Substance Name	CASRN	ER TA Activity ^{1,2}
Oxazepam	604-75-1	PN
Pimozide	2062-78-4	PN
Reserpine	50-55-5	PN
Spironolactone	52-01-7	PN
L-thyroxine	51-48-9	PN

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

1+++ Indicates that the substance was strongly active (EC₅₀ value was <0.001 M);

++ indicates that the substance was moderately active (EC₅₀ value was between 0.001 and 0.1 M); + indicates that the substance was weakly active (EC₅₀ value was >0.1 M), or a positive response was reported without an EC₅₀ value. The EC₅₀ is the effective concentration that causes half-maximal activation of the receptor.

2PP = Presumed Positive; PN - Presumed Negative

3Included on the ECVAM Provisional Chemicals Selection List

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2. SUBSTANCES FOR EACH PHASE OF THE VALIDATION OF ANTAGONIST PROTOCOLS

2.1 Phase I

- Training and laboratory qualification/protocol refinement by testing reference standards and controls
- Establish historical database for standards and controls by conducting ten independent experiments

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Substance Name	CASRN
Raloxifene HCl	82640-04-8
(Reference Standard) ¹	82040-04-8
Flavone	525-82-6
(Weak Positive Control)	323-62-0
17ß estradiol	50-28-2
(Negative Control)	30-28-2

72 73 Abbreviations: CASRN = Chemical Abstracts Service Registry Number ¹In the LUMI-CELL antagonist assay

75 76 ¹In the LUMI-CELL antagonist assay the reference standard, positive control and all test substances are run against a fixed concentration of 17ß-estradiol.

77 **2.2** Phase IIa

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- Four substances from ER minimum list tested independently three times in each laboratory for antagonism (12 total experiments)
- Substances to be well-characterized for ER TA antagonist activity (one strong positive, one moderately positive, one weakly positive and one negative); having no anticipated difficulties relating to solubility or cytotoxicity

Substance Name	CASRN	ER TA Antagonist Activity ¹
Tamoxifen ²	10540-29-1	###
Dibenzo[a.h]anthracene ²	53-70-3	##
p-n-nonylphenol ²	104-40-5	#
Progesterone ^{2,3}	57-83-0	

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

1### Indicates that the substance was uniformly positive in multiple assays,

indicates that the substance was positive in the majority of assays in which it was tested;

indicates that the substance was positive in the single assay in which it was tested;

#- indicates the substance was positive in one assay but was also negative in one or more assays;
- indicates that the substance was uniformly negative in multiple assays; PP = Presumed Positive;
PN - Presumed Negative

²Tested for antagonism during LUMI-CELL protocol standardization.

³Included on the ECVAM Provisional Chemicals Selection List

2.3 Phase IIb

- Eight substances from ER minimum list tested independently three times in each laboratory for antagonism (24 total experiments)
- Substances to be well-characterized for ER TA antagonist activity (a mix of strong positive, moderately positive, weakly positive and negative); with some anticipated difficulties relating to solubility or cytotoxicity

Substance Name	CASRN	ER TA Antagonist Activity ¹	Anticipated Difficulty
Flavone ²	525-82-6	###	
Apigenin	520-36-5	#	
Resveratrol	501-36-0	#	Withhold Control
Atrazine	1912-24-9	-	
Butylbenzyl phthalate ²	85-68-7		***************************************
Corticosterone	50-22-6	-	

Substance Name	CASRN	ER TA Antagonist Activity ¹	Anticipated Difficulty
o.p-DDT ^{2,3}	789-02-6	#	Cytotoxic, can potentially "stick" to plastic tissue cultureware
Genistein ^{2,3}	446-72-0	#	Relatively insoluble

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

1### Indicates that the substance was uniformly positive in multiple assays;

indicates that the substance was positive in the majority of assays in which it was tested;

indicates that the substance was positive in the single assay in which it was tested;

#- indicates the substance was positive in one assay but was also negative in one or more assays; - indicates that the substance was uniformly negative in multiple assays;

PP = Presumed Positive; PN - Presumed Negative

2.4 Phase III

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 Remaining 41 substances from ER minimum list tested once in each laboratory for antagonism (41 total experiments)

