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LUMI-CELL® ER ASSAY

ANTAGONIST PROTOCOL

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National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

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Developed by:

Xenobiotic Detection Systems, Inc.

1601 E. Geer St., Suite S

Durham, NC 27704

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27   **TABLE OF CONTENTS**

71	LIST OF ACRONYMS AND ABBREVIATIONS	
72	13 mm test tube	13 x 100 mm glass test tubes
73	DMEM	Dulbecco's Modification of Eagle's Medium
74	DMSO	Dimethyl Sulfoxide
75	DMSO Control	1% v/v dilution of DMSO in tissue culture media
76		used as a vehicle control
77	E2	17β-estradiol
78	E2 Control	2.5×10^{-5} μg/mL E2 used as a control.
79	ER	Estrogen Receptor
80	Estrogen-free DMEM	DMEM (phenol red free), supplemented with 1 %
81		Penicillin/Streptomycin, 2 % L-Glutamine, and 5%
82		Charcoal-dextran treated FBS
83	FBS	Fetal Bovine Serum
84	Flavone/E2 Control	25 μg/mL flavone + 2.5×10^{-5} μg/mL E2,
85		used as a positive control.
86	G418	Gentamycin
87	IC_{50} Value	Concentration of a substance that decreases the measured
88		activity in an antagonist assay to 50% of the maximum
89		activity induced by the reference substance
90	Ral/E2 Reference Standard	Nine point dilution of raloxifene HCl + 2.5×10^{-5} 17β-
91		estradiol reference standard for the LUMI-CELL® ER
92		antagonist assay
93	RPMI	RPMI 1640 growth medium
94	TA	Transcriptional Activation

95   T150   150 cm^2 tissue culture flask

96

97 **1.0   PURPOSE**

98 This protocol is designed to evaluate coded test substances for potential estrogen receptor (ER)

99 antagonist activity using the LUMI-CELL® ER assay.

100 **2.0   DEFINITIONS**

101 • **Dosing Solution:** The test substance, control substance, or reference standard

102   solution which is to be placed into the tissue culture wells for experimentation.

103 • **Raw Data:** Raw data includes information that has been collected but not

104   formatted or analyzed, and consists of the following:

105   o Data recorded in the Study Notebook

106   o Computer printout of initial luminometer data

107   o Other data collected as part of GLP compliance, e.g.:

108   ▪ Equipment logs and calibration records

109   ▪ Test substance and tissue culture media preparation logs

110   ▪ Cryogenic freezer inventory logs

111 • **Soluble:** Test substance exists in a clear solution without visible cloudiness or

112   precipitate.

113 • **Study Notebook:** The study notebook contains recordings of all activities related

114   to the conduct of the LUMI-CELL® ER TA antagonist assay.

115 • **Test Substances:** Substances supplied to the testing laboratories that are coded

116   and distributed such that only the Project Officer, Study Management Team

117   (SMT), and the Substance Inventory and Distribution Management have

118   knowledge of their true identity. The test substances will be purchased, aliquoted,

119   coded, and distributed by the Supplier under the guidance of the NIEHS/NTP

120   Project Officer and the SMT.

121 **3.0   CONTROLS AND REFERENCE STANDARDS**

122 Controls for the ER antagonist protocol are as follows:

123   *Vehicle control (dimethyl sulfoxide [DMSO])*: 1% v/v dilution of DMSO (CASRN 67-68-5)

124   diluted in tissue culture media.

125   *Ral/E2 reference standard for range finder testing*: Three concentrations (1.25×10^{-1}, 1.25×10^{3},

126   and 5.00×10^{-5} μg/mL) of raloxifene HCl (raloxifene, CASRN 84449-90-1) plus a fixed

127   concentration (2.5×10^{-5} μg/mL) of 17β-estradiol (E2, CASRN: 50-28-2).

128   *Ral/E2 reference standard for comprehensive testing*: A serial dilution of raloxifene HCl

129   (raloxifene, CASRN 84449-90-1) plus a fixed concentration (2.5×10^{-5} μg/mL) of 17β-estradiol

130   (E2, CASRN: 50-28-2), consisting of nine concentrations of Ral/E2 in duplicate wells.

131   *E2 control*: *17β-estradiol*, CASRN: 50-28-2, 2.5×10^{-5} μg/mL in tissue culture media used as a

132   base line negative control.

