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**NOMINATION OF THE LUMI-CELL™ ER
HIGH-THROUGHPUT SYSTEM FOR SCREENING
ESTROGEN-LIKE CHEMICALS FOR VALIDATION STUDIES**

DRAFT EVALUATION

NICEATM

August 16, 2004

TABLE OF CONTENTS

38			
39			
40			Page
41	EXECUTIVE SUMMARY		i
42	1.0 INTRODUCTION		1
43	1.1 XDS Nomination		1
44	1.2 SACATM Review (March 10-11, 2004).....		4
45	1.3 NICEATM <i>Federal Register</i> Notice.....		4
46	1.4 XDS Pre-validation Background Review Document.....		5
47	2.0 EVALUATION OF THE ABILITY OF THE LUMI-CELL™ ER		
48	BIOASSAY TO DETECT SUBSTANCES WITH ER AGONISM AND		
49	ANTAGONISM ACTIVITY		6
50	2.1 To What Extent Does the Nomination and Proposed Test Method		
51	Address the ICCVAM Prioritization Criteria?		6
52	2.2 Do the LUMI-CELL™ Pre-Validation Agonist and Antagonist		
53	Studies Adhere to the Recommendations of the <i>ICCVAM</i>		
54	<i>Evaluation of In Vitro Test Methods for Detecting Potential</i>		
55	<i>Endocrine Disruptors</i> (NIH Publ. No. 03-4503), Especially Those		
56	Regarding Essential Test Method Components (Previously Known		
57	as Minimum Procedural Standards) and Recommended Validation		
58	Substances?.....		7
59	2.2.1 Essential Test Method Components		7
60	2.2.2 ICCVAM Recommended Validation Substances		9
61	2.3 Does LUMI-CELL™ Show Adequate Performance (Reliability		
62	and Accuracy) During Pre-Validation to Warrant Consideration for		
63	Validation Studies?		10
64	2.3.1 Reliability (Repeatability and Intra- and Inter-laboratory		
65	Reproducibility) of the LUMI-CELL™ ER Bioassay for		
66	Detecting ER Agonist Activity		10
67	2.3.2 The Accuracy of the LUMI-CELL™ ER Bioassay for		
68	Detecting ER Agonist Activity		11
69	2.3.3 Reliability (Repeatability and Intra- and Inter-laboratory		
70	Reproducibility) of the LUMI-CELL™ ER Bioassay for		
71	Detecting ER Antagonist Activity.....		14
72	2.3.4 The Accuracy of the LUMI-CELL™ ER Bioassay for		
73	Detecting ER Antagonist Activity.....		15
74	2.4 Does the BRD Adequately Provide the Information Requested in		
75	the Outline Provided in the <i>ICCVAM Guidelines for the</i>		

76		<i>Nomination and Submission of New, Revised, and Alternative Test</i>	
77		<i>Methods (NIH Publ. No. 03-4508)?</i>	18
78	3.0	NICEATM RECOMMENDATIONS:	20
79			
80			

EXECUTIVE SUMMARY

81
82

83 On January 22, 2004, NICEATM received a letter from Dr. George Clark of Xenobiotic
84 Detection Systems (XDS) nominating a cell based transcriptional method (trademarked as
85 LUMI-CELL™) for validation studies. The test method evaluates the endocrine disruptor
86 activity of chemicals by measuring whether and to what extent the chemical induces or
87 blocks transcription at the estrogen receptor (ER). The nomination requested that NICEATM
88 and ICCVAM aid in and manage the cross-laboratory validation studies needed to formally
89 evaluate the reliability and accuracy of the LUMICELL™ ER bioassay for its proposed use
90 as a regulatory test method for detecting chemicals with *in vitro* estrogenic agonist and
91 antagonist activity.

92

93 On April 21, 2004, NICEATM authored a *Federal Register (FR)* Notice (Vol. 69, No. 77, p.
94 21564), entitled “In Vitro Endocrine Disruptor Test Methods: Request for Comments and
95 Nominations.” The *FR* :

- 96 • identified *in vitro* endocrine disruptor screening methods that do not require
97 the use of animal tissues as an ICCVAM priority for validation studies;
- 98 • indicated the availability of published ICCVAM recommendations¹ for
99 standardization and validation of *in vitro* endocrine-disruptor estrogen and
100 androgen receptor binding and transcriptional activation assays; and
- 101 • invited the nomination for validation studies of *in vitro* test methods that meet
102 the recommendations and for which there are standardized test method
103 protocols, pre-validation data, and proposed validation study designs.

104

105 NICEATM received a pre-validation background review document (BRD) from XDS on
106 April 23, 2004, and a revised BRD on June 21, 2004. In accordance with the ICCVAM
107 nomination process, NICEATM conducted a pre-screen evaluation of the revised BRD and
108 proposal to determine the extent that the proposed nomination addresses the ICCVAM
109 prioritization criteria, ICCVAM submission guidelines, and ICCVAM recommendations for

¹ ICCVAM Evaluation of *In Vitro* Test Methods For Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. (2003). NIH Publication No. 03-4503. <http://iccvam.niehs.nih.gov/methods/endocrine.htm>

110 standardization and validation of *in vitro* endocrine disruptor test methods. The performance
111 of the test method based on pre-validation data was also reviewed to determine if this
112 performance warrants consideration for further validation. The revised BRD is the focus of
113 the NICEATM pre-screen evaluation.

114

115 The four areas considered in evaluating the pre-validation information provided by XDS in
116 their background review document (BRD) and the extent to which the criteria are met are as
117 follows:

118

119 **1. To what extent does the nomination and proposed test method address the**
120 **ICCVAM prioritization criteria?**

121

122 The LUMI-CELL™ ER bioassay meets all of the ICCVAM prioritization criteria. The test
123 method:

- 124 • is applicable to the needs of the US Environmental Protection Agency (EPA)
125 for a high throughput screening system to evaluate substances for their
126 potential estrogen disruptor activity, and may also be applicable to the US
127 Food and Drug Administration, Department of Agriculture, Department of
128 Defense, and Department of Homeland Security, since methodologies are being
129 developed to screen feed and food for potential estrogen disruptor chemicals.
- 130 • is warranted, based on the worldwide concern about the association between
131 exposure to endocrine disruptors and adverse health effects in human and
132 wildlife populations.
- 133 • is warranted, based on its potential to refine, reduce, or replace animal use
- 134 • is warranted, based on its demonstrated ability to detect estrogenic activity at
135 extremely low levels (i.e., some six to seven magnitudes lower than that
136 induced by β -estradiol, the endogenous estrogen).
- 137 • is warranted, based on its relatively low cost per substances tested (\$350) and
138 the relatively quick study duration (two days)

