatory agencies, can be used to evaluate the 516 reliability and relevance of other test methods that are based on similar scientific principles 517 and measure or predict the same biological or toxic effect. Two *in vitro* basal cytotoxicity 518 test methods, the BALB/c 3T3 mouse fibroblast (3T3) neutral red uptake (NRU) assay and 519 the normal human keratinocyte (NHK) NRU assay, underwent a validation process to 520 evaluate the correlation between in vitro cytotoxicity and acute lethality and the feasibility of 521 using *in vitro* NRU assays to predict starting doses for *in vivo* acute oral systemic toxicity 522 assays.

523

524 This section describes the three elements of performance standards identified by ICCVAM

525 (ICCVAM 2003) and the ICCVAM process used to develop performance standards during a

526 test method evaluation, These test method performance standards are proposed as initial

527 standards that can be used to evaluate future *in vitro* basal cytotoxicity test methods. If other

- 528 *in vitro* basal cytotoxicity test methods are adequately validated and demonstrate
- 529 significantly improved performance, then the test method performance standards may be 530 revised accordingly.

531

532

532	1.2	Elements of ICCVAM Performance Standards
533		
534	Performance	e standards are standards based on a validated test method that provide a basis for
535	evaluating th	he comparability of a proposed test method that is mechanistically and
536	functionally	similar (ICCVAM 2003). The three elements of performance standards are:
537	•	Essential test method components: These consist of essential structural,
538		functional, and procedural elements of a validated test method that should be
539		included in the protocol of a proposed, mechanistically and functionally similar
540		test method. Essential test method components include unique characteristics of
541		the test method, critical procedural details, and quality control measures.
542		Adherence to essential test method components will help to assure that a
543		proposed test method is structurally and functionally similar to the
544		corresponding validated test method.
545	•	A minimum list of reference substances: Reference substances are used to
546		assess the accuracy and reliability of a proposed, mechanistically and
547		functionally similar test method. These substances are a representative subset
548		of those used to demonstrate the reliability and the accuracy of the validated test
549		method. To the extent possible, this subset of substances should:
550		- be representative of the range of responses that the validated test method is
551		capable of measuring or predicting
552		- have produced consistent results in the validated test method and in the <i>in</i>
553		vivo reference test method and/or the species of interest
554		 have well-defined chemical structures
555		 be readily available
556		 not be associated with excessive hazard or prohibitive disposal costs
557		- have performance characteristics (accuracy, sensitivity, specificity, false
558		negative and false positive rates) of the validated test method for the subset
559		of reference chemicals should approximate the performance values obtained
560		during the validation process for all appropriate substances
561		

562		These reference substances are the minimum number that should be used to
563		evaluate the performance of a proposed, mechanistically and functionally similar
564		test method. Reference substances should not be used to develop the prediction
565		model for the proposed test method. However, if reference substances have been
566		used for this purpose, then they should be replaced with other substances of the
567		same chemical class and biological activity for the endpoint of interest and for
568		which adequate reference data are available. Similarly, if any of the recommended
569		reference substances are unavailable, other substances for which adequate reference
570		data are available could be substituted. Again, to the extent possible, the substituted
571		substance(s) should be of the same chemical class as the original reference
572		substance(s). If desired, additional substances representing other chemical or
573		product classes and for which adequate reference data are available can be used to
574		more comprehensively evaluate the accuracy of the proposed test method.
575		However, none of these additional substances should have been used to develop the
576		proposed test method.
577		• Accuracy and reliability values: These are the accuracy and reliability
578		characteristics that the proposed test method should be comparable to when
579		evaluated using the minimum list of reference chemicals.
580		
581	1.3	ICCVAM Process for the Development of Performance Standards
582		
583	The pr	ocess followed by ICCVAM for developing performance standards for new test
584	method	ds is as follows:
585		NICEATM and the appropriate ICCVAM working group develop proposed
586		performance standards for consideration during the ICCVAM evaluation
587		process. If performance standards are proposed by a test method sponsor, they
588		will be considered by ICCVAM at this stage. Generally, the proposed
589		performance standards are based on the information and data provided in the
590		test method submission or on other available applicable data.
591		The ICCVAM/NICEATM Peer Review Panel evaluates the proposed
592		performance standards for completeness and appropriateness during its

593		evaluation of the validation status of the proposed test method. The proposed		
594		performance standards, as well as the test method submission, are made		
595		available to the public for comment prior to and during the Peer Review Panel		
596		meeting.		
597		• The appropriate ICCVAM working group, with the assistance of NICEATM,		
598		prepares the final performance standards for ICCVAM approval, taking into		
599		consideration the recommendations of the Peer Review Panel and public		
600		comments.		
601				
602	Perforn	nance standards recommended by ICCVAM are incorporated into ICCVAM test		
603	method	evaluation reports, which are then provided to U.S. Federal agencies and made		
604	availab	le to the public. Regulatory authorities can then reference the performance standards		
605	in the I	CCVAM report when they communicate their acceptance of a new test method. In		
606	addition	n, performance standards adopted by U.S. Federal regulatory authorities can be		
607	provide	d in guidelines issued for new test methods. Availability of ICCVAM test method		
608	evaluation reports are announced routinely in the Federal Register, NTP Newsletters, and by			
609	e-mail t	to ICCVAM/NICEATM listserve groups.		
610				
611	1.4	ICCVAM Development of Recommended Performance Standards for		
612		In Vitro Acute Toxicity Test Methods		
613				
614	1.4.1	Current Regulatory Testing Requirements for Acute Systemic Toxicity		
615	The ma	jor regulatory requirement for acute systemic toxicity testing is for the hazard		
616	classifie	cation and labeling of products, which is intended to protect handlers and consumers		
617	from to	xic hazards. The LD_{50} results (i.e., median lethal dose) from acute systemic toxicity		
618	tests are	e used to place substances in various toxicity categories that, in turn, invoke the		
619	associat	ted hazard phrases to be used on product labels. Table 1-1 shows the current U.S.		
620	legislat	ion requiring the use of acute systemic toxicity testing for product labeling and the		
621	substan	ces regulated. Table 1-2 shows the statutory protocol requirements and classification		
622	systems	s used by each U.S. regulatory agency. Also included is an international guideline for		
623	labeling	g, the Harmonized Integrated Classification System for Human Health and		

- 624 Environmental Hazards of Chemical Substances and Mixtures (OECD 2001c), which
- 625 provides guidance to regulatory agencies on the use of the Globally Harmonized System of
- 626 Classification and Labelling of Chemicals (GHS; UN 2005) as a method for an
- 627 internationally comprehensible system for hazard communication.

628Table 1-1Summary of Current U.S. Legislation for Using Acute Systemic Toxicity629Data for Product Labeling

Legislation (Year of Initial Enactment)	U.S. Regulatory Agency	Substance
Federal Insecticide, Fungicide and Rodenticide Act (1947)	EPA	Pesticides
Federal Hazardous Substances Act (1964)	CPSC	Household products
Occupational Safety and Health Act (1970)	OSHA	Occupational materials
Federal Hazardous Material Transportation Act (1975)	DOT	Transported substances

Abbreviations: EPA = U.S. Environmental Protection Agency; CPSC = U.S. Consumer Product Safety
 Commission; OSHA = U.S. Occupational Safety and Health Administration; DOT = U.S. Department of
 Transportation. [Note: The U.S. Food and Drug Administration (FDA) does not require data for acute lethality
 testing, and in fact, discourages the use of animals for such testing (FDA 1993).]

634

635

636 1.4.2 Test Methods for Assessing Acute Systemic Toxicity

637 The current internationally recognized test methods for acute systemic toxicity testing are the

638 UDP (OECD 2001a; EPA 2002a), the ATC method (OECD 2001b), and the Fixed Dose

639 Procedure (FDP; OECD 2001d). Information on signs of acute toxicity and target organs can

640 be obtained using any of the three test methods. All three methods are sequential tests in

641 which the outcome of testing one or more animals at the first dose is used to determine the

- second dose that should be tested. The FDP differs from the UDP and ATC in that it
- 643 involves testing more animals per dose and the primary endpoint of interest is evident
- 644 toxicity³ rather than lethality. The ATC method provides a range for the LD_{50} for
- 645 classification purposes. The UDP generally provides a point estimate of the LD₅₀ with a
- 646 confidence interval (EPA 2002a).
- 647

³ *Evident toxicity* is a general term describing clear signs of toxicity following administration of test substance, such that an increase to the next highest fixed dose would result in the development of severe toxic signs and probably mortality (ICCVAM 2000).

Regulatory Agency (Authorizing Act)	Animals	Endpoint	Classification
EPA (Federal Insecticide, Fungicide and Rodenticide Act)	Use current EPA or OECD protocol	Death ¹	$\begin{split} I - LD_{50} &\leq 50 \text{ mg/kg} \\ II - 50 &< LD_{50} \leq 500 \text{ mg/kg} \\ III - 500 &< LD_{50} \leq 5000 \text{ mg/kg} \\ IV - LD_{50} &> 5000 \text{ mg/kg} \end{split}$
CPSC (Federal Hazardous Substances Act)	White rats, 200-300 g	Death ¹ within 14 days for \geq half of a group of \geq 10 animals	$\begin{array}{l} \mbox{Highly toxic} - \mbox{LD}_{50} \leq 50 \mbox{ mg/kg} \\ \mbox{Toxic} - 50 \mbox{ mg/kg} < \mbox{LD}_{50} < 5 \mbox{ g/kg} \end{array}$
OSHA (Occupational Safety and Health Act)	Albino rats, 200-300 g	Death ¹ , duration not specified.	Highly toxic - $LD_{50} \le 50 \text{ mg/kg}$ Toxic - 50 < $LD_{50} \le 500 \text{ mg/kg}$
DOT (Federal Hazardous Material Transportation Act)	Male and female young adult albino rats	Death ¹ within 14 days of half the animals tested. Number of animals tested must be sufficient for statistically valid results.	$\begin{array}{l} Packing \ Group \ I\\ LD_{50} \leq 5 \ mg/kg\\ \hline Packing \ Group \ II\\ 5 < LD_{50} \leq 50 \ mg/kg\\ \hline Packing \ Group \ III\\ LD_{50} < 500 \ mg/kg \ (liquid)\\ LD_{50} < 200 \ mg/kg \ (solid)\\ \hline \end{array}$
OECD Guidance for Use of GHS (2001a)	Protocol not specified	Protocol not specified	$\begin{array}{l} I - LD_{50} \leq 5 \mm{mg/kg} \\ II - 5 < LD_{50} \leq 50 \mm{mg/kg} \\ III - 50 < LD_{50} \leq 300 \mm{mg/kg} \\ IV - 300 < LD_{50} \leq 2000 \mm{mg/kg} \\ V - 2000 < LD_{50} \leq 5000 \mm{mg/kg} \\ Unclassified - LD_{50} > 5000 \mm{mg/kg} \end{array}$

648 Table 1-2 Regulatory Classification Systems for Acute Oral Toxicity

^TGuidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for
 Experimental Animals Used in Safety Evaluation calls for humane killing of moribund animals (OECD 2000).
 Moribund animals that are humanely euthanized are accepted as deaths.

652 Abbreviations: EPA = U.S. Environmental Protection Agency; CPSC = U.S. Consumer Product Safety

653 Commission; OECD = Organisation for Economic Co-operation and Development; OSHA = U.S. Occupational

654 Safety and Health Administration; DOT = U.S. Department of Transportation; GHS = Globally Harmonized

655 System of Classification and Labelling of Chemicals (UN 2005)

656

657 1.4.3 Intended Regulatory Uses for In Vitro Cytotoxicity Test Methods

658 *In vitro* cytotoxicity test methods are not recommended for the replacement of acute oral

659 toxicity tests in animals. Rather, such test methods are intended to serve as adjuncts for *in*

660 vivo acute systemic toxicity test methods. To select a starting dose for a test substance, the

- 661 current test guidelines for acute oral systemic toxicity recommend using information on
- 662 structurally-related substances and the results of any other toxicity tests, including *in vitro*
- 663 cytotoxicity results (OECD 2001a, b, d; EPA 2002a). In vitro basal cytotoxicity test methods

are intended to be used as part of the weight-of-evidence approach to select starting doses for

the UDP and ATC assays in order to reduce and refine the use of animals for *in vivo* acute

toxicity testing. Since the estimation of the true LD_{50} is irrelevant to setting doses for

667 measuring evident toxicity, the use of *in vitro* basal cytotoxicity test methods for setting

starting doses for the FDP was not considered in the NICEATM/ECVAM validation study.

669

670 1.4.4 <u>Similarities and Differences in the Endpoints of *In Vitro* Cytotoxicity Test Methods</u> 671 <u>and *In Vivo* Acute Oral Toxicity Test Methods</u>

672 The endpoint measured in the in vitro NRU cytotoxicity test methods is cell death (neutral 673 red [NR] is taken up only by live cells) and the major endpoint of interest is the concentration 674 at 50% inhibition of NRU (i.e., the IC_{50}). The endpoint measured in acute systemic toxicity 675 assays is usually animal death. Cell death and animal death may be similar since animals are 676 comprised of organ systems consisting of tissues, which are comprised of cells. All cells, 677 regardless of whether they are in animals or in vitro cell cultures, have similar cellular 678 mechanisms of energy production and utilization and maintenance of cell membrane integrity. Animal death and death of cells in culture due to toxicity are similar in that both 679 680 involve some type of cellular injury. For the animal, the cellular injury produces tissue and 681 organ injury to the most sensitive target organ, which may then cause the death of the whole 682 organism. Cell death in a culture system involves the death of a single cell type. Cell death 683 and animal death may be produced by the same mechanisms, such as disruption of membrane 684 structure or function, inhibition of mitochondrial function, disturbance of protein turnover, disruption of energy production, etc. (Gennari et al. 2004). 685

686

687 Animal and cell culture systems are different with respect to how a substance or toxin is 688 delivered to the cell and how it is distributed, metabolized, and excreted. After oral 689 administration, animals must absorb the toxin from the gastrointestinal tract, which involves 690 the passage of membranes. The toxin may or may not be heavily bound to serum proteins; 691 this would reduce the availability of the toxin to the target organ. The toxin may then be 692 metabolized during and/or after distribution to the target organs and then the toxin or its 693 metabolites are excreted. In a cell culture system, the only membranes that must be passed 694 are those of the target cell and cellular organelles. No absorption and distribution by other 695 cellular systems is required. Cell culture systems may or may not include serum proteins, 696 which could reduce the availability of toxin to act as its target site. Excretion from the cell

697	culture milieu cannot occur since cell culture systems have no excretory system. The
698	cultured cells are exposed to substances for the entire duration of exposure in the test system.
699	
700	Animal and cell culture systems may also be different with respect to the target on which a
701	toxin acts. If a toxin acts in a specialized organ system in a whole animal, it may not produce
702	a toxic effect by the same mechanism in cultured cells that are derived from tissue different
703	from the target organ. For example, a neurotoxin that acts by a neuroreceptor-mediated
704	pathway in animals, would be expected to produce toxicity by a different mechanism in 3T3
705	or NHK cells, which are derived from fibroblasts, and skin cells, respectively. Even if a
706	neurotoxin were applied to neuronal cells in culture, the cultured cells may not respond in the
707	same way as neuronal cells in a whole animal. Cultured cells may not retain the same
708	functionality as cells in vivo.
709	
710	2.0 IN VITRO ACUTE TOXICITY TEST METHODS
711	
712	2.1 Background
713	
714	Pre-validation and validation studies have been completed to evaluate the ability of the 3T3
715	and NHK NRU test methods to be used in a weight of evidence approach (and not as stand-
716	alone tests) to estimate acute rat oral LD_{50} values, which are to be used as the basis for
717	selecting the starting dose for acute systemic toxicity (i.e., lethality) studies. This section
718	briefly describes the principles of in vitro basal cytotoxicity test methods followed by the
719	recommended performance standards that would be used to evaluate test methods that are
720	functionally and mechanistically similar to the 3T3 and NHK NRU test methods. The
721	performance standards consist of 1) essential test method components, 2) reference
722	substances, and 3) the comparable accuracy and reliability that should be achieved.
723	Theoretically, any in vitro test capable of determining basal cytotoxicity could be used to
724	estimate the starting dose for acute systemic toxicity testing using the UDP (EPA 2002c,
725	OECD 2001a) or the ATC method (OECD 2001b).
726	
727	

728		
729	2.2	Principles of In Vitro Basal Cytotoxicity Assays to Predict Starting
730		Doses for Acute Oral Toxicity Tests
731		
732	The bas	ic steps of an <i>in vitro</i> basal cytotoxicity assay are as follows:
733 734		• the test substance is dissolved in an appropriate solvent and applied as a solution to a sub-confluent cell culture monolayer or to exponentially growing cultures
735		of non-adherent cells
736		• the test substance is incubated with the cells for a specified period of time
737		• the test substance is removed and an endpoint indicative of cell viability or
738		cytotoxicity is measured
739		• the IC_{50} value is calculated (i.e., the concentration at which cell viability or
740		growth is inhibited by 50% compared to control values)
741		
742	Many di	ifferent in vitro basal cytotoxicity methods can be used to estimate the acute rat oral
743	LD ₅₀ va	lue (the calculated value of the oral dose that produces lethality in 50% of test
744	animals) and, thus, to predict the starting dose for an acute lethality assay. In vitro basal
745	cytotoxi	city data determined in various primary cells, as well as in various permanent non-
746	differen	tiated finite or transformed cell lines, generally exhibit the same dose response
747	cytotoxi	city relationship in response to the same xenobiotic, regardless of the type of toxic
748	endpoin	ts investigated. The following endpoints are sufficiently characteristic of basal
749	cytotoxi	city (Spielmann et al. 1999; Halle 1998):
750		
751		• Inhibition of cell proliferation: cell number, cell protein, DNA content, DNA
752		synthesis, colony formation
753		• <u>Cell viability - metabolic markers</u> : metabolic inhibition test, mitochondrial
754		reduction of tetrazolium salts
755		• Decreased cell viability - membrane markers: NRU into cell lysosomes, Trypan
756		Blue exclusion, cell detachment for monolayer cultures
757		• <u>Differentiation markers</u> : functional differentiation within cell clusters,
758		morphological differentiation within cell clusters, intracellular morphology

759

760 Markers of the release of intracellular components, such as the enzyme lactate 761 dehydrogenase (i.e., LDH release test), or of dye introduced into the cells previous to 762 chemical exposure, as occurs, for example, in the fluorescein leakage [FL] test or the Neutral 763 Red Release [NRR] test, are not considered to be characteristic for basal cytotoxicity because 764 they specifically detect damage of the outer cell membrane and generally are associated with 765 short-term chemical exposure (ICCVAM 2001). A chemical that specifically damages only 766 cell membranes, however, will be detected correctly in one of the tests for basal cytotoxicity 767 listed above.

768

769 Investigators using an *in vitro* basal cytotoxicity system for prediction of the *in vivo* starting 770 dose for acute oral toxicity/lethality studies must be able to demonstrate that the assay is 771 valid for its intended use. This includes demonstrating that any modification to the existing 772 validated reference test method does not adversely affect its performance characteristics. In 773 *vitro* systems may be used to test solids, liquids, and emulsions of any chemical or product 774 class. The liquids can be aqueous or nonaqueous; solids can be soluble or insoluble in water. 775 The samples may be pure chemicals, dilutions, formulations, or waste. Test substances must 776 be soluble in cell culture medium, dimethyl sulfoxide (DMSO), or ethanol (ETOH). The test 777 method endpoint (i.e., percent of control values) will be used to generate an IC₅₀ value in 778 μ g/mL and this value will be used in the regression formula developed to estimate the LD₅₀ 779 value in mg/kg.

780

2.3 Essential Test Method Components for In Vitro Basal Cytotoxicity Assays to Predict Starting Doses for Acute Oral Toxicity (Lethality) Tests

784

Essential test method components consist of essential structural, functional, and procedural

- elements of a validated test method that should be included in the protocol of a
- 787 mechanistically and functionally similar proposed test method. These components include
- vinique characteristics of the test method, critical procedural details, and quality control

789	measures. Adherence to these components will help assure that a proposed test method is			
790	based on the same concepts as the corresponding validated test method.			
791				
792	The following is a description of the essential test method components for in vitro basal			
793	cytotoxicity assays to predict starting doses for acute oral toxicity/lethality tests.			
794				
795	2.3.1 In Vitro Cell Culture Conditions			
796	• Use a mammalian cell line (or primary cells) that divides rapidly with doubling			
797	times of less than 30 hours under standard culture conditions, preferably with			
798	calf serum [CS], newborn calf serum [NCS]), or serum-free medium (ICCVAM			
799	2001).			
800	• Propagate cells in sterile tissue culture vessels (e.g., flasks) and then subculture			
801	cells to other sterile tissue culture vessels (e.g., 96 well-plates) for use in testing.			
802	Initial cell seeding should be done at a density that allows rapid growth			
803	throughout the exposure period. However, cell density should not reach			
804	confluency by the end of the test exposure period.			
805	Maintain appropriate cell culture growth conditions throughout the testing			
806	period (e.g., $37^{\circ}C \pm 1^{\circ}C$, $90\% \pm 10\%$ humidity, $5.0\% \pm 1\%$ CO ₂ /air). The cell			
807	cultures should be free of contamination with bacteria, mycoplasma, or fungi.			
808				
809	Cell culture media should be prequalified by the testing laboratory via a standardized			
810	protocol before initiating the test to guarantee that the media provide cells with appropriate			
811	nutrients to meet the growth criteria needed for the test method.			
812				
813	2.3.2 <u>Application of the Test Substances</u>			
814	Test Substance Preparation			
815	• Test substance solutions should be prepared in cell culture medium within one			
816	hour (unless otherwise known stability conditions of the substance require			
817	different parameters) before application to the cell cultures.			
818	• Standard protocol methods for solubility procedures can include mixing the test			
819	substance by vortexing, sonication, warming, and stirring. Test substances			

820		should be fully solubilized (i.e., no visual observation of test substance in the
821		dosing solution) before application.
822	•	One inherent limitation to <i>in vitro</i> cytotoxicity is the testing of volatile
823		chemicals since the material may evaporate before application to the cells or
824		may not remain in the test vessel when incubated. If volatility is predicted or
825		identified for a test substance (e.g., by detection of cross-contamination of the
826		high concentrations of test substance in culture with lower concentrations or
827		controls in the test vessel), measures can be employed to test moderately
828		volatile chemicals (e.g., cover the test plate with a CO ₂ permeable plastic film
829		cover/sealer).
830		
831 832	Cytotoxicity •	<i>Test</i> Each cytotoxicity test should contain a range of test substance concentrations
833		such that the IC_{50} value can be determined with at least one cytotoxic point
834		between $0 - 50\%$ viability and at least one cytotoxic point between $50 - 100\%$
835		viability.
836	•	A minimum of three adequate data points should be collected for each test
837		substance concentration. Note: The NICEATM/ECVAM validation study
838		required the testing of six replicates for each test substance concentration with
839		at least four successful replicates].
840	•	Blanks (i.e., culture vessels without cells) should be available for assessing
841		background interference when measuring the endpoint.
842	•	Cell monolayers in tissue culture vessels should be adequately covered (e.g., a
843		minimum of 100 μ L of test substance solution per well in a 96-well test plate).
844	•	The chemical exposure period should be at least the duration of one cell cycle,
845		i.e., 24 – 72 hours (Riddell et al. 1986). [Note: The NICEATM/ECVAM
846		validation study required an exposure period of 48 hours for 3T3 and NHK
847		cells; the cell cycle duration for these cells is generally 17-19 and 10-22 hours
848		in the log phase, respectively.]
849	•	At the end of the exposure period, most endpoints require washing the test
850		substance from the cells with an appropriate buffering solution (e.g., Dulbecco's
851		Phosphate Buffered Saline [DPBS]) before applying the endpoint material (e.g.,

neutral red dye). Washing cells to remove the test substance is the default
recommendation unless it is known that washing would interfere with
measurement of the endpoint.

855

856 2.3.3 <u>Control Substances</u>

857 *Vehicle Controls:* The vehicle controls provide the reference for 100% cell growth in the test 858 vessel and, thus, the vehicle (or solvent) must be compatible with the cell culture system (i.e., 859 not cause cytotoxicity or reduce cell growth through other mechanisms) and should not alter 860 the properties of the test substance. The vehicle controls should contain the solvent at the 861 concentration applied to the cells. For example, DMSO and ethanol at a final concentration 862 $\leq 0.5\%$ [v/v] were demonstrated to be compatible with cell growth for 3T3 and NHK cells in 863 the NICEATM/ECVAM validation study. If the compatibility of the solvent with the cell 864 culture system is unknown, cultures with and without the solvent must be included in each 865 experiment.

866

867 *Positive Controls:* The purpose of a positive control chemical is to demonstrate that the cell 868 culture system is responding with adequate sensitivity to a cytotoxic agent for which the 869 magnitude of the cytotoxic response is well characterized. The positive control chemical 870 should be tested concurrently with (and independent of) the test substance. The positive 871 control should be well characterized for its cytotoxicity potential and each test should 872 generate a response that is comparable to the historic IC_{50} range generated by the laboratory. 873 A laboratory should perform a minimum of 10 cytotoxicity tests using the positive control 874 over a number of days to develop a minimum historical database of IC₅₀ data. Typically, for 875 biologically based test methods, suggested acceptable ranges for the positive control response 876 are within two to three standard deviations of the historical mean response, but developers of 877 proprietary test methods may establish tighter ranges. Sodium lauryl sulfate (SLS) is an 878 effective positive control substance for use in *in vitro* basal cytotoxicity test methods. [Note: 879 The NICEATM/ECVAM validation study used SLS as the positive control and required 2.5 880 standard deviations of the historical mean response as the acceptable range.]