Substance Name	CASRN	ER TA Antagonist Activity ¹
4-hydroxytamoxifen ²	68047-06-3	###
Raloxifene HCl ²	82640-04-8	###
Bisphenol A	80-05-7	-
Coumestrol	479-13-0	-
Daidzein	486-66-8	_
p.p '-DDE	72-55-9	-
Dicofol	115-32-2	_
Diethylstilbestrol	56-53-1	-
17α - ethinyl estradiol ²	57-63-6	-
Estrone	53-16-7	-
Fluoranthene	206-44-0	-
Kaempferol	520-18-3	-
p,p'-methoxychlor	72-43-5	-
Di - n -butyl phthalate ²	84-74-2	-
Vinclozolin ²	50471-44-8	PP
17α-Estradiol	57-91-0	PN
Actinomycin D	57-76-0	PN
4-androstenedione	63-05-8	PN
Bisphenol B	77-40-7	PN
2-sec-butylphenol	89-72-5	PN
Clomiphene citrate	50-41-9	PN
4-cumylphenol	599-64-4	PN

²Tested for antagonism during LUMI-CELL protocol standardization.

³Included on the ECVAM Provisional Chemicals Selection List

Substance Name	CASRN	ER TA Antagonist Activity ¹
Dexamethasone ²	50-02-2	PN
5α-dihydrotestosterone	521-18-6	PN
17ß-estradiol ²	50-28-2	PN
meso-hexestrol ²	84-16-2	PN
Hydroxyflutamide	52806-53-8	PN
Kepone	143-50-0	PN
Morin	480-16-0	PN
Norethy nodrel ²	68-23-5	PN
4-tert-octylphenol ²	140-66-9	PN
Ethyl paraben	120-47-8	PN
Phenobarbital	50-06-6	PN
Phenolphtalin	81-90-3	PN
Diethylhexyl phthalate	117-81-7	PN
Propylthiouracil	51-52 - 5	PN
Sodium azide	26628-22-8	PN
Testosterone ²	58-22-0	PN
Methyl testosterone	28-18-4	PN
12 - O - Tetradecanoylphorbol- 13-acetate	16561-29-8	PN
2,4,5- Trichlorophenoxyacetic acid	93-76-5	PN

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123 124 Abbreviations: CASRN = Chemical Abstracts Service Registry Number 1### Indicates that the substance was uniformly positive in multiple assays;

indicates that the substance was positive in the majority of assays in which it was tested;

indicates that the substance was positive in the single assay in which it was tested;

#- indicates the substance was positive in one assay but was also negative in one or more assays;

- indicates that the substance was uniformly negative in multiple assays; PP = Presumed Positive; PN - Presumed Negative

³Included on the ECVAM Provisional Chemicals Selection List

2.5 Phase IV

Testing of remaining 25 substances from ER list for antagonism tested once in one laboratory (may be divided between participating laboratories) (25 total experiments)

Substance Name	CASRN	ER TA Antagonist Activity ¹
Fenarimol	60168-88-9	#
Fluoxymestrone	76-43-7	-
Ammonium perchlorate	7790-98-9	PN
Apomorphine	58-00-4	PN

NICEATM Draft Proposed Distribution of Substances During Phased International ED Validation

		ER TA
Substance Name	CASRN	Antagonist
		Activity ¹
Bicalutamide	90357-06-5	PN
Chrysin	480-40-0	PN
Cycloheximide	66-81-9	PN
Cyproterone acetate	427-51-0	PN
Finasteride	98319-26-7	PN
Haloperidol	52-86-8	PN
4-hydroxy	566-48-3	PN
androstenedione ²	300-46-3	LIN
Ketoconazole	65277-42-1	PN
Linuron ²	330-55-2	PN
Medroxyprogesterone	71-58-9	PN
acetate		
Mifepristone ²	84371-65-3	PN
Nilutamide	63612-50-0	PN
19-nortestosterone	434-22-0	PN
Oxazepam	604-75-1	PN
Pimozide	2062-78-4	PN
Procy midone	32809-16-8	PN
Reserpine	50-55-5	PN
Spironolactone	52-01-7	PN
L-thyroxine	51-48-9	PN
17ß-trenbolone	10161-33-8	PN
Flutamide ²	13311-84-7	PP

