Align first-level headings flush left. Indent headings for each succeeding level 0.5 inches from the preceding level.

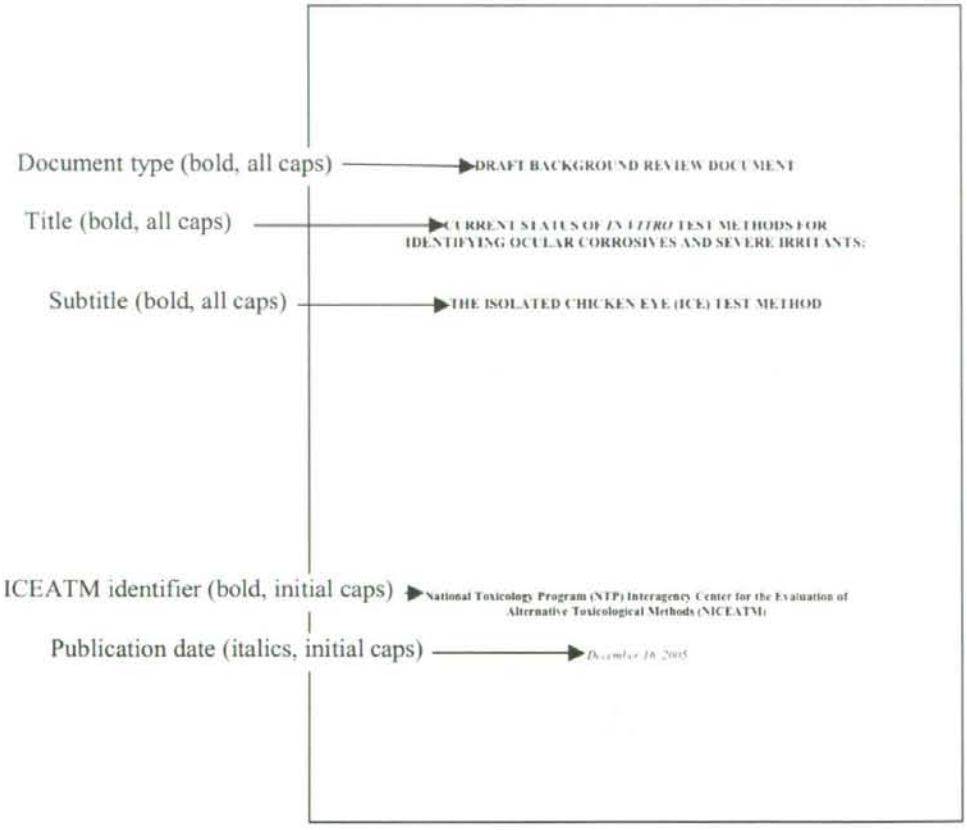
Set a right-aligned tab with a dotted leader at 6.25" for the page number.

12.44 Tabs

Use the TAB key instead of the space bar to set spacing across the page.

12.45 Title Page

Format the title page as in the following example (all text is Times New Roman, 12 pt.):



12.46 Trademark

Do not use a trademark to describe a generic (e.g., use the word *photocopy*, not *Xerox®).*

When using a trademark in the text, ensure that the proper symbol (i.e., ™ or ®) is used with the trademark. If unsure about which symbol is correct, verify with the owner of the trademark, either online or by contacting the company.

添付資料4

DRAFT

LUMI-CELL® ER ASSAY

Visual Observation Cell Viability Manual

20 April 2007

Procedure for Assessing Cell Viability Using the LUMI-CELL® ER Visual Observation Method

As per LUMI-CELL® ER assay agonist and antagonist protocols:

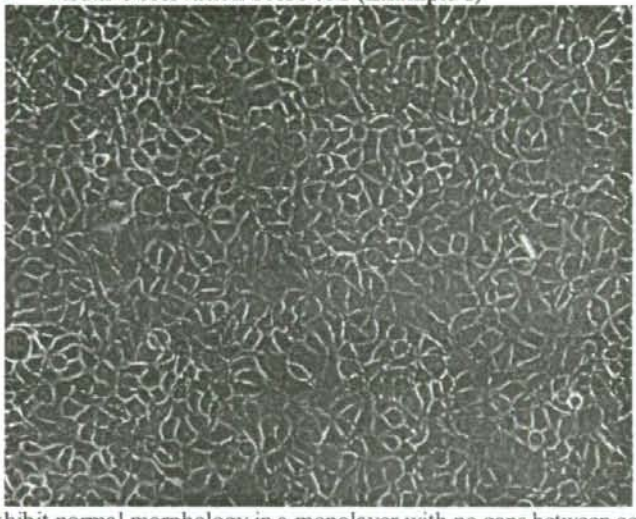
- Following nineteen to twenty four hours of incubation in test substances, remove the plates from the incubator and remove media by inverting the plate and lightly shaking over absorbent bench paper.
- Lightly tap the plate on the bench paper to remove excess liquid.
- Rinse the wells with 50 µl 1x PBS, and remove the PBS by inverting the plate and tapping it the plate on the bench paper to remove excess liquid.
- Examine all wells under an inverted microscope at 100X using phase contrast and assign cell viability scores for each well using the Visual Observation Scoring Table (**Table 1**) and **Figures 1 – 12** as reference.