881

A-27

882 *Benchmark Controls:* Benchmark controls may be useful to demonstrate that the test method 883 is functioning properly for detecting the cytotoxic potential of chemicals of a specific 884 chemical class or a specific range of responses, or for evaluating the relative cytotoxic 885 potential of a cytotoxic test substance. Appropriate benchmark controls should have the 886 following properties:

887		 consistent and reliable source(s) for the chemical
888		• structural and functional similarity to the class of the substance being tested
889		known physical/chemical characteristics
890		• supporting data on known effects in animal models
891		• known potency in the range of response (including moderate response)
892		
893	2.3.4	Viability Measurements
894		• Only standardized, quantitative methods should be used to measure cell
895		viability. The protocol should be compatible with laboratory apparatus such as
896		spectrophotometers that allow a quick and precise measurement of the endpoint.
897		• Non-specific dye binding must not interfere with the viability measurement. A
898		measurement endpoint that is well established and that has good interlaboratory
899		reproducibility should be used (ICCVAM 2001).
900		• A detailed concentration-response experiment should be conducted using a
901		progression factor that yields graded effects between no effect and total
902		cytotoxicity. Any desired toxicity measure can be derived from a well-designed
903		concentration-response experiment.
904		• Preference should be given to endpoints that determine either cell proliferation
905		or cell viability (e.g., NRU, MTT [3-(4,5,dimethylthiazol-2yl)2,5-diphenyl
906		tetrazolium bromide], XTT [Sodium 3,3,-[(Phenylamino)carbonyl]-3,4-
907		Tetrazolium-Bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate]) (ICCVAM
908		2001).
909		• Simple endpoints such as total protein content are not recommended, as they
910		may under-predict the toxicity of certain test chemicals by including protein
911		from dead cells.

- 912
- seek only to identify the highest tolerated dose or the lowest cytotoxic dose.

A lack of information and a low level of accuracy characterize experiments that

914

915 Colorimetric endpoints (e.g., NRU) should have the optical density (OD)

916 spectrascopically-measured at the appropriate wavelength (e.g., 540 nm for NRU) and

- 917 OD values for blanks should be subtracted from the vehicle control and test substance
- 918 ODs.
- 919

920 2.3.5 Interpretation of Results

٠

921 *IC*₅₀ Determination

The endpoint values obtained for each test sample can be used to calculate the percentage of cell viability or growth relative to the negative (vehicle) control, which is arbitrarily set at 100%. The cell viability criteria used to determine an IC_{50} value must be clearly defined and documented, and be shown to be appropriate. In general, such criteria are established during test optimization, tested during a prevalidation phase, and confirmed in a validation study.

927

928 Regression Formula

929 The regression formula for *in vitro* acute toxicity test methods in the NICEATM/ECVAM 930 validation study was determined from the chemical IC₅₀ values (μ g/mL) and rat LD₅₀ values 931 (mg/kg) from the Registry of Cytotoxicity (RC) database. The RC contains acute oral LD₅₀ 932 values for rats and mice obtained from the Registry of Toxic Effects for Chemical Substances (RTECS[®]) and IC₅₀ values from *in vitro* cytotoxicity assays using multiple cell lines and 933 934 cytotoxicity endpoints for chemicals with known molecular weights (Halle 1998). Of the 935 347 chemicals in the RC, 282 chemicals have rat LD_{50} values. Those chemicals without rat 936 data were excluded. Chemicals with mechanisms of action not expected to be active in 937 culture cells used for basal cytotoxicity test methods were excluded from the database prior 938 to deriving the regression line. The regression formula based upon rat data and excluding 939 chemicals with specific mechanisms other than cytotoxicity was developed from the data for 940 232 chemicals.

941

942 $\log (LD_{50} \text{ in mg/kg}) = 0.357 \text{ x } \log (IC_{50} \text{ in } (\mu \text{g/mL}) + 2.194)$

A-29

943	
944	Before using a candidate in vitro basal cytotoxicity test to predict starting doses, the
945	correlation between the <i>in vitro</i> test and the <i>in vivo</i> test must be established quantitatively.
946	This can be accomplished by testing in the new assay a minimum of 12 reference substances
947	from Table 2-1 that cover all six hazard classification categories (i.e., the entire range of
948	cytotoxicity) and that produce the same regression formula as the total database. After
949	testing, the IC_{50} data are used to calculate a regression formula (least square method) for the
950	selected reference substances using the corresponding LD_{50} values provided in Table 2-1.
951	The resulting regression is compared against the original regression. If the regressions are
952	not statistically significantly different (at $p < 0.05$; e.g., comparison of slope and intercept),
953	then the test is considered suitable to generate IC_{50} data to use with the recommended
954	regression formula for estimating starting doses for acute oral systemic toxicity/lethality
955	tests.
956	
957	2.3.6 <u>Test Report</u>
958	The test report should include the following information, if relevant to the conduct of the
959	study:
960	Test Substances and Control Substances
961	Chemical name(s) such as Chemical Abstracts Service Registry Number
962	(CASRN) and molecular weight (if known), followed by other names, if known
963	• Formulation (if available) of the test substance if the material is a mixture
964	• Purity and composition of the substance or preparation (in percentage[s] by
965	weight)
966	• Physicochemical properties such as physical state, volatility, pH, stability,
967	chemical class, water solubility relevant to the conduct of the study
968	• Treatment of the test/control substances prior to testing, if applicable (e.g.,
969	vortexing, sonication, warming; solvent used)
970	Stability, if known
971	Justification of the In Vitro Test Method and Protocol Used
972	Test Method Integrity

973	• The procedure used to ensure the integrity (i.e., accuracy and reliability) of the
974	test method over time
975	• If the test method employs proprietary components, documentation on the
976	procedure used to ensure their integrity from "lot-to-lot" and over time
977	• The procedures that the user may employ to verify the integrity of the
978	proprietary components
979 980	<i>Criteria for an Acceptable Test</i>Acceptable concurrent positive control ranges based on historical data
981	Acceptable negative and solvent/vehicle control data
982	Test Conditions
983	Cell system used
984	• Calibration information for measuring device used for measuring cell viability
985	(e.g., spectrophotometer)
986	Details of test procedure used
987	• Test doses used
988	• Description of any modifications of the test procedure
989	Reference to historical data of the model
990	Description of evaluation criteria used
991 992	<i>Results</i>Tabulation of data from individual test samples (e.g., OD values and calculated
993	percentage cell viability data for the test substance and the positive, negative,
994	and benchmark controls, reported in tabular form, including data from replicate
995	repeat experiments as appropriate, and means and \pm the standard deviation for
996	each trial)
997	• Calculated IC ₅₀ value
998	• Calculated starting dose (i.e., LD ₅₀ value) using IC ₅₀ value in regression formula
999	Regression formula (prediction model) used
1000	Description of Other Effects Observed
1001	Discussion of the Results
1002	Conclusion
1003	
1004	

1005	2.4 Reference Substances for In Vitro Basal Cytotoxicity Assays to Predict
1006	Starting Doses for Acute Oral Toxicity Tests
1007	
1008	Reference substances are used to assess the accuracy and reliability of a proposed,
1009	mechanistically and functionally similar test method and are a representative subset of those
1010	used to demonstrate the reliability and the accuracy of the validated test method. These
1011	substances are:
1012	• representative of the range of responses that the validated test method is capable
1013	of measuring or predicting
1014	 have produced consistent results in the validated test method
1015	• reflect the accuracy of the validated test method
1016	have well-defined chemical structures
1017	• are readily available
1018	• are not associated with excessive hazard or prohibitive disposal costs
1019	
1020	To demonstrate technical proficiency with the validated test method, the user should evaluate
1021	his/her ability calculating IC50 values of at least 12 of the reference substances (minimum of
1022	two unclassified chemicals and two from each from the five GHS hazard categories) listed in
1023	Table 2-1. The resulting IC_{50} values should be within 2.5 standard deviations of the
1024	reported RC IC ₅₀ values in the table.
1025	
1026	This subset of 25 reference substances was chosen from the 72 reference substances used in
1027	the NICEATM/ECVAM in vitro acute toxicity test methods validation study and are
1028	common to the RC database. Criteria used for selecting the substances in this subset are:
1029	• they must be in common with the RC database and have rat LD_{50} data
1030	• they cannot be the same substances as those that were removed from the revised
1031	RC regression formula because of mechanism of action
1032	• they cannot be volatile or insoluble (determined by the NICEATM/ECVAM
1033	study)
1034	

- 1035 The low number of substances in the 5 < LD_{50} \leq 50 mg/kg and 300 < LD_{50} \leq 2000 mg/kg
- 1036 GHS categories is due to the exclusionary criteria described above. Reference substances
- 1037 that exhibited solubility difficulties (with 3T3 medium) or were volatile are included as a
- 1038 secondary subset and are recommended for investigational purposes only.
- 1039
- 1040 The substances in this list represent the following types of chemical classes: alcohols;
- 1041 amides; boron compounds; cadmium compounds; carboxylic acids; copper compounds;
- 1042 cyclic hydrocarbons; heterocyclics; mercury compounds; organometalics; potassium
- 1043 compounds; sodium compounds; and sulfur compounds. Chemicals that exhibited solubility
- 1044 difficulties (with 3T3 medium) or were volatile are included as a secondary subset and are to
- 1045 be used for investigational purposes only.
- 1046
- 1047

1047Table 2-1Recommended Reference Substances for Evaluation of In Vitro

1048Basal Cytotoxicity Methods for Predicting Starting Dose for Acute

1049Systemic Toxicity Tests

Chemical	CASRN ^a	LD50 ^b (mg/kg)	IC50x ^c (µg/mL)
	LD ₅₀ ≤ 5 1	mg/kg	·
Mercury II chloride	7487-94-7	1	4.07
Triethylenemelamine	51-18-3	1	0.159
Cycloheximide	66-81-9	2	0.166
Busulfan	55-98-1	2	11.3
Phenylthiourea	103-85-5	3	82.2
	$5 < LD_{50} \le 5$	0 mg/kg	
Triphenyltin hydroxide	76-87-9	44	0.0180
Sodium bichromate	10588-01-9	50	0.244
	$50 < LD_{50} \le 3$	00 mg/kg	
Hexachlorophene	70-30-4	61	3.21
Cadmium II chloride	10108-64-2	88	1.17
Sodium oxalate	62-76-0	155	59.0
Sodium fluoride	7681-49-4	180	77.7
Diquat dibromide	85-00-7	231	55.1
Cupric sulfate pentahydrate	7758-99-8	300	82.4
	$300 < LD_{50} \le 2$	000 mg/kg	1
Acetylsalicylic acid	50-78-2	1000	409.0
Propranolol HCl ³	350-60-90	470	35.5
	$2000 < LD_{50} \le 3$	5000 mg/kg	·
Acetaminophen	103-90-2	2404	409.7
Potassium chloride	7447-40-7	2602	6113
Dimethylformamide	68-12-2	2800	8335
Sodium chloride	7647-14-5	2998	4436
Chloramphenicol	56-75-7	3393	255.3
Lactic acid	50-21-5	3730	5946
Trichloroacetic acid	76-03-9	4999	1338
	$LD_{50} > 5000$	0 mg/kg	
Ethylene glycol	107-21-1	8567	34454
Dibutyl phthalate	84-74-2	11998	211.6
Glycerol	56-81-5	12691	57477

	Chemical	CASRN ^a	LD50 ^b (mg/kg)	IC50x ^c (µg/mL)	
	Secondary Subset		(1116/116)		
	Precipitating Chemicals ¹				
		$LD_{50} \leq 5$			
	Arsenic trioxide	1327-53-3	20	1	
	Parathion	56-38-2	2	27.1	
		$\frac{300 < \text{LD}_{50} \le 2}{77.0 \le 5}$	2 2		
	Giberrellic Acid	77-06-5	6305	797	
		Volatile Ch			
	Phenol	$\frac{300 < \text{LD}_{50} \le 2}{108-95-2}$	414	283.3	
		$LD_{50} > 500$		200.0	
	Ethanol	64-17-5	14008	17464	
1050	2-Propanol ^a Chemical Abstracts Service Regist	67-63-0	5843	10038	
1052 1053 1054 1055 1056 1057 1058 1059 1060 1061 1062 1063 1064 1065 1066	^c Reference substance concentration endpoint measured (i.e., cell viabilit ¹ Reference substances expected to p ² Reference substances expected to c ³ Propranol HCl data were not used i lacked rat data (RC LD ₅₀ value = 46 470 mg/kg in the literature and deci representative of the 300 < LD ₅₀ \leq 2 2.5 Accuracy and Reliability The third element of the performance sta as relevance) and reliability values. The mechanistically similar to the 3T3 NRU to assess accuracy and reliability.	by: contaminate at cyt contaminate neig in developing th 66 mg/kg [from 1 ded that this refe 2000 mg/kg cate andards is the e proposed tes	otoxic concentrations hboring wells at high e regression formula mouse]). NICEATM erence substance wou gory. determination of at method, functio	a concentrations. because the compound found a rat LD ₅₀ value of ild be an acceptable	
1067	to assess accuracy and rendomity.				
1068	2.5.1 <u>Accuracy</u>				
1069	When evaluated using the minimum list	of recommer	ded reference sub	ostances (Table 2-1),	
1070	the proposed test method should have pe	erformance cl	naracteristics that	are comparable to the	
1071	performance of the validated 3T3 NRU	test method	Accuracy is defin	ed as the closeness of	
1072	agreement between a test method result		•		
1073	Substances, ranging in toxicity activity	1		× , , , , , , , , , , , , , , , , , , ,	
		-		-	
1074	chemical classes across all GHS hazard	categories are	e included so that	the performance of the	

proposed test method can be determined and compared to that of the validated reference testmethod.

1077

1078 Although the *in vitro* basal cytotoxicity test methods are not intended as replacements for

acute systemic toxicity assays, the accuracy of these assays to predict LD₅₀ values in the

1080 correct GHS acute oral toxicity hazard classification category (UN 2005) was evaluated.

1081 This accuracy evaluation characterizes the extent that additional test methods will be

1082 necessary to achieve accurate *in vitro* predictions of acute oral toxicity hazards for regulatory

- 1083 classification and labeling purposes.
- 1084

1085 The log IC₅₀ data from the 3T3 and NHK NRU test methods and the corresponding reference

1086 log LD₅₀ values (mg/kg) values were used to calculate linear regressions for each validation

1087 study laboratory. The slopes for all regressions were statistically significant (p < 0.0001),

1088 which indicated a significant relationship between IC₅₀ and LD₅₀ values. The higher adjusted

1089 R^2 values for the 3T3 regressions, compared with the NHK regressions indicated that the 3T3

1090 IC₅₀ data provided a better fit to the LD_{50} data than the corresponding NHK data (see **Table**

- 1091 **2-2).**
- 1092

	Weig	Weight Unit Regressions ²				
Laboratory	Slope Intercept		Adjusted R ²			
	3T3 NRU T	est Method				
Lab 1	0.509	1.552	0.420			
Lab 2	0.453	1.513	0.307			
Lab 3	0.515	1.542	0.421			
Combined ³	0.516	1.498	0.409			
	NHK NRU T	Cest Method				
Lab 1	0.425	1.679	0.319			
Lab 2	0.375	1.798	0.276			
Lab 3	0.424	1.704	0.318			
Combined ³	0.424	1.720	0.322			

1093 Table 2-2 Linear Regression Analyses of *In Vitro* and *In Vivo* Results¹

¹Data for 70 reference substances in the 3T3 assay and 71 reference substances in the NHK assay.

 $^{2}\log IC_{50}$ in µg/mL; log LD₅₀ in mg/kg.

³Single regression for the test method using the geometric mean of the laboratory-specific IC_{50} values for each reference substance.

1098 1099

1094

1095

1096

17 Mar 2006

Table 2-3 shows that the accuracy of the 3T3 and NHK NRU test methods for predicting
GHS acute oral toxicity categories was similar. The accuracy for the 3T3 regression was
46% while the accuracy for the NHK regression was 38%.

1103

1104 2.5.2 <u>Reliability</u>

Reliability is the degree to which a test method can be performed reproducibly within and
among laboratories over time (ICCVAM 2003) and was assessed in the NICEATM/ECVAM

1107 validation study by determining both intra- and inter-laboratory reproducibility.

1108 Intralaboratory reproducibility is the agreement of results produced when qualified people

1109 within the same laboratory perform the test method using the same test protocol at different

1110 times. Interlaboratory reproducibility is the agreement of results from different qualified

1111 laboratories using the same protocol and reference substances. Interlaboratory

1112 reproducibility indicates the extent to which a test method can be transferred successfully

among laboratories. Intra- and inter-laboratory reproducibility of the 3T3 and NHK NRU

1114 test methods were determined using Analysis of Variance (ANOVA) and Coefficient of

1115 Variation (CV) analysis. Interlaboratory reproducibility of the 3T3 and NHK NRU test

1116 methods was also assessed by comparing the laboratory-specific *in vitro-in vivo* regressions

1117 (for each test method) to one another.

1118 Table 2-3 Prediction of GHS Toxicity Category¹ by RC Rat-Only Weight Regression Excluding Chemicals with

1119

Specific Mechanisms of Toxicity

Reference	3T3 NRU-Predicted Toxicity Category								Toxicity	Toxicity
Rodent LD ₅₀ ²	< 5	5 - 50	50 - 300	300-2000	2000-5000	> 5000	Total	Accuracy	Overpredicted	Underpredicted
< 5	0	0	2	2	0	0	4 ³	0%	100%	0%
5 - 50	0	1	4	2	0	0	7^{4}	14%	86%	0%
50 - 300	0	0	4	1	0	0	5 ⁵	80%	20%	0%
300 - 2000	0	1	1	7	0	0	9 ⁶	78%	0%	22%
2000 - 5000	0	0	0	3	6	0	9 ⁷	67%	0%	33%
> 5000	0	0	0	5	4	3	128,9	25%	0%	75%
Total	0	2	11	20	10	3	46	46%	24%	30%
Predictivity	0%	50%	36%	35%	60%	100%				
Category Underpredicted	0%	50%	9%	40%	40%	0%				
Category Overpredicted	0%	0%	55%	25%	0%	0%				
Reference	NHK NRU-Predicted Toxicity Category								Torioity	Torioity
Rodent LD ₅₀ ²	< 5	5 - 50	50 - 300	300 - 2000	2000 - 5000	> 5000	Total	Accuracy	Toxicity Overpredicted	Toxicity Underpredicted
< 5	0	0	2	2	0	0	4 ³	0%	100%	0%
5 - 50	0	1	4	2	0	0	7^{4}	14%	86%	0%
50 - 300	0	1	3	1	0	0	5 ⁵	60%	20%	20%
300 - 2000	0	1	0	8	0	0	9 ⁶	89%	0%	11%
2000 - 5000	0	0	0	5	4	0	9 ⁷	44%	0%	56%
> 5000	0	0	0	4	7	2	13 ⁹	15%	0%	85%
Total	0	3	9	22	11	2	47	38%	23%	38%
Predictivity	0%	33%	33%	36%	36%	100%				
Category Underpredicted	0%	67%	0%	41%	64%	0%				
Category Overpredicted	0%	33%	67%	23%	0%	0%				

1120

¹GHS-Globally Harmonized System of Classification and Labelling of Chemicals with LD₅₀ in mg/kg (UN 2005). The RC rat-only weight regression

1121 excluding chemicals with specific mechanisms of toxicity is $\log LD_{50}$ (mg/kg) = $\log IC_{50}$ (µg/mL) X 0.357 + 2.194.

1122

- 125 < 5: $LD_{50} \le 5 \text{ mg/kg}$
- 1126 5-50: $5 < LD_{50} \le 50 \text{ mg/kg}$
- 1127 50 300: 50 <LD₅₀ \leq 300 mg/kg
- 1128 300 2000: $300 < LD_{50} \le 2000 \text{ mg/kg}$
- 1129 2000 5000: $2000 < LD_{50} \le 5000 \text{ mg/kg}$
- 1130 > 5000: $LD_{50} > 5000 mg/kg$
- 1131 Numbers in table represent number of reference substances.
- 1132 2 *In vivo* reference LD_{50} values
- 1133

Goodness of fit F-tests indicated that the laboratory-specific regressions for both test methods were not significantly different from one another (p = 0.796 for comparison of the 3T3 regressions and p = 0.985 for comparison of the NHK regressions).

The reliability of the proposed test method for the reference substances should be comparable to or better than that of the validated 3T3 NRU test method. **Table 2-4** illustrates intra- and inter-laboratory CV results obtained from the NICEATM/ECVAM *in vitro* acute toxicity test methods validation study.

	3T3 NRU Test Method	Ν	NHK NRU Test Method	Ν
	Intralaboratory	/ CV		
Range	1-122%	202	1-129%	208
Mean CV (and Range)	26%	202	26%	208
Lab 1	23% (2-95%)	68	23% (2-76%)	69
Lab 2	33% (1-98%)	66	42%(1-129%)	69
Lab 3	21% (1-122%)	68	14% (1-38%)	70
	Interlaboratory	/ CV		
Range	2-135%	68	1-99%	69
Mean	46%	68	28%	69

Table 2-4Summary of CV Results for the 3T3 and NHK NRU Test Methods

CV - coefficient of variation; N - number of values

3.0 REFERENCES

EPA. 2002. Health Effects Test Guidelines OPPTS 870.1100 Acute Oral Toxicity. EPA 712–C–02–190. Washington, DC: U.S. Environmental Protection Agency.

FDA. 1993. Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food. Center for Food Safety and Applied Nutrition. Washington, DC: U.S. Food and Drug Administration.

Gennari A, van den Berghe C, Casati S, Castell J, Clemedson C, Coecke S, et al. 2004. Strategies to Replace In Vivo Acute Systemic Toxicity Testing. The Report and Recommendations of ECVAM Workshop 50. Altern Lab Anim 32:437-459.

Halle W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD₅₀) zur Einsparung von Tierversuchen. Life Sciences/ Lebenswissenschaften, Volume 1, 94 pp., Jülich: Forschungszentrum Jülich. <u>English</u> <u>translation</u>: Halle W. 2003. The Registry of Cytotoxicity: Toxicity testing in cell cultures to predict acute toxicity (LD50) and to reduce testing in animals. Altern Lab Anim 31:89-198.

ICCVAM. 1997. Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication No. 97-3981. Research Triangle Park, NC. National Institute of Environmental Health Sciences. Available: <u>http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf</u> [accessed 14 March 2006].

ICCVAM. 2001. Guidance Document On Using In Vitro Data To Estimate In Vivo Starting Doses For Acute Toxicity. NIH Publication No. 01-4500. National Institute for Environmental Health Sciences, Research Triangle Park, NC. Available: <u>http://iccvam.niehs.nih.gov/</u>. [accessed 14 March 2006].

ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508. National Institute for Environmental Health Sciences, Research Triangle Park, NC. Available: <u>http://iccvam.niehs.nih.gov/</u>. [accessed 14 March 2006].

OECD. 1996. Final Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Methods. (Solna Report) Paris: Organisation for Economic Co-operation and Development. Available: <u>http://www.oecd.org</u> [accessed 14 March 2006].

OECD. 2000. Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment No. 19. Paris France: OECD. Available:

http://www.oecd.org/document/22/0,2340,en 2649 34377 1916054 1 1 1 1,00.html

[accessed 14 March 2006]

OECD. 2001a. Guideline for Testing of Chemicals, 425, Acute Oral Toxicity – Up-and-Down Procedure. Paris France: OECD. Available: <u>http://www.oecd.org</u> [accessed 14 March 2006].

OECD. 2001b. Guideline For Testing of Chemicals, 423, Acute Oral Toxicity – Acute Toxic Class Method. Paris France: OECD.

OECD. 2001c. OECD Series on Testing and Assessment, 33, Harmonized Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures. ENV/JM/MONO(2001)6. Paris France: OECD. Available: <u>http://www.oecd.org</u> [accessed 12 September 2004].

OECD. 2001d. Guideline for Testing of Chemicals, 420, Acute Oral Toxicity – Fixed Dose Method. Paris France: OECD. Available: <u>http://www.oecd.org</u> [accessed 12 September 2004].

OECD. 2002. Report of the Stockholm Conference on Validation and Regulatory Acceptance of New and Updated Methods in Hazard Assessment. Paris: Organisation for Economic Co-operation and Development. Available: <u>http://www.oecd.org</u> [accessed 14 March 2006].

Riddell RJ, Panacer DS, Wilde SM, Clothier RH, Balls M. 1986. The importance of exposure period and cell type in *in vitro* cytotoxicity tests. Altern Lab Anim 14:86-92.

Spielmann H, Genschow E, Liebsch M, Halle W. 1999. Determination of the starting dose for acute oral toxicity (LD_{50}) testing in the up and down procedure (UDP) from cytotoxicity data. Altern Lab Anim 27:957-966.

UN. 2005. Globally Harmonized System of Classification and Labelling of Chemicals (GHS), First Revised Edition. [ST/SG/AC.10/30/Rev.1]. United Nations, New York and Geneva. Available:

http://www.unece.org/trans/danger/publi/ghs/ghs_rev01/01files_e.html [accessed 14 March 2006].