Abbreviations: CASRN = Chemical Abstracts Service Registry Number ¹### Indicates that the substance was uniformly positive in multiple assays; 125 126

indicates that the substance was positive in the majority of assays in which it was tested;

indicates that the substance was positive in the single assay in which it was tested;

#- indicates the substance was positive in one assay but was also negative in one or more assays;

- indicates that the substance was uniformly negative in multiple assays; PP = Presumed Positive;

PN - Presumed Negative

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²Included on the ECVAM Provisional Chemicals Selection List

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6	
7	VALIDATION OF THE LUMI-CELL® ESTROGEN RECEPTOR
8	TRANSCRIPTIONAL ACTIVATION ASSAYS FOR THE DETECTION OF
9	ESTROGEN RECEPTOR AGONISTS AND ANTAGONISTS
10	
11	
12	
13	
14	
15	
16	Prepared by
17	
18	Integrated Laboratory Systems, Inc.
19	for Contract (TBA) Supporting
20	The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative
21	Toxicological Methods (NICEATM)
22	
23	1 November 2006

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111		STATEMENT OF WORK			
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113		Validation of the LUMI-CELL® Estrogen Receptor (ER)			
114		Transcriptional Activation (TA) Assay for the Detection of			
115		ER Agonists and Antagonists			
116					
117	1.0 P	ROJECT OBJECTIVES AND GENERAL REQUIREMENTS			
118	1.1 P	roject Objectives			
119	This Statem	ent of Work specifies the procedures that Xenobiotic Detection Systems, Inc. (XDS)			
120	will use as a participating laboratory in the conduct of an international validation study of the				
121	LUMI-CELL® estrogen receptor (ER) transcriptional activation (TA) assay (LUMI-CELL® ER				
122	assay) for the detection of ER agonists and antagonists. The list of 78 ICCVAM recommended				
123	substances, which possess varying degrees of ER agonist and/or antagonist activity (ICCVAM				
124	2002; ICCVAM 2003; Federal Register, Vol. 71, No. 51, pp. 13597-13598, March 16, 2006;),				
125	will be used in this validation study to characterize the reliability and relevance of the LUMI-				
126	CELL® ER	assay.			
127	1.2 G	Seneral Capabilities			
128		there to this Statement of Work throughout the validation study and is capable of the			
129	following:				
130	1.	Transfer of the second			
131		performance of the LUMI-CELL® ER TA agonist and antagonist assays			
132	2.	Conducting all aspects of the study in accordance with Good Laboratory Practices			
133		(GLP)			
134	3.	Providing study reports and all associated data from studies outlined in this			
135		document (e.g.,) to the Study Management Team (SMT) through the designated			
136		contacts listed in Section 2.2.			
127					

137	1.3	Guidelines		
138	The Proj	ect Officer and designated members of the SMT may inspect the XDS testing facilities		
139	and audi	t any procedures. XDS should notify the SMT of any changes in Key Personnel (see		
140	Section	3.1.1)		
141	1.4	Definitions		
142	Good L	aboratory Practices (GLPs): Regulations governing the conduct, procedures, and		
143	operation	ns of toxicology laboratories developed to assure the quality and integrity of the data and		
144	to addre	ss such matters as organization and personnel, facilities, equipment, facility operations,		
145	and stud	y conduct (OECD, 1998).		
146				
147	Standar	rd Operating Procedures (SOPs): Written documents that describe in sufficient detail		
148	the routi	ne procedures to be followed for a specific operation, analysis, or action. Consistent use		
149	of an approved SOP ensures conformance with organizational practices; reduced work effort;			
150	reduction in error occurrences; and improved data comparability, credibility, and defensibility,			
151	SOPs al	so serve as resources for training and for ready reference and documentation of proper		
152	procedu	res.		
153				
154	Stateme	ent of Work: A description of all phases of the validation study and the purpose of the		
155	procedu	res; also provides guidance for the preparation of reports.		
156				
157	Test Mo	ethod Protocols: Specific and detailed guides for performing the LUMI-CELL® ER		
158	assay fo	r the detection of ER agonists and antagonists.		
159				
160	Test Su	bstances: Chemicals supplied to XDS that are coded and distributed such that only the		
161	Project	Officer, the SMT, and the Substance Inventory and Distribution Management (identified		
162	in Secti	on 2.2.2) have knowledge of the identity of each test substance. The test substances will		
163	be purcl	nased, aliquoted, coded, and distributed by the Substance Inventory and Distribution		
164	Manage	ment, under the guidance of the Project Officer and the SMT.		