139

140 **2. Do the LUMI-CELL™ pre-validation agonist and antagonist studies adhere to**

141 **the recommendations of the ICCVAM Evaluation of In Vitro Test Methods for**
142 **Detecting Potential Endocrine Disruptors (NIH Publ. No. 03-4503), especially**
143 **those regarding essential test method components (called minimum procedural**
144 **standards in this document) and recommended validation substances?**

145

146 *Essential Test Method Components:* With a few exceptions, the agonist and antagonist
147 protocols for the LUMI-CELL™ ER bioassay incorporates the recommended essential test
148 method components for both agonist and antagonist studies. These exceptions do not appear
149 to adversely impact on the performance (accuracy and reliability) of the assay. Examples of
150 exceptions include the preferential use of dimethylsulfoxide (DMSO), rather than water or
151 ethanol (95 to 100%) as the preferred solvent; using 40 pg and not the recommended
152 maximum test substance concentration of 1 mM for agonism and antagonism assays; and
153 incorporating qualitative rather than quantitative measures of cytotoxicity in the assay.

154

155 *ICCVAM Recommended Validation Substances:* For the validation of ER TA agonist assays,
156 ICCVAM recommended 78 substances (35 positive/presumed positive, 43
157 negative/presumed negative). The BRD provided data on 108 substances, 56 of which were
158 included in the ICCVAM recommended validation list (29 classified by ICCVAM as
159 positive/presumed positives by ICCVAM, 27 classified by ICCVAM as negatives/presumed
160 negatives for ER TA activity). This number of substances is considered sufficient for the
161 pre-validation of the agonist version of the LUMI-CELL™ ER bioassay.

162

163 **3. Does LUMI-CELL™ show adequate performance (reliability and accuracy)**
164 **during pre-validation to warrant consideration for validation studies?**

165

166 *Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-*
167 *CELL™ ER Bioassay for Detecting ER Agonist Activity:* In their BRD, XDS provided
168 coefficient of variation (CV) data for LUMI-CELL™ agonist test results with respect to what
169 they classified as well-to-well variability² within an experiment for 12 ICCVAM

² In LUMI-CELL™, a substance is tested at up to 11 concentrations, with each concentration tested in triplicate wells on a 96-well plate. To evaluate well-to-well variability, XDS determined the CV for the EC50 values

170 recommended positive reference substances and plate-to-plate (plate = experiment; minimum
171 of three independent experiments) for 33 ICCVAM recommended validation substances
172 reported as positive in LUMI-CELL™. An evaluation of interlaboratory agonist
173 reproducibility has not been conducted; this evaluation would be conducted as part of a
174 multi-laboratory validation effort. XDS did not use coded chemicals in the collection of
175 these data. The mean and median CV values for within experiment EC50 values for the 12
176 ICCVAM recommended positive reference substances was 28 and 29%, respectively. The
177 mean and median CV values for plate-to-plate (i.e., experiment-to-experiment) EC50 values
178 for 33 ICCVAM recommended reference substances that induced a positive response in
179 LUMI-CELL™ was 45 and 38%, respectively. These levels of repeatability and
180 intralaboratory reproducibility are considered adequate for screening assays by NICEATM.

181

182 *Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity:* There is no
183 agreed-upon animal or human data set to serve as a reference for determining the accuracy of
184 *in vitro* test methods for identifying substances with estrogen activity *in vivo*. As an
185 alternative, the compilation of published mammalian cell *in vitro* ER TA results, as
186 summarized in Appendix D of the ICCVAM report was compared with the LUMI-CELL™
187 ER bioassay test results reported in Appendix D of the XDS BRD. Fifty-six of the 78
188 substances recommended by ICCVAM for the validation of *in vitro* TA test methods were
189 tested for agonist activity by XDS in the LUMI-CELL™ ER Bioassay. Based on the LUMI-
190 CELL™ agonism test results, the concordance was 0.82, the sensitivity was 1.00, the
191 specificity was 0.66, the false negative rate was 0, and the false positive rate was 0.34. The
192 high “false positive” rate was due to ten of 29 ICCVAM recommended ER negative
193 substances producing a positive or weak positive ER agonist response in LUMI-CELL™.
194 However, due to the mechanistic basis of this test system, false positives are highly unlikely.
195 These ten substances most likely have very weak transcriptional activity that is producing the
196 weak positive response. Compared to the EC50 value for estradiol, all ten substances
197 exhibited EC50 values that were six to seven fold orders of magnitude weaker. For these ten
198 false positive substances, ICCVAM did not have supporting negative ER TA data for seven

(i.e., the concentration that induces a half-maximal agonist response) calculated using the first, the second, or the third sets of wells.

199 substances, and had single test data only for two substances. Only one substance, atrazine,
200 had been reported as negative for ER TA activity in three studies. Thus, it is entirely
201 possible that all ten of these substances are capable of producing weak ER transcriptional
202 activation and that that increased TA activity represents “true” positives for the type and
203 distribution of estrogen receptors in this test system. Furthermore, these responses may
204 indicate that this test system is capable of detecting ER activity over a broad dynamic range,
205 including very weak activity. Nonetheless, such results will need confirmation in a multi-
206 laboratory validation study and, if possible, in other transcriptional assays with comparable
207 receptor composition and sensitivity. Finally, the quantitative nature of the response will
208 likely need to be considered when using this data for weight-of-evidence decisions in the
209 EPA’s Tier 1 Endocrine Disruptor Screening Program, with possibly less weight given to
210 very weak acting substances, especially those that do not demonstrate an *in vivo* effect at
211 established limit doses.

212

213 Another approach to evaluating the performance of the LUMI-CELL™ ER Bioassay, in
214 terms of the ICCVAM recommended validation substances, is to compare the relative
215 quantitative agonist activity of substances reported as positive in both data sets. Due to the
216 lack of EC50 data for many of the substances recommended in the ICCVAM report, this
217 analysis was limited to nine substances with ER TA activity. The regression correlations (r^2)
218 for EC50 values and relative rankings were 0.607 ($p = 0.013$) and 0.903 ($p < 0.001$),
219 respectively. Thus, the relative ER TA activities of these nine agonist substances are
220 significantly correlated between the LUMI-CELL™ ER bioassay and the data summarized in
221 the ICCVAM report.

222

223 *Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-*
224 *CELL™ ER Bioassay for Detecting ER Antagonist Activity:* XDS did not provide CV data
225 for LUMI-CELL™ antagonist test results with respect to well-to-well variability within an
226 experiment but did provide plate-to-plate (plate = experiment; minimum of three experiments
227 conducted on different days) for eight ICCVAM recommended substances reported as
228 positive in LUMI-CELL™. An evaluation of interlaboratory antagonist reproducibility has
229 not been conducted; this evaluation would be conducted as part of a multi-laboratory

230 validation effort. The mean and median CV values for plate-to-plate (i.e., experiment-to-
231 experiment) IC₅₀³ values for eight ICCVAM recommended reference substances that
232 induced a positive antagonist response in LUMI-CELL™ was 24 and 25%, respectively.
233 This level of intralaboratory reproducibility is considered adequate by NICEATM for
234 screening assays.