Appendix **B**

Draft Recommended Test Method Protocols

B-1	Test Method Protocol for the BALB/c 3T3 Neutral Red Uptake				
	(NRU) Cytotoxicity TestB-1-3				
B-2	Test Method Protocol for the Normal Human Epidermal				
	Keratinocyte (NHK) Neutral Red Uptake (NRU) Cytotoxicity				
	TestB-2-3				

[This Page Intentionally Left Blank]

Appendix B-1

Test Method Protocol for the BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test

This draft recommended 3T3 NRU test method protocol is substantially the same as the protocol used in Phase III of the NICEATM/ECVAM validation study. Revisions were made based on recommendations from NICEATM and the study directors involved in the study. The changes are as follows:

- Explanations and directions for the use of the revised Hill function for determining IC50 values are included in the protocol.
- The range for relative humidity values for the cell culture incubators was changed from 90 % \pm 5 % humidity to 90 % \pm 10 % humidity.
- An additional step was added to the test substance solubility protocol to allow testing of higher concentrations of test material.
- The spreadsheet templates used in the NICEATM/ECVAM validation study are incorporated into this protocol as an annex (ANNEX I).
- The stand-alone solubility protocol is incorporated into this protocol as an annex (ANNEX II).

[This Page Intentionally Left Blank]

1		Test Method Protocol
2 3 4	Т	he BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test A Test for Basal Cytotoxicity
5 6		
7 8 9	I.	PURPOSE
10 11 12 13 14 15 16		This test method is used to evaluate the cytotoxicity of test substances using the BALB/c 3T3 Neutral Red Uptake (NRU) <i>in vitro</i> cytotoxicity test. The data generated from the <i>in vitro</i> cytotoxicity assays are used to evaluate the effectiveness of the assay to predict the starting doses for rodent acute oral systemic toxicity assays. This test method protocol outlines the procedures for performing the basal cytotoxicity test and is the result of the <i>in vitro</i> validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM).
17 18 19 20 21		If changes or modifications are made to this protocol, the testing laboratory should prove that the results are comparable to those obtained when using the original protocol.
22	П.	TEST SYSTEM
23 24 25 26 27 28 29 30 31 22		The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.
32 33 34 35 36 37 38 39		Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after chemical exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.
40	III.	KEY PERSONNEL
41 42 43 44 45		 A. Laboratory Study Director Laboratory Technician(s)

46		В.	Testing Facility
47 48 49 50 51			 Scientific Advisor Quality Assurance Director Safety Manager Facility Management
52	IV.	DE	CFINITIONS
53 54 55 56 57		3	<i>Hill function</i> : a four parameter logistic mathematical model relating the concentration of test substance to the response being measured in a sigmoidal shape.
58			$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(logEC50-X)HillSlope}}$
59 60 61 62 63 64			where Y= response, X is the logarithm of dose (or concentration), Bottom is the minimum response, Top is the maximum response, logEC50 is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve. When Top = 100 and Bottom = 0, the EC ₅₀ is the concentration at 50% viability (i.e., the IC ₅₀).
65 66 67 68 69 70 71		4	Documentation : all methods and procedures will be noted in a Study Workbook; logs will be maintained for general laboratory procedures and equipment (e.g., media preparation, test substance preparation, incubator function); all optical density data obtained from the spectrophotometer plate reader will be saved in electronic and paper formats; all calculations of ICx values and other derived data will be in electronic and paper format; all data will be archived.
71 72 73 74		5	IC_{50} : test substance concentration producing 50% inhibition of the endpoint measured (i.e., cell viability).
75 76	V.	ID	ENTIFICATION OF CONTROL SUBSTANCES
77 78 79		А.	Positive Control (PC) Sodium Lauryl Sulfate (SLS)
80 81 82 83		B.	Vehicle Control (VC) Assay medium (DMEM containing 5% NBCS, 4 mM L-Glutamine, 100 IU/mL Penicillin, 100 μg/mL Streptomycin)
83 84 85 86		C.	Solvent Control VC control with solvent (i.e., assay medium, dimethyl sulfoxide [DMSO], or ethanol [ETOH])

87			(DMSO is the preferred solvent for substances that are not water [i.e., assay
88			medium] soluble.)
89			
90	VI.	PR	OCEDURES
91			
92		A.	Materials
93			1. Cell Line
94			BALB/c 3T3 cells, clone A31
95			(e.g., CCL-163, American Type Culture Collection [ATCC], Manassas, VA,
96			USA)
97			
98			2. Technical Equipment
99			[Note: Suggested brand names/vendors are listed in parentheses. Equivalents
100			may be used.]
101			
102			4 Incubator: $37^{\circ}C \pm 1^{\circ}C$, $90 \% \pm 10 \%$ humidity, $5.0 \% \pm 1 \% CO_2/air$
103			5 Laminar flow clean bench/cabinet (standard: "biological hazard")
104			6 Waterbath: $37^{\circ}C \pm 1^{\circ}C$
105			7 Inverse phase contrast microscope
106			8 Sterile glass tubes with caps (e.g., 5 mL)
107			9 Centrifuge
108			10 Laboratory balance
109			11 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm \pm
110			10 nm filter
111			12 Shaker for microtiter plates
112			13 Cell counter or hemocytometer
113			14 Pipetting aid
114			15 Pipettes, pipettors (multi-channel and single channel; multichannel repeater
115			pipette), dilution block
116			16 Cryotubes
117			17 Tissue culture flasks (e.g., $75 - 80 \text{ cm}^2$, 25 cm^2)
118			18 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008;
119			Falcon tissue culture-treated)
120			19 pH paper (wide and narrow range)
121			20 Multichannel reagent reservoir
122			21 Waterbath sonicator
123			22 Magnetic stirrer
124			23 Antistatic bar ionizer/antistatic gun (optional for neutralizing static on 96-
125			well plates)
126			24 Dry heat block (optional) 25 Adharing film plate angless (a.e., Freed Scientific ScalPlate M Cat # STP
127			25 Adhesive film plate sealers (e.g., Excel Scientific SealPlate [™] , Cat # STR-
128			SEAL-PLT or equivalent)
129			26 Vortex mixer
130			27 Filters/filtration devices
131			

133 ensure that they adequately support the growth of 3T3 cells. Multi-channel 134 repeater pipettes may be used for plating cells in the 96-well plates, dispensing 135 plate rinse solutions, NR medium, and desorb solution. Do not use the repeater 136 pipette for dispensing test substances to the cells.] 137 138 3. Chemicals, Media, and Sera 139 a) Dulbecco's Modification of Eagle's Medium (DMEM) without L-140 Glutamine; should have high glucose [4.5gm/l] (e.g., ICN-Flow Cat. No. 12-141 332-54) 142 b) L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49) 143 c) New Born Calf Serum (NCS) (e.g., Biochrom # SO 125) d) 0.05 % Trypsin/0.02 % EDTA solution (e.g., SIGMA T 3924, ICN-Flow, # 144 16891-49) 145 e) Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (for trypsinization) 146 f) Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and $Mg^{2+}(CMF-$ 147 148 HBSS) 149 g) Dulbecco's Phosphate Buffered Saline (D-PBS) [formulation containing 150 calcium and magnesium cations; glucose optional] (for rinsing) h) Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49) 151 i) Neutral Red (NR) Dye – tissue culture-grade; liquid form (e.g., SIGMA N 152 153 2889); powder form (e.g., SIGMA N 4638) i) DMSO, U.S.P. analytical grade (Store under nitrogen @ -20°C) 154 k) ETOH, U.S.P. analytical grade (100 %, non-denatured for test substance 155 preparation; 95 % can be used for the desorb solution) 156 1) Glacial acetic acid, analytical grade 157 m) Distilled H₂O or any purified water suitable for cell culture and NR desorb 158 159 solution (sterile) 160 n) Sterile/non-sterile paper towels (for blotting 96-well plates) 161 162 [Note: Due to lot variability of NCS, first check a lot for growth stimulating properties with 3T3 cells (approximately 20-24 hours doubling time) and then 163 164 reserve a sufficient amount of NCS.] 165 166 **B.** Preparations of Media and Solutions [Note: All solutions (except NR stock solution, NR medium and NR desorb), 167 168 glassware, pipettes, etc., shall be sterile and all procedures should be carried out 169 under aseptic conditions and in the sterile environment of a laminar flow cabinet 170 (biological hazard standard). All methods and procedures will be adequately 171 documented.] 172 173 1. Media 174 DMEM (buffered with sodium bicarbonate) supplemented with (final 175 concentrations in DMEM are quoted): 176

Note: Tissue culture flasks and microtiter plates should be prescreened to

177 178 179 180 181 182 183 184		freezing solution 40 % No 20 % Di b) Routine Culture 10 % No	on CS MSO	entration of NCS and DMSO of final
185 186 187 188 189 190		200 IU/mL Pe 200 μg/mL St	lutamine enicillin reptomycin	
191 192 193 194 195 196			CS lutamine enicillin	
197 198 199 200 201 202		concentration of the proteins may mask excluded because c	e Routine Culture M the toxicity of the to cell growth is marke	h with test substance will dilute the serum Medium in the test plate to 5 %. Serum test substance, but serum cannot be totally edly reduced in its absence.]
202 203 204 205	2.	stored for no longe Neutral Red (NR)	r than two weeks.	be kept at approximately 2-8°C and
206 207 208 209 210 211 212 213 214		SIGMA #N2889, 3 Solution at the stor manufacturer. A stock solution ca Dye powder in 100	3.3 mg/mL). Store l age conditions and on be made with power that H_2O if the liq	IR Solution is the first choice (e.g., iquid tissue culture-grade NR Stock shelf-life period recommended by the wder NR dye and water (e.g., 0.25 g NR uid stock form is not available. The stock mperature for up to two months.
215	3.	Neutral Red (NR)	Medium	
216 217 218 219 220		EXAMPLE: 0.758 mL (3.3 mg 1 99.242 mL	NR dye/mL sol.)	NR Stock Solution NR Dilution Medium (pre-warmed to 37°C)

221 222		I concentration of the NR Medium is $25 \ \mu g \ NR \ dye/mL$ and aliquots will be prepared on the day of application.
 223 224 225 226 227 228 229 230 	s a r 3	The NR Medium shall be filtered (e.g., Millipore filtering, 0.2 – 0.45 µm pore size) to reduce NR crystals. Aliquots of the NR Medium should be maintained at 37°C (e.g., in a waterbath) before adding to the cells and used within 60 minutes of preparation but also used within 15 minutes after removing from 37°C storage. Examine the solution for crystals.]
231 232 233 234	5	Glacial acetic acid solution50 %ETOH49 %H2O
235	C. Met	
 236 237 238 239 240 241 242 243 244 245 	E f 9 u a 2. H U	Cell Maintenance and Culture Procedures BALB/c 3T3 cells are routinely grown as a monolayer in tissue culture grade flasks (e.g., 75 - 80 cm ²) at $37^{\circ}C \pm 1^{\circ}C$, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO ₂ /air. The cells should be examined on a daily (i.e., on workdays) basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted in a Study Workbook. Receipt of Cryopreserved BALB/c 3T3 Cells Upon receipt of cryopreserved BALB/c 3T3 cells, the vial(s) of cells shall be stored in a liquid nitrogen freezer until needed.
246	2 1	
247		Thawing Cells
248249250	5	y putting ampules into a waterbath at $37^{\circ}C \pm 1^{\circ}C$. Leave for as brief a time as possible.
250 251 252 253 254 255 256 257 258 259 260	3 4 5 6	 transfer into pre-warmed Routine Culture Medium in a tissue-culture flask. Incubate at 37°C ± 1°C, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO₂/air. When the cells have attached to the bottom of the flask (within 4 to 24 hours), decant the supernatant and replace with fresh pre-warmed (37°C) medium. Culture as described above.
261 262		f frozen cells from the stock lot of cells should be thawed out and cultured approximately every two months. This period resembles a sequence of about 18

263 264		passages.								
264 265	4.	Routine Cultu	re of BALB/C 3T	3 Cells						
266 267 268		When cells exceed 50 % confluence (but less than 80 % confluent) they should be removed from the flask by trypsinization:								
269 270 271 272 273 274 275 276 277 278 279 280 281		 (without Ca by gentle ag action of th 4 Discard the washing so 5 Add 1-2 ml seconds (e.g 6 Remove ex temperature 	 <u>a²⁺, Mg²⁺)</u> per 25 cm gitation to remove e trypsin. washing solution. lution. trypsin-EDTA so g., 15-30 seconds). cess trypsin-EDTA inutes, lightly tap 	cultures with 5 mL m ² flask (15 mL per any remaining serur Repeat the rinsing dution per 25 cm ² to a solution and incub the flask to detach t	75 cm ² flask). Wa m that might inhibi procedure and disc the monolayer for ate the cells at roon	ash cells t the card the c a few m				
282	5.	Cell Counting								
283 284 285 286 287 288 289	6	Medium/cm ² to monolayer by g counting. Count hemocytometer	b the flask (e.g., 2.5 gentle trituration to nt a sample of the of or cell counter (e.	0.2 mL of pre-warn 5 mL for a 25 cm ² fl obtain a single cell cell suspension obta g., Coulter counter)	ask). Disperse the suspension for exa ined using a					
289	0.	Subculture of				_				
290 291 292 293 294 295 296		After determination of cell number, the culture can be sub-cultured into other flasks or seeded into 96-well microtiter plates (see Section VI.E.1 for 96-well test plate configuration). BALB/c 3T3 cells are routinely passaged at suggested cell densities as listed in the table (approximate doubling time is 20-24 hours). Laboratories must determine and adjust the final density to achieve appropriate growth.								
297		Table 1. Cell	Density Guideline	s for Subculturing						
298		Days in	Seeding Density	Total Cells per	Total Calls par	1				
		Culture	(cells/cm ²)	$25 \text{ cm}^2 \text{ flask}$	Total Cells per 75 cm ² flask					
		2	16800	4.2×10^5	1.26×10^6					
		3	8400	2.1×10^5	6.3×10^5					
		4	4200	1.05×10^5	3.15×10^5					

300[Note: It is important that cells have overcome the lag growth phase when they301are used for the test.]

303	7.	Freezing Cells (procedure required only if current stock of cells is depleted)
304		Stocks of BALB/c 3T3 cells can be stored in sterile, freezing tubes in a
305		liquid nitrogen freezer. DMSO is used as a cryoprotective agent.
306		
307		4 Centrifuge trypsinized cells at approximately 200 x g.
308		5 Suspend the cells in cold Routine Culture Medium (half the final
309		freezing volume) so a final concentration of $1-5 \times 10^6$ cells/mL can be
310		attained.
311		6 Slowly add cold Freeze Medium to the cells so that the solvent will
312		equilibrate across the cell membranes. Bring the cell suspension to the
313		final freezing volume. The final cell suspension will be 10 % DMSO.
314		Aliquot the cell suspension into freezing tubes and fill to 1.8 mL.
315		7 Place the tubes into an insulated container (e.g., styrofoam trays) and
316		place in a freezer (-70 to -80°C) for 24 hours (~freezing rate of
317		1°C/minutes). The laboratory needs to ensure that the freezing protocol
318		is applicable to the 3T3 cells and that the cells are viable when removed
319		from cryopreservation.
320		8 Place the frozen tubes into liquid nitrogen for storage.
321		
322	8.	Preparation of Cells for Assays
323		• Cultured cells that will be used in seeding the 96-well plates should be fed
324		fresh medium the day before subculturing to the plates. On the day of plate
325		seeding, prepare a cell suspension of $2.0 - 3.0 \times 10^4$ cells/mL in Routine
326		Culture Medium. Using a multi-channel pipette, dispense 100 µl Routine
327		Culture Medium only into the peripheral wells (blanks) of a 96-well tissue
328		culture microtiter plate (See Section VI.E.1). In the remaining wells,
329		dispense 100 µl of a cell suspension of $2.0 - 3.0 \times 10^4$ cells/mL (= 2.0×10^4 cells/mL (= 2.0×10^4 cells/mL (= 2
330		3.0×10^3 cells/well). The seeding density should be noted to ensure that the
331		cells in the control wells are not overgrown after three days (i.e., 24 hour
332		incubation in step b and 48 hour exposure to test substances). Prepare one
333		plate per substance to be tested.
334		• Incubate cells for 24 hours ± 2 hours (37°C $\pm 1°$ C, 90 % ± 10 % humidity,
335		$5.0\% \pm 1\%$ CO ₂ /air) so that cells form a less than half (< 50%) confluent
336		monolayer. This incubation period assures cell recovery and adherence and
337		progression to exponential growth phase.
338		• Examine each plate under a phase contrast microscope to assure that cell
339		growth is relatively even across the microtiter plate. This check is
340		performed to identify experimental and systemic cell seeding errors. Record
341		observations in the Study Workbook.
342		
343	9.	Determination of Doubling Time
344		a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for
345		subculture. Resuspend cells in NR Dilution Medium (5 % NBCS/NCS).
346		Seed cells at 4200 cells/cm^2 .

347	b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15
348	tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture
349	medium for the culture vessels. Note number of cells placed into each
350	culture dish. Place dishes into the incubators $(37^{\circ}C \pm 1^{\circ}C, 90\% \pm 10\%)$
351	humidity, $5.0\% \pm 1\%$ CO ₂ /air).
352	c) After 4 - 6 hours (use the same initial measurement time for each subsequent
353	doubling time experiment), remove three culture dishes and trypsinize cells.
354	Count cells using a cell counter or hemocytometer. Study Director may
355	determine cell viability by dye exclusion (e.g., Trypan Blue; Nigrosin). Use
356	appropriate size exclusion limits if using a Coulter counter. Determine the
357	total number of cells and document. Repeat sampling at 24 hours, 48 hours,
358	72 hours, and 96 hours post inoculation. Change culture medium at 72
359	hours or sooner in remaining dishes if indicated by pH drop.
360	d) Plot cell concentration (per mL of medium) on a log scale against time on a
361	linear scale. Determine lag time and population doubling time. Additional
362	dishes and time are needed if the entire growth curve is to be determined
363	(lag phase, log phase, plateau phase).
364	
365	D. Preparation of Test Substances
366	[Note: Preparation under red or yellow light is recommended to preserve substances
367	that degrade upon exposure to light.]
368	
369	Test substance solubility should be determined by following the procedures
370	outlined in ANNEX II of this protocol.
371	
372	1. Test Substances in Solution
373	a) Allow test substances to equilibrate to room temperature before dissolving
374	and diluting.
375	b) Prepare test substance immediately prior to use rather than preparing in bulk
376	for use in subsequent tests. Ideally, the solutions must not be cloudy nor
377	have noticeable precipitate. Each stock dilution should have at least 1-2 mL
378	total volume to ensure adequate solution for the test wells in a single 96-well
379	plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest
380	2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C)
381	for use in future substance analyses.
382	c) For substances dissolved in DMSO or ETOH, the final DMSO or ETOH
383	concentration for application to the cells must be 0.5 % (v/v) in the vehicle
384	controls and in all of the eight test concentrations.
385	d) The stock solution for each test substance should be prepared at the highest
386	concentration found to be soluble in the solubility test conducted per
387	ANNEX II: Test Method Procedure - Solubility Determination of Test
388	Substances. Thus, the highest test concentration applied to the cells in each
389	range finding experiment is:
390	4 0.5 times the highest concentration found to be soluble in the solubility
391	test, if the substance was soluble in Chemical Dilution Medium, or

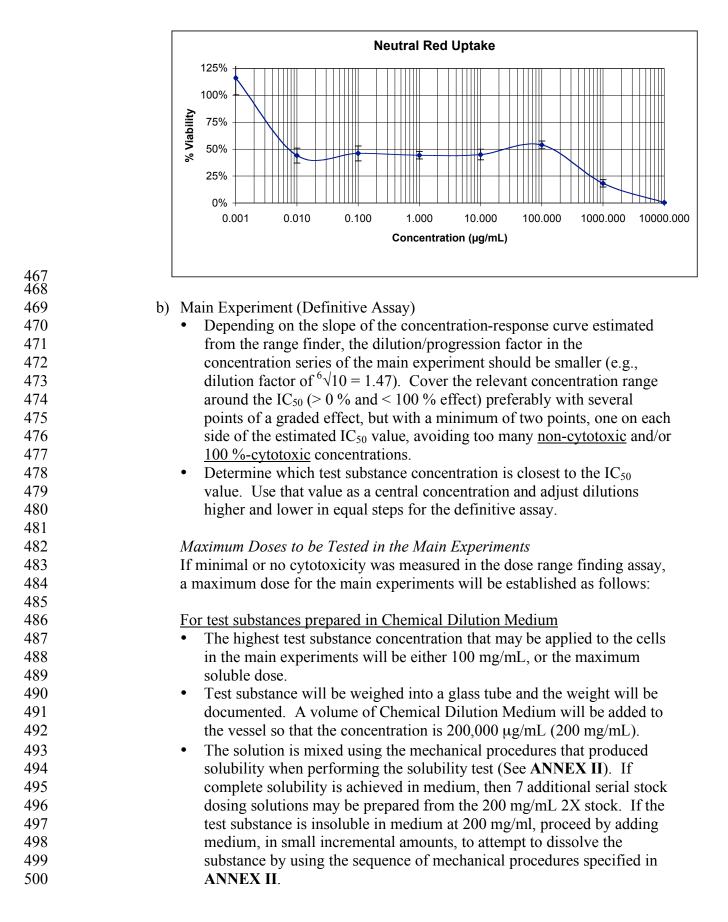
392	5 1/200 the highest concentration found to be soluble in the solubility test
393	if the substance was soluble in ETOH or DMSO.
394	e) The seven lower concentrations in the range finding experiment are
395	prepared by successive dilutions that decrease by one log unit each. The
396	following example illustrates the preparation of test substance in solvent and
397	the dilution of dissolved test substance in Chemical Dilution Medium before
398	application to 3T3 cells.
399	
400	Example: Preparation of Test Substance in Solvent Using a Log Dilution
401	<u>Scheme</u>
402	If DMSO is determined to be the preferred solvent at Tier 3 of the solubility test
403	(i.e., 200,000 μ g/mL), dissolve the substance in DMSO at 200,000 μ g/mL for
404	the chemical stock solution.
405	
406	1) Label eight tubes 1 – 8. Add 0.9 mL solvent (e.g., DMSO) to tubes 2 8.
407	2) Prepare stock solution of 200,000 µg test substance/mL solvent in tube # 1.
408	3) Add 0.1 mL of 200,000 µg/mL dilution from tube #1 to tube #2 to make a
409	1:10 dilution in solvent (i.e., $20,000 \ \mu g/mL$).
410	4) Add 0.1 mL of 20,000 μ g/mL dilution from tube #2 to tube #3 to make
411	another 1:10 dilution (i.e., 1:100 dilution from stock solution) in solvent
412	(i.e., $2,000 \ \mu g/mL$)
413	5) Continuing making serial 1:10 dilutions in the prepared solvent tubes.
414	6) Since each concentration is 200 fold greater than the concentration to be
415	tested, make a 1:100 dilution by diluting 1 part dissolved test substance in
416	each tube with 99 parts of Chemical Dilution Medium (e.g., 0.1 mL test
417	substance in DMSO + 9.9 mL Chemical Dilution Medium) to derive the
418	eight 2X concentrations for application to 3T3 cells. Each 2X test substance
419	concentration will then contain 1 % v/v solvent. The 3T3 cells will have
420	0.05 mL Routine Culture Medium in the wells prior to application of the test
421	substance. By adding 0.05 mL of the appropriate 2X test substance
422	concentration to the appropriate wells, the test substance will be diluted
423	appropriately (e.g., highest concentration in well will be 1,000 μ g/mL) in a
424	total of 0.1 mL and the solvent concentration in the wells will be $0.5\% \text{ v/v}$.
425	7) A test substance prepared in Chemical Dilution Medium, DMSO, or ETOH
426	may precipitate upon transfer into the Routine Culture Medium. The 2X
427	dosing solutions should be evaluated for precipitates and the results
428	recorded in the Study Workbook. It is permissible to test all of the dosing
429	solutions in the dose range finding assay and main experiments. However,
430	doses containing test substance precipitates should be avoided because it
431	creates doubt about the concentration of test substance exposed to the cells.
432	
433	Document all test substance preparations in the Study Workbook.
434	

2. pH of Test Substance Solutions

Prior to or immediately after application of the test substance to the 96-well plate, measure the pH of the highest 2X dosing concentration of the test substance (i.e., C1 in the test plate, see **Figure 1**) in culture medium. Use pH paper (e.g., pH 0 - 14 to estimate and pH 5 - 10 to determine more precise value; or Study Director's discretion) for measurements. The pH paper should be in contact with the solution for approximately one minute. Document the pH and note the color of the 2X concentration medium (i.e., in the Microsoft Excel **®** template; see **ANNEX 1** for an example template). Medium color for all dosing dilutions should be noted in the Study Workbook. Do not adjust the pH.