166	2.0	ORGANIZATION
100	A-0	VIOANIZATION

- 167 2.1 Validation Study Sponsors
- 168 The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative
- 169 Toxicological Methods (NICEATM)
- 170 The European Centre for the Validation of Alternative Methods (ECVAM)
- 171 The Japanese Center for the Validation of Alternative Methods (JaCVAM)

- 173 2.2 Study Management
- 174 2.2.1 <u>International Study Management Team</u>
- 175 2.2.1.1 NICEATM
- 176 Dr. William Stokes (NICEATM/NIEHS) Co-Chair/Project Officer
- 177 Dr. Raymond Tice (NICEATM/NIEHS) Co-Chair
- 178 Dr. David Allen (NICEATM/ILS) NICEATM Principal Investigator
- 179 Mr. Frank Deal (NICEATM/ILS) Project Coordinator
- 180 Ms. Patricia Ceger (NICEATM/ILS) Assistant Project Coordinator
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- 183 Bldg. 4401, MD-EC-17
- 184 3rd Floor, Room 3126
- 185 P.O. Box 12233
- 186 Research Triangle Park, NC 27709
- 187 2.2.1.2 *ECVAM*
- 188 Dr. Susanne Bremer
- 189 Dr. Miriam Jacobs
- 190 Mailing Address:
- 191 Joint Research Center European Commission
- 192 21020 Ispra (VA), Italy

193	2.2.1.3	JaCVAM
エンン	4.4.1.	04407711171

- 194 Dr. Hajime Kojima
- d 0 ~
- 195 Dr. Jun Kanno
- 196 Mailing Address:
- 197 National Institute of Health Sciences
- 198 Kamiyouga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan
- 199 2.2.2 Substance Inventory and Distribution Management
- 200 Dr. Cynthia Smith
- 201 Chemistry Resources Group Leader
- 202 Mailing Address:
- 203 NIEHS
- 204 111 Alexander Dr.
- 205 Research Triangle Park, NC 27709
- 206 2.2.3 Contract Management
- 207 Mr. Doug Winters (NICEATM/ILS) NICEATM Project Manager
- 208 Ms. Kelly Inman (ILS) Contract Specialist
- 209 Mailing Address:
- 210 ILS, Inc.
- 211 P.O. Box 13501
- 212 Research Triangle Park, NC 27709
- 213 3.0 TESTING FACILITY AND KEY PERSONNEL
- 214 3.1 Competence and Capabilities
- 215 XDS should be competent in the conduct of the LUMI-CELL® ER assay and will provide
- 216 competent personnel, adequate facilities, equipment, supplies, proper health and safety
- 217 guidelines, and quality assurance procedures.

- 218 3.1.1 Personnel
- 219 3.1.1.1 Facility Management
- 220 XDS facility management is responsible for establishing scientific guidelines and procedures.
- training and supervision of technical staff, and evaluation of results. The facility manager must
- 222 maintain training files that include qualifications, experience, and a job description for each
- 223 individual involved in the LUMI-CELL® ER assay validation study.
- 224 3.1.1.2 Study Director
- 225 The Study Director has the overall responsibility for the LUMI-CELL® ER assay validation
- study conducted at XDS. The Study Director should be responsible for providing GLP compliant
- 227 Standard Operating Procedures (OECD 1998) for use during the validation study.
- 228 3.1.1.3 Director of Quality Assurance (QA)
- 229 The Director of QA should monitor the validation study to assure compliance with GLP
- 230 requirements for all aspects of the validation study.
- 231 3.1.1.4 *Consultant(s)*
- 232 Consultants are scientists or other professionals of appropriate education, training, and
- 233 experience with the LUMI-CELL® ER assay who provide scientific guidance to XDS.
- 234 3.1.1.5 Laboratory Technician(s)
- Each individual engaged in the conduct of or responsible for the supervision of the assay should
- have education, training, and experience, or combination thereof, to enable that individual to
- perform the assigned duties. Technical ability must be documented as per GLP requirements.
- 238 3.1.1.6 Safety Officer
- A designated Safety Officer (someone not involved in the actual conduct of the validation study)
- 240 will receive the blinded (coded) test substances from Substance Inventory and Distribution
- 241 Management and transfer the substances to the Study Director. A sealed health and safety
- 242 information package will accompany the coded test substances and the Safety Officer should
- retain the package until the completion of the validation study. The Safety Officer will promptly
- 244 notify the SMT Project Coordinator if this is opened at any time during the validation study.