235

236 *The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity:*

237 Sixteen of the 78 substances recommended by ICCVAM for the validation of *in vitro* TA test
238 methods were tested for antagonist activity by XDS in the LUMI-CELL™ ER bioassay. In
239 their list of 78 recommended substances, ICCVAM identified eight substances with
240 demonstrated antagonist activity, three with anticipated antagonist activity, 10 with
241 demonstrated negative antagonist activity, and 57 with anticipated negative antagonist
242 activity. Of the 16 substances listed by XDS as being tested for antagonist activity in the
243 LUMI-CELL™ ER bioassay, ICCVAM had classified eight as positive for ER antagonist
244 activity and eight without ER antagonist activity. Based on the LUMI-CELL™ antagonism
245 test results, the concordance was 0.50, the sensitivity was 1.00, the specificity was 0, the
246 false negative rate was 0, and the false positive rate was 1.00. All eight ICCVAM validation
247 substances presumed to be ER antagonists induced a positive or weak positive antagonist
248 response in LUMI-CELL™. However, ICCVAM did not have supporting ER antagonism
249 data for six of these substances. Only eight ICCVAM validation substances with known or
250 predicted ER antagonist activity were tested by XDS in the LUMI-CELL™ ER bioassay.
251 However, the list of validation substances recommended by ICCVAM only contains 11 ER
252 antagonist substances (eight with supporting data, three without *in vitro* ER TA antagonist
253 supporting data). Due to the limited number of antagonists tested by XDS and the limited
254 number of studies reported by ICCVAM with quantitative data, a comparative analysis of
255 potency could not be conducted. While additional LUMI-CELL™ ER antagonist data would
256 be useful in clarifying the performance of this assay for identifying substances with
257 antagonist activity, the lack of such studies is not considered to be a significant detriment to
258 conducting cross laboratory validation studies.

³ The concentration of the test substance calculated to inhibit the estrogenic activity of a specified concentration of the reference estrogen by 50%.

259

260 **4. Does the BRD adequately provide the information requested in the outline**
261 **provided in the ICCVAM Guidelines for the Nomination and Submission of New,**
262 **Revised, and Alternative Test Methods (NIH Publ. No. 03-4508)?**

263

264 The XDS BRD adheres to the recommended outline and provides nearly all of the requested
265 information. However, additional information should be provided if the BRD is to be
266 released beyond ICCVAM. The lack of this information did not adversely impact on the
267 evaluation of Criteria 1 through 3.

268

269 *NICEATM Recommendations:* Based on the data provided in the XDS BRD on the LUMI-
270 CELL™ ER bioassay, NICEATM recommends to the EDWG that:

- 271 • LUMI-CELL™ be considered as a high priority for validation studies as an *in*
272 *vitro* test method for the detection of test substances with ER agonist and
273 antagonist activity.
- 274 • To facilitate independent and timely standardization and validation studies,
275 NICEATM should manage the needed studies by exercising a validation
276 coordination option in its support contract. Such studies should include
277 coordination and collaboration with ECVAM and JCVAM, and ideally
278 include one laboratory in each of the three respective geographic regions
279 supported by these three Centers.
- 280 • During finalization of their BRD and in preparation for the interlaboratory
281 validation study, XDS conduct additional antagonist studies to more
282 comprehensively demonstrate the suitability of LUMI-CELL™ as an assay
283 for the detection of substances with ER antagonist activity.

284

285 **1.0 INTRODUCTION**

286

287 **1.1 XDS Nomination**

288

289 On January 22, 2004, NICEATM received a letter from Dr. George Clark of Xenobiotic
290 Detection Systems (XDS) nominating for validation a cell based transcriptional method
291 (trademarked as LUMI-CELL™) for the evaluation of the endocrine disruptor activity of
292 chemicals for the estrogen receptor (ER). In its nomination, Dr. Clark stated that the LUMI-
293 CELL™ ER Bioassay was a standardized test procedure in a stably transfected recombinant
294 cell line that was sensitive, robust, and reproducible in detecting estrogen active chemicals,
295 and summarized the extent to which this *in vitro* test method met each of the ICCVAM
296 prioritization criteria (ICCVAM, 2003⁴). The ICCVAM prioritization criteria and the extent
297 to which these criteria were stated to be met by the LUMI-CELL™ ER Bioassay are:

298 • ***The Extent To Which The Proposed Test Method Is Applicable To***
299 ***Regulatory Testing Needs***

300 "The LUMI-CELL™ ER bioassay will meet the need for a high throughput
301 screening (HTPS) system of chemicals for their potential estrogen disruptor
302 activity. The US Environmental Protection Agency (EPA) identified a need
303 for this technology in the Endocrine Disruptor Steering and Testing Advisory
304 Committee (EDSTAC) recommendations in order to meet a mandate of the
305 Food Quality Protection Act of 1996 and the Safe Drinking Water Act of
306 1996. This test method is also in response to Federal Register Notice (Vol. 66,
307 No. 57/Friday, March 23, 2001) as a HTPS method for estrogen active
308 compounds".

309

310 • ***The Extent To Which The Proposed Test Method Is Applicable To Multiple***
311 ***Agencies/Programs***

312 "The LUMI-CELL™ ER bioassay technology may also be applicable to the

⁴ ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No: 03-4508. Research Triangle Park, North Carolina: NIEHS (<http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm>)

313 US Food and Drug Administration, Department of Agriculture, Department of
314 Defense, and Department of Homeland Security, since methodologies are being
315 developed to screen feed and food for potential estrogen disruptor chemicals.
316 Both food and feed are a potential source for exposure to EDCs".

317

- 318 • ***The Extent To Which The Proposed Test Method Is Warranted, Based On***
319 ***The Extent Of Expected Use Or Application And Impact On Human,***
320 ***Animal, Or Ecological Health***

321 "The association of exposure to EDCs and adverse health effects in human and
322 wildlife populations has led to worldwide concern. Some of the health effects
323 that have led to this concern include global increases in testicular cancer,
324 regional declines in sperm counts, altered sex ratios in wildlife populations,
325 increases in the incidence of breast cancer and endometriosis, and accelerated
326 puberty in females that are expected to result from exposure to chemicals that
327 adversely affect steroid hormone action".