3. Concentrations of Test Substance

- a) Range Finder Experiment
 - Test eight concentrations of the test substance by diluting the stock solution using log dilutions (e.g., 1:10, 1:100, 1:1000, etc.).
 - If a range finder experiment does not generate enough cytotoxicity, then higher doses should be attempted. If cytotoxicity is limited by solubility, then more stringent solubility procedures to increase the stock concentration (to the maximum concentration specified in **Section VI.D.3.b**.) should be employed.
 - Place the test substance concentration into an incubator (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air) and stir or rock for up to 3 hours, if necessary, to facilitate dissolution. For stocks prepared in medium, vessel caps should be loose to allow for CO₂ exchange. Proceed with dosing solution preparation and dosing.
 - If a range finding test produces a biphasic curve, then the doses selected for the subsequent main experiments should cover the most toxic dose-response range (see **Example 1** the most toxic range is $0.001 0.1 \mu g/mL$) that reduces viability to 50 %.
- Example 1 Biphasic Curve



501		• Mo	re strir	ngent se	olubilit	y proc	edures	may be	e emplo	oyed if	needeo	l based	
502		on	results	from t	he rang	ge finde	er expe	riment	(Section	on VI.l	D.3.a.).	The	
503							will be						
504		-			ng solu								
505					C								
506		For tes	t substa	ances p	repare	d in eit	her DN	ASO or	ETOF	I			
507			The highest test substance concentration that may be applied to the cells										
508		in t	he mai	n expe	riments	s will b	$be \leq 2.5$	i mg/m	L or le	ss, dep	ending	upon	
509								U		· 1	C	1	
510			the maximum solubility in solvent. Weigh the test substance into a glass tube and document the weight.										
511			-				-					ility tes	st)
512			-				ntratior			-		-	<i>.</i>
513									•	-		pecifie	t
514					•	-	lubility			1	-		
515												rom the	2
516							e test s		2		-		
517			-									ounts, t	0
518			-		•	-	-					mixin	
519							ole stoc						<u> </u>
520		the	seven	additio	nal ser	ial stoc	ck dosi	ng solu	tions.		-	-	
521								C					
522		If preci	pitates	are ob	served	in the	2X dil	utions,	contin	ue with	the		
523		experir	nent ar	nd mak	e the a	ppropr	iate obs	servatio	ons and	l docur	nentati	on.	
524		-			-								
525	c)	Test Su	ıbstanc	e Dilut	tions								
526		The do	sing fa	ctor of	3.16 (=	$= 2\sqrt{10}$) divide	es a log	; into tv	vo equ	idistan	t steps,	
527		2.15 (=							o six st	eps, 1.'	78 (⁴ √1	0) into	
528		four ste	eps, and	d 1.21	$(= 12\sqrt{1})$	0) into	12 ste	ps.					
529													
530	EXAN	MPLE:											
531								-	-		-		
	10						31.6						100
	10				21.5				46.4				100
	10		14.7		21.5		31.6		46.4		68.1		100
	10	12.1	14.7	17.8	21.5	26.1	31.6	38.3	46.4	56.2	68.1	82.5	100
532													
533		An exa	mple o	of decir	nal geo	metric	conce	ntratio	n series	for fa	ctor 1.4	7:	
534		Dilute	-		•								
535		diluent											7

535 536

- 537
- 538

volumes of diluent...(etc.).

538 539 540 541 542 543 544		as sl	Well P 3T3 N hown i	late C IRU as n Figu	ssay for ire 1.	r test s te Con	ubstan	tion fo	or Posi				figuratio and Test	
545		1	2	3	4	5	6	7	8	9	10	11	12	
	А	VCb	VCb	C ₁ b	C ₂ b	C ₃ b	C ₄ b	C ₅ b	C ₆ b	C ₇ b	C ₈ b	VCb	VCb	
	В	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	С	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	D	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	Е	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	F	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	G	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	Н	VCb	VCb	C ₁ b	C ₂ b	C ₃ b	C ₄ b	C ₅ b	C ₆ b	C ₇ b	C ₈ b	VCb	VCb	
546 547 548 549 550 551 552 553	VC1 and VC2 = VEHICLE CONTROL C ₁ - C ₈ = Test Substances or PC (SLS) at eight concentrations (C1 = highest, C8 = lowest) b = BLANKS (Test substance or PC, but contain no cells) VCb = VEHICLE CONTROL BLANK (contain no cells) 2. Application of Test Substance													
554 555 556 557 558 559 560 561 562 563 564 565 566 566 567		9	96-wel 1) Ad Co res 8-c 2) Us hol (wi dis app test	l plate d each rning/(ervoirs hanne e a "du d the c th cell pensec blied to	s may of the Costar s; or Cu l; or ot ummy" dosing (s). Th l into t o the pl (i.e., g	be util 2X do model orning/ her mu plate solutione test solutione test solutione he dun late con	ized. sing so 4870 s (Transt iltichan (i.e., an ons imp substar hmy pl ntainin	blution sterile car moo nel re n empt nediate ace and ate in t g cells	is into polyst del 487 servoin y steri ely pri l contro the san s. Mor	labelec yrene 5 78 disp rs). le 96-v or to tr ol dosi ne patt e volu	l, steril 50 mL osable vell pla eatmen ng solu ern/orc me that	le reserver reagent reserver nte) pre nt of th utions s ler as v	pared to e test pla hould be vill be ed for the	g., s, ite

568		
569		At the time of treatment initiation, a multi-channel micropipettor is used to
570		transfer the 2X dosing solutions, from the reservoirs or dummy plate, to the
571		appropriate wells on the treatment plate (as described in step c. below).
572		These methods will ensure that the dosing solutions can be transferred
573		rapidly to the appropriate wells of the test plate to initiate treatment times
574		and to minimize the range of treatment initiation times across a large
575		number of treatment plates, and to prevent "out of order" dosing. Do not
576		use a multichannel repeater pipette for dispensing test substance to the
577		plates.
578		7.0 After 24 hours \pm 2 hours incubation of the cells, remove Routine Culture
579		Medium from the cells by careful inversion of the plate (i.e., "dump") over
580		an appropriate receptacle. Gently blot the plate on a sterile paper towel so
581		that the monolayer is minimally disrupted. Do not use automatic plate
582		washers for this procedure nor vacuum aspiration.
582		8.0 Immediately add 50 μ L of fresh pre-warmed Routine Culture Medium to all
584		of the wells, including the blanks. Fifty microliters (50 μ L) of dosing
585		solution will be rapidly transferred from the 8-channel reservoir (or dummy
586		plate) to the appropriate wells of the test plate using a single delivery multi-
587		channel pipettor. For example, the VC may be transferred first (into
588		columns 1, 2, 11, and 12), followed by the test substance dosing solutions
589		from lowest to highest dose, so that the same pipette tips on the multi-
590		channel pipettor can be used for the whole plate. [The Vehicle Control
591		blank (VCb) wells (column 1, column 12, wells A2, A11, H2, H11) will
592		receive the Vehicle Control dosing solutions (which should include any
593		solvents used)]. Blanks for wells $A3 - A10$ and $H3 - H10$ shall receive the
594		appropriate test substance solutions for each concentration (e.g., wells A3
595		and H3 receive C_1 solution).
596		9.0 Incubate cells for 48 hours \pm 0.5 hours (37°C \pm 1°C, 90 % \pm 10 % humidity,
597		and $5.0 \% \pm 1 \%$ CO ₂ /air).
598		10.0 Positive Control : For each set of test substance plates used in an
599		assay, prepare a separate plate of positive control concentrations. If multiple
600		sets of test substance plates are set up, then clearly designate the positive
601		control plates for each set; each set will be an individual entity. The Study
602		Director will decide how many test substance plates will be run with a
603		positive control plate. This plate will follow the same schedule and
604		procedures as used for the test substance plates (including appropriate test
605		substance concentrations in the appropriate wells and meeting test
606		acceptance criteria – see sections VI.E.1, E.2, and E.5).
607		
608	3.	Microscopic Evaluation
609		After at least 46 hours of treatment, examine each plate under a phase contrast
610		microscope to identify systematic cell seeding errors and growth characteristics
611		of control and treated cells. Record any changes in morphology of the cells due
612		to the cytotoxic effects of the test substance, but <u>do not use these records for any</u>
613		<u>quantitative measure of cytotoxicity</u> . Undesirable growth characteristics of

614control cells may indicate experimental error and may be cause for rejection of615the assay. Use the following Visual Observations Codes in the description of616cell culture conditions. Numerical scoring of the cells (see Section VI.E.3)617should be determined and documented in the Study Workbook and in the618appropriate section of the Microsoft Excel® template.

Note Code	Note Text
1	Normal Cell Morphology
2	Low Level of Cell Toxicity
3	Moderate Level of Cell Toxicity
4	High level of Cell Toxicity
1P	Normal Cell Morphology with Precipitate
2P	Low Level of Cell Toxicity with Precipitate
3P	Moderate Level of Cell Toxicity with Precipitate
4P	High level of Cell Toxicity with Precipitate
5P	Unable to View Cells Due to Precipitate

Visual Observations Codes

4. Measurement of NRU

- a) Carefully remove (i.e., "dump") the medium with test substance and rinse the cells very carefully with 250 μL pre-warmed D-PBS. Remove the rinsing solution by dumping and remove excess by gently blotting on paper towels. Add 250 μL NR medium (to all wells including the blanks) and incubate (37°C ± 1°C, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO₂/air) for 3 hours ± 0.1 hour. Observe the cells briefly during the NR incubation (e.g., between 2 and 3 hours Study Director's discretion) for NR crystal formation. Record observations in the Study Workbook. Study Director can decide to reject the experiment if excessive NR crystallization has occurred.
 b) After incubation, remove the NR medium, and carefully rinse cells with 250
- µl pre-warmed D-PBS.
 - c) Decant and blot D-PBS from the plate.
- d) Add exactly 100 μl NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.
- e) Shake microtiter plate rapidly on a microtiter plate shaker for 20 45 minutes to extract NR from the cells and form a homogeneous solution. Plates should be protected from light by using a cover during shaking.
- f) Plates should be still for at least five minutes after removal from the plate shaker (or orbital mixer). If any bubbles are observed, assure that they have been ruptured prior to reading the plate. Measure the absorption (within 60 minutes of adding NR Desorb solution) of the resulting colored solution at 540 nm ± 10 nm in a microtiter plate reader (spectrophotometer), using the blanks as a reference.

648 649 650 651 652	[Note: A mean $OD_{540 \pm 10nm}$ of 0.031 - 0.065 for the VC blanks is a target range of ODs but not a test acceptance criterion (range = mean OD \pm 2.5 standard deviations; mean = 0.048; SD = 0.007; N = 233).] Save raw data in the Microsoft Excel® template.
653	5. Quality Check of 3T3 NRU Assay
654 655 656 657 658 659 660 661	 a) Test Acceptance Criteria All acceptance criteria (i.e., criteria 1, 2, and 3) must be met for a test to be acceptable. 1) The PC (SLS) IC₅₀ must be within ± two and a half (2.5) standard deviations (SD) of the historical mean established by the Test Facility, and must meet criteria 2 and 3, and must have an r² (coefficient of determination) value calculated for the Hill model fit (i.e., from
662	PRISM® software) ≥ 0.85 . NICEATM/ECVAM study generated the
663 664 665	 following PC data: IC₅₀ mean = 41.5 μg/mL; SD = 4.8 (n = 233) range for IC₅₀ mean ± 2.5 SD = 29.5 μg/mL - 53.5 μg/mL
666 667	2) The left and right mean of the VCs do not differ by more than 15% from the mean of all VCs.
668 669 670	 At least one calculated cytotoxicity value > 0 % and ≤ 50 % viability and at least one calculated cytotoxicity value > 50 % and < 100 % viability must be present.
671 672 673 674	<i>Exception:</i> If a test has only one point between 0 and 100 % and the smallest dilution factor (i.e., 1.21) was used and all other test acceptance criteria were met, then the test will be considered acceptable.
675 676 677 678 679	Stopping Rule for Insoluble Substances : If the most rigorous solubility procedures have been performed and the assay cannot achieve adequate toxicity to meet the test acceptance criteria after three definitive trials, then the Study Director may end all testing for that particular substance.
680 681 682 683 684	[Note: A corrected mean $OD_{540 \pm 10nm}$ of 0.183 - 0.769 for the VCs is a target range of ODs but not a test acceptance criterion (range = mean OD \pm 2.5 standard deviations; mean = 0.476; SD = 0.117; N = 233).]
685 686 687 688 689 690 691 692	 b) Checks for Systematic Cell Seeding Errors To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row 2) and the right side (row 11 for the test plates) of the 96- well plate. Aberrations in the cell monolayer for the VCs may reflect a volatile and toxic test substance present in the assay. If volatility is suspected, then proceed to Section VI.E.6. Checks for cell seeding errors may also be performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent.

693	
693 694	C Testing Veletile Substances
695	6. Testing Volatile Substances
	Although this test method is not suitable for highly volatile substances, mildly
696	volatile substances may be tested with some success. Volatile test substances
697	may generate vapors from the treatment medium during the test substance
698	treatment incubation period. These vapors may become resorbed into the
699	treatment medium in adjacent wells, such that culture wells nearest the highest
700	doses may become contaminated by exposure. If the test substance is
701	particularly toxic at the doses tested, the cross contamination may be evident as
702	a significant reduction in viability in the VC cultures (i.e., VC1) adjacent to the
703	highest test substance doses.
704	
705	If potential test substance volatility is suspected (e.g., for low density liquids) or
706	if the initial range finder test (non-sealed plate) results show evidence of toxic
707	effects in the control cultures (i.e., > 15 % difference in viability between VC1
708	[column 2] and VC2 [column 11]), then seal the subsequent test plates using the
709	following procedure.
710	Tono wing procodure.
711	a) Plates and substances will be prepared as usual according to Sections
712	VI.D and VI.E.
712	b) Immediately after the 96-well culture plate has been treated with the
713	suspected volatile substance (Section VI.E.2.b), apply the adhesive
714	
	plate sealer (e.g., using a hand, microplate roller, etc.) directly over the
716	culture wells. Assure that the sealer adheres to each culture well (well
717	tops should be dry). Place the 96-well plate cover over the sealed plate
718	and incubate the plate under specified conditions (Section VI.E.2.b).
719	[Note: Do not jam the plate lid over the film to avoid deforming the
720	sealer and causing the sealer to detach from culture wells. Loose fit of
721	the plate lid is acceptable.]
722	c) At the end of the treatment period, the plate sealer should be carefully
723	removed to avoid spillage. Continue with the NRU assay as per Section
724	VI.E.4 .
725	
726	F. Data Analysis
727	• The Study Director will use good biological/scientific judgment for determining
728	"unusable" wells that will be excluded from the data analysis and provide
729	explanations for the removal of any data from the analysis.
730	• A calculation of cell viability expressed as NRU is made for each concentration
731	of the test substance by using the mean NRU of the six replicate values
732	(minimum of four acceptable replicate well) per test concentration (blanks will
733	be subtracted). This value is compared with the mean NRU of all VC values.
734	Relative cell viability is then expressed as percent of untreated VC. If
735	achievable, the eight concentrations of each substance tested will span the range
736	of no effect up to total inhibition of cell viability.
737	• Data from the microtiter plate reader should be transferred to a spreadsheet
738	template (e.g., Microsoft Excel®) that will automatically determine cell

viability, calculate IC₅₀ values by linear interpolation, and perform statistical analyses (including statistical identification of outliers) (see **ANNEX 1** for an example spreadsheet template).

- A Hill function analysis should be performed using statistical software (e.g., GraphPad PRISM® 3.0) and a template to calculate IC₂₀, IC₅₀, and IC₈₀ values (and the associated confidence limits) for each test substance.
- 745• Dose-responses for which the toxicity plateaus as concentration increases do not746fit the Hill function well when Bottom =0. To obtain a better model fit,747unconstrain the Bottom parameter so that the model calculates the Bottom748value. However, when Bottom $\neq 0$, the EC₅₀ reported by the Hill function \neq 74950% viability since the Hill function defines EC₅₀ as the point midway between750Top and Bottom. To obtain the appropriate IC₅₀ when Bottom $\neq 0$, use the751following rearranged Hill function:

$$X = \log EC_{50} - \frac{\log \left(\frac{Top - Bottom}{Y - Bottom} - 1\right)}{HillSlope}$$

753 754

755

756

757

758 759

760

761

763

767

773

778

752

739

740

741

- X is the logarithm of concentration at 50% response, $logEC_{50}$ is logarithm of concentration at the response midway between Top and Bottom, Top is the maximum response, Bottom is the minimum response, Y = 50 (i.e., 50% response), and HillSlope describes the steepness of the curve.
 - [Note: IC₅₀ values are used in a regression formula to predict the LD₅₀ value of a test substance as an estimate of the starting dose for an acute oral toxicity test.]

762 VII. REFERENCES

Hackenberg, U. and H. Bartling. 1959. Messen und Rechnen im pharmakologischen
Laboratorium mit einem speziellen Zahlensystem (WL24-System). Arch. Exp. Pathol.
Pharmakol. 235: 437-463.

Spielmann, H., S. Gerner, S. Kalweit, R. Moog, T. Wirnserberger, K. Krauser, R.
Kreiling, H. Kreuzer, N.P. Luepke, H.G. Miltenburger, N. Müller, P. Murmann, W.
Pape, B. Siegmund, J. Spengler, W. Steiling, and F.J. Wiebel. 1991. Interlaboratory
assessment of alternatives to the Draize eye irritation test in Germany. Toxicol. *In Vitro* 5: 539-542.

- *Test Method Protocol for Solubility Determination. In Vitro* Cytotoxicity Validation
 Study. Phase III. August 29, 2003. Prepared by The National Toxicology Program
 (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods
 (NICEATM). (http://iccvam.niehs.nih.gov/methods/invitro.htm)
- U. S. Environmental Protection Agency. 1996. Product Properties Test Guidelines.
 OPPTS 803.7840. Water Solubility: Column Elution Method; Shake Flask Method.
 EPA712-C-96-041, Prevention, Pesticides and Toxic Substances, Washington DC.

782	
783	Test Method Protocol for the 3T3 Neutral Red Uptake Cytotoxicity Test. A
784	Test for Basal Cytotoxicity for an In Vitro Validation Study. November 4,
785	2004. Prepared by the National Toxicology Program (NTP) Interagency
786	Center for the Evaluation of Alternative Toxicological Methods (NICEATM).
787	(http://iccvam.niehs.nih.gov/methods/invitro.htm)
788	

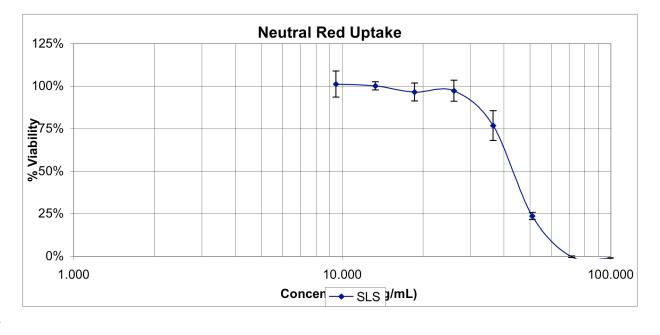
789

790

ANNEX 1 Microsoft EXCEL® Example Spreadsheet Template

	st Facility :						/ Number.:					
	cal Code :						I Plate ID :					
2nd Che	m. Code*:	11				Expe	riment ID :	XX				
					00.14/5							
						LL PLAT						
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
В	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
С	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
D	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
E	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
 F	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
G	Blank	VC1 VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2 VC2	Blank
Н	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
				RAW	ABSOR	BANCE D	DATA (OD350)				
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.044	0.044	0.045	0.045	0.045	0.046	0.051	0.057	0.057	0.043	0.041	0.044
В	0.042	0.456	0.043	0.043	0.130	0.300	0.395	0.414	0.418	0.402	0.401	0.042
С	0.043	0.407	0.042	0.041	0.130	0.294	0.383	0.382	0.413	0.375	0.385	0.044
D	0.043	0.438	0.042	0.043	0.147	0.337	0.409	0.404	0.438	0.436	0.391	0.047
E	0.044	0.448	0.041	0.045	0.132	0.321	0.429	0.414	0.416	0.420	0.441	0.042
F	0.045	0.411	0.040	0.042	0.127	0.375	0.397	0.402	0.422	0.447	0.403	0.043
G	0.041	0.405	0.043	0.040	0.124	0.361	0.444	0.442	0.425	0.448	0.405	0.044
Н	0.041	0.041	0.048	0.042	0.042	0.044	0.042	0.042	0.040	0.044	0.041	0.041
Max	0.045	0.456	0.043	0.045	0.147	0.375	0.444	0.442	0.438	0.448	0.441	0.047
Min	0.041	0.405	0.040	0.040	0.124	0.294	0.383	0.382	0.413	0.375	0.385	0.041
Next Max	0.044	0.448	0.042	0.043	0.132	0.361	0.429	0.414	0.425	0.447	0.405	0.044
Next Min	0.042	0.407	0.041	0.041	0.102	0.300	0.395	0.402	0.416	0.402	0.391	0.042
Rmax	-0.250	-0.157	-0.333	-0.400	-0.652	-0.173	-0.246	-0.467	-0.520	-0.014	-0.643	-0.500
	0.250	0.039				0.173	0.240	0.333	0.320	0.370		
Rmin	0.250	0.039	0.333	0.200	0.130	0.074	0.197	0.333	0.120	0.370	0.107	0.167
		COF	RECTER	D ABSO	RBANCI	E (Samp	le OD550	- Mean	Blank O	D550)		
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.001	-0.002	0.002	0.002	0.001	0.005	0.008	0.009	-0.001	-0.002	0.001
В	-0.001	0.413	-0.004	-0.001	0.087	0.255	0.349	0.365	0.370	0.359	0.358	-0.001
С	0.000	0.364	-0.005	-0.003	0.087	0.249	0.337	0.333	0.365	0.332	0.342	0.001
D	0.000	0.395	-0.005	-0.001	0.104	0.292	0.363	0.355	0.390	0.393	0.348	0.004
E	0.001	0.405	-0.006	0.002	0.089	0.276	0.383	0.365	0.368	0.377	0.398	-0.001
F	0.002	0.368	-0.007	-0.001	0.084	0.330	0.351	0.353	0.374	0.404	0.360	0.000
G	-0.002	0.362	-0.004	-0.004	0.081	0.316	0.398	0.393	0.377	0.405	0.362	0.001
H	-0.002	-0.002	0.002	-0.001	-0.001	-0.001	-0.005	-0.008	-0.009	0.001	-0.002	-0.002
Mean Blank =	0.043		0.047	0.044	0.044	0.045	0.047	0.050	0.049	0.044		
Bank -	0.040		0.047	0.044	0.044	0.040	0.047	0.000	0.043	0.044		
					IABILITY			E CONT				
	1	2	3	4	5	(70 OF			9	10	11	12
	1	2	3	4	Ð	Ø	1	Ø	Э	10	11	12
A B	r	110.7%	-0.9%	-0.1%	23.2%	68.4%	93.4%	97.7%	99.0%	96.1%	96.0%	
C B		97.6%	-0.9%	-0.1%	23.2%	66.7%	93.4%	<u>97.7%</u> 89.1%	99.0%	88.9%	90.0%	
D		105.9%	-1.2%	-0.1%	27.7%	78.3%	90.2%	95.0%	104.4%	105.2%	93.3%	
E		103.5%	-1.5%	0.4%	23.7%	74.0%	102.5%	97.7%	98.5%	100.9%	106.7%	
F		98.7%	-1.7%	-0.4%	22.4%	88.5%	94.0%	94.5%	100.1%	108.2%	96.5%	
G		97.1%	-0.9%	-0.9%	21.6%	84.7%	106.5%	105.2%	100.9%	108.4%	97.1%	
Н												

	st Facility :						y Number.					
Chemi	ical Code :	SLS				96-We	II Plate ID	: A11				
2nd Che	m. Code*:	11				Expe	riment ID	XX				
		VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	
Conc	. (µg/mL) :	0.0	100	71.4	51.0	36.4	26.0	18.6	13.3	9.49	0.0	
Mean	Corr. OD :	0.385	-0.005	-0.001	0.088	0.286	0.363	0.360	0.374	0.378	0.361	
	SD :	0.023	0.001	0.002	0.008	0.033	0.023	0.020	0.009	0.029	0.020	
Mean Vehi	cle Control :	0.373										
Me	an Blank :	0.043										
% of Vehi	icle Control :	103.1%	-1.3%	-0.3%	23.6%	76.8%	97.3%	96.5%	100.1%	101.3%	96.9%	
	SD :	6.0%	0.3%	0.5%	2.1%	8.7%	6.2%	5.3%	2.4%	7.7%	5.2%	
	% CV :	5.86%	-25.1%	-150%	9.09%	11.4%	6.33%	5.47%	2.39%	7.59%	5.41%	
Mean VC	- VC1 (%) :	-3.1%										
Mean VC	- VC2 (%) :	3.1%										
Mean At	osolute OD :	0.416										
					Visu	al Observa	tions					
		VC	C1	C2	C3	C4	C5	C6	C7	C8		
ENTER	R CODES:	1	4	4	3	2	1	1	1	1		
						4.00						
			Ir	nterpolat	ed IC ₅₀ :	4.32	=+01	µg/mL				