Table 1 Visual Observation Scoring Table

Viability Score	Brief Description[1]
1	Normal Cell Morphology and Cell Density
2	Altered Cell Morphology and/or Small Gaps between Cells
3	Altered Cell Morphology and/or Large Gaps between Cells
4	Few (or no) Visible Cells
P	Wells containing precipitation are to be noted with "P"

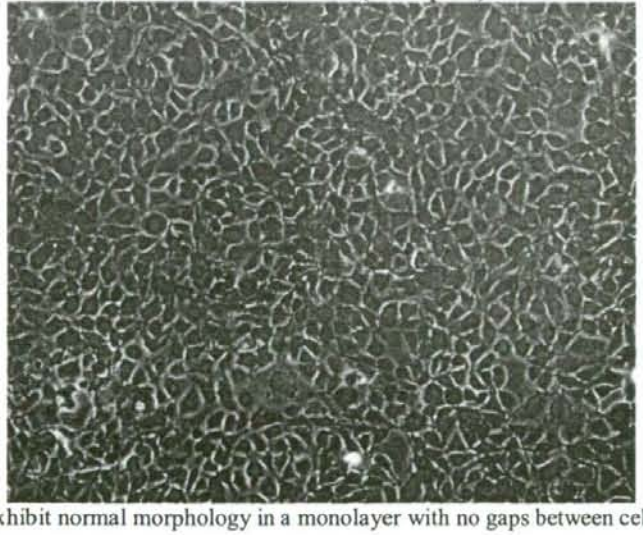
The following (**Figures 1-12**) are photomicrographs of individual wells from 96-well plates used in the LUMI-CELL® ER assay showing BG-1 cells observed through an inverted microscope at 100X using phase contrast after exposure to various substances. The scores were assigned using the visual inspection viability scoring system in **Table 1**.

Figure 1 Visual Observation Score of 1 (Example 1)



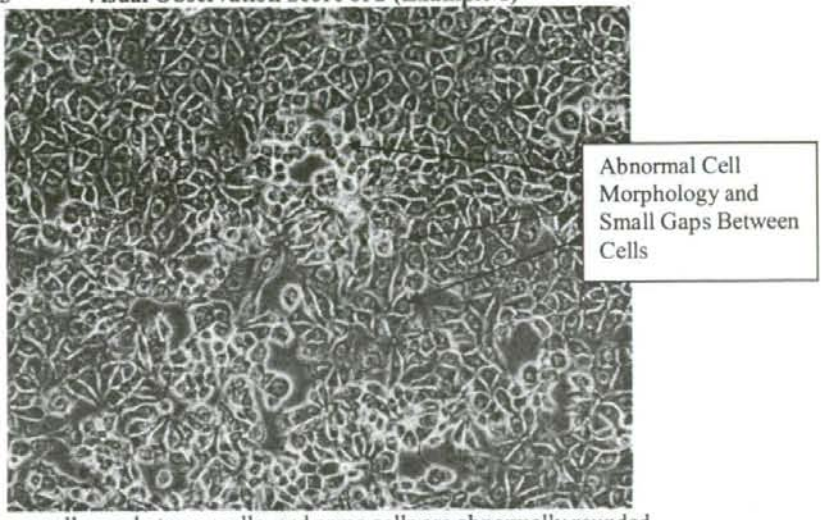
Cells exhibit normal morphology in a monolayer with no gaps between cells.

Figure 2 Visual Observation Score of 1 (Example 2)



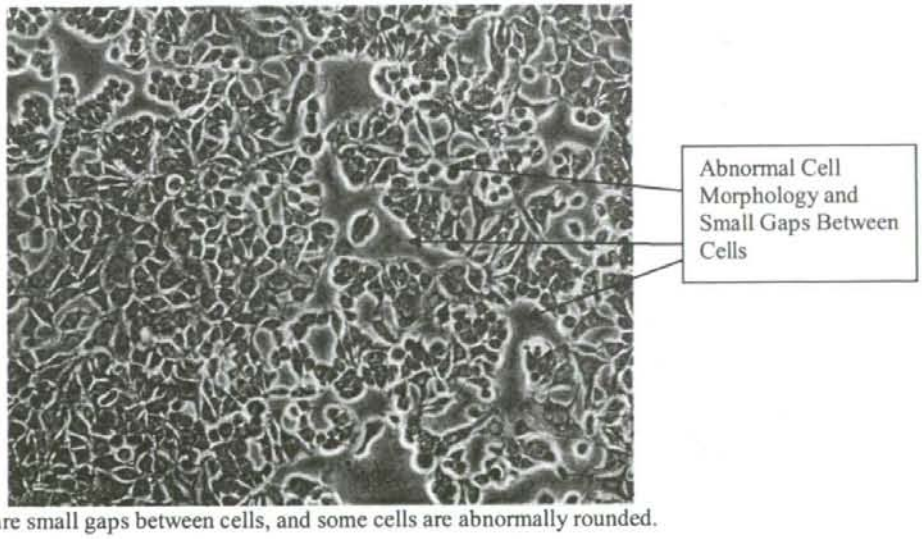
Cells exhibit normal morphology in a monolayer with no gaps between cells.