328

- 329 • ***The Potential For The Proposed Test Method, Compared To Current Test***
330 ***Methods Accepted By Regulatory Agencies, To Refine, Reduce, or Replace***
331 ***Animal Use***

332 "There are no currently accepted methods that are being used to screen for
333 EDCs but some have been proposed and are in the process of validation by
334 the EPA. Most of these methods require substantial use of animals to evaluate
335 endocrine disruptor activity. The LUMI-CELL™ ER bioassay method would
336 allow for a rapid process to screen and set priorities on testing chemicals for
337 disruption of estrogenic activity in other animal models. This would
338 consequently result in a significant reduction in animal use in the screening
339 process".

340

341 • ***The Potential For The Proposed Test Method To Provide Improved***
342 ***Prediction Of Adverse Health Or Environmental Effects, Compared To***
343 ***Current Test Methods Accepted By Regulatory Agencies***

344 "There are no current methods approved for the detection of ECDs by any
345 federal agency. However, the LUMI-CELL™ ER bioassay shows tremendous
346 potential for prediction of adverse health and environmental effects. This is
347 shown by the very high correlation between agonist response data collected
348 using our test method and the historical data available in the database
349 developed by NICEATM on these compounds. The LUMI-CELL™ ER
350 bioassay is sensitive enough to allow for an extremely low detection limit
351 (ppq), which should be lower than federal regulations are likely to mandate.
352 Unlike ELISA detection limits which have a lower limit of >1 ppb. The
353 LUMI-CELL™ ER bioassay will give a measure of bioavailability, being a
354 biological system itself.

355
356 • ***The Extent To Which The Test Method Provides Other Advantages (e.g.,***
357 ***Reduced Cost And Time To Perform) Compared To Current Methods***

358 "The LUMICELL™ ER bioassay is an extremely rapid in vitro method that
359 can evaluate the estrogenic activity of chemicals within two days. The method
360 also provides relative activity of a chemical to the standard, beta-estradiol, and
361 provides dose response activity of the chemical. The standardized protocol
362 developed allows for a very robust system with low variability and high
363 sensitivity. The cost of the LUMI-CELL™ ER bioassay is a few hundred
364 dollars per chemical, which is substantially less than any animal base method.
365 The LUMI-CELL™ ER bioassay is a transcriptionally based assay capable of
366 testing for antagonistic responses of EDCs, which is not possible using
367 binding assays".

368
369

370 In the XDS letter, Dr. Clark requested that NICEATM and ICCVAM aid in and manage the
371 cross-laboratory validation studies needed to formally evaluate the reliability and accuracy of
372 the LUMI-CELL™ ER bioassay and its use as a regulatory test method for detecting
373 chemicals with estrogenic agonist and antagonist activity. Dr. Clark stated that “the pre-
374 validation and method development steps for this test method are essentially complete and
375 data on the screening of 120 chemicals for estrogenic agonist activity can be made available
376 to NICEATM and ICCVAM.” Further, Dr. Clark proposed that XDS “act as the primary
377 laboratory providing training and technical support to other participating laboratories.”
378

379 **1.2 SACATM Review (March 10-11, 2004)**

380
381 NICEATM and ICCVAM presented for consideration two nominated *in vitro* endocrine
382 disruptor test methods, one of which was the XDS LUMI-CELL™ ER bioassay, to the
383 Scientific Advisory Committee on Alternative Toxicological Methods (SACTAM) on March
384 10-11, 2004. The SACATM was supportive of the nominations and raised no objections to
385 these assays being evaluated by NICEATM and considered by the EDWG and ICCVAM for
386 future validation studies.
387

388 **1.3 NICEATM Federal Register Notice**

389
390 On April 21, 2004, NICEATM sponsored a *Federal Register (FR)* Notice (Vol. 69, No. 77, p.
391 21564), entitled “In Vitro Endocrine Disruptor Test Methods: Request for Comments and
392 Nominations.” This *FR* Notice stated that:

- 393 • ICCVAM and the SACATM had identified *in vitro* endocrine disruptor
394 screening methods as a priority for validation.
- 395 • ICCVAM had published guidelines for development of *in vitro* endocrine-
396 disruptor estrogen and androgen receptor binding and transcriptional
397 activation assays. In these guidelines, ICCVAM recommended that priority
398 be given to assays that
 - 399 1. do not require the use of animal tissue as the receptor source, but
400 rather use recombinant-derived proteins, and

- 401 2. do not use radioactive materials.
- 402 • On behalf of ICCVAM, NICEATM invited the nomination for validation
- 403 studies of *in vitro* test methods that meet these recommendations and for
- 404 which there are standardized test method protocols, pre-validation data, and
- 405 proposed validation study designs.
- 406 • At this time, ICCVAM had received nominations for two *in vitro* endocrine-
- 407 disruptor screening methods (one was the nomination from XDS) purported to
- 408 meet these recommendations.
- 409 • ICCVAM will consider nominations and comments received in response to
- 410 this notice and develop recommended priorities for proposed evaluation and
- 411 validation studies of endocrine disruptor screening methods.
- 412 • Prior to the initiation of such studies, the proposed validation studies would be
- 413 evaluated for adherence to relevant recommendations in the report:
- 414 “ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential
- 415 Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and
- 416 Transcriptional Activation Assays” (NIH Publication No. 03–4503;
- 417 <http://iccvam.niehs.nih.gov/methods/endocrine.htm>) by the ICCVAM
- 418 Endocrine Disruptor Working Group (EDWG) and NICEATM.

419

420 NICEATM did not receive any comments on the XDS nomination in response to this *FR*

421 Notice.

422

423 **1.4 XDS Pre-validation Background Review Document**

424

425 On April 23, 2004, NICEATM received a pre-validation background review document

426 (BRD) from XDS. A request for clarification of the structure of the appendices was

427 submitted to XDS on May 12, 2004, with comments and questions submitted on May 28,

428 2004. In response to these comments and questions, XDS submitted a revised BRD on June

429 21, 2004. This revised BRD is the focus of this evaluation by NICEATM.

