

Annex B

ICCVAM/NICEATM Evaluation – BG1Luc ER TA Submission

This page intentionally left blank

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32

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

EVALUATION

NICEATM

August 16, 2004

DO NOT CITE, QUOTE, OR DISTRIBUTE

TABLE OF CONTENTS

33			
34			
35			Page
36	EXECUTIVE SUMMARY		i
37	1.0 INTRODUCTION		1
38	1.1 XDS Nomination		1
39	1.2 SACATM Review (March 10-11, 2004)		3
40	1.3 NICEATM <i>Federal Register</i> Notice.....		3
41	1.4 XDS Pre-validation Background Review Document		4
42	2.0 EVALUATION OF THE ABILITY OF THE LUMI-CELL™ ER		
43	BIOASSAY TO DETECT SUBSTANCES WITH ER AGONISM AND		
44	ANTAGONISM ACTIVITY		5
45	2.1 To What Extent Does the Nomination and Proposed Test Method		
46	Address the ICCVAM Prioritization Criteria?		5
47	2.2 Do the LUMI-CELL™ Pre-Validation Agonist and Antagonist		
48	Studies Adhere to the Recommendations of the <i>ICCVAM</i>		
49	<i>Evaluation of In Vitro Test Methods for Detecting Potential</i>		
50	<i>Endocrine Disruptors</i> (NIH Publ. No. 03-4503), Especially Those		
51	Regarding Essential Test Method Components (Previously Known		
52	as Minimum Procedural Standards) and Recommended Validation		
53	Substances?		6
54	2.2.1 Essential Test Method Components.....		6
55	2.2.2 ICCVAM Recommended Validation Substances.....		7
56	2.3 Does LUMI-CELL™ Show Adequate Performance (Reliability		
57	and Accuracy) During Pre-Validation to Warrant Consideration for		
58	Validation Studies?		8
59	2.3.1 Reliability (Repeatability and Intra- and Inter-laboratory		
60	Reproducibility) of the LUMI-CELL™ ER Bioassay for		
61	Detecting ER Agonist Activity		8
62	2.3.2 The Accuracy of the LUMI-CELL™ ER Bioassay for		
63	Detecting ER Agonist Activity		8

64	2.3.3	Reliability (Repeatability and Intra- and Inter-laboratory	
65		Reproducibility) of the LUMI-CELL™ ER Bioassay for	
66		Detecting ER Antagonist Activity	12
67	2.3.4	The Accuracy of the LUMI-CELL™ ER Bioassay for	
68		Detecting ER Antagonist Activity	12
69	2.4	Does the BRD Adequately Provide the Information Requested in	
70		the Outline Provided in the <i>ICCVAM Guidelines for the</i>	
71		<i>Nomination and Submission of New, Revised, and Alternative Test</i>	
72		<i>Methods</i> (NIH Publ. No. 03-4508)?.....	15
73	3.0	NICEATM RECOMMENDATIONS:.....	16
74			
75			

76 **EXECUTIVE SUMMARY**

77

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

87 On April 21, 2004, NICEATM authored a *Federal Register (FR)* Notice (Vol. 69, No. 77, p.
88 21564), entitled “In Vitro Endocrine Disruptor Test Methods: Request for Comments and
89 Nominations.” The *FR* :

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

98 NICEATM received a pre-validation background review document (BRD) from XDS on
99 April 23, 2004, and a revised BRD on June 21, 2004. In accordance with the ICCVAM
100 nomination process, NICEATM conducted a pre-screen evaluation of the revised BRD and
101 proposal to determine the extent that the proposed nomination addresses the ICCVAM
102 prioritization criteria, ICCVAM submission guidelines, and ICCVAM recommendations for
103 standardization and validation of *in vitro* endocrine disruptor test methods. The performance
104 of the test method based on pre-validation data was also reviewed to determine if this
105 performance warrants consideration for further validation. The revised BRD is the focus of
106 the NICEATM pre-screen evaluation.