Figure 3 Visual Observation Score of 2 (Example 1)



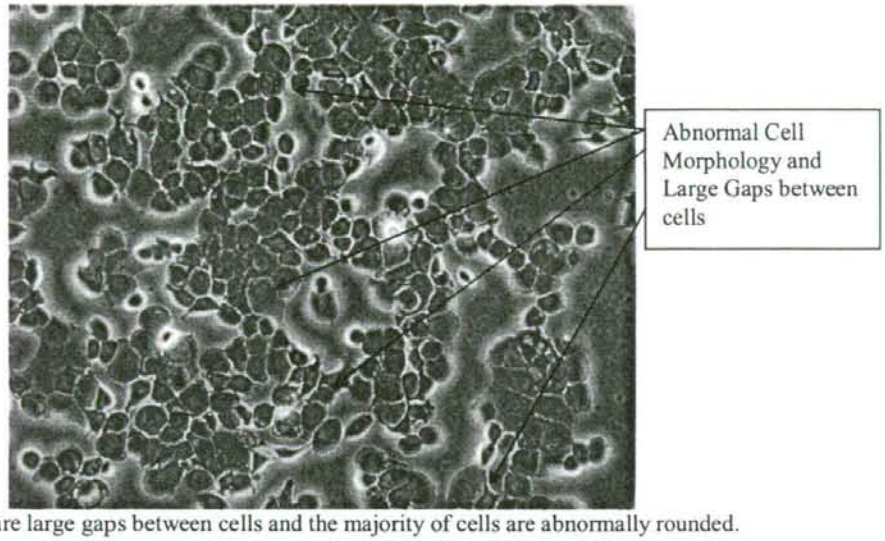
There are small gaps between cells, and some cells are abnormally rounded.

Figure 4 Visual Observation Score of 2 (Example 2)



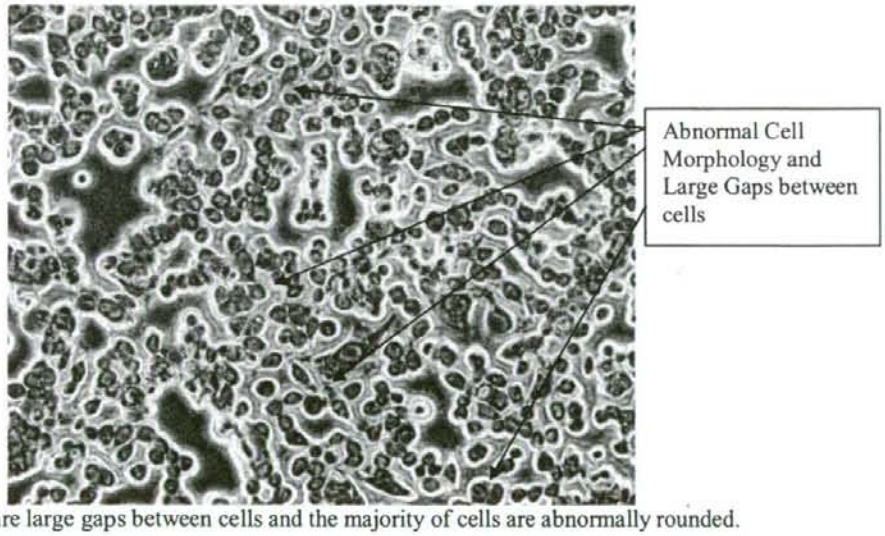
There are small gaps between cells, and some cells are abnormally rounded.

Figure 5 Visual Observation Score of 3 (Example 1)



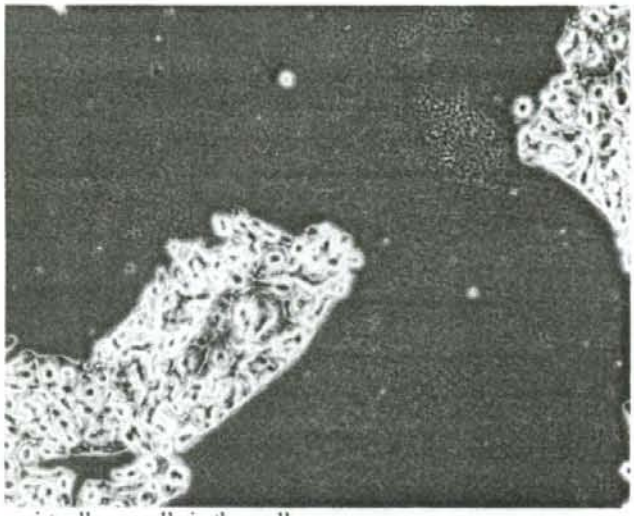
There are large gaps between cells and the majority of cells are abnormally rounded.

Figure 6 Visual Observation Score of 3 (Example 2)



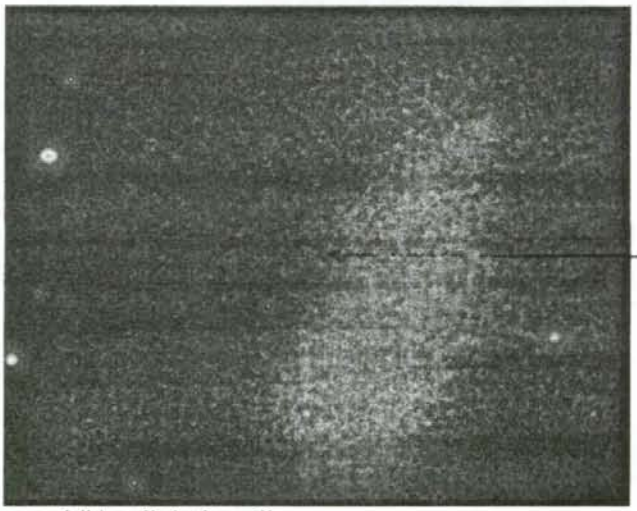
There are large gaps between cells and the majority of cells are abnormally rounded.