133   *Flavone/E2 Control*: Flavone, CASRN 525-82-6, 25 μg/mL, with E2 2.5×10^{-5} μg/mL in tissue

134   culture media used as a weak positive control.

135   **4.0   OVERVIEW OF GENERAL PROCEDURES FOR ANTAGONIST TESTING**

136   All experimental procedures are to be carried out under aseptic conditions and all solutions,

137   glassware, plastic ware, pipettes, etc., shall be sterile. All methods and procedures shall be

138   documented in the study notebook.

139   Antagonist range finder testing is conducted on 96-well plates using three concentrations of

140   Ral/E2 (1.25×10^{-1}, 1.25×10^{-3}, and 5.00×10^{-5} μg/mL raloxifene with 2.50×10^{-5} μg/mL E2) in

141   duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls.

142   Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in

143   duplicate as the reference standard (**Table 4-1**). Three replicate wells for the DMSO control,

144   Flavone/E2 and E2 controls are included on each plate.

145 **Table 4-1 Concentrations of Ral/E2 Reference Standard Used in Comprehensive Testing**

Raloxifene Concentrations[1]	E2 Concentrations
1.25×10^{-2}	2.5×10^{-5}
6.25×10^{-3}	2.5×10^{-5}
3.13×10^{-3}	2.5×10^{-5}
1.56×10^{-3}	2.5×10^{-5}
7.81×10^{-4}	2.5×10^{-5}
3.91×10^{-4}	2.5×10^{-5}
1.95×10^{-4}	2.5×10^{-5}
9.77×10^{-5}	2.5×10^{-5}
4.88×10^{-5}	2.5×10^{-5}

146 [1]Concentrations are presented in μg/mL.
147

148 Visual observations for cell viability are conducted for all experimental plates just prior to

149 LUMI-CELL® ER assay evaluation.

150 Luminescence data, measured in relative light units (RLUs), is corrected for background

151 luminescence by subtracting the mean RLU value of the vehicle control (DMSO) wells from the

152 RLU measurements for each of the other wells of the 96-well plate. Data is then graphed, and

153 evaluated as follows:

154 • A response is considered positive for antagonist activity when the average
155 adjusted RLU for a given concentration is less than the mean RLU value minus
156 three times the standard deviation for the E2 control.

157 • Any luminescence at or above this threshold is considered a negative response.

158 Where possible, the concentration that causes a half-maximal response (the IC_{50}) is calculated

159 using a Hill function analysis for substances that are positive. The Hill function is a four-

160 parameter logistic mathematical model relating the substance concentration to the response

161 (typically following a sigmoidal curve) using the equation below

162 $$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log IC50 - X)\text{HillSlope}}}$$

163   where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the

164   minimum response; Top = the maximum response; log IC_{50} = the logarithm of X as the response

165   midway between Top and Bottom; and HillSlope describes the steepness of the curve. The model

166   calculates the best fit for the Top, Bottom, HillSlope, and IC_{50} parameters.

167   Acceptance or rejection of a test is based on evaluation of reference standard and control results

168   from each experiment conducted on a 96-well plate. Results for these controls are compared to

169   historical results compiled in the historical database.

170   **4.1   Range Finder Testing**

171   Antagonist range finding for coded substances consists of a seven point, logarithmic serial

172   dilution using duplicate wells per concentration. Concentrations for comprehensive testing are

173   selected based on the response observed in range finder testing. If necessary, a second range

174   finder test can be conducted to clarify the optimal concentration range to test.

175   **4.2   Comprehensive Testing**

176   Comprehensive antagonist testing for coded substances consists of 11 point, double serial

177   dilutions, with each concentration tested in triplicate wells of the 96-well plate.

178   **5.0   MATERIALS FOR LUMI-CELL® ER ASSAY ANTAGONIST TESTING**

179   This section provides the materials needed to conduct LUMI-CELL® ER testing, with associated

180   brand names/vendors[1] in brackets.

181   **5.1   BG1Luc4E2 Cells:**

182   Human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response

183   element (**Figure 5-1**) [XDS].

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[1]Brand names and vendors should not be considered an endorsement by the U.S. Government or any member of the
U.S. Government; such information is provided as examples.