430

431

432

432 **2.0 EVALUATION OF THE ABILITY OF THE LUMI-CELL™ ER BIOASSAY**
433 **TO DETECT SUBSTANCES WITH ER AGONISM AND ANTAGONISM**
434 **ACTIVITY**

435

436 Four criteria were considered in evaluating the XDS pre-validation information provided in
437 their BRD:

438

- 439 1. To what extent does the nomination and proposed test method address the
440 ICCVAM prioritization criteria?
441
- 442 2. Do the LUMI-CELL™ pre-validation agonist and antagonist studies adhere to
443 the recommendations of the *ICCVAM Evaluation of In Vitro Test Methods for*
444 *Detecting Potential Endocrine Disruptors* (NIH Publ. No. 03-4503,
445 <http://iccvam.niehs.nih.gov/methods/endocrine.htm>), especially those
446 regarding essential test method components (previously known as minimum
447 procedural standards) and recommended validation substances?
448
- 449 3. Does LUMI-CELL™ show adequate performance (reliability and accuracy)
450 during pre-validation to warrant consideration for validation studies?
451
- 452 4. Does the BRD adequately provide the information requested in the outline
453 provided in the *ICCVAM Guidelines for the Nomination and Submission of*
454 *New, Revised, and Alternative Test Methods* (NIH Publ. No. 03-4508)?
455

456 **2.1 To What Extent Does the Nomination and Proposed Test Method Address the**
457 **ICCVAM Prioritization Criteria?**

458

459 The LUMI-CELL™ ER bioassay meets all of the ICCVAM prioritization criteria. The test
460 method:

- 461 • is applicable to the needs of the US Environmental Protection Agency (EPA)
462 for a high throughput screening system to evaluate substances for their

- 463 potential estrogen disruptor activity, and may also be applicable to the US
464 Food and Drug Administration, Department of Agriculture, Department of
465 Defense, and Department of Homeland Security, since methodologies are being
466 developed to screen feed and food for potential estrogen disruptor chemicals.
- 467 • is warranted, based on the worldwide concern about the association between
468 exposure to endocrine disruptors and adverse health effects in human and
469 wildlife populations.
 - 470 • is warranted, based on its potential to refine, reduce, or replace animal use
 - 471 • is warranted, based on its demonstrated ability to detect estrogenic activity at
472 extremely low levels (i.e., some six to seven magnitudes lower than that
473 induced by β -estradiol, the endogenous estrogen).
 - 474 • is warranted, based on its relatively low cost per substances tested (\$350) and
475 the relatively quick study duration (two days)

476

477 **2.2 Do the LUMI-CELL™ Pre-Validation Agonist and Antagonist Studies Adhere**
478 **to the Recommendations of the ICCVAM Evaluation of In Vitro Test Methods for**
479 **Detecting Potential Endocrine Disruptors (NIH Publ. No. 03-4503), Especially**
480 **Those Regarding Essential Test Method Components (Previously Known as**
481 **Minimum Procedural Standards) and Recommended Validation Substances?**

482

483 The ICCVAM recommendations in regard to essential test method components and
484 substances to be used in the validation of ER transcriptional activation (TA) assays are
485 described in Sections 4.1 and 4.2, respectively, of the ICCVAM report.

486

487 **2.2.1 Essential Test Method Components**

488 The ER TA section in the ICCVAM report contained essential test method component
489 recommendations in regard to:

- 490 • the reference estrogen and associated TA response
- 491 • preparation of test substances and the volume of the administered solvent
- 492 • the concentration range of test substances that should be tested
- 493 • solvent and positive controls

- 494 • the number of within-test replicates
- 495 • methods for data analysis
- 496 • the need for Good Laboratory Practice (GLP) compliance
- 497 • study acceptance criteria
- 498 • interpretation of results
- 499 • repeat studies
- 500 • the study report

501

502 The agonist and antagonist protocols for the LUMI-CELL™ ER bioassay incorporates the
503 recommended essential test method components for both agonist and antagonist studies, with
504 few exceptions, and these exceptions do not appear to adversely impact on the performance
505 (accuracy and reliability) of the assay. Examples of exceptions include the following:

506

507 *ICCVAM Report Section 4.1.2 (Preparation of Test Substances and Volume of Administered*
508 *Solvent)*: The report indicates that the preferred solvent is water, ethanol (95-100%), or
509 dimethylsulfoxide (DMSO), in that order. Members of the ICCVAM Expert Panel stated
510 that water or ethanol (95 to 100%) were preferred to DMSO because some substances, when
511 dissolved in DMSO, might exhibit reduced agonist activity. In the LUMI-CELL™ ER
512 Bioassay, DMSO is the solvent of choice. Based on the performance of the assay (see
513 **Section 2.2** of this BRD), the use of DMSO does not appear to have impacted on the
514 performance of this assay.

515

516 *ICCVAM Report Section 4.1.3 (Concentration Range of the Test Substances)*: In the absence
517 of solubility or cytotoxicity constraints, the recommended maximum test substance
518 concentration (i.e., the limit dose) for agonism and antagonism assays should be 1 mM for
519 negative test substances. However, as the LUMI-CELL™ ER bioassay was developed
520 originally to test complex mixtures, the approach XDS uses is to test to a maximum
521 concentration of 40 pg. For many, but not all, single chemicals evaluated by XDS that were
522 negative for estrogenic activity, this level exceeds the recommended 1 mM limit
523 concentration (*note: this information is provided in the data appendices to the XDS BRD*).

524

525 The ICCVAM report states that an evaluation of cell cytotoxicity should be included in each
526 study, and only those dose levels not associated with toxicity greater than 10% of the
527 concurrent solvent control considered in the analysis of the data. In the LUMI-CELL™ ER
528 bioassay, XDS evaluates several measures of cytotoxicity. The first is a visual inspection of
529 the cells. If the cells morphology is abnormal, or there appears to be some cell death (i.e.,
530 some cells have become detached), or if the cells are no longer attached at all and have been
531 washed away in the PBS rinse, the data from those wells are not used. The second method of
532 assessing cell toxicity is to use, for substances that are negative in the agonist assay, two
533 positive response assays. This is accomplished by mixing the highest concentration and
534 1/10th of the highest concentration of the test substance tested with the EC₅₀⁵ concentration
535 of β-estradiol (*note: there is discordance between the BRD and the correspondence from*
536 *XDS in how toxicity is evaluated – the information provided here is based on clarification*
537 *from XDS*). If toxicity is absent, one or both of these sets of wells should result in an positive
538 response for the reference estrogen (*note: this viability assay may be of limited use if the*
539 *substances being evaluated are ER antagonists*). These approaches appear to be useful but
540 less quantitative than what was recommended by the ICCVAM Expert Panel.

541

542 2.2.2 ICCVAM Recommended Validation Substances

543 To facilitate the validation of *in vitro* ER TA assays, ICCVAM provided a list of 78
544 recommended substances (35 substances were classified as positive or presumed positive and
545 43 substances were classified as presumed negative for ER TA agonist activity). It was
546 recommended further that, at a minimum, 53 of these substances should be tested for agonist
547 activity (34 substances were classified as positive or presumed positive, 19 substances were
548 classified as presumed negative). Data on 108 substances were provided in the XDS BRD.
549 Of the 108 substances, 29 were substances classified as positive or presumed positives by
550 ICCVAM and 27 were substances classified by ICCVAM as presumed negatives for ER TA
551 activity (i.e., for a total of 56 of 78 recommended substances). The remaining 22 of the 78
552 ICCVAM recommended substances were not tested due to a lack of availability, cost
553 considerations, or because they were controlled substances for which XDS did not have a

⁵ The concentration that is calculated to induce a response that is 50% of the maximally induced agonist response by that substance.