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>

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

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

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

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

129 **2. Do the LUMI-CELL™ pre-validation agonist and antagonist studies adhere to**
130 **the recommendations of the ICCVAM Evaluation of In Vitro Test Methods for**
131 **Detecting Potential Endocrine Disruptors (NIH Publ. No. 03-4503), especially**
132 **those regarding essential test method components (called minimum procedural**
133 **standards in this document) and recommended validation substances?**

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

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

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

151 *Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-*
152 *CELL™ ER Bioassay for Detecting ER Agonist Activity:* In their BRD, XDS provided
153 coefficient of variation (CV) data for LUMI-CELL™ agonist test results with respect to what
154 they classified as well-to-well variability² within an experiment for 12 ICCVAM
155 recommended positive reference substances and plate-to-plate (plate = experiment; minimum
156 of three independent experiments) for 33 ICCVAM recommended validation substances
157 reported as positive in LUMI-CELL™. An evaluation of interlaboratory agonist
158 reproducibility has not been conducted; this evaluation would be conducted as part of a
159 multi-laboratory validation effort. XDS did not use coded chemicals in the collection of
160 these data. The mean and median CV values for within experiment EC50 values for the 12
161 ICCVAM recommended positive reference substances was 28 and 29%, respectively. The
162 mean and median CV values for plate-to-plate (i.e., experiment-to-experiment) EC50 values
163 for 33 ICCVAM recommended reference substances that induced a positive response in
164 LUMI-CELL™ was 45 and 38%, respectively. These levels of repeatability and
165 intralaboratory reproducibility are considered adequate for screening assays by NICEATM.

166 *Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity:* There is no
167 agreed-upon animal or human data set to serve as a reference for determining the accuracy of
168 *in vitro* test methods for identifying substances with estrogen activity *in vivo*. As an
169 alternative, the compilation of published mammalian cell *in vitro* ER TA results, as
170 summarized in Appendix D of the ICCVAM report was compared with the LUMI-CELL™
171 ER bioassay test results reported in Appendix D of the XDS BRD. Fifty-six of the 78
172 substances recommended by ICCVAM for the validation of *in vitro* TA test methods were
173 tested for agonist activity by XDS in the LUMI-CELL™ ER Bioassay. Based on the LUMI-
174 CELL™ agonism test results, the concordance was 0.82, the sensitivity was 1.00, the

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

175 specificity was 0.66, the false negative rate was 0, and the false positive rate was 0.34. The
176 high “false positive” rate was due to ten of 29 ICCVAM recommended ER negative
177 substances producing a positive or weak positive ER agonist response in LUMI-CELL™.
178 However, due to the mechanistic basis of this test system, false positives are highly unlikely.
179 These ten substances most likely have very weak transcriptional activity that is producing the
180 weak positive response. Compared to the EC50 value for estradiol, all ten substances
181 exhibited EC50 values that were six to seven fold orders of magnitude weaker. For these ten
182 false positive substances, ICCVAM did not have supporting negative ER TA data for seven
183 substances, and had single test data only for two substances. Only one substance, atrazine,
184 had been reported as negative for ER TA activity in three studies.

185 Thus, it is entirely possible that all ten of these substances are capable of producing weak ER
186 transcriptional activation and that that increased TA activity represents “true” positives for
187 the type and distribution of estrogen receptors in this test system. Furthermore, these
188 responses may indicate that this test system is capable of detecting ER activity over a broad
189 dynamic range, including very weak activity. Nonetheless, such results will need
190 confirmation in a multi-laboratory validation study and, if possible, in other transcriptional
191 assays with comparable receptor composition and sensitivity. Finally, the quantitative nature
192 of the response will likely need to be considered when using this data for weight-of-evidence
193 decisions in the EPA’s Tier 1 Endocrine Disruptor Screening Program, with possibly less
194 weight given to very weak acting substances, especially those that do not demonstrate an *in*
195 *vivo* effect at established limit doses.

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

205 *Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the LUMI-*
206 *CELL™ ER Bioassay for Detecting ER Antagonist Activity:* XDS did not provide CV data
207 for LUMI-CELL™ antagonist test results with respect to well-to-well variability within an
208 experiment but did provide plate-to-plate (plate = experiment; minimum of three experiments
209 conducted on different days) for eight ICCVAM recommended substances reported as
210 positive in LUMI-CELL™. An evaluation of interlaboratory antagonist reproducibility has

211 not been conducted; this evaluation would be conducted as part of a multi-laboratory
212 validation effort. The mean and median CV values for plate-to-plate (i.e., experiment-to-
213 experiment) IC₅₀³ values for eight ICCVAM recommended reference substances that
214 induced a positive antagonist response in LUMI-CELL™ was 24 and 25%, respectively.
215 This level of intralaboratory reproducibility is considered adequate by NICEATM for
216 screening assays.