184 **Figure 5-1   pGudLuc7.ERE Plasmid.**
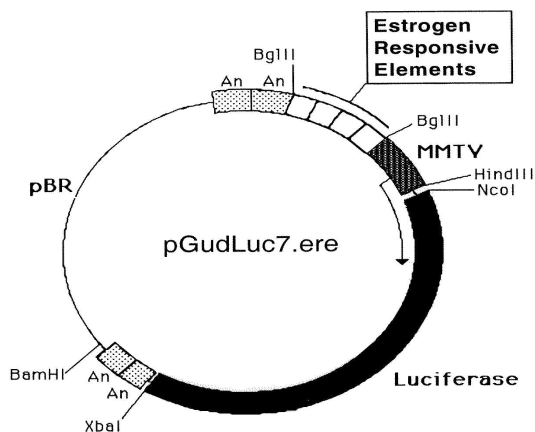
185

186 **5.2   Equipment and Supplies:**

187 General cell culture equipment, media and supplies suitable to a cell culture facility are needed.

188 Equipment, media, and supplies specific to the LUMI-CELL® ER assay are specified below.

189 Equivalent materials from other commercial sources can be used.

190 • Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or
191 equivalent and dedicated computer

192 • Shaker for 96-well plates

193 • BackSeal-96/384, white adhesive bottom seal for 96-well and 384-well microplate
194 [Perkin-Elmer, Cat. No. 6005199]

195 • 17 β-estradiol (CAS RN: 50-28-2)

196 • Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38]

197 • DMSO, U.S.P. analytical grade

198 • Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L
199 glucose, with sodium pyruvate, without phenol red or L-glutamine

200 • Fetal Bovine Serum

201 • Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 μm sterile filtered

202 • Flavone (CASRN: 525-82-6) [Sigma-Aldrich, Cat. No. F2003]

203   •   Gentamycin Sulfate (G418), 50 mg/mL

204   •   L-glutamine, 29.2 mg/mL

205   •   Luciferase Assay System (10-Pack) [Promega Cat. No. E1501]

206   •   Lysis Solution 5X [Promega, Cat. No. E1531]

207   •   Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 µg/mL streptomycin

208   •   Phosphate buffered saline (PBS, 1X) without calcium and magnesium

209   •   Raloxifene (CASRN 84449-90-1) [Sigma-Aldrich Cat. No. R1402]

210   •   RPMI 1640 medium, containing L-glutamine

211   •   Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium

212   and magnesium, without phenol red

213 Equipment should be maintained and calibrated as per GLP guidelines and individual laboratory

214 SOPs.

215 **6.0   PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS**

216 **6.1   RPMI 1640 Growth Medium (RPMI)**

217 RPMI 1640 is supplemented with 0.9% Pen-Strep and 8.0% FBS to make RPMI growth medium

218 (RPMI).

219   1.   Remove FBS from -70°C freezer, and Pen-Strep from -20°C freezer and allow to

220   equilibrate to room temperature.

221   2.   Add 44 mL of FBS and 5 mL Pen-Strep to the bottle of RPMI 1640.

222 *Store at 2-8℃ for no longer than six months or until the shortest expiration date of any media*

223 *component.*

224 **6.2   Estrogen-Free DMEM Medium**

225 DMEM is supplemented to contain 4.5% charcoal/dextran treated FBS, 1.9% L-glutamine, 0.9%

226 Pen-Strep.

227 Procedure for one 539 mL bottle:

228   1. Remove charcoal/dextran treated FBS from -70°C freezer, and L-glutamine and
229   Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.

230   2. Add 24 mL of charcoal/dextran treated FBS, 10 mL L-glutamine, and 5 mL Pen-
231   Strep to one 500 mL bottle of DMEM.

232 *Store at 2-8℃ for no longer than six months or until the shortest expiration date of any media*
233 *component..*

234 **6.3   1X Trypsin Solution**

235 1X Trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock
236 solution should be stored in 10 mL aliquots in a -20°C freezer.

237 Procedure for making 100 mL of 1X trypsin:

238   1. Remove a 10 mL aliquot of 10X trypsin from -20°C freezer and allow to
239   equilibrate to room temperature.

240   2. Aliquot 1 mL Trypsin (10X) along with 9 mL of 1X PBS into ten 15 mL sterile
241   centrifuge tubes.