- 245 3.1.2 Facilities, Equipment, and Supplies
- 246 3.1.2.1 *Cell Culture Laboratory*
- 247 A designated cell culture laboratory should be available to ensure that the LUMI-CELL® ER
- 248 assay can be performed using good cell culture practice (Coecke et al. 2005). Access to the
- validation study assays and reference substances should be restricted to appropriate personnel as
- 250 determined by XDS management.
- 251 3.1.2.2 Equipment
- 252 Appendices B and C detail the types of equipment that are required for conducting the LUMI-
- 253 CELL® ER agonist and antagonist assays. All equipment maintenance and calibration should be
- routinely performed and documented as per GLP guidelines (OECD. 1998).
- 255 3.1.3 Health and Safety
- 256 XDS should conform to all local, state, and federal statutes in effect at the time of this validation
- 257 study. The designated Safety Officer should be the point of contact for health and safety issues.
- 258 3.1.4 Ouality Assurance
- 259 XDS should conduct this validation study in compliance with Good Laboratory Practice (GLP)
- 260 Standards (OECD 1998). The QA unit (as per GLPs) should review the protocol and audit the in-
- life phase, study workbook, and final report data.
- 262 The final reports for all phases of the validation study should be audited by the XDS QA unit for
- 263 GLP compliance and a QA Statement should be provided with each final report. Each final report
- should identify: 1) the phases and data inspected, 2) the dates of inspection, and 3) the dates
- 265 findings were reported to the Study Director and XDS management. The QA Statement should
- 266 identify whether the methods and results described in the final report accurately reflect the raw
- 267 data produced during the validation study.

268 4.0 TEST PHASES AND SCHEDULE

4.1 Study Timeline and Deliverables

270 4.1.1 Study Timeline

269

TASK	ACTIVITIES	TIMELINE
Phase I	 Development of automated testing procedures (XDS) Qualification/protocol refinement by testing reference standards and controls Establish historical database for standards and controls by conducting independent experiments (10 each for the agonist and antagonist protocols) Submission of draft report and review by SMT 	Nov. 06 – Mar. 07
Phase IIa	Four substances from ER minimum list tested independently three times for agonism and antagonism (24 total experiments to include the quantitative assessment of cell viability in parallel plates in the agonist and antagonist assays) Submission of draft report and review by SMT	Apr. 07 – May 07
Phase IIb	Eight substances from ER minimum list tested independently three times (48 total experiments) Submission of draft report and review by SMT	Jun. 07 – Aug. 07
Phase III	Remaining 41 substances from ER minimum list tested once for agonism and antagonism (82 total experiments) Submission of draft report and review by SMT	Sep. 07 – Oct. 07
Phase IV	 Testing of remaining 25 substances from ER list for agonism and antagonism (XDS only), (50 total experiments) Submission of draft report and review by SMT 	Nov. 07

271

272 4.1.2 <u>Study Deliverables</u>

- 273 4.1.2.1 Test Results (Phases I-IV)
- 274 XDS will provide raw and quality control data in electronic format (i.e., email with attachments)
- 275 to the SMT Project Coordinator on a weekly basis during in-life (i.e., during those weeks when
- 276 LUMI-CELL® ER bioassay data is being collected and/or analyzed) portions of the study.