- 796

- 802

TEST CHI									
	Test Facility :	Α		Stu	udy Number.:	A1			
Che	emical Code :	SLS		96-V	Vell Plate ID :	A11			
2 nd C	Chem. Code*:	11		Ex	periment ID :	XX			
* Testing F	Facility Acces	sion Code, if a	pplicable						
PREPAR	ATION OF TE	ST CHEMICA	L						
			Solvent:	Medium			[Dilution factor:	
Solvent Co	onc. (%, v/v) i	in dosing soluti	ons :	N/A		Highest	Stock Conc.:	20,000	µg/mL
Aids used	to dissolve :	Vorte	exing	so	nication	h	eating to 37C		
	pH (highest	medium stock	or 2X dosir	ng solution) :	8.0				
	Medium Cla	rity/Color (high	iest 2X dosi	ng solution):	clear red		lf ppt, note	lowest conc.:	
					tration Series	(µg/mL)			
	C1	C2	C3	C4	C5	C6	C7	C8	
	100	71.4	51.0	36.4	26.0	18.6	13.3	9.49	
		Control (SLS)							
CELL LIN		<u> </u>							
		BALB/c 3T3		Supplier:	ATCC		Lot No.	not provided	
ſ	Passage No.:	69			No. in Assay:	75		erating/frozen	24-May-
				1 doodgo	no. mr toouy.	10	110	oradingriozoni	21 1103
	Medium:			Supplier:			Lot No.:		
	Serum:			Supplier:		Lot No.:			
ç	Serum Conc.:		Grov	wth Medium:	10%	Treatm	ent Medium:	0%	
			Grou		1070	rioutin			
		% and <100%:	3	No. c	of values >0%	and <50%	1	Accept?	YES
110.0					een Col 2 and			Accept?	YES
		PC: Hill Fi		alue of SLS:			-070	Accept?	YES
		10.111110		IC50 of SLS:	43.2	µg/mL		Accept?	YES
TIMELINE	-		F 0.	1050 01 323.	43.2	µg/m∟		Accept	TL3
		Seeding Date		Dose Apr	olication Date			mination Date	
		Security Date		<u>0036 A</u>			<u>OD550 Deter</u>		
TEST RES									
		corrected OD ₅₅₀ :	0.373				Hill Eur	nction R ² Value:	0.9869
	log IC20 :			log IC50 :	1.635E+00	ua/ml	log IC80 :	1.718E+00	
	IC20 :			IC50 :	4.32E+00		IC80 :	5.22E+01	
	1020.	3.30ETUT	µу/ш∟	1000.	4.32ETUI	µg/111∟	1000.	J.22ETUI	µg/m∟
			Test Ch	emical E W ·	288.4				
	10.00	0 10001100		emical F.W. :			1000	0.18113599	
	IC20 :	0.12331183		IC50 :	0.1496252		IC80 :	0.10113399	111111

809	ANNEX II
810	
811	TEST METHOD PROCEDURE
812	Solubility Determination of Test Substances
813	
814	BDODOG A L
815	PROPOSAL
816	This procedure was designed to identify the solvent that would provide the highest soluble
817	concentration of a test substance so there would be uniform availability of the substance to
818 819	cells used for <i>in vitro</i> basal cytotoxicity testing. The solubility exercises can be performed in
	a routine and repeatable manner and provide guidelines to effectively prepare test substances
820 821	for toxicity testing in the NRU test methods.
821	TEST SYSTEM
822	The solubility test procedure is based on attempting to dissolve substances in various
823	solvents with increasingly rigorous mechanical techniques. The solvents to be used, in the
825	order of preference, are cell culture medium, DMSO, and ETOH. Determination of whether
825	a test substance has dissolved is based entirely on visual observation for the purposes of this
827	protocol. A test substance has dissolved if the solution is clear and shows no signs of
828	cloudiness or precipitation.
829	
830	PROCEDURES
831	Preparation of the 3T3 medium will follow all procedures in the 3T3 NRU protocol.
832	
833	Materials – see Section VI.A
834	
835	Preparations of Media and Solutions – see Section VI.B
836	All solutions glassware, pipettes, etc., should be sterile and all procedures should be carried
837	out under aseptic conditions and in the sterile environment of a laminar flow cabinet
838	(biological hazard standard). All methods and procedures should be adequately documented.
839	
840	Determination of Solubility
841	• Solubility should be determined in a step-wise procedure that involves attempting to
842	dissolve a test substance at a relatively high concentration with the sequence of
843	mechanical procedures specified in Mechanical Procedures. Table 1 and Figures 1 and
844	2 illustrate the step-wise procedures. The hierarchy of preference of solvent for
845	dissolving test substances is medium, DMSO, and then ETOH. If the substance does not
846	dissolve in the solvent, the volume of solvent is increased so as to decrease the test
847	substance concentration by a factor of 10, and then the sequence of mechanical
848	procedures are repeated in an attempt to solubilize the substance at the lower
849	concentrations.
850	• For testing solubility in medium, the starting concentration is $200,000 \mu g/ml$ (i.e., 200
851	mg/mL) in Tier 1, but for DMSO and ETOH the starting concentration is 200,000 μ g/ml

852 (i.e., 200 mg/mL) in Tier 3.

853 854	М	tha	da
854 855		tho Ti	
856	A.		er 1 begins with testing 200 mg/mL in Chemical Dilution Medium (see Table 1). Weigh approximately 100 mg (100,000 μ g) of the test substance into a glass tube.
857			Document the test substance weight.
858		2.	Add approximately 0.5 mL of medium into the tube so that the concentration is
859			200,000 µg/ml (200 mg/mL).
860		3.	Mix the solution as specified in Mechanical Procedures . If complete solubility is
861			achieved, then additional solubility procedures are not needed.
862			
863	B.	If t	the test substance is insoluble in Tier 1 at 200 mg/mL, then proceed to Tier 2.
864			Weigh approximately 10 mg (10,000 μ g) of the test substance into a glass tube.
865			Document the substance weight.
866		2	Add approximately 0.5 mL of medium into the tube so that the concentration is
867			$20,000 \ \mu g/ml$ (20 mg/mL).
868		3	Mix the solution as specified in Mechanical Procedures . If complete solubility is
869		5.	achieved, then additional solubility procedures are not needed.
870			demoved, then additional solution procedures are not needed.
871	C.	If t	the test substance is insoluble in Chemical Dilution Medium, proceed to Tier 3.
872		1.	Add enough medium, approximately 4.5 mL, to attempt to dissolve the substance at 2
873			mg/mL by using the sequence of mixing procedures. If the test substance dissolves in
874			medium at 2 mg/mL, no further procedures are necessary.
875		2.	If the test substance does NOT dissolve in medium, weigh out approximately 100 mg
876			test substance in a second glass tube and add enough DMSO to make the total volume
877			approximately 0.5 mL (for 200 mg/mL) and mix the solution as specified in
878			Mechanical Procedures.
879		3.	If the test substance does not dissolve in DMSO, weigh out approximately 100 mg
880			test substance in another glass tube and add enough ETOH to make the total volume
881			approximately 0.5 mL (for 200 mg/mL) and mix the solution as specified in
882			Mechanical Procedures.
883		4	If the substance is soluble in either solvent, no additional solubility procedures are
884			needed.
885			
886	D.	If t	the substance is NOT soluble in Chemical Dilution Medium, DMSO, or ETOH at Tier
887		3, 1	then continue to Tier 4 in Table 1 .
888		1.	Add enough solvent to increase the volume of the three (or four) Tier 2 solutions by
889			10 and attempt to solubilize again using the sequence of mixing procedures. If the
890			test substance dissolves, no additional solubility procedures are necessary.
891		2.	If the test substance does NOT dissolve, continue with Tier 5 and, if necessary, Tier 6
892			using DMSO and ETOH.
893		3.	Tier 5 begins by diluting the Tier 4 samples with DMSO or ETOH to bring the total
894			volume to 50 mL. The mixing procedures are again followed to attempt to solubilize
895			the substance.

- 896
 897
 897
 898
 4. Tier 6 is performed, if necessary, by weighing out another two samples of test substance at ~10 mg each and adding ~50 mL DMSO or ETOH for a 200 μg/mL solution, and following the mixing procedures.
- 899

900 <u>Example</u>

- 901 If complete solubility is not achieved at 20,000 μg/mL in Chemical Dilution Medium at
 902 Tier 2 using the mixing procedures, then the procedure continues to Tier 3 by diluting the
 903 solution to 5 mL with medium and mixing again.
- If the substance is not soluble in Chemical Dilution Medium, two samples of ~ 100 mg test substance are weighed to attempt to solubilize in DMSO and ETOH at 200,000 µg/mL (i.e., 200 mg/mL). Solutions are mixed following the sequence of procedures prescribed in Mechanical Procedures in an attempt to dissolve.
- If solubility is not achieved at Tier 3, then the solutions prepared in Tier 3 are diluted by
 10 so as to test 200 μg/mL in media, and 20,000 μg/mL in DMSO and ETOH. This
- advances the procedure to Tier 4. Solutions are again mixed in an attempt to dissolve.
- If solubility is not achieved in Tier 4, the procedure continues to Tier 5, and to Tier 6 if
 necessary (see Figures 1 and 2 and Table 1).

914 MECHANICAL PROCEDURES

- A. The following hierarchy of mixing procedures will be followed to dissolve the test substance:
- 917
- 9181. Add test substance to solvent as in Tier 1 of Table 1. (Test substance and solvent919should be at room temperature.)
- 920 2. Gently mix at room temperature. Vortex the tube (1 2 minutes).
- 921 3. If test substance has not dissolved, use waterbath sonication for up to 5 minutes.
- 922
 923
 924
 925
 925
 925
 926
 927
 928
 929
 929
 929
 920
 920
 920
 921
 921
 922
 922
 923
 924
 924
 925
 925
 925
 926
 927
 928
 929
 929
 929
 920
 920
 920
 920
 921
 921
 922
 923
 924
 925
 924
 925
 925
 925
 926
 927
 928
 929
 929
 929
 920
 920
 920
 921
 921
 921
 922
 922
 923
 924
 925
 924
 925
 925
 925
 926
 927
 928
 929
 929
 929
 920
 920
 920
 921
 921
 922
 922
 923
 924
 924
 925
 925
 924
 925
 925
 925
 925
 925
 926
 927
 928
 928
 929
 929
 929
 920
 920
 920
 921
 921
 921
 922
 922
 924
 925
 925
 925
 925
 925
 925
 926
 926
 927
 928
 928
 928
 928
 929
 929
 929
 929
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
 920
- 5. Proceed to Tier 2 (and Tiers 3-6, if necessary of **Table 1** and repeat procedures 2-4).
- B. The preference of solvent for dissolving test substances is Chemical Dilution Medium,
 DMSO, and then ETOH. Thus, if all solvents for a particular tier are tested
 simultaneously and a test substance dissolves in more than one solvent, then the choice of
 solvent follows this hierarchy. For example, if, at any tier, a substance were soluble in
- 932 Chemical Dilution Medium and DMSO, the choice of solvent would be medium. If the
 933 substance were insoluble in medium, but soluble in DMSO and ETOH, the choice of
 934 solvent would be DMSO.

935

927

936 Table 1 Determination of Solubility in Chemical Dilution Medium, DMSO, or

937

ETOH

938

Tier	1	2	3	4	5	6
Total Volume Chemical Dilution Medium	0.5 mL	0.5 mL	5 mL	50 mL		
Concentration of Test Substance <u>Tier 1</u> : Add ~ 100 mg to a tube. Add enough medium to equal Tier 1 volume. If insoluble, go to Tier 2. <u>Tier 2</u> : Add ~10 mg to another tube. Add enough medium to equal the first volume. Dilute to subsequent volumes if necessary.	200,000 μg/mL (200 mg/mL)	20,000 μg/mL (20 mg/mL)	2,000 μg/mL (2 mg/mL)	200 μg/mL (0.20 mg/mL)		
Total Volume DMSO/ETOH			0.5 mL	5 mL	50 mL	
Concentration of Test Substance (Add ~100 mg to a large tube. Add enough DMSO or ETOH to equal the first volume. Dilute with subsequent volumes if necessary.)			200,000 μg/mL (200 mg/mL)	20,000 μg/mL (20 mg/mL)	2,000 μg/mL (2 mg/mL)	
Total Volume DMSO/ETOH						50 mL
Concentration of Test Substance (Add ~10 mg to a large tube. Add enough DMSO or ETOH						200 μg/mL (0.2 mg/mL)
to equal 50 mL.) EQUIVALENT	100,000 μg/mL	10,000 μg/mL	1000 μg/mL	100 μg/mL	10 µg/mL	1 μg/mL
CONCENTRATION ON CELLS	(100 mg/mL)	(10 mg/mL)	(1 mg/mL)	(0.1 mg/mL)	(0.01 mg/mL)	(0.001 mg/mL)

939

940 [NOTE: The amounts of test substance weighed and Chemical Dilution Medium added may

941 be modified from the amounts given above, provided that the targeted concentrations

942 specified for each tier are tested.]

FIGURE 1 SOLUBILITY STEP-WISE (TIERED) PROCEDURE

	TIER 1
STEP 1:	200 mg/mL test substance (TS) in 0.5 mL Chemical Dilution Medium
	• if TS soluble in medium, then <u>STOP</u> .
	• if TS insoluble in medium, then go to STEP 2.
	TIER 2
STEP 2:	20 mg/mL TS in 0.5 mL Chemical Dilution Medium
	• if TS soluble, then <u>STOP</u> .
	• if TS insoluble, then go to STEP 3.
	TIER 3
STEP 3:	200 mg/mL TS in DMSO
	4 if TS soluble, then <u>STOP</u> .
	5 if TS insoluble, test at 200 mg/mL in ETOH.
	6 if TS soluble, then <u>STOP.</u>
	7 If TS insoluble, go to STEP 4.
	TIER 4
STEP 4:	0.2 mg/mL TS in medium (one or both) – increase volume from STEP 2 by 10 (i.e., to 50 mL)
	• if TS soluble in both media, then <u>STOP</u> .
	• if TS insoluble in one medium, test at 20 mg/mL in DMSO – increase volume from STEP 3
	by 10 (i.e., to 5 mL).
	• if TS soluble, then <u>STOP.</u>
	• if TS insoluble, test at 20 mg/mL in ETOH – increase volume from STEP 3 by 10 (i.e., to
	5 mL).
	• if TS soluble, then <u>STOP.</u>
	• if TS insoluble, then go to STEP 5.
	TIER 5
STEP 5:	2 mg/mL TS in DMSO – increase volume from STEP 4 by 10 (i.e., to 50 mL)
	• if TS soluble, then <u>STOP.</u>
	• if TS insoluble, test at 2 mg/mL in ETOH – increase volume from STEP 4 by 10 (i.e., to 50
	mL).
	• if TS soluble, then <u>STOP.</u>
	• if TS insoluble, then go to STEP 6.

TIER 6

STEP 6:	0.2 mg/mL TS in 50 mL DMSO	
	• if TS soluble, then <u>STOP.</u>	
	• if TS insoluble, test at 0.2 mg/mL in 50 mL ETOH	
	• <u>STOP</u>	

Figure 2Solubility Flow Chart

Tier	1		2		3			4			5		6
Concentration in Medium	Start Here 200 mg/mL	Incomplete solubility	20 mg/mL	Incomplete solubility	2 mg/mL			• 0.20 mg/mL					
					Incomplete solubility			Incomplete solubility					
Concentration in DMSO					200 mg/mL			20 mg/mL			2 mg/mL		►0.2 mg/mL
					Incomplete solubility			Incomplete solubility			Incomplete solubility		Incomplete solubility
Concentration in ETOH					200 mg/mL [−]	Incom	1plete bility	20 mg/mL -	Incon solu	nplete bility	2 mg/mL	Incomplete	0.2 mg/mL End
Concentration on Cells	100 mg/mL		10 mg/mL		1 mg/mL			0.1 mg/mL			0.01 mg/mL		0.001 mg/mL

Notes: 3T3 Medium - Dulbecco's Modification of Eagle's Medium, with supplements, for 3T3 mouse fibroblasts

[This Page Intentionally Left Blank]

40	
42	
43	
44	
45	
46	
47	
48	Appendix B-2
49	
50	
51	Test Method Protocol for the Normal Human Epidermal Keratinocyte
52	(NHK) Neutral Red Uptake (NRU) Cytotoxicity Test
53	
54	
55	This draft recommended NHK NRU test method protocol is substantially the same as the
56	protocol used in Phase III of the NICEATM/ECVAM validation study. Revisions were made
57	based on recommendations from NICEATM and the study directors involved in the study.
58	The changes are as follows:
59	• Explanations and directions for the use of the revised Hill function for determining
60	IC50 values are included in the protocol.
61	• The range for relative humidity values for the cell culture incubators was changed
62	from 90 % \pm 5 % humidity to 90 % \pm 10 % humidity.
63	• An additional step was added to the test substance solubility protocol to allow testing
64	of higher concentrations of test material.
65	• The spreadsheet templates used in the NICEATM/ECVAM validation study are
66	incorporated into this protocol as an annex (ANNEX I).
67	• The stand-alone solubility protocol is incorporated into this protocol as an annex
68	(ANNEX II).
69	• The stand-alone NHK media prequalification protocol is incorporated into this
70	protocol as an annex (ANNEX III).
71	

71	
72	
73	
74	
75	
76	
77	
78	
79	
80	
81	
82	
83	[This Page Intentionally Left Blank]
84	

84	TEST METHOD PROTOCOL
85	
86	THE NORMAL HUMAN KERATINOCYTE (NHK) NEUTRAL RED
87	UPTAKE (NRU) CYTOTOXICITY TEST
88	A Test for Basal Cytotoxicity
89	
90	
91 92	I. PURPOSE
92 93	I. I UNI USE
94	This test method is used to evaluate the cytotoxicity of test substances using the Normal
95	Human Keratinocyte (NHK) Neutral Red Uptake (NRU) in vitro cytotoxicity test. The
96	data generated from the <i>in vitro</i> cytotoxicity assays are used to evaluate the effectiveness
97	of the cytotoxicity assay to predict the starting doses for rodent acute oral systemic
98	toxicity assays. This test method protocol outlines the procedures for performing the
99	basal cytotoxicity test and is the result of the <i>in vitro</i> validation study organized by
100	NICEATM and the European Centre for the Validation of Alternative Methods
101	(ECVAM).
102	If abanges or modifications are made to this protocol, the testing laboratory should prove
103 104	If changes or modifications are made to this protocol, the testing laboratory should prove that the results are comparable to those obtained when using the original protocol.
104	that the results are comparable to those obtained when using the original protocol.
105	II. TEST SYSTEM
107	
108	The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay
109	based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital
110	dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic
111	diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or
112	the sensitive lysosomal membrane lead to lysosomal fragility and other changes that
113	gradually become irreversible. Such changes brought about by the action of xenobiotics
114 115	result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.
115	viable, damaged, of dead cens, which is the basis of this assay.
117	Healthy mammalian cells, when maintained in culture, continuously divide and multiply
118	over time. A toxic substance, regardless of site or mechanism of action, will interfere
119	with this process and result in a reduction of the growth rate as reflected by cell number.
120	Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR
121	after substance exposure thus providing a sensitive, integrated signal of both cell integrity
122	and growth inhibition.
123	
124	III. KEY PERSONNEL
125	
126	A. Laboratory
127 128	 Study Director Laboratory Technician(s)
128	2) Laboratory Technician(s)
147	

130	B.	Testing Facility
131		1) Scientific Advisor
132		2) Quality Assurance Director
133		3) Safety Manager
134		4) Facility Management
135		+) I denity Management
136	IV DI	CFINITIONS
130	11.01	
138	٨	Hill function: a four parameter logistic mathematical model relating the
138	А.	concentration of test substance to the response being measured in a sigmoidal
140		
140		shape.
141		$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(logEC50-X)HillSlope}}$
142		$1+10^{(logEC50-X)HillSlope}$
143		
144		where Y= response, X is the logarithm of dose (or concentration), Bottom is
145		the minimum response, Top is the maximum response, logEC50 is logarithm
146		of X at the response midway between Top and Bottom, and HillSlope
147		describes the steepness of the curve. When $Top = 100$ and $Bottom = 0$, the
148		EC_{50} is the concentration at 50% viability (i.e., the IC ₅₀).
149		
150	B.	Documentation: all methods and procedures will be noted in a Study Workbook; logs
151		will be maintained for general laboratory procedures and equipment (e.g., media
152		preparation, test substance preparation, incubator function); all optical density data
153		obtained from the spectrophotometer plate reader will be saved in electronic and
154		paper formats; all calculations of ICx values and other derived data will be in
155		electronic and paper format; all data will be archived.
156		
157	C.	IC_{50} : test substance concentration producing 50% inhibition of the endpoint measured
158		(i.e., cell viability).
159		
160	V. ID	ENTIFICATION OF CONTROL SUBSTANCES
161		
162	А.	Positive Control (PC)
163		Sodium Lauryl Sulfate (SLS)
164		
165	B.	Vehicle Control (VC)
166		Keratinocyte assay medium
167		
168	C.	Solvent Control
169		VC control with solvent (i.e., keratinocyte assay medium, dimethyl sulfoxide
170		[DMSO], or ethanol [ETOH])
171		(DMSO is the preferred solvent for substances that are not water [i.e., assay medium]
172		soluble.)

173 174 175	VI. PRO	CEDURES
176 177		aterials Cell Line
178 179 180 181 182 183	2.	Normal Human Epidermal Keratinocytes (NHK) Non-transformed cells; from cryopreserved primary or secondary cells (e.g., Clonetics #CC-2507 or equivalent - <i>Cambrex</i> [Cambrex Bio Science, 8830 Biggs Ford Road, Walkersville, MD). Cells will be Clonetics NHK cells. Technical Equipment
184 185 186		[Note: Suggested brand names/vendors are listed in parentheses. Equivalents may be used.]
187 188 189		 8 Incubator: 37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air 9 Laminar flow clean bench (standard: "biological hazard") 10 Waterbath: 37°C ± 1°C
190 191 192		11 Inverse phase contrast microscope12 Sterile glass tubes with caps (e.g., 5 mL)13 Centrifuge
193 194		 14 Laboratory balance 15 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm ± 10
195 196 197		nm filter 16 Shaker for microtiter plates 17 Cell counter or hemocytometer
198 199		18 Pipetting aid19 Pipettes, pipettors (multi-channel and single channel; multichannel repeater
200 201 202		pipette), dilution block 20 Cryotubes 21 Tissue culture flasks (75 - 80 cm ² , 25 cm ²)
203 204 205		 22 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Corning/COSTAR tissue culture-treated) 22 pL paper (wide and perrow range)
205 206 207		23 pH paper (wide and narrow range)q) Multichannel reagent reservoirr) Waterbath sonicator
208 209 210		 28 Magnetic stirrer 29 Antistatic bar ionizer/antistatic gun (optional for neutralizing static on 96-well plates)
210 211 212		30 Dry heat block (optional) 31 Adhesive film plate sealers (e.g., Excel Scientific SealPlate [™] ,Cat # STR-
213 214 215		SEAL-PLT or equivalent) 32 Vortex mixer 33 Filters/filtration devices
215		

[Note: Tissue culture flasks and microtiter plates should be prescreened to ensure that they adequately support the growth of NHK. Multi-channel repeater pipettes may be used for plating cells in the 96-well plates, dispensing plate rinse solutions, NR medium, and desorb solution. Do not use the repeater pipette for dispensing test substances to the cells.]

223

217

218

219

220

221

222

231

232

234 235

236

237

238

239

240

241

242 243

244

245

246

247

3. Chemicals, Media, and Sera

- Keratinocyte Basal Medium without Ca⁺⁺ (e.g., KBM®, Clonetics CC-3104) 224 225 that is completed by adding supplements (e.g., KBM® SingleQuots®, 226 Clonetics CC-4131) to achieve the proper concentrations of epidermal growth 227 factor, insulin, hydrocortisone, antimicrobial agents, bovine pituitary extract, and 228 calcium (e.g., Clonetics Calcium SingleQuots®, 300 mM CaCl₂, Clonetics CC-229 4202). 230
 - 10 HEPES Buffered Saline Solution (HEPES-BSS) (e.g., Clonetics # CC-5022)
 - 11 0.025 % Trypsin/EDTA solution (e.g., Clonetics # CC-5012)
 - 12 Trypsin Neutralizing Solution (TNS) (e.g., Clonetics # CC-5002)
- 233 13 Phosphate Buffered Saline (PBS)
 - 14 Dulbecco's Phosphate Buffered Saline (D-PBS) [formulation containing calcium and magnesium cations; glucose optional] (for rinsing)
 - 15 Neutral Red (NR) Dye tissue culture-grade; liquid form (e.g., SIGMA N 2889); powder form (e.g., SIGMA N 4638)
 - 16 DMSO, U.S.P analytical grade (Store under nitrogen @ -20°C)
 - 17 ETOH, U.S.P. analytical grade (100 %, non-denatured for test substance preparation; 95 % can be used for the desorb solution)
 - 18 Glacial acetic acid, analytical grade
 - 19 Hanks' Balanced Salt Solution without Ca²⁺ or Mg²⁺ (CMF-HBSS) (e.g., Invitrogen # 14170)
 - 20 Distilled H₂O or any purified water suitable for cell culture and NR desorb solution (sterile)
 - 21 Sterile/non-sterile paper towels (for blotting 96-well plates)
- 248 **B.** Preparations of Media and Solutions

249 [Note: All solutions (except NR stock solution, NR medium and NR desorb), 250 glassware, pipettes, etc., shall be sterile and all procedures should be carried out 251 under aseptic conditions and in the sterile environment of a laminar flow cabinet 252 (biological hazard standard). All methods and procedures will be adequately 253 documented.] 254

- 255 1. Media
- 256 [Note: This protocol is based on the use of Clonetics KBM® medium and supplements. Other media may be acceptable if proper cell growth conditions can 257 258 be maintained as per this protocol. Prequalify candidate media by using the 259 keratinocyte medium prequalification in ANNEX III.]