554 license. The 52 other substances tested by XDS were those not recommended by ICCVAM.
555 For the purpose of evaluating the performance of the LUMI-CELL™ ER bioassay as a
556 screen for the detection of substances with ER agonist activity, the number of ICCVAM
557 recommended substances tested by XDS was deemed adequate.

558

559 **2.3 Does LUMI-CELL™ Show Adequate Performance (Reliability and Accuracy)** 560 **During Pre-Validation to Warrant Consideration for Validation Studies?**

561

562 2.3.1 Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the 563 LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity

564 In their BRD, XDS provided coefficient of variation (CV) data for LUMI-CELL™ agonist
565 test results with respect to well-to-well variability⁶ within an experiment for 12 ICCVAM
566 recommended positive reference substances and plate-to-plate (plate = experiment; minimum
567 of three independent experiments) for 33 ICCVAM recommended validation substances
568 reported as positive in LUMI-CELL™. An evaluation of interlaboratory agonist
569 reproducibility has not been conducted; this evaluation would be conducted as part of a
570 multi-laboratory validation effort. XDS did not use coded chemicals in the collection of
571 these data.

572

573 *Test Method Repeatability:* The mean and median CV values for within experiment EC50
574 values for the 12 ICCVAM recommended agonists were 28 and 29%, respectively. This
575 level of repeatability is considered adequate by NICEATM for screening assays.

576

577 *Test Method Intralaboratory Reproducibility:* The mean and median CV values for plate-to-
578 plate (i.e., experiment-to-experiment) EC50 values for 33 ICCVAM recommended reference
579 substances that induced a positive response in LUMI-CELL™ was 45 and 38%, respectively.
580 This level of intralaboratory reproducibility is considered adequate by NICEATM for
581 screening assays.

⁶ In LUMI-CELL™, a substance is tested at up to 11 concentrations, with each concentration tested in triplicate wells on a 96-well plate. To evaluate well-to-well variability, XDS determined the CV for the EC50 values calculated using the first, the second, or the third sets of wells.

582

583 2.3.2 The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity

584 There is no agreed-upon animal or human data set to serve as a reference for determining the
 585 accuracy of *in vitro* test methods for identifying substances with estrogen activity *in vivo*. As
 586 an alternative, the compilation of published mammalian cell *in vitro* ER TA results, as
 587 summarized in Appendix D of the ICCVAM report was compared with the LUMI-CELL™
 588 ER bioassay test results reported in Appendix D of the XDS BRD. One difficulty in using
 589 the ICCVAM compilation as a reference data base is the lack of agreement among published
 590 studies regarding the positive or negative responses of a number of the substances
 591 recommended by ICCVAM for *in vitro* ER TA validation studies. This lack of agreement
 592 among laboratories is largely due to the diversity of test methods and the varied decision
 593 criteria developed by different investigators to evaluate ER TA activity. Another concern
 594 with using the list of ICCVAM recommended validation substances is that the classification
 595 of some substances is based on a single test in a single laboratory using a system that may not
 596 have been well-defined or was based on theory rather than experimentally obtained data.

597

598 *Evaluation of Concordance:* Fifty-six of the 78 substances recommended by ICCVAM for
 599 the validation of *in vitro* TA test methods were tested for agonist activity by XDS in the
 600 LUMI-CELL™ ER Bioassay. ICCVAM has classified 29 of these 56 substances as positive
 601 or presumed positive⁷ and 27 as negative or presumed negative for *in vitro* ER TA activity.

602 The results obtained by XDS for the 56 substances tested in LUMI-CELL™ are as follows:

- | | | | |
|-----|---|--|----------------------------|
| 603 | • | Positive in LUMI-CELL™ and ICCVAM Positive | 25 substances |
| 604 | • | Weak Positive ⁸ in LUMI-CELL™ and ICCVAM Positive | 2 substances |
| 605 | • | Negative in LUMI-CELL™ and ICCVAM Positive | 0 substances |
| 606 | • | Positive in LUMI-CELL™ and ICCVAM Negative | 9 substances |
| 607 | • | Weak Positive in LUMI-CELL™ and ICCVAM Negative | 1 substances |
| 608 | • | Negative in LUMI-CELL™ and ICCVAM Negative | 19 substances ⁹ |

609

⁷ Two of these substances are well-known ER antagonist reported as positive in some ER agonist assays.

⁸ XDS classifies substances as positive even if the nature of the agonist response is such that an EC50 cannot be calculated. NICEATM has designated these substances as weak positives.

⁹ This number includes two well-known ER antagonists (tamoxifen and 4-hydroxytamoxifen) that are listed in the ICCVAM report as being positive in some agonist assays.

610 Using these data, the concordance, sensitivity, specificity, positive and negative predictivity,
 611 and false negative and false positive rates for the LUMI-CELL™ ER bioassay were
 612 calculated (see **Table 1**). Substances classified as weak positives were included in the
 613 analysis of accuracy.

614

615

	ICCVAM Classification			<i>total</i>
		+	-	
results	+	27	10	37
	-	0	19	19
<i>total</i>		27	29	56

616

617 *Concordance* = 0.82618 *Sensitivity* = 1.00 *False negative rate* = 0.00619 *Specificity* = 0.66 *False positive rate* = 0.34620 *Positive predictivity* = 0.73 *Negative predictivity* = 1.00

621

622

623 The LUMI-CELL™ ER bioassay correctly identified all 27 ICCVAM recommended ER
 624 positive agonists tested by XDS. Among the 29 (including the two antagonists) ICCVAM
 625 recommended ER negative substances, ten induced a positive agonist TA response in LUMI-
 626 CELL™. Compared to the EC50 value for estradiol, all nine of these “false positive”
 627 substances exhibited EC50 values that were six to seven fold orders of magnitude weaker.