217 *The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity:*
218 Sixteen of the 78 substances recommended by ICCVAM for the validation of *in vitro* TA test
219 methods were tested for antagonist activity by XDS in the LUMI-CELL™ ER bioassay. In
220 their list of 78 recommended substances, ICCVAM identified eight substances with
221 demonstrated antagonist activity, three with anticipated antagonist activity, 10 with
222 demonstrated negative antagonist activity, and 57 with anticipated negative antagonist
223 activity. Of the 16 substances listed by XDS as being tested for antagonist activity in the
224 LUMI-CELL™ ER bioassay, ICCVAM had classified eight as positive for ER antagonist
225 activity and eight without ER antagonist activity. Based on the LUMI-CELL™ antagonism
226 test results, the concordance was 0.50, the sensitivity was 1.00, the specificity was 0, the
227 false negative rate was 0, and the false positive rate was 1.00. All eight ICCVAM validation
228 substances presumed to be ER antagonists induced a positive or weak positive antagonist
229 response in LUMI-CELL™. However, ICCVAM did not have supporting ER antagonism
230 data for six of these substances. Only eight ICCVAM validation substances with known or
231 predicted ER antagonist activity were tested by XDS in the LUMI-CELL™ ER bioassay.
232 However, the list of validation substances recommended by ICCVAM only contains 11 ER
233 antagonist substances (eight with supporting data, three without *in vitro* ER TA antagonist
234 supporting data).

235 Due to the limited number of antagonists tested by XDS and the limited number of studies
236 reported by ICCVAM with quantitative data, a comparative analysis of potency could not be
237 conducted. While additional LUMI-CELL™ ER antagonist data would be useful in
238 clarifying the performance of this assay for identifying substances with antagonist activity,
239 the lack of such studies is not considered to be a significant detriment to conducting cross
240 laboratory validation studies.

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

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

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

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

- 250 • LUMI-CELL™ be considered as a high priority for validation studies as an *in*
251 *vitro* test method for the detection of test substances with ER agonist and
252 antagonist activity.
- 253 • To facilitate independent and timely standardization and validation studies,
254 NICEATM should manage the needed studies by exercising a validation
255 coordination option in its support contract. Such studies should include
256 coordination and collaboration with ECVAM and JCVAM, and ideally include
257 one laboratory in each of the three respective geographic regions supported by
258 these three Centers.
- 259 • During finalization of their BRD and in preparation for the interlaboratory
260 validation study, XDS conduct additional antagonist studies to more
261 comprehensively demonstrate the suitability of LUMI-CELL™ as an assay for the
262 detection of substances with ER antagonist activity.
- 263 .

264 **1.0 INTRODUCTION**

265 **1.1 XDS Nomination**

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

- 275 • ***The Extent To Which The Proposed Test Method Is Applicable To Regulatory***
276 ***Testing Needs***
277 "The LUMI-CELL™ ER bioassay will meet the need for a high throughput
278 screening (HTPS) system of chemicals for their potential estrogen disruptor
279 activity. The US Environmental Protection Agency (EPA) identified a need for
280 this technology in the Endocrine Disruptor Steering and Testing Advisory
281 Committee (EDSTAC) recommendations in order to meet a mandate of the Food
282 Quality Protection Act of 1996 and the Safe Drinking Water Act of 1996. This
283 test method is also in response to Federal Register Notice (Vol. 66, No. 57/Friday,
284 March 23, 2001) as a HTPS method for estrogen active compounds".
- 285 • ***The Extent To Which The Proposed Test Method Is Applicable To Multiple***
286 ***Agencies/Programs***
287 "The LUMI-CELL™ ER bioassay technology may also be applicable to the US
288 Food and Drug Administration, Department of Agriculture, Department of
289 Defense, and Department of Homeland Security, since methodologies are being
290 developed to screen feed and food for potential estrogen disruptor chemicals.
291 Both food and feed are a potential source for exposure to EDCs".
- 292 • ***The Extent To Which The Proposed Test Method Is Warranted, Based On The***
293 ***Extent Of Expected Use Or Application And Impact On Human, Animal, Or***
294 ***Ecological Health***
295 "The association of exposure to EDCs and adverse health effects in human and

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>)

296 wildlife populations has led to worldwide concern. Some of the health effects that
297 have led to this concern include global increases in testicular cancer, regional
298 declines in sperm counts, altered sex ratios in wildlife populations, increases in
299 the incidence of breast cancer and endometriosis, and accelerated puberty in
300 females that are expected to result from exposure to chemicals that adversely
301 affect steroid hormone action".