Figure 8 Visual Observation Score of 4 (Example 1)



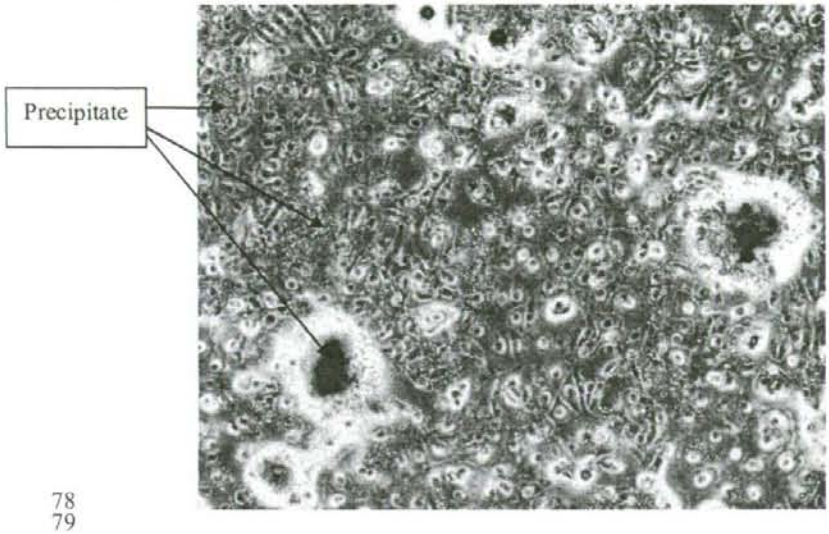
There are virtually no cells in the well.

Figure 9 Visual Observation Score of 4 (Example 2)



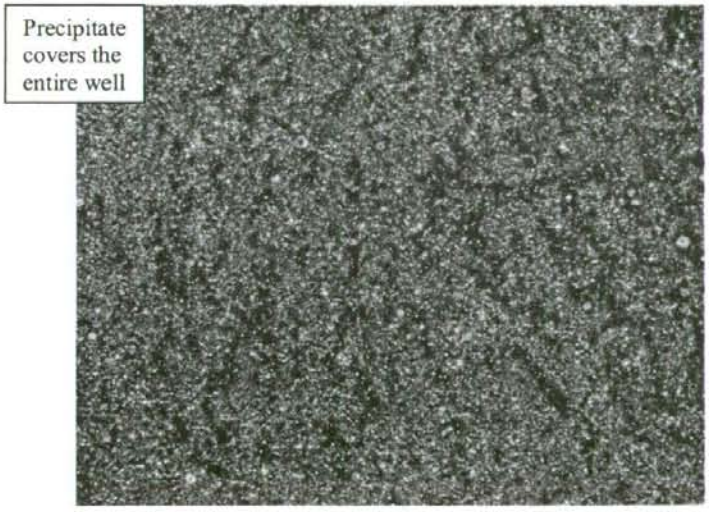
There are no visible cells in the well.

77 **Figure 10 Visual Observation noted with P for Precipitation (Example 1)**



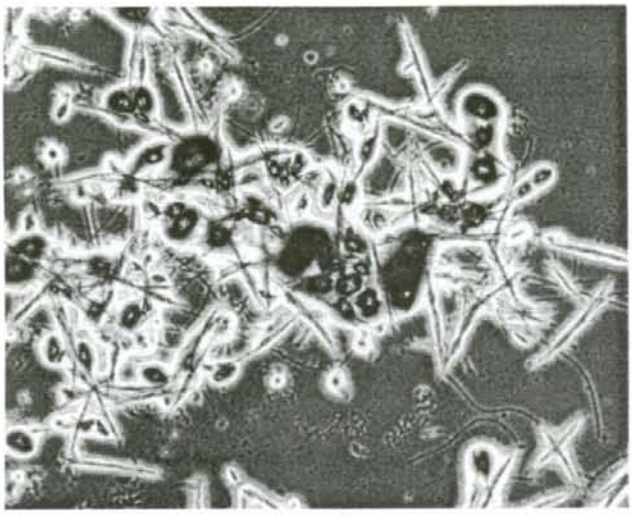
78
79

80
81 **Figure 11 Visual Observation noted with P for Precipitation (Example 2)**



82
83

Figure 12 Visual Observation noted with P for Precipitation (Example 3)



添付資料５

Amendment

LUMI-CELL® ER Assay Validation Study Design and Work Plan

Agonist and Antagonist Comprehensive Test Plate Acceptance Criteria

Amendment:

The amendment modifies the currently used criteria for acceptance of plates used for the comprehensive testing of substances for agonist and antagonist activity in order to reduce test plate failure rates in future validation study phases. Proposed modifications were agreed to by the Validation Study Management Team (SMT) on 1 October 2008 and are as follows:

- Current criteria used for acceptance of **agonist test plates**:
  - The mean plate dimethyl sulfoxide (DMSO) control relative light unit (RLU) value must be within 2.5 times the standard deviation (SD) (i.e., within 99% confidence limits) of the historical mean RLU value for the DMSO control.
  - Plate induction, as measured by dividing the highest mean estradiol (E2) reference RLU value by the mean DMSO control RLU value, must be greater than three-fold.
  - The mean plate E2 reference standard EC_{50}[1] value must be within 2.5 times the SD of the historical mean for EC_{50} values.
  - The mean plate methoxychlor control RLU value must be within 2.5 times the standard deviation of the historical mean RLU value for the methoxychlor control.

- Current criteria used for acceptance of **antagonist test plates**:
  - The mean plate DMSO control RLU value must be within 2.5 times the SD of the historical mean RLU value for the DMSO control.

[1] Half–maximal effective concentration

- Plate reduction, as measured by dividing the highest mean raloxifene (Ral)/E2 reference standard RLU value by the lowest mean Ral/E2 reference standard RLU value, must be greater than three fold.
- The mean plate Ral/E2 reference standard IC_{50}[2] value must be within 2.5 times the SD of the historical mean for IC_{50} values.
- The mean plate E2 control RLU value must be within 2.5 times the SD of the historical mean RLU value for the E2 control.