242 *1X Trypsin should be stored at -20°C.*

243 **6.4   1X Lysis Solution**

244 Lysis solution is prepared by dilution from a 5X premixed stock solution. Both the 5X and 1X
245 solutions can be repeatedly freeze-thawed.

246 The procedure for making 10 mL of 1X lysis solution:

247   1. Thaw the 5X Promega Lysis solution and allow it to reach room temperature.

248   2. Remove 2 mL of 5X solution and place it in a 15 mL conical centrifuge tube.

249   3. Add 8 mL of distilled, de-ionized water to the conical tube.

250   4. Cap and shake gently until solutions are mixed.

251 *Store at -20℃ for no longer than 1 year from receipt.*

252

252 **6.5   Reconstituted Luciferase Reagent**

253 Luciferase reagent consists of two components, luciferase buffer and lyophilized luciferase
254 substrate.

255 *For long term storage, unopened containers of the luciferase buffer and lyophilized luciferase*
256 *substrate can be stored at -70℃ for up to one year.*

257 To reconstitute luciferase reagent:

258   1.   Remove luciferase buffer and luciferase substrate from -70°C freezer and allow
259   them to equilibrate to room temperature.

260   2.   Add 10 mL of luciferase buffer solution to luciferase substrate container and swirl
261   or vortex gently to mix; the Luciferase substrate should readily go into solution.

262   3.   After solutions are mixed, aliquot to a 15mL centrifuge tube.

263   4.   Store complete solution at –20°C.

264 *Reconstituted luciferase reagent is stable for up to 1 month at – 20°C.*

265 **7.0   OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF**
266 **   BG1Luc4E2 CELLS**

267 BG-1 cells are grown as a monolayer in tissue culture flasks in a dedicated tissue culture
268 incubator at 37ºC ± 1ºC, 90% ± 5% humidity, and 5.0% ± 1% CO_2/air. The cells should be
269 examined, on a daily basis during working days, under an inverted phase contrast microscope
270 and any changes in morphology and/or adhesive properties must be noted in the study notebook.

271 Two T150 flasks containing cells at 80 to 90% confluence will usually yield a sufficient number
272 of cells to fill four 96-well plates for use in experiments.

273 **7.1   Conditioning in Estrogen-free Medium, and Plating Cells for Experimentation**

274 Cells must be conditioned to an estrogen-free environment 48 to 72 hours prior to plating the
275 cells in 96-well plates for analysis of estrogen dependent induction of luciferase activity. Cells
276 conditioned in estrogen-free medium are then plated (in estrogen-free medium) into 96-well
277 plates at a plating density of 200,000 cells/mL.

278   **8.0   PREPARATION OF TEST SUBSTANCES**

279   The solvent used for dissolution of test substances is 100% DMSO. All test substances should be

280   allowed to equilibrate to room temperature before being dissolved and diluted. Test substance

281   solutions (except for reference standards and controls) should not be prepared in bulk for use in

282   subsequent tests. Test substances are to be used within 24 hours of preparation. Solutions should

283   not have noticeable precipitate or cloudiness.

284   All information on weighing, solubility testing, and calculation of final concentrations for test

285   substances, reference standards and controls is to be recorded in the study notebook.

286   **8.1   Preparation of Reference Standard and Control Stock Solutions**

287   E2, raloxifene, and flavone are prepared separately and then combined into stocks, which are

288   then used to prepare dosing solutions in **Section 9.0**.

289   8.1.1   Preparation of Initial Stock Solutions

290   Prepare an initial E2 stock solution of 5.0×10^{-3} µg/mL.

291   Prepare an initial 2.5 µg/mL raloxifene stock solution.

292   Prepare an initial 10 mg/mL stock of flavone.

293   8.1.2   Preparation of Range Finder Ral/E2 Working Stock Solutions

294   Prepare working stock solutions of Ral/E2 containing the following concentrations (**Table 8-1**):

295   **Table 8-1   Concentrations of Raloxifene and E2 in the**
296   **Ral/E2 Range Finder Stock Solution**

Tube #	Raloxifene (µg/ml)	E2 (µg/ml)
1	1.25	2.5×10^{-3}
2	1.25×10^{-1}	2.5×10^{-3}
3	5.00×10^{-3}	2.5×10^{-3}

297

298   8.1.3   Preparation of Comprehensive Testing Ral/E2 Working Stock Solutions

299   Use the raloxifene and E2 solutions prepared in **Section 8.1.1** to make a 9 point serial dilution of

300   raloxifene plus E2 as shown in **Table 8-2**.