302 • ***The Potential For The Proposed Test Method, Compared To Current Test***
303 ***Methods Accepted By Regulatory Agencies, To Refine, Reduce, or Replace***
304 ***Animal Use***

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

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

315 "There are no current methods approved for the detection of EDCs by any federal
316 agency. However, the LUMI-CELL™ ER bioassay shows tremendous potential
317 for prediction of adverse health and environmental effects. This is shown by the
318 very high correlation between agonist response data collected using our test
319 method and the historical data available in the database developed by NICEA™
320 on these compounds. The LUMI-CELL™ ER bioassay is sensitive enough to
321 allow for an extremely low detection limit (ppq), which should be lower than
322 federal regulations are likely to mandate. Unlike ELISA detection limits which
323 have a lower limit of >1 ppb. The LUMI-CELL™ ER bioassay will give a
324 measure of bioavailability, being a biological system itself.

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

327 "The LUMI-CELL™ ER bioassay is an extremely rapid in vitro method that can
328 evaluate the estrogenic activity of chemicals within two days. The method also
329 provides relative activity of a chemical to the standard, beta-estradiol, and
330 provides dose response activity of the chemical. The standardized protocol
331 developed allows for a very robust system with low variability and high
332 sensitivity. The cost of the LUMI-CELL™ ER bioassay is a few hundred dollars

333 per chemical, which is substantially less than any animal base method. The
334 LUMI-CELL™ ER bioassay is a transcriptionally based assay capable of testing
335 for antagonistic responses of EDCs, which is not possible using binding assays".

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

344 **1.2 SACATM Review (March 10-11, 2004)**

345 NICEATM and ICCVAM presented for consideration two nominated *in vitro* endocrine
346 disruptor test methods, one of which was the XDS LUMI-CELL™ ER bioassay, to the
347 Scientific Advisory Committee on Alternative Toxicological Methods (SACTAM) on March
348 10-11, 2004. The SACATM was supportive of the nominations and raised no objections to
349 these assays being evaluated by NICEATM and considered by the EDWG and ICCVAM for
350 future validation studies.

351 **1.3 NICEATM Federal Register Notice**

352 On April 21, 2004, NICEATM sponsored a *Federal Register (FR)* Notice (Vol. 69, No. 77, p.
353 21564), entitled “In Vitro Endocrine Disruptor Test Methods: Request for Comments and
354 Nominations.” This *FR* Notice stated that:

- 355 • ICCVAM and the SACATM had identified *in vitro* endocrine disruptor screening
356 methods as a priority for validation.
- 357 • ICCVAM had published guidelines for development of *in vitro* endocrine-
358 disruptor estrogen and androgen receptor binding and transcriptional activation
359 assays. In these guidelines, ICCVAM recommended that priority be given to
360 assays that
 - 361 1. do not require the use of animal tissue as the receptor source, but rather use
362 recombinant-derived proteins, and
 - 363 2. do not use radioactive materials.
- 364 • On behalf of ICCVAM, NICEATM invited the nomination for validation studies
365 of *in vitro* test methods that meet these recommendations and for which there are
366 standardized test method protocols, pre-validation data, and proposed validation
367 study designs.

- 368 • At this time, ICCVAM had received nominations for two *in vitro* endocrine-
369 disruptor screening methods (one was the nomination from XDS) purported to
370 meet these recommendations.
- 371 • ICCVAM will consider nominations and comments received in response to this
372 notice and develop recommended priorities for proposed evaluation and
373 validation studies of endocrine disruptor screening methods.
- 374 • Prior to the initiation of such studies, the proposed validation studies would be
375 evaluated for adherence to relevant recommendations in the report: ‘‘ICCVAM
376 Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors:
377 Estrogen Receptor and Androgen Receptor Binding and Transcriptional
378 Activation Assays’’ (NIH Publication No. 03–4503;
379 <http://iccvam.niehs.nih.gov/methods/endocrine.htm>) by the ICCVAM Endocrine
380 Disruptor Working Group (EDWG) and NICEATM.

381 NICEATM did not receive any comments on the XDS nomination in response to this *FR*
382 Notice.

383 **1.4 XDS Pre-validation Background Review Document**

384 On April 23, 2004, NICEATM received a pre-validation background review document
385 (BRD) from XDS. A request for clarification of the structure of the appendices was
386 submitted to XDS on May 12, 2004, with comments and questions submitted on May 28,
387 2004. In response to these comments and questions, XDS submitted a revised BRD on June
388 21, 2004. This revised BRD is the focus of this evaluation by NICEATM.