628 The nine false positive substances included:

- 629 • 4-Androstene (ICCVAM reported as reported as presumed negative for ER
 630 agonist activity and as a strong androgen receptor [AR] agonist)
- 631 • Atrazine (ICCVAM reported as negative in three of three different ER agonist
 632 assays)
- 633 • 2-sec-Butylphenol (ICCVAM reported as presumed negative for ER agonist
 634 activity)
- 635 • Corticosterone (ICCVAM reported as negative in one ER agonist study and as
 636 binding weakly to the AR)

- 637 • Linuron (ICCVAM reported as negative in one ER agonist study and as a
638 weak AR agonist and antagonist)
- 639 • Medroxyprogesterone acetate (ICCVAM reported as presumed negative for
640 ER agonist activity and as a weak AR agonist)
- 641 • Morin (ICCVAM reported as presumed negative for ER agonist activity but as
642 binding weakly to the ER)
- 643 • Phenolphthalin (ICCVAM reported as presumed negative for ER agonist
644 activity)
- 645 • Spironolactone (ICCVAM reported as presumed negative for ER agonist
646 activity and as an AR agonist and antagonist)
- 647 • L-Thyroxine (ICCVAM reported as expected to be negative for ER agonist
648 activity)

649

650 Of the ten ICCVAM recommended negative ER TA substances reported as positive for
651 agonist activity in LUMI-CELL™, ICCVAM did not have supporting negative ER TA data
652 for seven substances, and had single test data only for two substances. Only one substance,
653 atrazine, had been reported as negative for ER TA activity in multiple (three) studies.

654 However, due to the mechanistic basis of this test system, false positives are highly unlikely.
655 These ten substances most likely have very weak transcriptional activity that is producing the
656 weak positive response. Thus, it is entirely possible that all ten of these substances are
657 capable of producing weak ER transcriptional activation and that that increased TA activity
658 represents “true” positives for the type and distribution of estrogen receptors in this test
659 system. Furthermore, these responses may indicate that this test system is capable of
660 detecting ER activity over a broad dynamic range, including very weak activity.

661 Nonetheless, such results will need confirmation in a multi-laboratory validation study and, if
662 possible, in other transcriptional assays with comparable receptor composition and
663 sensitivity. Finally, the quantitative nature of the response will likely need to be considered
664 when using this data for weight-of-evidence decisions in the EPA’s Tier 1 Endocrine
665 Disruptor Screening Program, with possibly less weight given to very weak acting
666 substances, especially those that do not demonstrate an *in vivo* effect at established limit
667 doses.

668

669 *Evaluation of Comparative Activity:* Another approach to evaluating the performance of the
 670 LUMI-CELL™ ER Bioassay, in terms of the ICCVAM recommended validation substances,
 671 is to compare the relative agonist activity of substances reported as positive in both data sets.
 672 Due to the lack of EC50 data for many of the substances recommended in the ICCVAM
 673 report, this analysis was limited to nine substances with ER TA activity. **Table 2** presents
 674 the EC50 values for these substances obtained in LUMI-CELL™ and the median EC50
 675 values reported by ICCVAM (*note: the EC50 values reported by ICCVAM were generated*
 676 *by varied test methods and protocols; where multiple studies were conducted for the same*
 677 *substance, the median value was used*). Also presented in **Table 2** are the relative rankings
 678 (from most to least potent) for the nine substances. The regression correlations (r^2) for EC50
 679 values and relative rankings were 0.607 ($p = 0.013$) and 0.903 ($p < 0.001$), respectively. Thus,
 680 the relative ER TA activities of these nine agonist substances are significantly correlated
 681 between the LUMI-CELL™ ER bioassay and the data summarized in the ICCVAM report.

682

683 **Table 2. Correlation Between Positive LUMI-CELL™ and Positive ICCVAM**
 684 **Substances with Agonist Activity**

685

Substance	ICCVAM*		LUMI-CELL™	
	Median EC50 Value (μM)	Ranking	EC50 Value (μM)	Ranking
Diethylstilbestrol	0.000019	1	0.0000000311	1
Estrone	0.0032	3	0.00000061	2
17 α -Estradiol	0.0001	2	0.00000316	3
Coumestrol	0.015	4	0.000043	4
n-Nonylphenol	0.085	6	0.000236	5
Genistein	0.062	5	0.00079	6
Bisphenol A	0.4	8	0.00107	7
Daidzein	0.29	7	0.0026	8
Methoxychlor	8.85	9	0.00353	9

686 * The ICCVAM EC50 data are generated by different investigators using different test ER TA test
 687 methods

688

689

690 2.3.3 Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the 691 LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity

692 XDS did not provide CV data for LUMI-CELL™ antagonist test results with respect to well-
 693 to-well variability within an experiment but did provide plate-to-plate (plate = experiment;

694 minimum of three experiments conducted on different days) for eight ICCVAM
 695 recommended substances reported as positive in LUMI-CELL™. An evaluation of
 696 interlaboratory antagonist reproducibility has not been conducted; this evaluation would be
 697 conducted as part of a multi-laboratory validation effort.

698

699 *Test Method Intralaboratory Reproducibility:* The mean and median CV values for plate-to-
 700 plate (i.e., experiment-to-experiment) IC50 values for eight ICCVAM recommended
 701 reference substances that induced a positive antagonist response in LUMI-CELL™ was 24
 702 and 25%, respectively. This level of intralaboratory reproducibility is considered adequate.

703

704 2.3.4 The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist
 705 Activity

706 The discussion in **Section 2.2.2** about approaches for evaluating the accuracy of the agonist
 707 version of the LUMI-CELL™ ER bioassay are relevant also to approaches for evaluating the
 708 accuracy of the antagonist version of the same assay.

709

710 *Evaluation of Concordance:* Sixteen of the 78 substances recommended by ICCVAM for
 711 the validation of *in vitro* TA test methods were tested for antagonist activity by XDS in the
 712 LUMI-CELL™ ER bioassay. In their list of 78 recommended substances, ICCVAM
 713 identified eight substances with demonstrated antagonist activity, three with anticipated
 714 antagonist activity, 10 with demonstrated negative antagonist activity, and 57 with
 715 anticipated negative antagonist activity. Of the 16 substances listed by XDS as being tested
 716 for antagonist activity in the LUMI-CELL™ ER bioassay, ICCVAM had classified eight as
 717 positive for ER antagonist activity and eight without ER antagonist activity. The results
 718 obtained by XDS for these 16 substances are as follows:

- | | | | |
|-----|---|---|--------------|
| 719 | • | Positive in LUMI-CELL™ and ICCVAM Positive | 6 substances |
| 720 | • | Weak Positive ¹⁰ in LUMI-CELL™ and ICCVAM Positive | 2 substances |
| 721 | • | Negative in LUMI-CELL™ and ICCVAM Positive | 0 substances |
| 722 | • | Positive in LUMI-CELL™ and ICCVAM Negative | 3 substances |

¹⁰ XDS classifies substances as positive even if the nature of the antagonist response is such that an IC50 cannot be calculated. NICEATM has designated these substances as weak positives.

- 723 • Weak Positive in LUMI-CELL™ and ICCVAM Negative 5 substances
- 724 • Negative in LUMI-CELL™ and ICCVAM Negative 0 substances

725

726 Using these antagonist data, the concordance, sensitivity, specificity, positive and negative
 727 predictivity, and false negative and false positive rates for the LUMI-CELL™ ER bioassay
 728 were calculated (see **Table 3**). Substances classified as weak positives were included in the
 729 analysis of accuracy.