261	3	a) Routine Cult	ure Medium/Treatment Medium	
262 263		KRM® (Clar	netics CC-3104) supplemented with KBM® S	ingleQuote®
263			C-4131) and Clonetics Calcium SingleQuots®	•
265			ium. Final concentrations of supplements in r	· · · · · · · · · · · · · · · · · · ·
203		300 mL mea	ium. Timai concentrations of supplements in f	
267	0.00	01 ng/mI Hums	an recombinant epidermal growth factor	
268	0.00	5 μg/mL	Insulin	
269			Hydrocortisone	
270		30 μg/mL		
270			Amphotericin B	
271 272		0.10 mM	Calcium	
272	2		ne pituitary extract	
273	-	ομg/IIIL Dovi	ic pituliary extract	
275		-	dia should be kept at 2-8°C and stored for	no longer than two
276		weeks.		
277				
278			® SingleQuots® contain the following stock	concentrations and
279		volumes:		
280				
281		0.1 ng/mL	hEGF	0.5 mL
282		5.0 mg/mL	Insulin	0.5 mL
283		0.5 mg/mL	Hydrocortisone	0.5 mL
284		30 mg/mL	Gentamicin, 15 µg/mL Amphotericin-B	0.5 mL
285		7.5 mg/mL	Bovine Pituitary Extract (BPE)	2.0 mL
286				
287		Clonetics Cal	cium SingleQuots® are 2 mL of 300 mM calc	cium.
288				
289			ution per 500 mL calcium-free medium equal	s 0.10 mM calcium
290		in the mediur	n.	
291	•			
292		,	R) Stock Solution	
293		1	culture-grade stock NR Solution will be the f	
294	-	-	ssay (e.g., SIGMA #N2889, 3.3 mg/mL). Sto	-
295		-	Stock Solution at the storage conditions and	shelf-life period
296	ľ	recommended by	the manufacturer.	
297				<pre>/</pre>
298			can be made with powder NR dye and water	
299			00 mL H_2O) if the liquid stock form is not av	
300	S	should be stored	in the dark at room temperature for up to two	months.
301	.			
302	3. I	Neutral Red (N	K) Wealum	
303	_	EXAMPLE:		
304	1	1.0 mL (3.3 mg 1	NR dye/mL) NR Stock Solution	

305		99 ml 99.0 mL	Routine Culture Medium (pre-warmed to 37°C)
306 307 308 309		The final concentration of be prepared on the day of	the NR Medium is <u>33 μg NR dye/mL</u> and aliquots will application.
310 311 312 313 314 315		pore size) used to reduce I maintained at 37°C (e.g., i within 60 minutes of prepa	hall be filtered (e.g., Millipore filtering, $0.2 - 0.45 \mu m$ NR crystals. Aliquots of the NR Medium should be n a waterbath) before adding to the cells and used aration but also used within 15 minutes after removing ine the solution for crystals.]
316	4.	ETOH/Acetic Acid Solut	ion (NR Desorb)
317 318 319 320		1 % Glacial acetic a 50 % ETOH 49 % H ₂ O	icid solution
320 321	C. M	ethods	
322	1.	Cell Maintenance and C	ulture Procedures
323 324 325 326 327 328		25 cm^2) at $37^\circ\text{C} \pm 1^\circ\text{C}$, 90 cells should be examined	rown as a monolayer in tissue culture grade flasks (e.g., $\% \pm 10 \%$ humidity, and $5.0 \% \pm 1 \%$ CO ₂ /air. The on a daily (i.e., on workdays) basis under a phase any changes in morphology or their adhesive properties Workbook.
329	2.	Receipt of Cryopreserve	d Keratinocytes
330		Upon receipt of cryoprese	rved keratinocytes, the vial(s) of cells shall be stored in
331		a liquid nitrogen freezer u	ntil needed.
332			
333	3.	Thawing Cells and Estab	olishing Cell Cultures
334 335 336 337 338 339 340 341 342 343		 possible. Do not thaw cells into culture flasks 5 Slowly (taking approx Culture Medium to the transfer cells into flask Table 1). 6 Incubate the cultures at CO₂/air until the cells a 	ampules into a water bath at 37°C for as brief a time as cells at room temperature or by hand. Seed the thawed as quickly as possible and with minimal handling. imately 1-2 minutes) add 9 mL of pre-warmed Routine cells suspended in the cryoprotective solution and s containing pre-warmed Routine Culture Medium (See $37^{\circ}C \pm 1^{\circ}C$, 90 % ± 10 % humidity, 5.0 % ± 1 % attach to the flask (within 4 to 24 hours), at which time the um should be removed and replaced with fresh Routine
344		Culture Medium.	-

- 3457Unless otherwise specified, the cells should be incubated at $37^{\circ}C \pm 1^{\circ}C$, 90 % ±34610 % humidity, 5.0 % ± 1 % CO₂/air and fed every 2-3 days until they exceed34750 % confluence (but less than 80 % confluent).
- 348 349

349 350		Table 1. Guidelines for Establi	ishing Cell Cult	ures	
550		Cells/25 cm ² flask	6.25×10^4	1.25×10^5	2.25×10^5
		(in approximately 5 mL)	$(2500/cm^2)$	$(5000/cm^2)$	$(9000/cm^2)$
		1 flask each cell concentration	(2000,000)		() 000,011)
		Approximate Time to	96+ hours	72 - 96 hours	48 - 72 hours
		Subculture	<i>y</i> 0 × 110 u 15	7 2 90 Hours	
		Cells to 96-Well Plates	6-8 plates	6-8 plates	6 – 8 plates
351			F		
352		Cell growth guidelines – actual g	rowth of individ	lual cell lots may	varv.
353		5 5 6	, ,	5	5
354	4. S	bubculture of NHK Cells to 96-W	ell Plates		
355	[]	Note: It is important that cells have	e overcome the	lag growth phase	e when they
356	a	re used for the test. Keratinocytes	will be passage	d only into the 9	6-well plates
357	a	nd will not be subcultured into flas	sks for use in lat	er assays]	
358			2		
359	8	J			
360		less than 80 % confluent), remov			
361		5 mL HEPES-BSS. The first rin		1	
362		and the second rinse should rema	ain on the cells for	or approximately	5 minutes.
363		Discard the washing solutions.			
364	9	51			
365		seconds. Incubate the flask at ro	1		
366		than 50 % of the cells become dis	slodged, rap the	flask sharply aga	ainst the palm
367	1	of the hand.	1 4 1 1 6	4 6	· 4 0 1
368	1	0 When most of the cells have been		,	
369		with 5 mL of room temperature T			,
370 371	1	same 5 mL of TNS may be used 1 Then rinse the flask with 5 mL C		-	
371	1	centrifuge tube.	IVIT-FIDSS and	transfer the cell s	suspension to a
373	1	2 Pellet the cells by centrifugation	for 5 minutes at	annrovimately?	20 v a
373	1	Remove the supernatant by aspir		approximatory 2	20 A E.
375	1	3 Resuspend the keratinocyte pelle		ration (to have s	ingle cells) in
376	1	Routine Culture Medium. It is in			e ,
377		exact counting. Count a sample	1	•	1
378		or cell counter.		<i></i>	
379	1	4 Prepare a cell suspension –1.6 –	-2.0×10^4 cells/n	nL in Routine C	ulture Medium
380	1	Using a multi-channel pipette, d			
381		into the peripheral wells (blanks			
382		In the remaining wells, dispense	· · · · · · · · · · · · · · · · · · ·		<u> </u>
383		2.5×10^3 cells/well). Prepare one			
384		1, Section VI.E.1).	Prate per subst		. (See Figure
385	1	5 Incubate cells $(37^{\circ}C \pm 1^{\circ}C, 90^{\circ})$	$\% \pm 10\%$ humic	lity. and $5\% \pm 1$	% CO ₂ /air) so
386	1	that cells form a $20+\%$ monolar		•	
*				,	- r **

Table 1. Guidelines for Establishing Cell Cultures

 assures cell recovery and adherence and progression to exponential growth phase. 16 Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook. 5. Determination of Doubling Time a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for subculture. Resuspend cells in appropriate culture medium. Usc Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture mediam at 72 hours and 96 hours post inoculation doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined by absec, plateau phase). D Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance is tock dilution should have at least 1-20. Itotal volume to ensure adequate solutio			
 16 Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook. 393 5. Determination of Doubling Time a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 2 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pl drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log cup resports to light.] D Preparation of Test Substances t. Test Substance in Solution Allow test substances to equilibrate			
 growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook. 5. Determination of Doubling Time a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, l			1
 i to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook. 5. Determination of Doubling Time a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dve exclusion (e.g., Typan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture mediuma 172 hours or soner in remaining dishes if indicated by pl drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log the cord y yellow light is recommended to preserve substances that degrade upon exposure to light.] D. Preparation of Test Substances (Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance in Solution Allow test substances to equilibrate to room te	389		16 Examine each plate under a phase contrast microscope to assure that cell
 observations in the Study Workbook. 5. Determination of Doubling Time a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances I. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance is to equilibrate to room temperature before dissolving and diluting. Prepare test substance is to equilibrate to room temperature before dissolving and diluting. Prepare test substance is a could by prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipi	390		growth is relatively even across the microtiter plate. This check is performed
 observations in the Study Workbook. 5. Determination of Doubling Time a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances I. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance is to equilibrate to room temperature before dissolving and diluting. Prepare test substance is to equilibrate to room temperature before dissolving and diluting. Prepare test substance is a could by prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipi	391		to identify experimental and systemic cell seeding errors. Record
 5. Determination of Doubling Time a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance solution of the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the bighest 2X stock solution (e.g., low so	392		
 5. Determination of Doubling Time a) Establish cells in culture and trypsinize cells as per Section VI.C.4 for subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances I. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance is cultility should be determined by following the procedures outlined in ANNEX II of this protocol. 			y
 subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Not: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance is Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance solution for the test wells in a single 96-well plate. The Study Director may store an aliquo (e.g., 1 mL) of the highest 2X stock solution (e.g., 10w Col) for use in 		5.	Determination of Doubling Time
 subculture. Resuspend cells in appropriate culture medium. Use Table 1 to determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Not: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance is Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance solution for the test wells in a single 96-well plate. The Study Director may store an aliquo (e.g., 1 mL) of the highest 2X stock solution (e.g., 10w Col) for use in 	205		a) Establish calls in culture and transinize calls as nor Section VIC 4 for
 determine seeding densities. b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue, Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance is mediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e			
 b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time vill be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. Prepare test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			
 issue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37°C ± 1°C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solution smust not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 90-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			6
400medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators $(37^{\circ}C \pm 1^{\circ}C, 90\% \pm 10\%$ humidity, 5.0 $\% \pm 1\%$ CO ₂ /air).401c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop.400d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase).415D. Preparation of Test Substances (Integrade upon exposure to light.]426Test substance isolubility should be determined by following the procedures outlined in ANNEX II of this protocol.427Allow test substance is to equilibrate to room temperature before dissolving and diluting.426Prepare test substance is coulibrate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in			
401dish. Place dishes into the incubators $(37^{\circ}C \pm 1^{\circ}C, 90^{\circ}\% \pm 10^{\circ}\%$ humidity, 5.0402 $\% \pm 1^{\circ}\%$ CO ₂ /air).403c) After 4-6 hours (use the same initial measurement time for each subsequent404doubling time experiment), remove three culture dishes and trypsinize cells.405Count cells using a cell counter or hemocytometer. Cell viability may be406determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the407total number of cells and document. Repeat sampling at 24 hours, 48 hours,40872 hours, and 96 hours post inoculation. Change culture medium at 72 hours409or sooner in remaining dishes if indicated by pH drop.410d) Plot cell concentration (per mL of medium) on a log scale against time on a411linear scale. Determine lag time and population doubling time. The doubling412time will be in the log (exponential) phase of the growth curve. Additional413dishes and time are needed if the entire growth curve is to be determined (lag414phase, log phase, plateau phase).415D.416D. Preparation of Test Substances417[Note: Preparation under red or yellow light is recommended to preserve substances418that degrade upon exposure to light.]429Test substance in Solution421• Allow test substances to equilibrate to room temperature before dissolving and422diluting.423• Allow test substance inmediately prior to use and not in bulk for use in424• Allow test substance inmediately prior to use and not in bu			E 3/ 11 1
 402 % ± 1 % CO₂/air). c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in	400		
 403 c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. 422 1. Test Substance in Solution • Allow test substances to equilibrate to room temperature before dissolving and diluting. • Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in	401		dish. Place dishes into the incubators $(37^{\circ}C \pm 1^{\circ}C, 90\% \pm 10\%$ humidity, 5.0
 doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. 423 Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance is neediately prior to use and not in bulk for use in subsequent tests. Ideally, the solution should have at least 1-2 mL total volume to ensure adequate solution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 	402		$\% \pm 1 \% CO_2/air).$
 405 Count cells using a cell counter or hemocytometer. Cell viability may be 406 determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the 407 total number of cells and document. Repeat sampling at 24 hours, 48 hours, 408 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours 409 or sooner in remaining dishes if indicated by pH drop. 410 d) Plot cell concentration (per mL of medium) on a log scale against time on a 411 linear scale. Determine lag time and population doubling time. The doubling 412 time will be in the log (exponential) phase of the growth curve. Additional 413 dishes and time are needed if the entire growth curve is to be determined (lag 414 phase, log phase, plateau phase). 415 416 D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances 418 that degrade upon exposure to light.] 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 1. Test Substance in Solution 424 Allow test substances to equilibrate to room temperature before dissolving and 425 diluting. 426 Prepare test substance in mediately prior to use and not in bulk for use in 427 subsequent tests. Ideally, the solution should have at least 1-2 mL total 428 volume to ensure adequate solution for the test wells in a single 96-well plate. 429 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock 430 solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 	403		c) After 4-6 hours (use the same initial measurement time for each subsequent
 405 Count cells using a cell counter or hemocytometer. Cell viability may be 406 determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the 407 total number of cells and document. Repeat sampling at 24 hours, 48 hours, 408 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours 409 or sooner in remaining dishes if indicated by pH drop. 410 d) Plot cell concentration (per mL of medium) on a log scale against time on a 411 linear scale. Determine lag time and population doubling time. The doubling 412 time will be in the log (exponential) phase of the growth curve. Additional 413 dishes and time are needed if the entire growth curve is to be determined (lag 414 phase, log phase, plateau phase). 415 416 D. Preparation of Test Substances 417 [Note: Preparation under red or yellow light is recommended to preserve substances 418 that degrade upon exposure to light.] 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 424 • Allow test substances to equilibrate to room temperature before dissolving and 425 diluting. 426 • Prepare test substance in mediately prior to use and not in bulk for use in 427 subsequent tests. Ideally, the solution should have at least 1-2 mL total 428 volume to ensure adequate solution for the test wells in a single 96-well plate. 429 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock 430 Stoal (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 	404		doubling time experiment), remove three culture dishes and trypsinize cells.
 determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop. d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. Prepare test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 	405		· · ·
 407 total number of cells and document. Repeat sampling at 24 hours, 48 hours, 408 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours 409 or sooner in remaining dishes if indicated by pH drop. 410 d) Plot cell concentration (per mL of medium) on a log scale against time on a 411 linear scale. Determine lag time and population doubling time. The doubling 412 time will be in the log (exponential) phase of the growth curve. Additional 413 dishes and time are needed if the entire growth curve is to be determined (lag 414 phase, log phase, plateau phase). 415 416 D. Preparation of Test Substances 417 [Note: Preparation under red or yellow light is recommended to preserve substances 418 that degrade upon exposure to light.] 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 423 424 • Allow test substances to equilibrate to room temperature before dissolving and 425 diluting. • Prepare test substance immediately prior to use and not in bulk for use in 426 subsequent tests. Ideally, the solutions must not be cloudy nor have 427 noticeable precipitate. Each stock dilution should have at least 1-2 mL total 428 volume to ensure adequate solution for the test wells in a single 96-well plate. 429 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock 431 			
 408 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours 409 or sooner in remaining dishes if indicated by pH drop. 410 d) Plot cell concentration (per mL of medium) on a log scale against time on a 411 linear scale. Determine lag time and population doubling time. The doubling 412 time will be in the log (exponential) phase of the growth curve. Additional 413 dishes and time are needed if the entire growth curve is to be determined (lag 414 phase, log phase, plateau phase). 415 416 D. Preparation of Test Substances 417 [Note: Preparation under red or yellow light is recommended to preserve substances 418 that degrade upon exposure to light.] 419 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 1. Test Substance in Solution 424 • Allow test substances to equilibrate to room temperature before dissolving and 410 diluting. • Prepare test substance in solution smust not be cloudy nor have 428 noticeable precipitate. Each stock dilution should have at least 1-2 mL total 429 volume to ensure adequate solution for the test wells in a single 96-well plate. 430 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock 431 			
 409 or sooner in remaining dishes if indicated by pH drop. 410 d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). 415 D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] 419 420 Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. 423 1. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substances to equilibrate to room temperature before dissolving and diluting. Prepare tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			
 d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. 1. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in			
 411 linear scale. Determine lag time and population doubling time. The doubling 412 time will be in the log (exponential) phase of the growth curve. Additional 413 dishes and time are needed if the entire growth curve is to be determined (lag 414 phase, log phase, plateau phase). 415 D. Preparation of Test Substances 417 [Note: Preparation under red or yellow light is recommended to preserve substances 418 that degrade upon exposure to light.] 419 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 1. Test Substance in Solution • Allow test substances to equilibrate to room temperature before dissolving and 425 diluting. • Prepare test substance immediately prior to use and not in bulk for use in 426 subsequent tests. Ideally, the solutions must not be cloudy nor have 428 noticeable precipitate. Each stock dilution should have at least 1-2 mL total 429 volume to ensure adequate solution for the test wells in a single 96-well plate. 430 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock 431 			
 time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance in Solution Prepare test substance in mediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in			
 dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase). D Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. 1. Test Substance in Solution • Allow test substances to equilibrate to room temperature before dissolving and diluting. • Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in			
 414 phase, log phase, plateau phase). 415 416 D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances 418 that degrade upon exposure to light.] 419 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 1. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			
 415 416 D. Preparation of Test Substances [Note: Preparation under red or yellow light is recommended to preserve substances 417 [Note: Preparation under red or yellow light is recommended to preserve substances 418 that degrade upon exposure to light.] 419 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 1. Test Substance in Solution 424 Allow test substances to equilibrate to room temperature before dissolving and diluting. 426 Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. 430 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			
 416 D. Preparation of Test Substances 417 [Note: Preparation under red or yellow light is recommended to preserve substances 418 that degrade upon exposure to light.] 419 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 1. Test Substance in Solution 424 Allow test substances to equilibrate to room temperature before dissolving and diluting. 426 Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			phase, log phase, plateau phase).
 In the second second		Due	maration of Test Substances
 that degrade upon exposure to light.] Test substance solubility should be determined by following the procedures outlined in ANNEX II of this protocol. 1. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			
 419 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 1. Test Substance in Solution 424 425 Allow test substances to equilibrate to room temperature before dissolving and diluting. 426 426 Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. 430 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 		-	
 420 Test substance solubility should be determined by following the procedures outlined 421 in ANNEX II of this protocol. 422 423 1. Test Substance in Solution 424 • Allow test substances to equilibrate to room temperature before dissolving and 425 diluting. • Prepare test substance immediately prior to use and not in bulk for use in 427 subsequent tests. Ideally, the solutions must not be cloudy nor have 428 noticeable precipitate. Each stock dilution should have at least 1-2 mL total 429 volume to ensure adequate solution for the test wells in a single 96-well plate. 430 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock 431 		that	degrade upon exposure to light.
 in ANNEX II of this protocol. 1. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 		T	
 422 423 424 424 425 426 426 426 427 428 428 428 428 429 429 420 420 420 421 422 423 424 425 426 427 428 428 429 429 429 420 420 420 421 421 422 423 424 424 425 425 426 427 428 428 429 429 429 430 430 431 431 430 431 431			
 1. Test Substance in Solution Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 		ın A	NNEX II of this protocol.
 Allow test substances to equilibrate to room temperature before dissolving and diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			
 diluting. Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 		1.	
 Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 			 Allow test substances to equilibrate to room temperature before dissolving and
 427 subsequent tests. Ideally, the solutions must not be cloudy nor have 428 noticeable precipitate. Each stock dilution should have at least 1-2 mL total 429 volume to ensure adequate solution for the test wells in a single 96-well plate. 430 The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock 431 solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 	425		diluting.
428noticeable precipitate. Each stock dilution should have at least 1-2 mL total429volume to ensure adequate solution for the test wells in a single 96-well plate.430The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock431solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in	426		• Prepare test substance immediately prior to use and not in bulk for use in
 volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 	427		subsequent tests. Ideally, the solutions must not be cloudy nor have
 volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in 	428		noticeable precipitate. Each stock dilution should have at least 1-2 mL total
430The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock431solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in	429		
431 solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in			

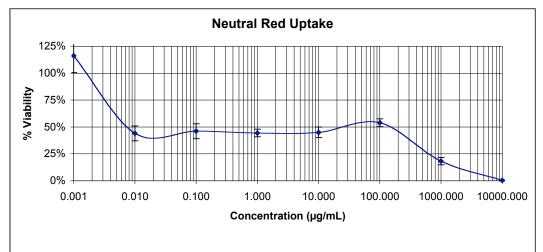
433	• For substances dissolved in DMSO or ETOH, the final DMSO or ETOH
434	concentration for application to the cells must be 0.5 % (v/v) in the vehicle
435	controls and in all of the eight test concentrations.
436	
437	• The stock solution for each test substance should be prepared at the highest
438	concentration found to be soluble in the solubility test (ANNEX II: Test
439	Method Procedure - Solubility Determination of Test Substances). Thus, the
440	highest test concentration applied to the cells in each range finding experiment
441	is:
442	• 0.5 times the highest concentration found to be soluble in the
443	solubility test, if the substance was soluble in medium, or
444	• 1/200 the highest concentration found to be soluble in the
445	solubility test if the substance was soluble in ETOH or DMSO.
446	• The seven lower concentrations in the range finding experiment are prepared
447	by successive dilutions that decrease by one log unit each. The following
448	example illustrates the preparation of test substance in solvent and the dilution
449	of dissolved test substance in medium before application to NHK cells.
450	
451	Example: Preparation of Test Substance in Solvent Using a Log Dilution Scheme
452	If DMSO is determined to be the preferred solvent at Tier 3 of the solubility test
453	(i.e., $200,000 \ \mu g/mL$), dissolve the substance in DMSO at $200,000 \ \mu g/mL$ for the
454	substance stock solution.
455	substance stock solution.
456	8) Label eight tubes 1 – 8. Add 0.9 mL solvent (e.g., DMSO) to tubes 2 8.
457	 9) Prepare stock solution of 200,000 µg test substance/mL solvent in tube # 1.
458	10) Add 0.1 mL of 200,000 μ g/mL dilution from tube #1 to tube #2 to make a
459	1:10 dilution in solvent (i.e., 20,000 μ g/mL).
460	11) Add 0.1 mL of 20,000 μ g/mL dilution from tube #2 to tube #3 to make
461	another 1:10 dilution (i.e., 1:100 dilution from stock solution) in solvent (i.e.,
462	$2,000 \ \mu g/mL)$
463	12) Continuing making serial 1:10 dilutions in the prepared solvent tubes.
464	13) Since each concentration is 200 fold greater than the concentration to be
465	tested, make a 1:100 dilution by diluting 1 part dissolved substance in each
466	tube with 99 parts of culture medium (e.g., 0.1 mL of test substance in DMSO
467	+ 9.9 mL culture medium) to derive the eight 2X concentrations for
468	application to NHK cells. Each 2X test substance concentration will then
469	contain 1 % v/v solvent. The NHK cells will have 0.125 mL of culture
470	medium in the wells prior to application of the test substance. By adding
471	0.125 mL of the appropriate 2X test substance concentration to the appropriate
472	wells, the test substance will be diluted appropriately (e.g., highest
473	concentration in well will be 1,000 μ g/mL) in a total of 0.250 mL and the
474	solvent concentration in the wells will be 0.5% v/v.
475	14) A test substance prepared in DMSO or ETOH may precipitate upon transfer
476	into the Routine Culture Medium. The 2X dosing solutions should be
477	evaluated for precipitates and the results recorded in the Study Workbook. It