- 277 4.1.2.2 Study Status Reports (Phases I-IV)
- 278 XDS will provide study status reports during each phase of the study to the SMT Project
- 279 Coordinator on a biweekly basis. These reports will be provided in electronic format (i.e., email
- with attachments) and will include raw and quality control data as the study progresses. Reports
- should contain the information outlined in Appendix A.
- 282 4.1.2.3 Draft Reports (Phases I-IV)
- 283 At the conclusion of each phase of the study, a draft report will be provided by the Study
- 284 Director to the SMT Project Coordinator. The draft report will be provided electronically in
- Word[®]. Reports should contain the information outlined in **Appendix A** and should follow the
- 286 recommended formats and styles provided in the "Style Guide for LUMI-CELL® ER Validation
- 287 Study Laboratory Reports and Documents" (Appendix D).
- 288 4.1.2.4 Final Reports (Phases I-IV)
- 289 Each draft report that is approved by the SMT will be followed by a final report, which has been
- 290 reviewed by the QA Officer for GLP compliance, for each phase of the study. The final report
- will be provided electronically in Word® by the Study Director to the SMT Project Coordinator.
- 292 Copies of the audited Study Workbook pages should submitted in electronic format (i.e., pdf
- 293 files) as an attachment to the report. However, completion of the final report is not required prior
- 294 to initiation of the next phase of the validation study.

295 4.1.3 Estimated Due Dates for Reports

ESTIMATED DUE DATES					
REPORTS	PHASE I	PHASE IIa	PHASE IIb	PHASE III	PHASE IV
Study Status	*	*	*	*	*
Draft	Mar., 2007	May, 2007	Aug., 2007	Oct., 2007	Nov., 2007
Final	Apr., 2007	Jun., 2007	Sep., 2007	Nov., 2007	Dec., 2007

*Study status reports will be provided biweekly during each phase of the study.

4.2 Phase I

- This phase will be used for initial laboratory qualification/protocol refinement by all participating laboratories and is limited to the testing of reference standards, positive controls,
- and the solvent control. The results will be used to establish an historical database in each
- 302 laboratory for reference standards and controls.

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303	4.2.1 <u>Initial Laboratory Qualification/Protocol Refinement</u>		
304	Repetitive testing of agonist and antagonist reference standards and positive/solvent controls will		
305	be used to demonstrate proficiency with the LUMI-CELL® ER assay, demonstrate		
306	intralaboratory repeatability and intra- and inter-laboratory reproducibility, and establish an		
307	historical database. Results will be compared to historical control data established during the		
308	LUMI-CELL® ER Protocol Standardization Study. If there is excessive variation of reference		
309	standard and control data within or among the participating laboratories, the SMT (through the		
310	designated contacts) will work with the laboratories to determine cause and recommend		
311	appropriate actions needed to reduce variation. Statements of Work, Test Method Protocols, and		
312	SOPs will be revised, if necessary, and testing repeated until acceptable proficiency is		
313	demonstrated (i.e., acceptable intralaboratory repeatability and intra- and inter-laboratory		
314	reproducibility). The SMT may convene a teleconference with appropriate participants of the		
315	validation study to discuss information concerning the progression of the validation study.		
316	4.2.2 <u>Criteria for Advancing to Phase II</u>		
317	The SMT will decide when all laboratories will advance to Phase II of the validation study,		
318	based on the following criteria:		
319	 Data, reviewed by the QA Officer (or independent reviewer), has been received 		
320	by the SMT		
321	 All participating laboratories have submitted acceptable draft reports as outlined 		
322	in Section 4.1.2.2 .		
323	 Acceptable intralaboratory repeatability and intra- and inter-laboratory 		
324	reproducibility has been demonstrated by the participating laboratories		
325	A suitable historical negative and positive control database has been established		
326	4.3 Phase II		
327	Phase II provides for initial laboratory qualification using procedures that have been refined in		
328	Phase I, but is also the initial phase for testing substances from the ICCVAM list of 78 reference		
329	substances recommended for validation of ER TA assays. In this phase, four coded test		
330	substances (Phase IIa) and then eight coded test substances (Phase IIb) will be tested in all three		
331	participating laboratories. Acceptance criteria for experimental data for Phase IIa will be based		
332	on the historical database established in Phase I for reference standards and controls. Reference		

