Estrogen Receptor (ER) Bioassay

Environmental Monitoring for
Endocrine-Disrupting Chemical (EDC) Pollutants

96-well Format Assays
Product # EM00431

▪▪▪

Technical Manual
(version 7.2EM)

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INDIGO BIOSCIENCES
Nuclear Receptor & *In Vitro* Toxicology Solutions™

Estrogen Receptor (ER) Bioassay
96-well Format Assay

I. Description

▪ The Assay System ▪

The principal application of this Estrogen Receptor bioassay is to screen processed environmental samples, with the aim of quantifying the aggregate bioactivities, if any, of contaminating endocrine disrupting chemical (EDC) entities.

This assay utilizes proprietary mammalian cells expressing the native human Estrogen Receptors 1 & 2, ligand-dependent transcription factors commonly referred to as ERα and ERβ. The cells also include the luciferase reporter gene functionally linked to an ER-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in total ER activity.

The ER Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields exceptional cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for cumbersome intermediate steps such as spin-and-rinse steps, viability determinations, or cell titer adjustments prior to assay setup.

This ER Assay is an all-inclusive assay system. In addition to the Reporter Cells, included are two optimized media for use during cell culture and in diluting the user's test samples, the reference agonist 17β-estradiol (E2), Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor reporter assays capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg^{+2}-dependent reaction that consumes O_2 and ATP as co-substrates and yields as products oxyluciferin, AMP, PP_i, CO_2, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer and is reported in terms of Relative Light Units (RLU's).

INDIGO's Nuclear Receptor Reporter Assays feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating at least a 5-minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

▪ Preparation of Vehicle, E2-Standare Curve, Test-, and Control-Sample Media Treatment ▪

Preparation of E2 treatment media for Standard Curve Generation. The provided stock of 17β-estradiol (E2) is used to generate a series of serial dilutions that will be used to generate an E2 Standard Curve, thus allowing one to correlate measured Test Sample-induced ER activity to an E2 Bioequivalent (E2-BEq) concentration. Depending on the preference of the local regulatory authority, E2 concentration will be reported in either units of "pM" or "ng/L". This Technical Manual adopts pM as the default unit of concentration, however, **APPENDIX 1** references both units of concentration.

Preparation of Test Sample treatment media: Test samples are derived from collected volumes of environmental water. These are then processed by solid phase extraction (SPE) and provided in the form of highly concentrated DMSO stocks.

> *NOTE:* For all SPE processed Test- and Control- samples, when performing the final DMSO solvent-exchange we recommend striving for a 5,000-fold *or greater* enrichment factor (EF) relative to the original volume of water sample collected.

Preparation of Vehicle and various Control Sample treatment media:

a.) **'Vehicle only'** treatment media. This is Compound Screening Medium (CSM) supplemented with 0.4% DMSO. The averaged RLU values measured from the vehicle-treated assay wells are background signal, which will then be subtracted from the RLU values of all other Control and Test sample treatments.

b.) **Other Controls** treatment media. These control samples include those mandated by the local regulatory authority. They are SPE-processed water samples prepared in final form as concentrated DMSO stocks. Often, three types of Control Samples are required:

> *1.* **'Method Blank'**: Ultra-pure water → SPE → concentrated DMSO stock
>
> *2.* **'Method Spike'**: Ultra-pure water spiked with the reference analyte → SPE → concentrated DMSO stock. For the ER assay, 17β-estradiol (E2; MW 272.4) is the reference used.
>
> *3.* **'Matrix Spike'**. This is an environmental (or 'field') water sample that has been collected and similarly spiked with the reference analyte.

As detailed in *Step 7* of the following assay protocol, the provided E2 DMSO stock, and the SPE-processed Test- and Control-Sample DMSO stocks will ultimately be diluted 250-fold to achieve their respective final assay concentrations. This scheme ensures that the final concentration of DMSO in each assay well does not exceed 0.4% (see NOTE 1).

Due to the hydrophobic nature of many industrial and agrichemical pollutants, we recommend adopting the 3-step dilution strategy depicted in **APPENDICES 1** & **2**.