- Modified criteria to be used for acceptance of **agonist test plates**:
  - The mean plate DMSO control RLU value must be within 2.5 times the SD of the historical mean RLU value for the DMSO control.
  - Plate induction, as measured by dividing the highest mean E2 reference standard RLU value by the mean DMSO control RLU value, must be greater than three-fold.
  - The E2 reference standard curve should be sigmoidal in shape and have at least three values within the linear portion of the curve.
  - The mean plate methoxychlor control RLU value must be greater than three times the SD of the mean plate RLU value of the DMSO control.
- Modified criteria to be used for acceptance of **antagonist test plates**:
  - The mean plate DMSO control RLU value must be within 2.5 times the SD of the historical mean RLU value for the DMSO control.
  - Plate reduction, as measured by dividing the highest mean Ral/E2 reference standard RLU value by the lowest mean Ral/E2 reference standard RLU value, must be greater than three fold.
  - The Ral/E2 reference standard curve should be sigmoidal in shape and have at least three values within the linear portion of the curve.
  - The mean E2 control RLU value must be within 2.5 times the SD of the historical mean RLU value for the E2 control.

[2]Concentration of the test substance that inhibits E2 response by 50%

- The mean plate flavone/E2 control RLU value must be less than three times the SD of the mean plate E2 control value.
- The mean plate flavone/E2 control RLU value must be within 2.5 times the SD of the historical mean RLU value for the flavone/E2 control.

These modifications will be incorporated into LUMI-CELL® ER Assay validation study protocols and will be provided to the participating laboratories prior to the initiation of Phase IIb comprehensive testing.

Rationale for Amendment:

Following the completion of Phase IIa of the LUMI-CELL® ER validation study, the failure rates of test plates used for the comprehensive testing of the four coded agonist and four coded antagonist substances used in this phase were calculated. The percentages of agonist and antagonist test plates that failed acceptance criteria across the three participating laboratories were 61% (33/54) and 38% (13/34), respectively. At Hiyoshi Corporation, 11% (1/9) of agonist test plates and 0% (0/6) of antagonist test plates failed acceptance criteria; at XDS, Inc., 53% (8/15) of agonist plates and 43% (6/14) of antagonist test plates failed acceptance criteria, and at the ECVAM laboratory, 80% (24/30) of agonist test plates and 50% (7/14) of antagonist test plates failed acceptance criteria.

To determine if changes to the current acceptance criteria could reduce the failure rates of comprehensive test plates without compromising the ability of the assay to detect and quantify test substance agonist or antagonist activity, the SMT compared the qualitative (i.e., classification as an agonist or antagonist) and quantitative (i.e., agonist EC_{50} or antagonist IC_{50} value, if one could be calculated) outcomes for test plates that met all acceptance criteria versus those that failed to meet one or more criterion. Failure to meet the agonist assay acceptance criteria based on the DMSO vehicle control RLU values and the E2 reference standard minimum fold-increase induction values were not considered in this evaluation because they are essential for monitoring background activity and reference estrogen performance. In the same manner, antagonist assay acceptance criteria based on the DMSO vehicle control RLU values and the Ral/E2 minimum fold-reduction values were not considered for modification because they are

essential for monitoring background activity and reference anti-estrogen performance. In addition, the antagonist assay acceptance criterion for the E2 control RLU values was not considered for modification, because it is essential for determining test substance anti-estrogenic activity. Therefore, the acceptance criteria that were considered for modification were the E2 EC_{50} and methoxychlor RLU control values in the agonist assay, and the Ral/E2 IC_{50} and flavone/E2 RLU control values in the antagonist assay.

The coded reference substances tested in Phase IIa (four tested for agonist activity and four tested for antagonist activity) and their agonist or antagonist classification (based on ICCVAM published reference substance estrogen receptor transcriptional activation data; NIH Publication No: 03-4503 "ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors", 2003) are listed in **Table 1**.

The results for all Phase IIa comprehensive plates tested, including test substance EC_{50} or IC_{50} values and reasons for test plate failure are provided in **Appendix A** and a summary of test plate failure rates, by acceptance criterion and laboratory, is provided in **Tables 2** (agonist test plates) and **3** (antagonist test plates). Of the 54 agonist plates tested across the three laboratories, 33 (61%) failed acceptance criteria and for those that failed, 58% (19/33) did not meet the acceptance criterion for E2 EC_{50} values only (100% [8/8] at XDS, and 46% [11/24] at ECVAM), 12% (4/33) did not meet the acceptance criterion for methoxychlor control RLU values only (13% [3/24] at ECVAM, and 100% [1/1] at Hiyoshi), and 8% (2/24) did not meet acceptance criteria for both E2 EC_{50} and methoxychlor control RLU values. Of the 34 antagonist plates tested, 13 (38%) failed acceptance criteria and for those that failed, 46% (6/13) did not meet the acceptance criterion for Ral/E2 IC_{50} values only (100% [6/6] at XDS), and 15% (2/13) did not meet the acceptance criterion for flavone/E2 control RLU values only (100% [2/2] at ECVAM).