389
390
391

391 **2.0 EVALUATION OF THE ABILITY OF THE LUMI-CELL™ ER BIOASSAY**
392 **TO DETECT SUBSTANCES WITH ER AGONISM AND ANTAGONISM**
393 **ACTIVITY**

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

- 396 1. To what extent does the nomination and proposed test method address the
397 ICCVAM prioritization criteria?
- 398 2. Do the LUMI-CELL™ pre-validation agonist and antagonist studies adhere to the
399 recommendations of the *ICCVAM Evaluation of In Vitro Test Methods for*
400 *Detecting Potential Endocrine Disruptors* (NIH Publ. No. 03-4503,
401 <http://iccvam.niehs.nih.gov/methods/endocrine.htm>), especially those regarding
402 essential test method components (previously known as minimum procedural
403 standards) and recommended validation substances?
- 404 3. Does LUMI-CELL™ show adequate performance (reliability and accuracy)
405 during pre-validation to warrant consideration for validation studies?
- 406 4. Does the BRD adequately provide the information requested in the outline
407 provided in the *ICCVAM Guidelines for the Nomination and Submission of New,*
408 *Revised, and Alternative Test Methods* (NIH Publ. No. 03-4508)?

409 **2.1 To What Extent Does the Nomination and Proposed Test Method Address the**
410 **ICCVAM Prioritization Criteria?**

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

- 413 • is applicable to the needs of the US Environmental Protection Agency (EPA) for a
414 high throughput screening system to evaluate substances for their potential
415 estrogen disruptor activity, and may also be applicable to the US Food and Drug
416 Administration, Department of Agriculture, Department of Defense, and
417 Department of Homeland Security, since methodologies are being developed to
418 screen feed and food for potential estrogen disruptor chemicals.
- 419 • is warranted, based on the worldwide concern about the association between
420 exposure to endocrine disruptors and adverse health effects in human and wildlife
421 populations.
- 422 • is warranted, based on its potential to refine, reduce, or replace animal use
- 423 • is warranted, based on its demonstrated ability to detect estrogenic activity at
424 extremely low levels (i.e., some six to seven magnitudes lower than that induced
425 by β -estradiol, the endogenous estrogen).

- 426 • is warranted, based on its relatively low cost per substances tested (\$350) and the
427 relatively quick study duration (two days)

428 **2.2 Do the LUMI-CELL™ Pre-Validation Agonist and Antagonist Studies Adhere**
429 **to the Recommendations of the ICCVAM Evaluation of In Vitro Test Methods for**
430 **Detecting Potential Endocrine Disruptors (NIH Publ. No. 03-4503), Especially**
431 **Those Regarding Essential Test Method Components (Previously Known as**
432 **Minimum Procedural Standards) and Recommended Validation Substances?**

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

436 2.2.1 Essential Test Method Components

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

- 439 • the reference estrogen and associated TA response
440 • preparation of test substances and the volume of the administered solvent
441 • the concentration range of test substances that should be tested
442 • solvent and positive controls
443 • the number of within-test replicates
444 • methods for data analysis
445 • the need for Good Laboratory Practice (GLP) compliance
446 • study acceptance criteria
447 • interpretation of results
448 • repeat studies
449 • the study report

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

454 *ICCVAM Report Section 4.1.2 (Preparation of Test Substances and Volume of Administered*
455 *Solvent):* The report indicates that the preferred solvent is water, ethanol (95-100%), or
456 dimethylsulfoxide (DMSO), in that order. Members of the ICCVAM Expert Panel stated
457 that water or ethanol (95 to 100%) were preferred to DMSO because some substances, when
458 dissolved in DMSO, might exhibit reduced agonist activity. In the LUMI-CELL™ ER
459 Bioassay, DMSO is the solvent of choice. Based on the performance of the assay (see

460 **Section 2.2** of this BRD), the use of DMSO does not appear to have impacted on the
461 performance of this assay.

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

470 The ICCVAM report states that an evaluation of cell cytotoxicity should be included in each
471 study, and only those dose levels not associated with toxicity greater than 10% of the
472 concurrent solvent control considered in the analysis of the data. In the LUMI-CELL™ ER
473 bioassay, XDS evaluates several measures of cytotoxicity. The first is a visual inspection of
474 the cells. If the cells morphology is abnormal, or there appears to be some cell death (i.e.,
475 some cells have become detached), or if the cells are no longer attached at all and have been
476 washed away in the PBS rinse, the data from those wells are not used. The second method of
477 assessing cell toxicity is to use, for substances that are negative in the agonist assay, two
478 positive response assays. This is accomplished by mixing the highest concentration and
479 1/10th of the highest concentration of the test substance tested with the EC50⁵ concentration
480 of β -estradiol (*note: there is discordance between the BRD and the correspondence from*
481 *XDS in how toxicity is evaluated – the information provided here is based on clarification*
482 *from XDS*). If toxicity is absent, one or both of these sets of wells should result in a positive
483 response for the reference estrogen (*note: this viability assay may be of limited use if the*
484 *substances being evaluated are ER antagonists*). These approaches appear to be useful but
485 less quantitative than what was recommended by the ICCVAM Expert Panel.