First, use DMSO to prepare serial 2-fold dilutions to generate intermediate DMSO stocks.

Second, a portion of each intermediate DMSO stock is transferred into a volume of Compound Screening Medium (CSM) to make 125-fold dilutions. That manipulation generates '2x-concentrated' treatment media.

Finally, 100 µl of each 2x-concentrated media is dispensed into wells of the assay plate. The assay wells will already contain ER Reporter Cells and 100 µl of CSM. In this way, the desired series of respective final (1x) treatment concentrations are achieved in the assay wells.

> *NOTE 1*: Total DMSO (or any organic solvent) carried over into assay wells should not exceed 0.4%. Emerging cytotoxicity can be expected if above 0.4% DMSO.
>
> *NOTE 2:* Dilutions of the DMSO intermediate stocks into CSM to generate the 2x-concentrated media should be made just prior to assay setup, and then considered to be 'single-use' reagents.

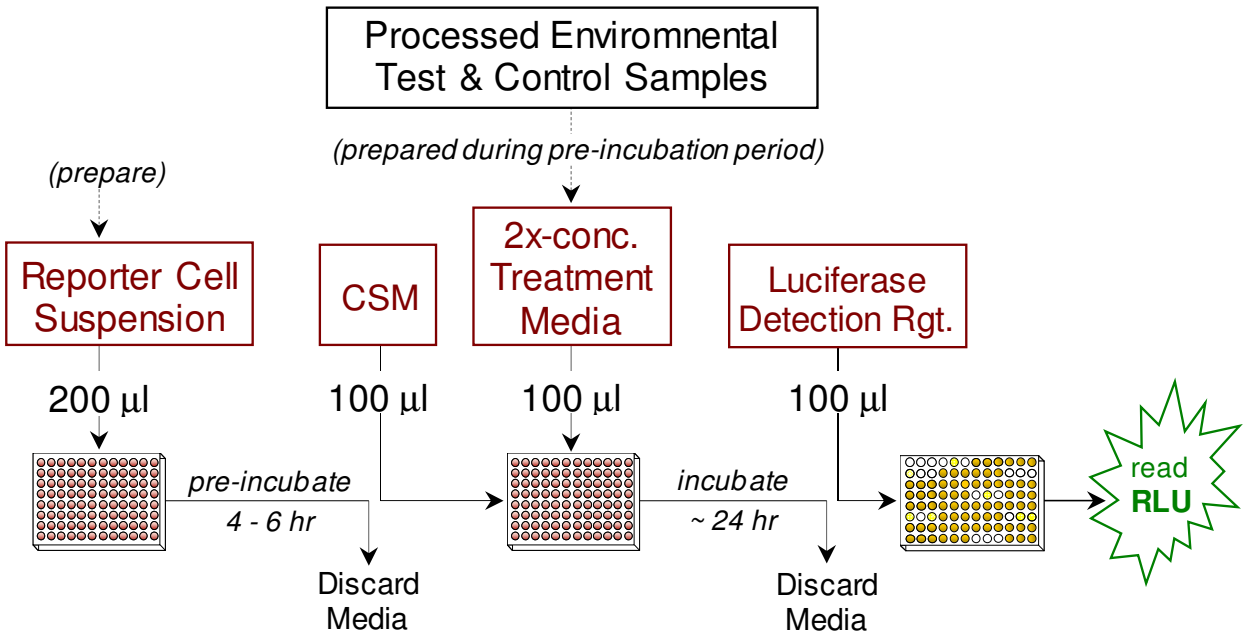

Figure 1. The ER Assay Workflow*. Environmental 'Test' and 'Control' samples are processed and concentrated in advance of the ER assay setup. Sample processing is most commonly performed *via* solid phase extraction (SPE), solvent 'blow-off', and a final solvent exchange into DMSO with a preferred relative enrichment target of ≥ 5,000-fold.