478 will be permissible to test all of the dosing solutions in the dose range finding 479 assay and main experiments. However, doses containing test substance 480 precipitates should be avoided because it creates doubt about the 481 concentration of test substance exposed to the cells. 482 483 Document all test substance preparations in the Study Workbook. 484 485 2. pH of Test Substance Solutions 486 Prior to or immediately after application of the test substance to the 96-well plate, measure the pH of the highest 2X dosing concentration of the test substance (i.e., 487 488 C1 in the test plate, see Figure 1) in culture medium. Use pH paper (e.g., pH 0 -489 14 to estimate and pH 5 - 10 to determine more precise value; or Study Director's The pH paper should be in contact with the solution for 490 discretion). 491 approximately one minute. Document the pH and note the color of the 2X 492 concentration medium (i.e., in the Microsoft Excel® template; see ANNEX 1 for 493 an example template). Medium color for all dosing dilutions should be noted in 494 the Study Workbook. Do not adjust the pH. 495 496 3. Concentrations of Test Substance 497 a) Range Finder Experiment Test eight concentrations of the test substance by diluting the stock 498 499 solution using log dilutions (e.g., 1:10, 1:100, 1:1000, etc.). If a range finder experiment does not generate enough cytotoxicity, then 500 • 501 higher doses should be attempted. If cytotoxicity is limited by solubility, 502 then more stringent solubility procedures to increase the stock 503 concentration (to the maximum concentration specified in Section 504 VI.D.3.b.) should be employed. Place the highest test substance concentration into an incubator $(37^{\circ}C \pm$ 505 1° C, 90 % ± 10 % humidity, 5.0 % ± 1 % CO₂/air) and stir or rock for up 506 to 3 hours, if necessary, to facilitate dissolution. For stocks prepared in 507 medium, vessel caps should be loose to allow for CO₂ exchange. Proceed 508 509 with dosing solution preparation and dosing. 510 If a range finding test produces a biphasic curve, then the doses selected 511 for the subsequent main experiments should cover the most toxic dose-512 response range (see Example 1 – the most toxic range is 0.001 - 0.1513 μ g/mL) that reduces viability to 50%. 514 515

515 Example 1 – Biphasic Curve



Example 1 Diphase Curve



517	
518	
519	b) Main Experiment (Definitive Assay)
520	• Depending on the slope of the concentration-response curve estimated
521	from the range finder, the dilution/progression factor in the concentration
522	series of the main experiment should be smaller (e.g., dilution factor of
523	$^{6}\sqrt{10} = 1.47$). Cover the relevant concentration range around the IC ₅₀ (> 0
524	% and < 100 % effect) preferably with several points of a graded effect,
525	but with a minimum of two points, one on each side of the estimated IC_{50}
526	value, avoiding too many non-cytotoxic and/or 100 %-cytotoxic
527	concentrations.
528	
529	• Determine which test substance concentration is closest to the IC ₅₀ value.
530	Use that value as a central concentration and adjust dilutions higher and
531	lower in equal steps for the definitive assay.
532	
533	Maximum Doses to be Tested in the Main Experiments
534	If minimal or no cytotoxicity was measured in the dose range finding assay, a
535	maximum dose for the main experiments will be established as follows:
536	
537	For test substances prepared in Routine Culture Medium
538	• The highest test substance concentration that may be applied to the cells in
539	the main experiments will be either 100 mg/mL, or the maximum soluble
540	dose.
541	• Test substance will be weighed into a glass tube and the weight will be
542	documented. A volume of Routine Culture Medium will be added to the
543	vessel so that the concentration is 200,000 μ g/mL (200 mg/mL).
544	 The solution is mixed using the mechanical procedures specified in
545	ANNEX II of this protocol. If complete solubility is achieved in medium,
546	then seven additional serial stock dosing solutions may be prepared from
547	the 200 mg/mL 2X stock. If the test substance is insoluble in medium at
548	200 mg/ml, proceed by adding medium, in small incremental amounts, to

549 550	attempt to dissolve the substance by using the sequence of mixing										
550	procedures specified in ANNEX II.										
551	• More stringent solubility procedures may be employed if needed based on										
552	results from the range finder experiment (Section VI.D.3.a.). The highest										
553	soluble stock solution will be used to prepare the seven additional serial										
554	stock dosing solutions.										
555											
556	For test substances prepared in either DMSO or ETOH										
557	11.0 The highest test substance concentration that may be applied to the										
558	cells in the main experiments will be ≤ 2.5 mg/mL, depending upon the										
559	maximum solubility in solvent.										
560	12.0 Test substance will be weighed into a glass tube and the weight will be										
561	documented. A volume of the appropriate solvent (determined from the										
562	original solubility test) will be added to the vessel so that the concentration										
563	is 500,000 μg/mL (500 mg/mL).										
564	13.0 The solution is mixed as specified ANNEX II . If complete solubility										
565	is achieved in the solvent, then seven additional serial stock dosing										
566	solutions may be prepared from the 500 mg/mL 200X stock. If the test										
567	substance is insoluble in solvent at 500 mg/ml, proceed by adding solvent,										
568	in small incremental amounts, to attempt to dissolve the substance by										
569	using the sequence of mixing procedures. The highest soluble stock										
570	solution will be used to prepare the seven additional serial stock dosing										
571	solutions.										
572											
573	If precipitates are observed in the 2X dilutions, continue with the experiment,										
574	make the appropriate observations and documentation, and report data to the										
575	SMT.										
576	01111.										
577	c) Test Substance Dilutions										
578	The dosing factor of 3.16 (= $\sqrt{10}$) divides a log into two equidistant steps,										
579	2.15 (= $\sqrt[3]{10}$) into three steps, 1.47 (= $\sqrt[6]{10}$) into six steps, 1.78 ($\sqrt[4]{10}$) into										
580	four steps, and 1.21 (= ${}^{12}\sqrt{10}$) into 12 steps.										
581											
582	EXAMPLE:										
583											
	10 31.6 100										
	10 21.5 46.4 100										
	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$										
	10 12.1 14.7 17.8 21.5 26.1 31.6 38.3 46.4 56.2 68.1 82.5 100										
584	10 12.1 14.7 17.0 21.3 20.1 51.0 50.5 40.4 50.2 00.1 02.5 100										
585	An example of decimal geometric concentration series for factor 1.47: Dilute										
586	<i>1 volume of the highest concentration by adding 0.47 volumes of diluent.</i>										
587	After equilibration, dilute 1 volume of this solution by adding 0.47 volumes of adding 0.47 volumes of										
588											
200	diluent(etc.).										

590 591 592 593 594	 E. Test Procedure 1. 96-Well Plate Configuration The NHK NRU assay for test substances will use the 96-well plate configuration shown in Figure 1. 								ion					
595 596 597		Figur			Plate (ce Ass		uratio	on for 1	Positiv	ve Con	trol (P	PC) and	d Test	
571		1	2	3	4	5	6	7	8	9	10	11	12	_
	А	VCb	VCb	C ₁ b	C ₂ b	C ₃ b	C ₄ b	C ₅ b	C ₆ b	C ₇ b	C ₈ b	VCb	VCb	
	В	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	С	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	D	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	Е	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	F	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	G	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb	
	Н	VCb	VCb	C ₁ b	C ₂ b	C ₃ b	C ₄ b	C ₅ b	C ₆ b	C ₇ b	C ₈ b	VCb	VCb	
598 599 600 601 602 603 604			VC1 a C ₁ – C b VCb	nd VC	=	(C1 = = BLA	Substa = highe NKS (inces o est, C8 Test s	or PC (= low ubstan	est) ce or P	PC, but		ntration n no ce o cells)	
605	2.	Appl	icatio	1 of Te	est Sub	ostance	e							
606 607 608			-		methoc may b		1 2	applyi	ing the	2X do	osing so	olution	s onto t	he
609 610 611 612 613 614 615 616 617		1) 2)	Corr or C othe) Use the c cells disp	ning/C orning r multi a "dur dosing s). The ensed	ostar n /Trans ichann nmy" p solutic e test su into the	nodel 4 tar mo el reser plate (i pns imi ubstance e dumr	870 st del 48' rvoirs) .e., an nediat ce and ny pla	erile p 78 disp empty ely pri contro te in th	olystyn oosable sterile or to tr l dosir ne same	e reserv 96-we reatmen ng solu e patter) mL re voir lin ell plate nt of th tions si rn/orde	eagent : lers, 8- e) prep le test p hould b er as wi		irs l; or hold vith

618		plate (i.e., greater than 125 μ l/well) should be in the wells of the dummy
619		plate.
620		
621		At the time of treatment initiation, a multi-channel micropipettor is used to
622		transfer the 2X dosing solutions, from the reservoirs or dummy plate, to the
623		appropriate wells on the treatment plate (as described in step c. below). These
624		methods will ensure that the dosing solutions can be transferred rapidly to the
625		appropriate wells of the test plate to initiate treatment times and to minimize
626		the range of treatment initiation times across a large number of treatment
627		plates, and to prevent "out of order" dosing. Do not use a multichannel
628		repeater pipette for dispensing test substance to the plates.
629		
630		2. After 48 - 72 hours (i.e., after cells attain 20+ % confluency [see Section
631		VI.C.4(h)]) incubation of the cells, add 125 μ l of the appropriate
632		concentration of test substance, the PC, or the VC (see Figure 1 for the
633		plate configuration) directly to the test wells. Do not remove Routine
634		Culture Medium for re-feeding the cells. The dosing solutions will be
635		rapidly transferred from the 8-channel reservoir (or dummy plate) to the
636		test plate using a single delivery multi-channel pipettor. For example, the
637		VC may be transferred first (into columns 1, 2, 11, and 12), followed by
638		the test substance dosing solutions from lowest to highest dose, so that the
639		same pipette tips on the multi-channel pipettor can be used for the whole
640		plate. [The Vehicle Control blank (VCb) wells (column 1, column 12,
641		wells A2, A11, H2, H11) will receive the Vehicle Control dosing solutions
642		(which should include any solvents used)]. Blanks for wells A3 – A10
643		and H3 – H10 shall receive the appropriate test substance solution for each
644		concentration (e.g., wells A3 and H3 receive C ₁ solution).] Incubate cells
645		for 48 hours \pm 0.5 hours (37°C \pm 1°C, 90 % \pm 10 % humidity, and 5.0 % \pm
646		$1 \% CO_2/air).$
647		3. <u>Positive Control</u> : For each set of test substance plates used in an assay,
648		prepare a separate plate of positive control concentrations. If multiple sets
649		of test substance plates are set up, then clearly designate the positive
650		control plates for each set; each set will be an individual entity. The Study
651		Director will decide how many test substance plates will be run with a
652		positive control plate. This plate will follow the same schedule and
653		procedures as used for the test substance plates (including appropriate
654		substance concentrations in the appropriate wells and meeting test
655		acceptance criteria see Sections VI.E.1, E.2, and E.5).
656	_	
657	3.	Microscopic Evaluation
658		After at least 46 hours treatment, examine each plate under a phase contrast
659		microscope to identify systematic cell seeding errors and growth characteristics of
660		control and treated cells. Record any changes in morphology of the cells due to
661		the cytotoxic effects of the test substance, but do not use these records for any
662		quantitative measure of cytotoxicity. Undesirable growth characteristics of
663		control cells may indicate experimental error and may be cause for rejection of

665 666

- 667
- 668
- 669
- 670

the assay. Use the following Visual Observations Codes in the description of cell culture conditions. Numerical scoring of the cells (see Section VI.E.3) should be determined and documented in the Study Workbook and in the appropriate section of the Microsoft Excel® template.

Note Code	Note Text
1	Normal Cell Morphology
2	Low Level of Cell Toxicity
3	Moderate Level of Cell Toxicity
4	High level of Cell Toxicity
1P	Normal Cell Morphology with Precipitate
2P	Low Level of Cell Toxicity with Precipitate
3P	Moderate Level of Cell Toxicity with Precipitate
4P	High level of Cell Toxicity with Precipitate
5P	Unable to View Cells Due to Precipitate

Visual Observations Codes

671	
672	

673

674 675

676

677 678

679

680

681 682

683

684

685

686

687

688

4. Measurement of NRU

- a) Carefully remove (i.e., "dump") the Routine Culture Medium (with test substance) and rinse the cells very carefully with 250 µL pre-warmed D-PBS. Remove the rinsing solution by dumping and remove excess by gently blotting on paper towels. Add 250 µL NR medium (to all wells including the blanks) and incubate $(37^{\circ}C \pm 1^{\circ}C, 90\% \pm 10\%$ humidity, and $5.0\% \pm 1\%$ CO_2/air) for 3 hours ± 0.1 hour. Observe the cells briefly during the NR incubation (e.g., between 2 and 3 hours - Study Director's discretion) for NR crystal formation. Record observations in the Study Workbook. Study Director can decide to reject the experiment if excessive NR crystallization has occurred.
- b) After incubation, remove the NR medium, and carefully rinse cells with 250 µL pre-warmed D-PBS.
 - c) Decant and blot D-PBS from the plate. (Optionally: centrifuge the reversed plate.)
- d) Add exactly 100 µL NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.
- 689 Shake microtiter plate rapidly on a microtiter plate shaker for 20 - 45 minutes e) 690 to extract NR from the cells and form a homogeneous solution. Plates should 691 be protected from light by using a cover during shaking.
- Plates should be still for at least five minutes after removal from the plate 692 f) 693 shaker (or orbital mixer). If any bubbles are observed, assure that they have been ruptured prior to reading the plate. Measure the absorption (within 60 694 695 minutes of adding NR Desorb solution) of the resulting colored solution at 696 540 nm \pm 10 nm in a microtiter plate reader (spectrophotometer), using the 697 blanks as a reference.

698 699 700 701 702 703	5. Q	[Note: A mean $OD_{540 \pm 10nm}$ of 0.043 - 0.059 for the VC blanks is a target range of ODs but not a test acceptance criterion (range = mean OD ± 2.5 standard deviations; mean = 0.054; SD = 0.003; N = 114)]. Save raw data in the Microsoft Excel® template. uality Check of Assay
704 705	a)	Test Acceptance Criteria All acceptance criteria (i.e., criteria 1, 2, and 3) must be met for a test to be
706 707		acceptable.
708		1) The PC (SLS) IC_{50} must be within two and a half (2.5) standard deviations
709 710		(SD) of the historical mean established by the Test Facility and must meet criteria 2 and 3, and must have an r^2 (coefficient of determination) value
711		calculated for the Hill model fit (i.e., from PRISM® software) ≥ 0.85 .
712 713		NICEATM/ECVAM study generated the following PC data: • IC_{50} mean = 3.11 µg/mL; SD = 0.72 (n = 114)
714		• range for IC ₅₀ mean ± 2.5 SD = 1.31 µg/mL - 4.91 µg/mL
715		2) The left and the right mean of the VCs do not differ by more than 15 %
716 717		from the mean of all VCs. 3) At least one calculated cytotoxicity value > 0 % and \leq 50 % viability and
718		at least one calculated cytotoxicity value > 50 % and < 100 % viability
719 720		must be present.
720		<i>Exception</i> : If a test has only one point between 0 and 100 % and the smallest
722		dilution factor (i.e., 1.21) was used <u>and</u> all other test acceptance criteria were
723 724		met, then the test will be considered acceptable.
725		Stopping Rule for Insoluble Substances: If the most rigorous solubility
726 727		procedures have been performed and the assay cannot achieve adequate toxicity to meet the test acceptance criteria after three definitive trials, then the
728		Study Director may end all testing for that particular substance.
729		
730 731		[Note: A corrected mean $OD_{540 \pm 10nm}$ of 0.205 - 1.645 for the VCs is a target range of ODs but will not be a test acceptance criterion (range = mean OD ±
732		2.5 standard deviations; mean = 0.685 ; SD = 0.175 ; N = 114).]
733 734	b)	Checks for Systematic Cell Seeding Errors
735	0)	To check for systematic cell seeding errors, untreated VCs are placed both at
736		the left side (row 2) and the right side (row 11 for the test plates) of the 96-
737 738		well plate. Aberrations in the cell monolayer for the VCs may reflect a volatile and toxic test substance present in the assay. If volatility is suspected,
739		then proceed to Section VI.E.6. Checks for cell seeding errors may also be
740 741		performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent.
742		
7.42		

743		Testing Volatile Substances
744		Although this test method is not suitable for highly volatile substances, mildly
745	•	volatile substances may be tested with some success. Volatile test substances
746	1	may generate vapors from the treatment media during the test substance treatment
747	i	incubation period. These vapors may become resorbed into the treatment medium
748		in adjacent wells, such that culture wells nearest the highest doses may become
749	(contaminated by exposure. If the test substance is particularly toxic at the doses
750		tested, the cross contamination may be evident as a significant reduction in
751		viability in the VC cultures (i.e., VC1) adjacent to the highest test substance
752		doses.
753		
754	1	If potential test substance volatility is suspected (e.g., for low density liquids) or if
755		the initial range finder test (non-sealed plate) results show evidence of toxic
756		effects in the control cultures (i.e., > 15 % difference in viability between VC1
757		[column 2] and VC2 [column 11]), then seal the subsequent test plates by the
758		following procedure.
759	1	tonowing procedure.
760		a) Plate Sealer Method
760 761	(
761		 d) Plates and substances will be prepared as usual according to Sections VI.D and VI.E.
763 764		e) Immediately after the 96-well culture plate has been treated with the
764		suspected volatile substance (Section VI.E.2.b), apply the adhesive plate
765		sealer (e.g., using a hand, microplate roller, etc.) directly over the culture
766		wells. Assure that the sealer adheres to each culture well (well tops should
767		be dry). Place the 96-well plate cover over the sealed plate and incubate
768		the plate under specified conditions (Section VI.E.2.b). [Note: Do not
769		jam the plate lid over the film to avoid deforming the sealer and causing
770		the sealer to detach from culture wells. Loose fit of the plate lid is
771		acceptable.]
772		f) At the end of the treatment period, the plate sealer should be carefully
773		removed to avoid spillage. Continue with the NRU assay as per Section
774		VI.E.4 .
775		
776	F. Data	a Analysis
777	•	The Study Director will use good biological/scientific judgment for determining
778	4	"unusable" wells that will be excluded from the data analysis and provide
779	(explanations for the removal of any data from the analysis.
780	•	A calculation of cell viability expressed as NRU is made for each concentration of
781	1	the test substance by using the mean NRU of the six replicate values (minimum of
782	t	four acceptable replicates wells) per test concentration. This value is compared
783	•	with the mean NRU of all VC values. Relative cell viability is then expressed as
784		percent of untreated VC. If achievable, the eight concentrations of each substance
785	-	tested will span the range of no effect up to total inhibition of cell viability.
786		Data from the microtiter plate reader should be transferred to a spreadsheet
787		template (e.g., Microsoft Excel®) that will automatically determine cell viability,
788		calculate IC_{50} values by linear interpolation, and perform statistical analyses
,		

- 789(including statistical identification of outliers). The template should also calculate790the concentrations associated with 20 %, 50 %, and 80 % viability using the Hill791slope and EC_{50} (i.e., IC_{50}) from the Hill function analysis (see ANNEX 1 for an792example spreadsheet template).
- A Hill function analysis should be performed using statistical software (e.g., GraphPad PRISM® 3.0) and a template to calculate IC₂₀, IC₅₀, and IC₈₀ values (and the associated confidence limits) for each test substance.
- 796 • Dose-responses for which the toxicity plateaus as concentration increases do not 797 fit the Hill function well when Bottom =0. To obtain a better model fit, 798 unconstrain the Bottom parameter so that the model calculates the Bottom value. 799 However, when Bottom $\neq 0$, the EC₅₀ reported by the Hill function $\neq 50\%$ 800 viability since the Hill function defines EC_{50} as the point midway between Top 801 and Bottom. To obtain the appropriate IC₅₀ when Bottom $\neq 0$, use the following 802 rearranged Hill function: 803

$$X = \log EC_{50} - \frac{\log\left(\frac{Top - Bottom}{Y - Bottom} - 1\right)}{HillSlope}$$

- X is the logarithm of concentration at 50% response, logEC₅₀ is logarithm of concentration at the response midway between Top and Bottom, Top is the maximum response, Bottom is the minimum response, Y = 50 (i.e., 50% response), and HillSlope describes the steepness of the curve.
 - [Note: IC_{50} values are used in a regression formula to predict the LD_{50} value of a test substance as an estimate of the starting dose for an acute oral toxicity test.]
- 814 VII. REFERENCES

810

811 812 813

815

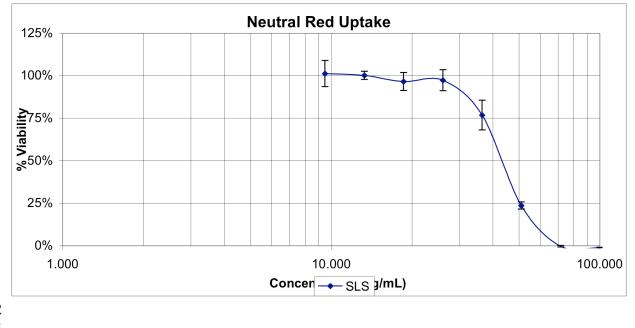
- 816Clonetics Normal Human Keratinocyte Systems Instructions for Use, AA-1000-4-817Rev.03/00. (http://www.clonetics.com).
- Hackenberg, U. and H. Bartling. 1959. Messen und Rechnen im pharmakologischen
 Laboratorium mit einem speziellen Zahlensystem (WL24-System). Arch. Exp. Pathol.
 Pharmakol. 235: 437-463.
- Triglia, D., P.T. Wegener, J. Harbell, K. Wallace, D. Matheson, and C. Shopsis. 1989.
 Interlaboratory validation study of the keratinocyte neutral red bioassay from
 Clonetics Corporation. In *Alternative Methods in Toxicology*, Volume 7. A.M.
 Goldberg, ed., pp. 357-365. Mary Ann Liebert, Inc., New York.
- 827
 828 *Test Method Protocol for Solubility Determination. In Vitro* Cytotoxicity Validation
 829 Study. Phase III. August 29, 2003. Prepared by The National Toxicology Program
 830 (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods
 831 (NICEATM). (http://iccvam.niehs.nih.gov/methods/invitro.htm)

832	U. S. Environmental Protection Agency. 1996. Product Properties Test Guidelines.
833	OPPTS 803.7840. Water Solubility: Column Elution Method; Shake Flask Method.
834	EPA712-C-96-041, Prevention, Pesticides and Toxic Substances, Washington DC.
835	
836	Test Method Protocol for the NHK Neutral Red Uptake Cytotoxicity Test. A
837	Test for Basal Cytotoxicity for an In Vitro Validation Study. November 4,
838	2004. Prepared by the National Toxicology Program (NTP) Interagency
839	Center for the Evaluation of Alternative Toxicological Methods (NICEATM).
840	(http://iccvam.niehs.nih.gov/methods/invitro.htm)
841	
842	Personal communication, Technical Services, Cambrex Bio Science,
843	Walkersville, MD
844	
845	
846	
847	
848	
849	

ANNEX 1 Microsoft EXCEL® Example Spreadsheet Template

To	st Facility :	^				- Study	/ Number.:	A1				
	cal Code :					96-Wel	Plate ID :	A1 A11				
	m. Code*:						riment ID :					
					96-WE	LL PLAT	E MAP					
	1	2	3	4	5	6	7	8	9	10	11	12
А	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
В	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
C	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
D	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
E	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
F	Blank	VC1 VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2 VC2	Blank
G	Blank	VC1 VC1	C1	C2	C3	C4 C4	C5	C6	C7	C8	VC2 VC2	Blank
			Blank									
Н	Blank	Blank	віалк	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
						BANCE D		OD\$50)				
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.044	0.044	0.045	0.045	0.045	0.046	0.051	0.057	0.057	0.043	0.041	0.044
B	0.042	0.456	0.043	0.043	0.130	0.300	0.395	0.414	0.418	0.402	0.401	0.042
С	0.043	0.407	0.042	0.041	0.130	0.294	0.383	0.382	0.413	0.375	0.385	0.044
D	0.043	0.438	0.042	0.043	0.147	0.337	0.409	0.404	0.438	0.436	0.391	0.047
E	0.044	0.448	0.041	0.045	0.132	0.321	0.429	0.414	0.416	0.420	0.441	0.042
F	0.045	0.411	0.040	0.042	0.127	0.375	0.397	0.402	0.422	0.447	0.403	0.043
G H	0.041	0.405	0.043	0.040	0.124	0.361	0.444	0.442	0.425	0.448	0.405	0.044
п	0.041	0.041	0.048	0.042	0.042	0.044	0.042	0.042	0.040	0.044	0.041	0.041
Max	0.045	0.456	0.043	0.045	0.147	0.375	0.444	0.442	0.438	0.448	0.441	0.047
Min	0.045	0.405	0.043	0.045	0.147	0.375	0.383	0.442	0.438	0.448	0.441	0.047
Next Max	0.041	0.403	0.040	0.040	0.124	0.294	0.383	0.362	0.413	0.375	0.385	0.041
Next Min	0.044	0.448	0.042	0.043	0.132	0.301	0.429	0.414	0.425	0.447	0.405	0.044
Rmax	-0.250	-0.157	-0.333	-0.400	-0.652	-0.173	-0.246	-0.467	-0.520	-0.014	-0.643	-0.500
Rmin	0.250	0.039	0.333	0.200	0.130	0.074	0.197	0.333	0.120	0.370	0.107	0.167
			RECTE	D ABSO		· ·		o - Mean				
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.001	-0.002	0.002	0.002	0.001	0.005	0.008	0.009	-0.001	-0.002	0.001
В	-0.001	0.413	-0.004	-0.001	0.087	0.255	0.349	0.365	0.370	0.359	0.358	-0.001
C	0.000	0.364	-0.005	-0.003	0.087	0.249	0.337	0.333	0.365	0.332	0.342	0.001
D E	0.000	0.395 0.405	-0.005 -0.006	-0.001 0.002	0.104 0.089	0.292	0.363	0.355 0.365	0.390 0.368	0.393	0.348	0.004
F	0.001	0.405	-0.007	-0.002	0.089	0.270	0.351	0.353	0.308	0.377	0.398	0.000
G	-0.002	0.362	-0.007	-0.004	0.004	0.316	0.398	0.393	0.377	0.405	0.362	0.000
H	-0.002	-0.002	0.002	-0.001	-0.001	-0.001	-0.005	-0.008	-0.009	0.001	-0.002	-0.002
Mean Blank =	0.043		0.047	0.044	0.044	0.045	0.047	0.050	0.049	0.044		
			RELA	ATIVE V	IABILITY	′ (% OF \	VEHICL	E CONT	ROL)			
					5	6	7	8	9	10	11	12
	1	2	3	4	5	•						
A	1		-				00.101	07 -01	00.001	00.101	00.001	
В	1	110.7%	-0.9%	-0.1%	23.2%	68.4%	93.4%	97.7%	99.0%	96.1%	96.0%	
B C	1	110.7% 97.6%	-0.9% -1.2%	-0.1% -0.7%	23.2% 23.2%	68.4% 66.7%	90.2%	89.1%	97.7%	88.9%	91.7%	
B C D	1	110.7% 97.6% 105.9%	-0.9% -1.2% -1.2%	-0.1% -0.7% -0.1%	23.2% 23.2% 27.7%	68.4% 66.7% 78.3%	90.2% 97.2%	89.1% 95.0%	97.7% 104.4%	88.9% 105.2%	91.7% 93.3%	
B C D E	1	110.7% 97.6% 105.9% 108.6%	-0.9% -1.2% -1.2% -1.5%	-0.1% -0.7% -0.1% 0.4%	23.2% 23.2% 27.7% 23.7%	68.4% 66.7% 78.3% 74.0%	90.2% 97.2% 102.5%	89.1% 95.0% 97.7%	97.7% 104.4% 98.5%	88.9% 105.2% 100.9%	91.7% 93.3% 106.7%	
B C D	1	110.7% 97.6% 105.9%	-0.9% -1.2% -1.2%	-0.1% -0.7% -0.1%	23.2% 23.2% 27.7%	68.4% 66.7% 78.3%	90.2% 97.2%	89.1% 95.0%	97.7% 104.4%	88.9% 105.2%	91.7% 93.3%	
B C D E F	1	110.7% 97.6% 105.9% 108.6% 98.7%	-0.9% -1.2% -1.2% -1.5% -1.7%	-0.1% -0.7% -0.1% 0.4% -0.4%	23.2% 23.2% 27.7% 23.7% 22.4%	68.4% 66.7% 78.3% 74.0% 88.5%	90.2% 97.2% 102.5% 94.0%	89.1% 95.0% 97.7% 94.5%	97.7% 104.4% 98.5% 100.1%	88.9% 105.2% 100.9% 108.2%	91.7% 93.3% 106.7% 96.5%	