301 **Table 8-2   Concentrations of Raloxifene and E2 in the Ral/E2 Working Stock Solution**

Tube #	Raloxifene (µg/mL)	E2 (µg/mL)
1	1.25	2.5×10^{-3}
2	6.25×10^{-1}	2.5×10^{-3}
3	3.13×10^{1}	2.5×10^{-3}
4	1.56×10^{-1}	2.5×10^{-3}
5	7.81×10^{2}	2.5×10^{-3}
6	3.91×10^{-2}	2.5×10^{-3}
7	1.95×10^{-2}	2.5×10^{-3}
8	9.77×10^{-3}	2.5×10^{-3}
9	4.88×10^{-3}	2.5×10^{-3}

302

303 8.1.4   Preparation of Fla/E2 Working Stock Solutions

304 Add 1 mL of the 5×10^{-3} µg/mL E2 (prepared as in **Section 8.1.1**) to the 10 mg/mL flavone.

305 Working stock concentration is working solution of 2.5 mg/mL flavone with 2.5×10^{-3} µg/mL E2

306 Store dosing solutions at room temperature. Use within 24 hours of preparation.

307 **8.2   Preparation of Reference Standard, and Control Substance Dosing Solutions for**
308 **Range Finder Testing**

309 Range finder testing is conducted on 96-well plates using three concentrations of Ral/E2 in

310 duplicate as the reference standard. Three replicate wells for the DMSO, and E2 controls are

311 included on each plate. Test substances are to be tested at 7 logarithmic dilutions starting at the

312 highest soluble concentration of test substance with a fixed amount of E2 (2.5×10^{-5} µg/mL)

313 8.2.1   Preparation of Ral/E2 Reference Standard Range Finder Dosing Solutions

314 Dilute the working stocks from **Section 8.1.2** 1 to 100 .

315 8.2.2   Preparation of E2 Control Range Finder Dosing Solution

316 Dilute the E2 stock from **Section 8.1.1** 1 to 200.

317 **8.3   Preparation of Reference Standard and Control Dosing Solutions for**
318 **Comprehensive Testing**

319 Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in

320 duplicate as the reference standard. Four replicate wells for the DMSO, E2 and flavone/E2

321 controls are included on each plate.

322   8.3.1   <u>Preparation of Ral/E2 Comprehensive Testing Dosing Solutions</u>

323   Dilute the working stock solution from **Section 8.1.3** 1 to 100.

324   8.3.2   <u>Preparation of E2 Control Comprehensive Testing Dosing Solutions</u>

325   Dilute the E2 stock from **Section 8.1.1** 1 to 200.

326   8.3.3   <u>Preparation of Flavone/E2 Control Comprehensive Testing Dosing Solutions</u>

327   Dilute the Fla/E2 stock from **Section 8.1.4** 1 to 200.

328   8.3.4   <u>Preparation of Test Substance Dosing Solutions for Comprehensive Testing</u>

329   Comprehensive testing experiments are used to determine whether a substance possesses ER

330   antagonist activity in the LUMI-CELL® ER test method. Antagonist comprehensive testing for

331   coded substances consists of 11 point, double serial dilutions of test substance with a fixed

332   amount of E2 (2.5×10^{-5} µg/mL), with each concentration tested in triplicate wells of the 96-well

333   plate. To prepare test substance dosing solutions for comprehensive testing, determine the

334   concentration at which maximal antagonism occurs in the range finder experiment. Start an 11-

335   point serial dilution curve at a 20-fold higher concentration than the concentration causing a

336   maximal antagonist response (i.e., if the maximum antagonist response occurred at 0.01 mg/mL,

337   start the serial dilution curve at 0.2 mg/mL).

338   **9.0**   **DATA ANALYSIS**

339   Prior to measurement of luminescence, remove treated plates from the incubator. Remove media,

340   then perform visual inspection of cell viability using the scoring in **Table 9-1**.

341   Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and

342   with software that controls the injection volume and measurement interval. Light emission from

343   each well is expressed as relative light units (RLU) per well.