730

731

	ICCVAM Classification			<i>total</i>
		+	-	
results		8	8	16
	+	0	0	0
	-	8	8	16
<i>total</i>		8	8	16

732

733	<i>Concordance</i>	= 0.50		
734	<i>Sensitivity</i>	= 1.00	<i>False negative rate</i>	= 0.00
735	<i>Specificity</i>	= 0.00	<i>False positive rate</i>	= 1.00
736	<i>Positive predictivity</i>	= 0.50	<i>Negative predictivity</i>	= not calculated

737

738

739 The LUMI-CELL™ ER bioassay correctly identified all eight ICCVAM recommended ER
 740 antagonist tested by XDS. Among the eight ICCVAM recommended ER TA validation
 741 substances presumed to be without antagonist activity, all eight induced a positive or weak
 742 positive antagonist ER response in LUMI-CELL™. The eight “false positive” substances
 743 included:

- 744 • Bisphenol A (ICCVAM reported as negative for ER antagonism activity in
 745 two of two antagonism studies)
- 746 • Corticosterone (ICCVAM reported as presumed negative for ER antagonism
 747 activity and as binding weakly to the AR)
- 748 • Daidzen (ICCVAM reported as negative for ER antagonist activity in two of
 749 two antagonism studies and as binding weakly to the AR)

- 750 • Diethylstilbestrol (ICCVAM reported as presumed negative for ER
751 antagonist activity and as strong ER agonist)
- 752 • 17 α -ethynyl estradiol (ICCVAM reported as presumed negative for ER
753 antagonist activity and as a strong ER agonist)
- 754 • Medroxyprogesterone acetate (ICCVAM reported as presumed negative for
755 ER antagonist activity and as a weak AR agonist)
- 756 • Spironolactone (ICCVAM reported as presumed negative for ER antagonist
757 activity and as an AR agonist and antagonist)
- 758 • Vinclozolin (ICCVAM reported as presumed negative for ER antagonist
759 activity and as an AR agonist and antagonist)

760

761 Thus, of the eight ICCVAM recommended negative antagonists reported as positive for
762 antagonist activity in LUMI-CELL™, ICCVAM did not have supporting ER antagonist
763 data for six substances; the other two substances were reported negative in two of two ER
764 antagonist studies. Daidzein was a weak antagonist in LUMI-CELL™ (i.e., reduced the
765 agonist activity of the reference estrogen but and IC50 could not be calculated).

766

767 Only eight ICCVAM recommended validation substances with known or predicted ER
768 antagonist activity were tested by XDS in the LUMI-CELL™ ER bioassay. However, the
769 list of validation substances recommended by ICCVAM contains only 11 ER antagonist
770 substances (eight with supporting data, three without *in vitro* ER TA antagonist supporting
771 data).

772

773 *Evaluation of Comparative Activity:* Another approach to evaluating the performance of the
774 LUMI-CELL™ ER bioassay for detecting antagonist activity, in terms of the ICCVAM
775 recommended validation substances, is to compare the relative antagonist activity of
776 substances reported as positive in both data sets. However, due to the limited number of
777 antagonists tested by XDS and the limited number of studies reported by ICCVAM with
778 quantitative data, this type of analysis could not be conducted.

779

780 Thus, while additional LUMI-CELL™ ER antagonist data would be useful in clarifying the
781 performance of this assay for identifying substances with antagonist activity, the lack of such
782 studies is not considered to be a significant detriment to conducting cross laboratory
783 validation studies.

784

785 **2.4 Does the BRD Adequately Provide the Information Requested in the Outline**
786 **Provided in the ICCVAM Guidelines for the Nomination and Submission of New,**
787 **Revised, and Alternative Test Methods (NIH Publ. No. 03-4508)?**

788

789 The XDS BRD adheres to the recommended outline and provides nearly all of the requested
790 information. However, additional information should be provided if the BRD is to be
791 released beyond ICCVAM. The lack of this information did not adversely impact on the
792 evaluation of Criteria 1 through 3. Examples of additional information or clarifications
793 that are needed include:

- 794 1. The information (or at least subsets of information) provided in the CD should
795 be included in the BRD.
- 796 2. In the Table of Contents, Appendices B-K should be identified and paginated,
797 and a lists of figures and tables and their locations should be included.
- 798 3. Lists of abbreviations should be in alphabetic order.
- 799 4. Figure numbers should be sequential within the main body and within each
800 Appendix.
- 801 5. Information is needed on the nature of the ER receptor in BG1Luc4E2 cell
802 line (subsequent communication from XDS indicated that ER α was the
803 primary active form but that ER β was also responsive in these cells).
- 804 6. More explanation is needed in the Appendices for some of the column
805 headings and for some of the symbols used in the various columns.
- 806 7. The approaches used by XDS to assess viability in the LUMI-CELL™ ER
807 bioassay and the way the results are presented in the various tables and
808 appendices requires clarification.
- 809 8. XDS has developed a LUMI-CELL™ historical control database for the
810 solvent controls, for the reference standard, 17 β -estradiol, and for concurrent

811 positive control chemicals. Although the relevant data appears to be the
812 subject of Appendix J (QC Charts), this information needs to be summarized
813 in **Section 7.3** of the BRD.

814 9. Appendix D-F. More information is needed on the source of the values for
815 the plate-to-plate and well-to-well CV values presented in these Appendices.

816 10. The criteria for an acceptable assay or for a positive result should be clarified.

817 11. A more comprehensive protocol (than the one provided) for both the agonist
818 and antagonist versions of LUMI-CELL™ is needed in Appendix A.

819

820

820 **3.0 NICEATM RECOMMENDATIONS:**

821

822 Based on the data provided in the XDS BRD on the LUMI-CELL™ ER bioassay,

823 NICEATM recommends to the EDWG that:

824 • LUMI-CELL™ be considered as a high priority for validation studies as an *in*
825 *vitro* test method for the detection of test substances with ER agonist and
826 antagonist activity.

827 • To facilitate independent and timely standardization and validation studies,
828 NICEATM should manage the needed studies by exercising a validation
829 coordination option in its support contract. Such studies should include
830 coordination and collaboration with ECVAM and JCVAM, and ideally
831 include one laboratory in each of the three respective geographic regions
832 supported by these three Centers.

833 • During finalization of their BRD and in preparation for the interlaboratory
834 validation study, XDS conduct additional antagonist studies to more
835 comprehensively demonstrate the suitability of LUMI-CELL™ as an assay
836 for the detection of substances with ER antagonist activity.

837

838