333	standard and control data collected during Phase IIa will also be included in the historical			
334	database, which will then be used to establish acceptance criteria for Phase IIb.			
335	4.3.1 Phase IIa Limited Testing of Protocol and Protocol Refinement			
336	After a range-finding assay is completed for each of the four coded test substances in Phase IIa,			
337	recommended starting concentrations for the comprehensive concentration-response experiment			
338	and the rationale for their selection are to be sent to the SMT for review and approval. The			
339	comprehensive concentration-response experiment for each test substance should not begin until			
340	the starting concentrations have been approved, and they should not be modified without			
341	approval from the SMT. The comprehensive concentration-response experiment should be			
342	performed three times, once on each of three different days. Laboratories will calculate EC_{50}			
343	values for the agonist reference standard or IC_{50} values for the antagonist reference standard (in			
344	$\mu g/mL$). Laboratories will also calculate EC $_{50}$ or IC $_{50}$ values (in $\mu g/mL$), when possible, for			
345	coded test substances. These data, along with all quality control, raw, derived and supporting			
346	data, will be reported to the SMT through the designated contacts. If there is excessive variation			
347	within or among participating laboratories, the SMT will work with the laboratories to determine			
348	the cause and recommend appropriate actions needed to reduce variation. Statements of Work,			
349	Test Method Protocols, and SOPs will be revised, if necessary, and testing repeated until			
350	acceptable proficiency is demonstrated (i.e., acceptable intralaboratory repeatability and intra-			
351	and inter-laboratory reproducibility). The SMT may convene a teleconference with appropriate			
352	participants of the validation study to discuss information concerning the progression of the			
353	validation study.			
354	4.3.2 <u>Criteria for Advancing to Phase IIb</u>			
355	The SMT will decide when all laboratories will advance to the Phase IIb of the validation study,			
356	based on the following criteria:			
357	Data, reviewed by the QA Officer (or independent reviewer), has been received			
358	by the SMT			
359	 All participating laboratories have submitted acceptable draft reports as outlined 			
360	in Section 4.1.2.2 .			
361	 Acceptable intralaboratory repeatability and intra- and inter-laboratory 			
362	reproducibility has been demonstrated by the participating laboratories			

363	4.3.3	Phase IIb Testing of Protocol and Protocol Refinement			
364	Phase IIb	includes the testing of eight coded substances and is the last phase for evaluating any			
365	protocol refinements from Phase I or IIA.				
366	After a ra	ange-finding assay is completed for each of the eight coded test substances in Phase IIb,			
367	recomme	nded starting concentrations for the comprehensive concentration-response experiment			
368	and the ra	ationale for their selection are to be sent to the SMT for review and approval. The			
369	comprehe	ensive concentration-response experiment for each test substance should not begin until			
370	the starting	ng concentrations have been approved and should not be modified without approval of			
371	the SMT.	The comprehensive concentration-response experiment should be performed three			
372	times, on	ce on each of three different days. Laboratories will calculate EC50 values for the			
373	agonist re	eference standard or IC ₅₀ values for the antagonist reference standard (in μ g/mL).			
374	Laborato	ries will also calculate EC ₅₀ or IC ₅₀ values (in μg/mL), when possible, for coded test			
375	substance	es. These data, along with all quality control, raw, derived and supporting data, will be			
376	reported	to the SMT through the designated contacts. If there is excessive variation within or			
377	among pa	articipating laboratories, the SMT will work with the laboratories to determine the cause			
378	and recor	nmend appropriate actions needed to reduce variation. Statements of Work, Test			
379	Method I	Protocols, and SOPs will be revised, if necessary, and testing repeated until acceptable			
380	proficiency is demonstrated (i.e., acceptable intralaboratory repeatability and intra- and inter-				
381	laborator	y reproducibility). The SMT may convene a teleconference with appropriate			
382	participa	nts of the validation study to discuss information concerning the progression of the			
383	validation	n study.			
384	4.3.4	Criteria for Advancing to Phase III			
385	The SMT	will decide when all laboratories will advance to the Phase III of the validation study,			
386		the following criteria:			
387		• Data, reviewed by the QA Officer (or independent reviewer), has been received			
388		by the SMT			
389		All participating laboratories have submitted acceptable draft reports as outlined			
390		in Section 4.1.2.2.			
391		Acceptable intralaboratory repeatability and intra- and inter-laboratory			
392		reproducibility has been demonstrated within and among the participating			
393		laboratories			