486 2.2.2 ICCVAM Recommended Validation Substances

487 To facilitate the validation of *in vitro* ER TA assays, ICCVAM provided a list of 78
488 recommended substances (35 substances were classified as positive or presumed positive and
489 43 substances were classified as presumed negative for ER TA agonist activity). It was
490 recommended further that, at a minimum, 53 of these substances should be tested for agonist
491 activity (34 substances were classified as positive or presumed positive, 19 substances were
492 classified as presumed negative). Data on 108 substances were provided in the XDS BRD.
493 Of the 108 substances, 29 were substances classified as positive or presumed positives by

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

494 ICCVAM and 27 were substances classified by ICCVAM as presumed negatives for ER TA
495 activity (i.e., for a total of 56 of 78 recommended substances). The remaining 22 of the 78
496 ICCVAM recommended substances were not tested due to a lack of availability, cost
497 considerations, or because they were controlled substances for which XDS did not have a
498 license. The 52 other substances tested by XDS were those not recommended by ICCVAM.
499 For the purpose of evaluating the performance of the LUMI-CELL™ ER bioassay as a
500 screen for the detection of substances with ER agonist activity, the number of ICCVAM
501 recommended substances tested by XDS was deemed adequate.

502 **2.3 Does LUMI-CELL™ Show Adequate Performance (Reliability and Accuracy)**
503 **During Pre-Validation to Warrant Consideration for Validation Studies?**

504 2.3.1 Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the
505 LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity

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

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

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

522 2.3.2 The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Agonist Activity

523 There is no agreed-upon animal or human data set to serve as a reference for determining the
524 accuracy of *in vitro* test methods for identifying substances with estrogen activity *in vivo*. As
525 an alternative, the compilation of published mammalian cell *in vitro* ER TA results, as

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.

526 summarized in Appendix D of the ICCVAM report was compared with the LUMI-CELL™
527 ER bioassay test results reported in Appendix D of the XDS BRD. One difficulty in using
528 the ICCVAM compilation as a reference data base is the lack of agreement among published
529 studies regarding the positive or negative responses of a number of the substances
530 recommended by ICCVAM for *in vitro* ER TA validation studies. This lack of agreement
531 among laboratories is largely due to the diversity of test methods and the varied decision
532 criteria developed by different investigators to evaluate ER TA activity. Another concern
533 with using the list of ICCVAM recommended validation substances is that the classification
534 of some substances is based on a single test in a single laboratory using a system that may not
535 have been well-defined or was based on theory rather than experimentally obtained data.

536 *Evaluation of Concordance:* Fifty-six of the 78 substances recommended by ICCVAM for
537 the validation of *in vitro* TA test methods were tested for agonist activity by XDS in the
538 LUMI-CELL™ ER Bioassay. ICCVAM has classified 29 of these 56 substances as positive
539 or presumed positive⁷ and 27 as negative or presumed negative for *in vitro* ER TA activity.
540 The results obtained by XDS for the 56 substances tested in LUMI-CELL™ are as follows:

- | | | |
|-----|--|----------------------------|
| 541 | • Positive in LUMI-CELL™ and ICCVAM Positive | 25 substances |
| 542 | • Weak Positive ⁸ in LUMI-CELL™ and ICCVAM Positive | 2 substances |
| 543 | • Negative in LUMI-CELL™ and ICCVAM Positive | 0 substances |
| 544 | • Positive in LUMI-CELL™ and ICCVAM Negative | 9 substances |
| 545 | • Weak Positive in LUMI-CELL™ and ICCVAM Negative | 1 substances |
| 546 | • Negative in LUMI-CELL™ and ICCVAM Negative | 19 substances ⁹ |

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

551

552

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.