Table 1 Phase IIa Reference Substances Tested for Agonist or Antagonist Activity

ER TA Activity Tested	Substance Name	CASRN	ER TA Agonist Classification[1,2]	ER TA Antagonist Classification[1,2]
Agonist	Diethylstilbestrol	56-53-1	+++	n.a.
	Bisphenol B	77-40-7	++	n.a.
	Bisphenol A	80-05-7	+	n.a.
	Corticosterone	50-22-6	-	n.a.
Antagonist	Tamoxifen	10540-29-1	n.a.	###
	Dibenzo[*a.h*]anthracene	53-70-3	n.a.	##
	p-n-nonylphenol	104-40-5	n.a.	#
	Progesterone	57-83-0	n.a.	-

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; ER = estrogen receptor; n.a. = not applicable to this Phase, since only tested for agonist or antagonist activity but not both; TA = transcriptional activation.

[1]ER TA classification is based on ICCVAM published reference substance estrogen receptor transcriptional activation data (NIH Publication No: 03-4503 "ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors", 2003).

[2]+++ Indicates that the substance was strongly active (half-maximal effective concentration [EC_{50}] value was <0.001 [M); ++ indicates that the substance was moderately active (EC_{50} value was between 0.001 and 0.1 [M); + indicates that the substance was weakly active (EC_{50} value was >0.1 [M), or a positive response was reported without an EC_{50} value; ### indicates that the substance was uniformly positive in multiple assays; ## indicates that the substance was positive in the majority of assays in which it was tested; # indicates that the substance was positive in the single assay in which it was tested; - indicates that the substance was uniformly negative in multiple assays

A graphical comparison of EC_{50} and IC_{50} values for reference agonists and antagonists, respectively, tested in Phase IIa calculated from test plates that met all acceptance criteria versus those that failed one or more acceptance criterion is provided in **Appendix B**. A visual inspection indicated that the EC_{50} values for bisphenol A (BPA), bisphenol B (BPB), and diethylstilbestrol (DES) were similar from test plates that either met all acceptance criteria or failed E2 EC_{50} or methoxychlor control RLU criteria when testing these substances for agonist activity, and that tamoxifen (TAM) IC_{50} values were similar from plates that either met all acceptance criteria or failed Ral/E2 IC_{50} or flavone/E2 control RLU criteria when testing this substance for antagonist activity.

A qualitative evaluation of concordance for test plate results, depending on whether the results were based on test plates that met all plate acceptance criteria or failed certain acceptance criteria, are provided for agonists and antagonist studies in **Tables 4** and **5**, respectively. For agonist studies, the classification of a test substance as an agonist appears to be independent of which test plates the data were generated from. For antagonist studies, the classification of a test

Table 2 Failure Rates of Plates used to Test Phase IIa Agonist Substances using Current Acceptance Criteria

Laboratory	Total Number of Plates Tested	Number of Plates Passing Acceptance Criteria	Number of Plates Failing Acceptance Criteria	Failed DMSO Only[1]	Failed E2 EC_{50} Only[2]	Failed Methoxychlor Only[3]	Failed Multiple Acceptance Criteria[4]
XDS	15	7 (47%)	8 (53%)	n.a.	8 (100%)	n.a.	n.a.
ECVAM	30	6 (20%)	24 (80%)[5]	1 (4%)	11 (46%)	3 (12.5%)	9 (37.5%)
Hiyoshi	9	8 (89%)	1 (11%)	n.a.	n.a.	1 (100)	n.a.
Overall	**54**	**21 (39%)**	**33 (61%)**	**1 (3%)**	**19 (58%)**	**4 (12%)**	**9 (27%)**

Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β-estradiol ; EC_{50} = half-maximal effective concentration; n.a. = not applicable

[1]DMSO = averaged DMSO control RLU value must be within 2.5 times the standard deviation (SD) of the historical DMSO control value.

[2]EC_{50} = E2 reference standard EC_{50} value must be within 2.5 times the SD of the historical E2 EC_{50} value.

[3]Methoxychlor = averaged methoxychlor control RLU value must be within 2.5 times the SD of the historical methoxychlor control RLU value.

[4]Nine plates failed multiple acceptance criteria, with two failing EC_{50} and methoxychlor; two failing induction and DMSO; one failing DMSO and methoxychlor; one failing induction, EC_{50} and methoxychlor; one failing DMSO, EC_{50}; and methoxychlor; and two failing induction, DMSO, EC_{50}, and methoxychlor.

Table 3 Failure Rates of Plates used to Test Phase IIa Antagonist Substances using Current Acceptance Criteria

Laboratory	Total Number of Experiments	Number of Plates Passing Acceptance Criteria	Number of Plates Failing Acceptance Criteria	Failed Ral\E2 IC_{50} Only[1]	Failed E2 Only[2]	Failed Flavone Only[3]	Failed Multiple Acceptance Criteria[4]
XDS	14	8 (57%)	6 (43%)	6 (100%)	n.a.	n.a.	n.a.
ECVAM	14	7 (50%)	7 (50%)[6]	n.a.	4 (57%)	2 (29%)	1 (14%)
Hiyoshi	6	6 (100%)	n.a.	n.a.	n.a.	n.a.	n.a.
Overall	**34**	**21 (62%)**	**13 (38%)**	**6 (46%)**	**4 (31%)**	**2 (15%)**	**1 (8%)**

Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β-estradiol; IC_{50} = concentration of the test substance that inhibits E2 response by 50%; n.a. = not applicable; Ral = raloxifene HCl

[1]IC_{50} = Ral/E2 reference standard IC_{50} value must be within 2.5 times the SD of the historical Ral/E2 IC_{50} value.

[2]E2 = averaged E2 control RLU value must be within 2.5 times the SD of the historical E2 control value.

[3]Flavone = averaged flavone/E2 control RLU value must be within 2.5 times the SD of the historical flavone/E2 control value.