In brief: 200 µl of Reporter Cells are dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 100 µl / well of CSM is added, followed immediately by dispensing 100 µl / well of the prepared 2x-concentrated sample treatment media. Following 22 - 24 hours incubation, treatment media are discarded, and Luciferase Detection Reagent is added. Light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.

* *NOTE:* If INDIGO's Live Cell Multiplex (LCM) Assay is to be incorporated, refer to the LCM Assay Technical Manual for a detailed description of the multiplexed assay workflow.

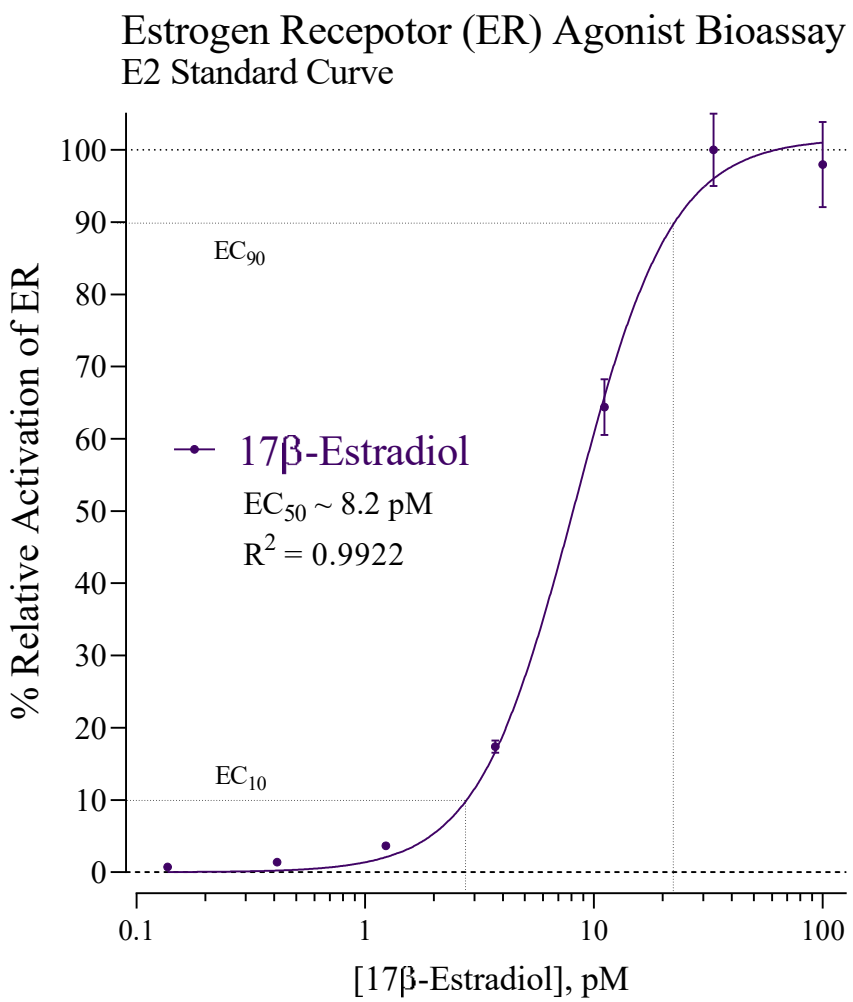

Figure 2. Generating a Standard Curve of ER activity.

The reference agonist 17β-estradiol (E2; provided) is used to generate a standard curve following the dilution scheme presented in APPENDIX 1. Average relative light units (RLU) and corresponding values of Percent Coefficient of Variation (%CV) were determined for each treatment concentration (n = 3). Values of Percent Activation (relative to the EC_{100} concentration of E2) were calculated for each treatment concentration of E2, as described in *Step 19e*. GraphPad Prism software was used to plot Percent Relative Activation *vs.* Log_{10}[E2, pM] using the 4-parameter, least squares method of non-linear regression; the bottom plateau of the sigmoidal curve is constrained to '0'.

II. Product Components & Storage Conditions

This ER Assay kit contains materials to perform assays within a single 96-well assay plate.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 3* of this protocol.