344   **Table 9-1**   **Visual Observation Scoring**

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"

348 Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and

349 with software that controls the injection volume and measurement interval. Light emission from

350 each well is expressed as RLU per well.

351 **9.1   Adjusting and Normalizing RLU Values**

352 Subtract background luminescence (average DMSO solvent control RLU value) from test

353 substance, reference standard and control RLU values. Plate induction is calculated using these

354 corrected RLU values. Test substance, reference standard, and control RLU values are then

355 adjusted relative to the highest E2 reference standard RLU value, which is set to 10,000.

356 9.1.1   Determination of Outliers

357 The Study Director will use good statistical judgment for determining "unusable" wells that will

358 be excluded from the data analysis and will provide an explanation in the study notebook for any

359 excluded data.

360 9.1.2   Acceptance Criteria

361 Acceptance or rejection of a test is based on evaluation of reference standard and control results

362 from each experiment conducted on a 96-well plate. Results are compared to quality controls

363 (QC) for these parameters derived from the historical database, which are summarized below.

364 • Reduction: Plate reduction, as measured by dividing the averaged highest Ral/E2

365   reference standard RLU value by the averaged lowest Ral/E2 reference standard

366   RLU value, must be greater than three fold.

367 • Reference standard results: Calculated Ral/E2 reference standard IC_{50} values must

368   be within 2.5 times the standard deviation of the historical database IC_{50} mean

369   value.

370 • Solvent control results: DMSO control RLU values must be within 2.5 times the

371   standard deviation of the historical solvent control mean RLU value.

372 • E2 control results: E2 control RLU values must be within 2.5 times the standard

373   deviation of the historical E2 control mean RLU value.

374   •   Positive control results: Flavone/E2 control RLU values must be within 2.5 times

375   the standard deviation of the historical database flavone/E2 control mean RLU

376   value.

377   An experiment that fails any single acceptance criterion will be discarded and repeated.

378   **10.0**   **RANGE FINDER TESTING**

379   To determine starting concentrations for comprehensive testing use the following criteria:

380   •   If there are no points on the test substance concentration curve that are less than

381   the mean value of the E2 control minus three times the standard deviation from

382   that mean, the highest concentration used in comprehensive testing is the limit

383   dose or the maximum soluble dose.

384   •   If there are points on the test substance concentration curve that are below the

385   mean value of the E2 standard control minus three times the standard deviation

386   from that mean, select a concentration that is a single log dilution higher than the

387   concentration giving the lowest adjusted RLU value in the range finder and use

388   that as the highest concentration for comprehensive testing.

389   •   If a substance exhibits a biphasic concentration curve, the range finder experiment

390   should be repeated unless the proposed concentration range for the comprehensive

391   studies will include all concentrations of the biphasic region in the range finding

392   study. If the range finder experiment is repeated and the substance still exhibits a

393   biphasic concentration curve, comprehensive testing must be conducted on the

394   peak of the biphasic curve at the lowest test substance concentration. If the

395   substance is negative at this lowest concentration, then test at the higher

396   concentration. For either peak of the concentration curve, select a concentration

397   that is a single log dilution higher than the concentration giving the lowest

398   adjusted RLU value in the range finder and use that as the highest concentration

399   for comprehensive testing.

400   **11.0**   **COMPREHENSIVE TESTING**

401   Evaluate whether comprehensive experiments have met acceptance criteria (**Section 10.1.2**).

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- If the substance has been tested up to the limit dose or the maximum soluble dose without causing a significant decrease in cell viability, and there are no points on the concentration curve that are below the line indicating the mean minus three times the standard deviation of the E2 control, the substance is considered negative for antagonism.

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- If the substance has been tested up to the limit dose and there are points on the concentration curve that are below the line indicating the mean minus three times the standard deviation of the E2 control, but cell viability, has a visual inspection score of 2 or greater at all points falling below the E2 line, the substance is considered negative for antagonism.

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- If there are points on the test substance concentration curve that are below the line indicating the mean minus three times the standard deviation of the E2 control that do not cause a visual inspection score of 2 or greater, the substance is positive for antagonism.

416
417

    o Points in the test substance concentration curve that cause a visual inspection score of 2 or greater, are not included in data analyses.

418