552 **Table 1** **Calculations**

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

553

554

Concordance = 0.82

555

Sensitivity = 1.00 *False negative rate* = 0.00

556

Specificity = 0.66 *False positive rate* = 0.34

557

Positive predictivity = 0.73 *Negative predictivity* = 1.00

558

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

565

- 4-Androstene (ICCVAM reported as reported as presumed negative for ER agonist activity and as a strong androgen receptor [AR] agonist)

566

- Atrazine (ICCVAM reported as negative in three of three different ER agonist assays)

567

- 2-sec-Butylphenol (ICCVAM reported as presumed negative for ER agonist activity)

568

- Corticosterone (ICCVAM reported as negative in one ER agonist study and as binding weakly to the AR)

569

- Linuron (ICCVAM reported as negative in one ER agonist study and as a weak AR agonist and antagonist)

570

- Medroxyprogesterone acetate (ICCVAM reported as presumed negative for ER agonist activity and as a weak AR agonist)

571

- Morin (ICCVAM reported as presumed negative for ER agonist activity but as binding weakly to the ER)

572

- Phenolphthalin (ICCVAM reported as presumed negative for ER agonist activity)

573

- Spironolactone (ICCVAM reported as presumed negative for ER agonist activity and as an AR agonist and antagonist)

574

575

576

577

578

579

580

581

- 582 • L-Thyroxine (ICCVAM reported as expected to be negative for ER agonist
583 activity)

584 Of the ten ICCVAM recommended negative ER TA substances reported as positive for
585 agonist activity in LUMI-CELL™, ICCVAM did not have supporting negative ER TA data
586 for seven substances, and had single test data only for two substances. Only one substance,
587 atrazine, had been reported as negative for ER TA activity in multiple (three) studies.
588 However, due to the mechanistic basis of this test system, false positives are highly unlikely.
589 These ten substances most likely have very weak transcriptional activity that is producing the
590 weak positive response. Thus, it is entirely possible that all ten of these substances are
591 capable of producing weak ER transcriptional activation and that that increased TA activity
592 represents “true” positives for the type and distribution of estrogen receptors in this test
593 system. Furthermore, these responses may indicate that this test system is capable of
594 detecting ER activity over a broad dynamic range, including very weak activity.
595 Nonetheless, such results will need confirmation in a multi-laboratory validation study and, if
596 possible, in other transcriptional assays with comparable receptor composition and
597 sensitivity. Finally, the quantitative nature of the response will likely need to be considered
598 when using this data for weight-of-evidence decisions in the EPA’s Tier 1 Endocrine
599 Disruptor Screening Program, with possibly less weight given to very weak acting
600 substances, especially those that do not demonstrate an *in vivo* effect at established limit
601 doses.

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

615

616

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

Substance	ICCVAM*		LUMI-CELL™	
	Median EC50 Value (µM)	Ranking	EC50 Value (µM)	Ranking
Diethylstilbestrol	0.000019	1	0.000000311	1
Estrone	0.0032	3	0.00000061	2
17a-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

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

620

621 2.3.3 Reliability (Repeatability and Intra- and Inter-laboratory Reproducibility) of the
 622 LUMI-CELL™ ER Bioassay for Detecting ER Antagonist Activity

623 XDS did not provide CV data for LUMI-CELL™ antagonist test results with respect to well-
 624 to-well variability within an experiment but did provide plate-to-plate (plate = experiment;
 625 minimum of three experiments conducted on different days) for eight ICCVAM
 626 recommended substances reported as positive in LUMI-CELL™. An evaluation of
 627 interlaboratory antagonist reproducibility has not been conducted; this evaluation would be
 628 conducted as part of a multi-laboratory validation effort.

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

633 2.3.4 The Accuracy of the LUMI-CELL™ ER Bioassay for Detecting ER Antagonist
 634 Activity

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

638 *Evaluation of Concordance:* Sixteen of the 78 substances recommended by ICCVAM for
 639 the validation of *in vitro* TA test methods were tested for antagonist activity by XDS in the
 640 LUMI-CELL™ ER bioassay. In their list of 78 recommended substances, ICCVAM
 641 identified eight substances with demonstrated antagonist activity, three with anticipated

642 antagonist activity, 10 with demonstrated negative antagonist activity, and 57 with
 643 anticipated negative antagonist activity. Of the 16 substances listed by XDS as being tested
 644 for antagonist activity in the LUMI-CELL™ ER bioassay, ICCVAM had classified eight as
 645 positive for ER antagonist activity and eight without ER antagonist activity. The results
 646 obtained by XDS for these 16 substances are as follows:

- 647 • Positive in LUMI-CELL™ and ICCVAM Positive 6 substances
- 648 • Weak Positive¹⁰ in LUMI-CELL™ and ICCVAM Positive 2 substances
- 649 • Negative in LUMI-CELL™ and ICCVAM Positive 0 substances
- 650 • Positive in LUMI-CELL™ and ICCVAM Negative 3 substances
- 651 • Weak Positive in LUMI-CELL™ and ICCVAM Negative 5 substances
- 652 • Negative in LUMI-CELL™ and ICCVAM Negative 0 substances

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

657 **Table 3** Calculations

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

658
 659 *Concordance* = 0.50
 660 *Sensitivity* = 1.00 *False negative rate* = 0.00
 661 *Specificity* = 0.00 *False positive rate* = 1.00
 662 *Positive predictivity* = 0.50 *Negative predictivity* = not calculated
 663

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

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.