[4]One plate failed multiple acceptance criteria, failing DMSO control, flavone/E2, and E2 control RLU values.

Table 4 Qualitative Evaluation of Agonist Test Results using Test Plates that Met All Acceptance Criteria or Failed Certain Acceptance Criteria

Agonist Test Substance	Laboratory	Passed All Acceptance Criteria	Failed E2 EC_{50} Only	Failed Methoxychlor Only	Failed both E2 EC_{50} and Methoxychlor
Bisphenol A	XDS	+ (3/3)	+ (4/4)	n.a.	n.a.
	ECVAM	+ (3/3)	+ (7/7)	+ (3/3)	n.a.
	Hiyoshi	+ (3/3)	n.a.	+ (1/1)	n.a.
Bisphenol B	XDS	+ (3/3)	+ (4/4)	n.a.	n.a.
	ECVAM	+ (3/3)	+ (4/4)	n.a.	+ (2/2)
	Hiyoshi	+ (3/3)	n.a.	+ (1/1)	n.a.
Corticosterone	XDS	+ (1/3)	+ (1/4)	n.a.	n.a.
	ECVAM	+ (3/3)	+ (5/7)	+ (3/3)	n.a.
	Hiyoshi	+ (0/4)	n.a.	n.a.	n.a.
Diethylstilbestrol	XDS	+ (3/3)	+ (4/4)	n.a.	n.a.
	ECVAM	+ (3/3)	+ (4/4)	n.a.	+ (2/2)
	Hiyoshi	+ (4/4)	n.a.	n.a.	n.a.

Abbreviations: E2 = 17β-estradiol ; EC_{50} = half-maximal effective concentration; n.a. = not applicable.
Data are presented as the number of studies with a positive result among the number of studies conducted

Table 5 Qualitative Evaluation of Antagonist Test Results using Test Plates that Met All Acceptance Criteria or Failed Certain Acceptance Criteria

Antagonist Test Substance	Laboratory	Passed All Acceptance Criteria	Failed Ral/E2 IC_{50} Only	Failed Flavone Only	Failed both Ral/E2 IC_{50} and Flavone
Dibenzo[*a.h*]anthracene	XDS	+ (3/3)	+(1/3)	n.a.	n.a.
	ECVAM	+ (3/3)	n.a.	n.a.	n.a.
	Hiyoshi	+ (3/3)	n.a.	n.a.	n.a.
***p*-n-nonylphenol**	XDS	+ (0/3)	+ (0/3)	n.a.	n.a.
	ECVAM	+ (3/3)	n.a.	n.a.	n.a.
	Hiyoshi	+ (3/3)	n.a.	n.a.	n.a.
Progesterone	XDS	+ (3/3)	+ (0/3)	n.a.	n.a.
	ECVAM	+ (3/3)	n.a.	n.a.	n.a.
	Hiyoshi	+ (3/3)	n.a.	n.a.	n.a.
Tamoxifen	XDS	+ (3/3)	+ (3/3)	n.a.	n.a.
	ECVAM	+ (3/3)	n.a.	+ (1/2)	n.a.
	Hiyoshi	+ (3/3)	n.a.	n.a.	n.a.

Abbreviations: E2 = 17β-estradiol; IC_{50} = concentration of the test substance that inhibits E2 response by 50%; n.a. = not applicable; Ral = raloxifene HCl
Data are presented as the number of studies with a positive result among the number of studies conducted

substance as an antagonist appears to be more variable across test plates. For example, XDS reported that Dibenzo[*a.h*]anthracene was positive as an antagonist in 3 of 3 plates that met all test plate acceptance criteria and in 1 of 3 plates that failed the Ral/E2 IC_{50} criterion. Also, at XDS, *p*-n-nonylphenol and progesterone were positive as antagonist in 3 of 3 plates that met all test plate acceptance criteria and in 0 of 3 plates that failed the Ral/E2 IC_{50} criterion. However,

tamoxifen when tested at XDS was positive as an antagonist in 3 of 3 plated that met all test plate acceptance criteria and also in 3 of 3 plates that failed the Ral/E2 IC_{50} criterion.

To conduct a quantitative analysis, a Mann-Whitney ranked sum test was conducted where feasible[3] to determine if there were significant differences between BPA, BPB, and DES EC_{50} values from plates that passed all acceptance criteria and plates that failed the E2 EC_{50} and/or the methoxychlor control RLU criteria, and between TAM IC_{50} values from plates that met all acceptance criteria and plates that failed the Ral/E2 IC_{50} criterion (**note:** number of plates failing for flavone/E2 control value criterion only or flavone/E2 control value and Ral/E2 IC_{50} criteria was less than three, therefore analysis could not be done for acceptance criteria that included the flavone/E2 control value criterion). Results of the Mann-Whitney analysis are provided in **Table 6**; the results indicated that:

- there were no significant differences ($P>0.05$) between BPA, BPB , and DES EC_{50} values from plates that met all acceptance criteria or plates that failed the E2 EC_{50} and/or methoxychlor control RLU value criteria at XDS
- there were no significant differences between BPA and BPB EC_{50} values from plates that met all acceptance criteria or plates that failed the E2 EC_{50} and/or methoxychlor control RLU value criteria at ECVAM
- there was not a significant difference between TAM IC_{50} values from plates that met all acceptance criteria or failed the Ral/E2 IC_{50} criterion at XDS

This quantitative analysis indicates that the potency of a test substance, where it could be evaluated, was independent of whether the test plates met all acceptance criteria or whether the test plates had failed one or more selected criterion.