Te	st Facility :	A				Stud	VNumber.:	A1				
Chemi	ical Code :	SLS				96-Wel	I Plate ID :	A11				
2nd Che	em. Code*:	11				Expe	riment ID :	XX				
		VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	
Conc	. (μg/mL) :	0.0	100	71.4	51.0	36.4	26.0	18.6	13.3	9.49	0.0	
Mean	Corr. OD :	0.385	-0.005	-0.001	0.088	0.286	0.363	0.360	0.374	0.378	0.361	
	SD :	0.023	0.001	0.002	0.008	0.033	0.023	0.020	0.009	0.029	0.020	
Mean Vehi	icle Control :	0.373										
Ме	ean Blank :	0.043										
% of Vehi	icle Control :	103.1%	-1.3%	-0.3%	23.6%	76.8%	97.3%	96.5%	100.1%	101.3%	96.9%	
	SD :	6.0%	0.3%	0.5%	2.1%	8.7%	6.2%	5.3%	2.4%	7.7%	5.2%	
	% CV :	5.86%	-25.1%	-150%	9.09%	11.4%	6.33%	5.47%	2.39%	7.59%	5.41%	
Mean VC	 C - VC1 (%) :	-3.1%										
	- VC2 (%) :	3.1%										
Mean At	bsolute OD :	0.416										
					Visu	ual Observat	ions					
		VC	C1	C2	C3	C4	C5	C6	C7	C8		
ENTER	R CODES:	1	4	4	3	2	1	1	1	1		
			lı	nterpolat	ed IC ₅₀ :	4.32E	+01	µg/mL				



)									
TEST CHE	MICAL								
	Test Facility :	А		St	udy Number.:	A1			
Che	Chemical Code : SLS			96-\	Vell Plate ID :	A11			
2 nd C	hem. Code*:	11		E	periment ID :	хх			
* Testing F	acility Acces	sion Code, if a	pplicable						
PREPARA	TION OF TE	ST CHEMICA	L		•				
			Solvent:	Medium			<u> </u>	ilution factor:	1.4
Solvent Co	onc. (%, v/v) i	n dosing soluti	ons :	N/A		Highest	Stock Conc.:	20,000	µg/mL
Aids used	to dissolve :	Vorte	exing	sc	nication	h	eating to 37C		
	pH (highest	medium stock	or 2X dosir	ng solution) :	8.0				
	Medium Cla	rity/Color (high	est 2X dosi	ng solution):	clear red		lf ppt, note	lowest conc.:	
				Concer	tration Series	(µg/mL)			
	C1	C2	C3	C4	C5	C6	C7	C8	
	100	71.4	51.0	36.4	26.0	18.6	13.3	9.49	
	Positive	Control (SLS)	100 - 9.49	µg/mL	•				
CELL LIN	E/TYPE								
	Name:	BALB/c 3T3		Supplier:	ATCC		Lot No.	not provided	
F	Passage No.:	69			No. in Assay:	75	Prolife	erating/frozen	24-May-02
	Medium:	DMEM		Supplier:			Lot No.:		
	Serum:	NCS		Supplier:			Lot No.:		
S	erum Conc.:		Growth Medium:		10%	Treatment Medium:		0%	
TEST ACC	CEPTANCE O	CRITERIA							
No. of	f values >50%	% and <100%:	3	No. (of values >0%	and <u><</u> 50%:	1	Accept?	YES
			VC: % Diff	erence betw	een Col 2 and	I mean VC.:	-3%	Accept?	YES
		PC: Hill Fu	unction R ² V	alue of SLS:	0.99			Accept?	YES
			PC:	IC50 of SLS:	43.2	µg/mL		Accept?	YES
TIMELINE									
	<u>Cell</u>	Seeding Date		Dose Ap	plication Date		OD ₅₅₀ Deterr	nination Date	
TEST RES	OLTS								
	VC: Mean C	orrected OD ₅₅₀ :	0.373				Hill Fun	ction R ² Value:	0.9869
	log IC20 :		µg/mL	log IC50 :	1.635E+00	µg/mL	log IC80 :	1.718E+00	µg/mL
	IC20 :	3.56E+01	µg/mL	IC50 :	4.32E+01	µg/mL	IC80 :	5.22E+01	µg/mL
			Test Ch	emical F.W.	288.4				
	IC20 :	0.12331183		IC50 :			IC80 :	0.18113599	mM
I									

859	ANNEX II
860	
861	TEST METHOD PROCEDURE
862	Solubility Determination of Test Substances
863	
864	
865	PROPOSAL
866	This procedure was designed to identify the solvent that would provide the highest soluble
867	concentration of a test substance so there would be uniform availability of the substance to
868	cells used for <i>in vitro</i> basal cytotoxicity testing. The solubility exercises can be performed in
869	a routine and repeatable manner and provide guidelines to effectively prepare test substances
870	for toxicity testing in the NRU test methods.
871	
872	TEST SYSTEM
873	The solubility test procedure is based on attempting to dissolve substances in various
874	solvents with increasingly rigorous mechanical techniques. The solvents to be used, in the
875	order of preference, are cell culture medium, DMSO, and ETOH. Determination of whether
876	a substance has dissolved is based entirely on visual observation for the purposes of this
877	protocol. A substance has dissolved if the solution is clear and shows no signs of cloudiness
878	or precipitation.
879	
880	PROCEDURES
881	Preparation of the keratinocyte medium and supplements will follow all procedures in the
882	NHK NRU protocol.
883	
884	Materials – see Section VI.A
885	
886	Preparations of Media and Solutions – see Section VI.B
887	All solutions glassware, pipettes, etc., should be sterile and all procedures should be carried
888	out under aseptic conditions and in the sterile environment of a laminar flow cabinet
889	(biological hazard standard). All methods and procedures should be adequately documented.
890	
891	Determination of Solubility
892	• Solubility should be determined in a step-wise procedure that involves attempting to
893	dissolve a test substance at a relatively high concentration with the sequence of
894	mechanical procedures specified in Mechanical Procedures. Table 1 and Figures 1 and
895	2 illustrate the step-wise procedures. The hierarchy of preference of solvent for
896	dissolving test substances is medium, DMSO, and then ETOH. If the substance does not
897	dissolve in the solvent, the volume of solvent is increased so as to decrease the test
898	substance concentration by a factor of 10, and then the sequence of mechanical
899	procedures are repeated in an attempt to solubilize the substance at the lower
900	concentrations.
901	• For testing solubility in medium, the starting concentration is 200,000 µg/ml (i.e., 200
902	mg/mL) in Tier 1, but for DMSO and ETOH the starting concentration is 200,000 μ g/ml
903	(i.e., 200 mg/mL) in Tier 3.

904 905 906 907 908 909 910 911 912	 Methods A. Tier 1 begins with testing 200 mg/mL in Routine Culture Medium (see Table 1). 1. Weigh approximately 100 mg (100,000 μg) of the test substance into a glass tube. Document the substance weight. 2. Add approximately 0.5 mL of medium into the tube so that the concentration is 200,000 μg/ml (200 mg/mL). 3. Mix the solution as specified in Mechanical Procedures. If complete solubility is achieved, then additional solubility procedures are not needed.
913 914 915 916 917 918 919 920 921	 B. If the test substance is insoluble in Tier 1 at 200 mg/mL, then proceed to Tier 2. 1. Weigh approximately 10 mg (10,000 µg) of the test substance into a glass tube. Document the substance weight. 2. Add approximately 0.5 mL of medium into the tube so that the concentration is 20,000 µg/ml (20 mg/mL). 3. Mix the solution as specified in Mechanical Procedures. If complete solubility is achieved, then additional solubility procedures are not needed.
922 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936	 C. If the test substance is insoluble in Routine Culture Medium, proceed to Tier 3. 1. Add enough medium, approximately 4.5 mL, to attempt to dissolve the substance at 2 mg/mL by using the sequence of mixing procedures. If the test substance dissolves in medium at 2 mg/mL, no further procedures are necessary. 2. If the test substance does NOT dissolve in medium, weigh out approximately 100 mg test substance in a second glass tube and add enough DMSO to make the total volume approximately 0.5 mL (for 200 mg/mL) and mix the solution as specified in Mechanical Procedures. 3. If the test substance does not dissolve in DMSO, weigh out approximately 100 mg test substance in another glass tube and add enough ETOH to make the total volume approximately 0.5 mL (for 200 mg/mL) and mix the solution as specified in Mechanical Procedures. 4. If the substance is soluble in either solvent, no additional solubility procedures are needed.
937 938 939 940 941 942 943 944 945 946	 D. If the substance is NOT soluble in Routine Culture Medium, DMSO, or ETOH at Tier 3, then continue to Tier 4 in Table 1. 1. Add enough solvent to increase the volume of the three (or four) Tier 2 solutions by 10 and attempt to solubilize again using the sequence of mixing procedures. If the test substance dissolves, no additional solubility procedures are necessary. 2. If the test substance does NOT dissolve, continue with Tier 5 and, if necessary, Tier 6 using DMSO and ETOH. 3. Tier 5 begins by diluting the Tier 4 samples with DMSO or ETOH to bring the total volume to 50 mL. The mixing procedures are again followed to attempt to solubilize the substance.

- 947
 948
 948
 948
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
 949
- 950

951 Example

- If complete solubility is not achieved at 20,000 μg/mL in Routine Culture Medium at
 Tier 2 using the mixing procedures, then the procedure continues to Tier 3 by diluting the
 solution to 5 mL with medium and mixing again.
- If the substance is not soluble in Routine Culture Medium, two samples of ~ 100 mg test substance are weighed to attempt to solubilize in DMSO and ETOH at 200,000 µg/mL
 (i.e., 200 mg/mL). Solutions are mixed following the sequence of procedures prescribed
- 958 in **Mechanical Procedures** in an attempt to dissolve.
- If solubility is not achieved at Tier 3, then the solutions prepared in Tier 3 are diluted by
 10 so as to test 200 μg/mL in media, and 20,000 μg/mL in DMSO and ETOH. This
 advances the procedure to Tier 4. Solutions are again mixed in an attempt to dissolve.
- If solubility is not achieved in Tier 4, the procedure continues to Tier 5, and to Tier 6 if necessary (see Figures 1 and 2 and Table 1).

965 MECHANICAL PROCEDURES

- A. The following hierarchy of mixing procedures will be followed to dissolve the test substance:
- Add test substance to solvent as in Tier 1 of Table 1. (Test substance and solvent should be at room temperature.)
- 970 2. Gently mix at room temperature. Vortex the tube (1 2 minutes).
- 971 3. If test substance has not dissolved, use waterbath sonication for up to 5 minutes.
- 972
 973
 974
 975
 975
 975
 976
 977
 978
 979
 979
 979
 970
 970
 970
 971
 972
 973
 974
 974
 974
 975
 975
 975
 975
 976
 976
 977
 978
 979
 979
 974
 974
 974
 974
 975
 974
 975
 975
 975
 975
 976
 976
 976
 977
 978
 978
 979
 979
 970
 970
 970
 971
 972
 972
 972
 974
 975
 974
 975
 975
 975
 975
 975
 976
 976
 976
 977
 978
 978
 978
 979
 979
 979
 970
 970
 970
 971
 972
 972
 972
 974
 975
 974
 975
 974
 975
 974
 975
 975
 974
 975
 974
 975
 974
 975
 974
 975
 974
 975
 974
 975
 974
 975
 974
 975
 974
 975
 975
 975
 975
 975
 975
 976
 976
 976
 977
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
 978
- 5. Proceed to Tier 2 (and Tiers 3-6, if necessary of **Table 1** and repeat procedures 2-4).
- B. The preference of solvent for dissolving test substances is Routine Culture Medium,
- DMSO, and then ETOH. Thus, if all solvents for a particular tier are tested
 simultaneously and a test substance dissolves in more than one solvent, then the choice of
 solvent follows this hierarchy. For example, if, at any tier, a substance were soluble in
 Routine Culture Medium and DMSO, the choice of solvent would be medium. If the
 substance were insoluble in medium, but soluble in DMSO and ETOH, the choice of
 solvent would be DMSO for both assays.
- 985
- 986

987 Table 1 Determination of Solubility in Routine Culture Medium, DMSO, or

988

ETOH

989

Tier	1	2	3	4	5	6
Total Volume Routine Culture Medium	0.5 mL	0.5 mL	5 mL	50 mL		
Concentration of Test Substance <u>Tier 1</u> : Add ~ 100 mg to a tube. Add enough medium to equal Tier 1 volume. If insoluble, go to Tier 2. <u>Tier 2</u> : Add ~10 mg to another tube. Add enough medium to equal the first volume. Dilute to subsequent volumes if necessary.	200,000 μg/mL (200 mg/mL)	20,000 μg/mL (20 mg/mL)	2,000 μg/mL (2 mg/mL)	200 μg/mL (0.20 mg/mL)		
Total Volume DMSO/			0.5 mL	5 mL	50 mL	
Concentration of Test Substance (Add ~100 mg to a large tube. Add enough DMSO or ETOH to equal the first volume. Dilute with subsequent volumes if necessary.)			200,000 μg/mL (200 mg/mL)	20,000 μg/mL (20 mg/mL)	2,000 μg/mL (2 mg/mL)	
Total Volume DMSO/ETOH						50 mL
Concentration of Test Substance (Add ~10 mg to a large tube. Add enough DMSO or ETOH						200 μg/mL (0.2 mg/mL)
to equal 50 mL.) EQUIVALENT CONCENTRATION ON	100,000 µg/mL	10,000 μg/mL	1000 μg/mL	100 μg/mL	10 µg/mL	1 μg/mL
CELLS	(100 mg/mL)	(10 mg/mL)	(1 mg/mL)	(0.1 mg/mL)	(0.01 mg/mL)	(0.001 mg/mL)

990

991 [NOTE: The amounts of test substance weighed and Routine Culture Medium added may be

992 modified from the amounts given above, provided that the targeted concentrations specified

993 for each tier are tested.]

FIGURE 1 SOLUBILITY STEP-WISE (TIERED) PROCEDURE

	TIER 1
STEP 1:	200 mg/mL test substance (TS) in 0.5 mL Routine Culture Medium
	• if TS soluble in medium, then <u>STOP</u> .
	• if TS insoluble in medium, then go to STEP 2.
	TIER 2
STEP 2:	20 mg/mL TS in 0.5 mL Routine Culture Medium
	• if TS soluble, then <u>STOP</u> .
	• if TS insoluble, then go to STEP 3.
	TIER 3
STEP 3:	200 mg/mL TS in DMSO
	• if TS soluble, then <u>STOP</u> .
	• if TS insoluble, test at 200 mg/mL in ETOH.
	• if TS soluble, then <u>STOP.</u>
	• If TS insoluble, go to STEP 4.
	TIER 4
STEP 4:	0.2 mg/mL TS in medium (one or both) – increase volume from STEP 2 by 10 (i.e., to 50 mL)
	• if TS soluble in both media, then <u>STOP</u> .
	• if TS insoluble in one medium, test at 20 mg/mL in DMSO – increase volume from STEP 3
	by 10 (i.e., to 5 mL).
	• if TS soluble, then <u>STOP</u> .
	 if TS insoluble, test at 20 mg/mL in ETOH – increase volume from STEP 3 by 10 (i.e., to 5 mL).
	• if TS soluble, then STOP.
	 if TS insoluble, then go to STEP 5.
	TIER 5
STEP 5:	2 mg/mL TS in DMSO – increase volume from STEP 4 by 10 (i.e., to 50 mL)
	• if TS soluble, then <u>STOP.</u>
	 if TS insoluble, test at 2 mg/mL in ETOH – increase volume from STEP 4 by 10 (i.e., to 50 mL).
	• if TS soluble, then STOP.
	• if TS insoluble, then go to STEP 6.
	~

TIER 6

STEP 6:	0.2 mg/mL TS in 50 mL DMSO
	• if TS soluble, then <u>STOP.</u>
	• if TS insoluble, test at 0.2 mg/mL in 50 mL ETOH
	• <u>STOP</u>

Figure 2Solubility Flow Chart

Tier	1		2		3		4		5		6
Concentration in Medium	Start Here 200 mg/mL	Incomplete solubility	20 mg/mL	Incomplete solubility	2 mg/mL		• 0.20 mg/mL				
					Incomplete solubility		Incomplete solubility				
Concentration in DMSO					200 mg/mL		20 mg/mL		· 2 mg/mL		▶0.2 mg/mL
					Incomplete solubility		Incomplete solubility		Incomplete solubility		Incomplete solubility
Concentration in ETOH					200 mg/mL	Incomplete	20 mg/mL -	Incomplete solubility	2 mg/mL	Incomplete	0.2 mg/mL End
Concentration on Cells	100 mg/mL		10 mg/mL		1 mg/mL		0.1 mg/mL		0.01 mg/mL		0.001 mg/mL

Notes: NHK medium - Keratinocyte Growth Medium (e.g., KGM® from Cambrex) for normal human keratinocytes.

ANNEX III

TEST METHOD PROCEDURE Prequalification of Normal Human Epidermal Keratinocyte Growth Medium

This annex provides the guidelines and testing requirements for prequalifying manufacturer lots of Keratinocyte Basal Medium and the medium supplements for use with the Test Method Protocol for the NHK Neutral Red Uptake (NRU) Cytotoxicity Test. The medium and supplements should be tested so as to demonstrate their ability to perform adequately in the recommended assay.

The Testing Facility should request the quality control (QC) test data from the manufacturer for each potential lot of medium and supplements. Based upon the QC test data, purchase and test the one or two most current lots of medium and supplements that appear to have the potential to support NHK cultures according to the requirements of the aforementioned protocol.

TEST SYSTEM

The NHK NRU test is performed to analyze NHK growth characteristics and the *in vitro* toxicity of sodium lauryl sulfate (SLS), as measured by the IC₅₀, with each NHK medium/supplements being tested.

Every combination of medium/supplements expected to be used should be tested. Potential medium testing/supplement combinations are:

- One lot of medium/one lot of supplements: Test the lot of medium using the lot of supplements.
- Two or more lots of medium/one lot of supplements: Test each lot of medium using the one lot of supplements.
- One lot of medium/two or more lots of supplements: Test the lot of medium using each lot of supplements.

NHK cultures should be established using each medium/supplement combination to be tested, and should be subcultured on three different days into 96-well plates (1 plate per day) for three subsequent SLS cytotoxicity tests using each test medium/supplement combination along with a control medium (if available) for which performance has been previously established.

PROCEDURES

Prequalification of the keratinocyte medium and supplements will follow all procedures in the NHK NRU protocol.

Materials – see Section VI.A

Preparations of Media and Solutions – see Section VI.B

Methods – *see Section VI.C*

- NHK cultures should be established with cryopreserved cells seeded into individual tissue culture 25 cm² flasks using a proven medium/supplement combination (i.e., the "control" medium) and each test medium/supplement combination.
- Suspend freshly thawed cells initially into 9 mL of control medium and then add the cell suspension to 25 cm² culture flasks containing pre-warmed control or test medium. Cell seeding densities (1 flask/density/medium) of 1 x 10⁴, 5 x 10³, and 2.5 x 10³ are recommended.
- The cells should be subcultured on three different days into 96-well plates for three subsequent NRU tests (three test plates total [one plate per day] for each medium/supplement combination and each control).

Flask	lture: 1 Test Plate and 1	Application of SLS			
	Control Plate				
$10^4 \text{ cells/mL})$	Day A	Day X			
10 ³ cells/mL)	Day B	Day Y			
x 10 ³ cells/mL)	Day C	Day Z			

• Subculturing the cells and application of the SLS will follow procedures in the protocol in reference to appropriate cell confluency. Cell numbers should be recorded for each flask prior to subculturing to the 96-well plates.

[Note: Use of a control medium assumes that the Testing Facility has recent experience with a medium/supplement combination proven to support adequate NHK growth and provide adequate sensitivity to SLS. It is not absolutely necessary to use a control medium.]

Doubling Time – see Section VI.C.5

A doubling time experiment may be considered as an additional quality assurance check.

Preparation of SLS – see Sections VI.D.1.a, b, and d

Preparation of SLS concentrations/dilutions should follow the main experiment (definitive assay) procedures specifically for testing compounds in Routine Culture Medium as outlined in **Section VI.D.3.b**. The concentrations/dilutions should be the same or similar to those used previously with control medium/supplements. SLS concentration ranges used by three laboratories in the NICEATM/ECVAM validation study were 20.0 μ g/mL - 1.4 μ g/mL and 10.0 μ g/mL - 0.6 μ g/mL.

Test Procedure – see Sections VI.E.1, E.2, and E.4

The C_1 test concentration will be the highest SLS concentration and C_8 the lowest concentration. Cells cultured in control medium and in each test medium/supplement combination should be tested in parallel for their sensitivity to SLS (see **Methods**). Each of the three test plates of the new medium/supplement combinations is considered a replicate test plate.

Microscopic Evaluation – see Section VI.E.3

Changes in morphology of the cells due to cytotoxic effects of the SLS (prior to

measurement of NRU) should be recorded as per procedures outlined in Section VI.E.3.

In addition to the general microscopic evaluation of the cell cultures, the Study Director

should make the following specific observations:

General culture observations

- rate of proliferation (e.g., rapid, fair, slow)
- percent confluence (e.g., daily estimate);
- number of mitotic figures (e.g., average per field);
- contamination (present/not present)

Cell morphology observations

- overall appearance (e.g., good, fair, poor)
- colony formation (e.g., tight/defined, fair, loose/migrating)
- distribution (e.g., even/uneven)
- abnormal cells (e.g., enlarged, vacuolated, necrotic, spotted, blebby [average per field])

Data Analysis and Test Evaluation see Sections VI.E.5 and VI.F

Test Acceptance Criteria in section VI.E.5 will be used to determine acceptability of a test plate. Other criteria that should be considered by the Study Director includes the following:

• Mean corrected $OD_{540-550}$ of the VCs [Note: The target range for corrected mean $OD_{540 \pm 10nm} = 0.248 - 1.123$ for the VCs, but it is not a test acceptance criterion (range = mean $OD \pm 2.5$ standard deviations; mean = 0.685; SD = 0.175; N = 114).]

• Cell morphology and confluence of the VCs at the end of the 48 hour treatment

• Doubling time

The Study Director should utilize all observed growth characteristics and test results in addition to comparison of results to the media manufacturer's QC data to determine whether the medium/supplements combinations perform adequately. The Testing Facility should request that the manufacturer reserve a portion of an acceptable lot based on estimates of media need.

[This Page Intentionally Left Blank]