- 669 • Bisphenol A (ICCVAM reported as negative for ER antagonism activity in two of
670 two antagonism studies)
- 671 • Corticosterone (ICCVAM reported as presumed negative for ER antagonism
672 activity and as binding weakly to the AR)
- 673 • Daidzein (ICCVAM reported as negative for ER antagonist activity in two of two
674 antagonism studies and as binding weakly to the AR)
- 675 • Diethylstilbestrol (ICCVAM reported as presumed negative for ER antagonism
676 activity and as strong ER agonist)
- 677 • 17 α -ethynyl estradiol (ICCVAM reported as presumed negative for ER
678 antagonism activity and as a strong ER agonist)
- 679 • Medroxyprogesterone acetate (ICCVAM reported as presumed negative for ER
680 antagonism activity and as a weak AR agonist)
- 681 • Spironolactone (ICCVAM reported as presumed negative for ER antagonism
682 activity and as an AR agonist and antagonist)
- 683 • Vinclozolin (ICCVAM reported as presumed negative for ER antagonism activity
684 and as an AR agonist and antagonist)

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

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

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

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

705 **2.4 Does the BRD Adequately Provide the Information Requested in the Outline**
706 **Provided in the ICCVAM Guidelines for the Nomination and Submission of New,**
707 **Revised, and Alternative Test Methods (NIH Publ. No. 03-4508)?**

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

- 713 1. The information (or at least subsets of information) provided in the CD should be
714 included in the BRD.
- 715 2. In the Table of Contents, Appendices B-K should be identified and paginated, and
716 a lists of figures and tables and their locations should be included.
- 717 3. Lists of abbreviations should be in alphabetic order.
- 718 4. Figure numbers should be sequential within the main body and within each
719 Appendix.
- 720 5. Information is needed on the nature of the ER receptor in BG1Luc4E2 cell line
721 (subsequent communication from XDS indicated that ER α was the primary active
722 form but that ER β was also responsive in these cells).
- 723 6. More explanation is needed in the Appendices for some of the column headings
724 and for some of the symbols used in the various columns.
- 725 7. The approaches used by XDS to assess viability in the LUMI-CELL™ ER
726 bioassay and the way the results are presented in the various tables and
727 appendices requires clarification.
- 728 8. XDS has developed a LUMI-CELL™ historical control database for the solvent
729 controls, for the reference standard, 17 β -estradiol, and for concurrent positive
730 control chemicals. Although the relevant data appears to be the subject of
731 Appendix J (QC Charts), this information needs to be summarized in **Section 7.3**
732 of the BRD.
- 733 9. Appendix D-F. More information is needed on the source of the values for the
734 plate-to-plate and well-to-well CV values presented in these Appendices.
- 735 10. The criteria for an acceptable assay or for a positive result should be clarified.
- 736 11. A more comprehensive protocol (than the one provided) for both the agonist and
737 antagonist versions of LUMI-CELL™ is needed in Appendix A.

738
739

739 **3.0 NICEATM RECOMMENDATIONS:**

740 Based on the data provided in the XDS BRD on the LUMI-CELL™ ER bioassay,
741 NICEATM recommends to the EDWG that:

- 742 • LUMI-CELL™ be considered as a high priority for validation studies as an *in*
743 *vitro* test method for the detection of test substances with ER agonist and
744 antagonist activity.
- 745 • To facilitate independent and timely standardization and validation studies,
746 NICEATM should manage the needed studies by exercising a validation
747 coordination option in its support contract. Such studies should include
748 coordination and collaboration with ECVAM and JaCVAM, and ideally include
749 one laboratory in each of the three respective geographic regions supported by
750 these three Centers.
- 751 • During finalization of their BRD and in preparation for the interlaboratory
752 validation study, XDS conduct additional antagonist studies to more
753 comprehensively demonstrate the suitability of LUMI-CELL™ as an assay for the
754 detection of substances with ER antagonist activity.

755
756