[3] Analysis requires test substance EC_{50} values from at least three plates that either passed all acceptance criteria or failed for E2 EC_{50} only or methoxychlor only, or IC_{50} values from at least three plates per test substance that either passed all acceptance criteria or failed for Ral/E2 IC_{50} only or flavone/E2 only

Table 6 Mann-Whitney Analysis of Test Substance EC_{50} or IC_{50} Values from Test Plates that either Passed or Failed E2 EC_{50}, Ral/E2 IC_{50} or Methoxychlor Control Acceptance Criteria

Laboratory and Substance Evaluated	Agonist Plates that Passed All Acceptance Criteria			Agonist Plates that did not Pass E2 EC_{50} and/or Methoxychlor Acceptance Criteria			P value[1]
	N	Mean EC_{50} value[2]	SD[2]	N	Mean EC_{50} value[2]	SD[2]	
XDS/BPA	3	8.8×10^{-2}	7.2×10^{-3}	4	9.9×10^{-2}	1.4×10^{-2}	0.40
ECVAM/BPA	3	1.9×10^{-1}	7.6×10^{-3}	10	1.6×10^{-1}	5.6×10^{-2}	0.16
XDS BPB	3	3.9×10^{-2}	6.0×10^{-3}	4	4.3×10^{-2}	1.1×10^{-2}	0.63
ECVAM/BPB	3	4.2×10^{-2}	1.3×10^{-2}	4	7.5×10^{-2}	1.7×10^{-2}	0.06
XDS/DES	4	1.4×10^{-5}	5.0×10^{-6}	4	2.6×10^{-5}	1.1×10^{-5}	0.20
Laboratory and Substance Evaluated	**Antagonist Plates that Passed All Acceptance Criteria**			**Antagonist Plates that did not Pass Ral/E2 IC_{50} Acceptance Criteria**			**P value[1]**
	N	**Mean IC_{50} value[2]**	**SD[2]**	**N**	**Mean IC_{50} value[2]**	**SD[2]**	
XDS/TAM	4	1.5×10^{-1}	5.7×10^{-2}	3	3.1×10^{-1}	8.8×10^{-2}	0.11

Abbreviations: BPA = bisphenol A; BPB = bisphenol B; DES = diethylstilbestrol; E2 = 17β-estradiol; EC_{50} = half-maximal effective concentration; IC_{50} = concentration of the test substance that inhibits E2 response by 50%; methoxychlor = weak positive methoxychlor control; N = number of plates; Ral = raloxifene HCl; SD = standard deviation; TAM = tamoxifen

[1]P>0.05 indicates that EC_{50} or IC_{50} values are not significantly different

[2]All are expressed in EC_{50} values (μg/mL) except for XDS/TAM, which is expressed in IC_{50} values (μg/mL)

A reevaluation of Phase IIa test plate failure rates using the amended acceptance criteria indicated that the overall rate of plate acceptance would have increased from 39% (21/54) to 80% (43/54), for agonist test plates and from 62% (21/34) to 85% (29/34) for antagonist test plates (see **Tables 7** and **8**).

Table 7 Failure Rates of Plates used to Test Phase IIa Agonist Substances using Revised Acceptance Criteria

Laboratory	Total Number of Plates Tested	Number of Plates Passing Acceptance Criteria	Number of Plates Failing Acceptance Criteria	Failed DMSO Only[1]	Failed E2 Reference Standard Only[2]	Failed Multiple Acceptance Criteria[3]
XDS	15	15 (100%)	n.a.	n.a.	n.a.	n.a.
ECVAM	30	19 (63%)	11 (37%)[5]	3 (27%)	3 (27%)	5 (46%)
Hiyoshi	9	9 (100%)	n.a.	n.a.	n.a.	n.a.
Overall	**54**	**43 (80%)**	**11 (20%)**	**3 (27%)**	**3 (27%)**	**5 (46%)**

Abbreviations: DMSO = dimethyl sulfoxide; E2 = 17β-estradiol ; n.a. = not applicable

[1]DMSO = averaged DMSO control RLU value must be within 2.5 times the standard deviation (SD) of the historical DMSO control RLU value.

[2]E2 Reference Standard = E2 reference standard concentration response curve must be sigmoidal in shape and have at least three values within
the linear portion of the curve.

[3]All five plates failed acceptance criteria for both induction and DMSO control RLU values.

Table 8 Failure Rates of Plates used to Test Phase IIa Antagonist Substances using Revised Acceptance Criteria

Laboratory	Total Number of Experiments	Number of Plates Passing Acceptance Criteria	Number of Plates Failing Acceptance Criteria	Failed E2 Only[1]	Failed Multiple Acceptance Criteria[2]
XDS	14	14 (100%)	n.a.	n.a.	n.a.
ECVAM	14	9 (64%)	5 (36%)[6]	4 (80%)	1 (20%)
Hiyoshi	6	6 (100%)	n.a.	n.a.	n.a.
Overall	**34**	**29 (85%)**	**5 (15%)**	**4 (80%)**	**1 (20%)**

Abbreviations: E2 = 17β-estradiol ; n.a. = not applicable;
[1]E2 = averaged E2 control value must be within 2.5 times the SD of the historical E2 control value..
[2]One plate failed acceptance criteria both DMSO and E2 controls RLU values.

Based on the results of the above evaluations, the SMT agreed that the amended acceptance criteria could reduce the failure rates of comprehensive test plates without compromising the ability of the assay to detect and quantify test substance agonist or antagonist activity. Therefore, the amended acceptance criteria will be incorporated into the validation study protocols that will be used to conduct Phase IIb comprehensive testing.