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

Kit Components	*Amount*	*Storage Temp.*
▪ ER Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ 17β-Estradiol, 25 nM (in DMSO) (E2, reference agonist for ER's)	1 x 30 µL	-20°C
▪ Luciferase Detection Reagent	1 x 12 mL	-20°C
▪ 96-well, *collagen-coated* assay plate (white, sterile, cell-culture ready)	1	-20°C

NOTE: This Assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or -80°C) until use.

III. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

DAY 1

▪ dry ice bucket (*Step 3*)

▪ cell culture-rated laminar flow hood.

▪ 37°C, humidified 5% CO_2 incubator for mammalian cell culture.

▪ 37°C water bath.

▪ 70% alcohol wipes

▪ 8-channel electronic, repeat-dispensing pipettes & sterile tips

▪ disposable media basins, sterile.

▪ Dimethyl Sulfoxide (DMSO), anhydrous.

▪ 0.5 mL snap-cap tubes or PCR Strip-tubes for generating low-volume, 250x-concentrated intermediate DMSO stocks of E2, Control, and Test Samples.

▪ sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* sterilized 96 deep-well blocks (*e.g.,* Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating treatment media comprising 2x-concentrated reference and test samples.

▪ *Optional:* clear 96-well assay plate, sterile, for viewing cells on *Day 2;* collagen-coating is not necessary for viewing applications.

▪ *Optional:* ER Antagonist reference as a '+ Control' when performing antagonist assays.

DAY 2 Plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-12* are performed on ***Day 1***, requiring less than 2 hours of actual bench work and a 4-hour incubation step to complete. *Steps 13-18* are performed on ***Day 2*** and require less than 1 hour to complete.

▪ A word about Antagonist-mode assay setup ▪

When setting up receptor inhibition assays the Reporter Cells are co-treated with a fixed sub-maximal concentration (typically between EC_{50} – EC_{85}) of the reference agonist AND varying concentrations of the test compound(s). This assay kit includes a 25 nM stock solution of **17β-estradiol (E2)**, a potent physiological agonist of the ER's, that may be used to set up such receptor inhibition studies. 16 pM E2 typically corresponds to ~EC_{80} in this assay and is a suitable concentration of challenge agonist to use when screening test samples for inhibitory activity to the ER's.

Add the challenge agonist, E2, to a bulk volume of **CSM** at a **2x-EC_{80}** concentration (*i.e.,* ~32 pM). This medium is then used to prepare the various 2x-concentrated treatment media to be dispensed into the wells of the assay plate. This is an efficient and precise method of setting up antagonist assays.

DAY 1 Assay Protocol: All steps must be performed using aseptic technique.

1.) Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Rapid Thaw of the Reporter Cells: *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from ***each* of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **21 ml**.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) Gently invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, dispense **200 µl / well** of cell suspension into the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

NOTE 4.3: Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear, *collagen-coated* 96-well assay plate. Continue to process the clear plate in an identical manner to the white assay plate.

5.) Pre-incubate reporter cells. Place the assay plate into a mammalian cell culture incubator (37°C, ≥70% humidity, 5% CO_2) for 4 - 6 hours.

6.) Remove Compound Screening Medium (CSM) from freezer storage and thaw in a 37°C water bath.

7.) Use Compound Screening Medium (CSM) to Prepare 2x-concentrated Treatment Media containing various concentrations of E2 for the Standard Curve, and all SPE processed Control and Test Samples. Refer to "Preparation of Test and Control Samples" on page 4. In *Step 9* of the assay protocol **100 µl** of these various 2x-concentrated treatment media preparations is dispensed into wells of the assay plate. These wells will already contain reporter cells and 100 µl of CSM. In this way, one will generate a series of treatment media at their respective final assay concentrations. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO (or any organic solvent) carried over into assay wells should not exceed 0.4%. Therefore, work with no less than 250x-concentrated DMSO stocks of test and control samples.

a.) **Prepare 2x-concentrated Treatment Media for the E2 Standard Curve.** This ER assay kit includes a 25 nM stock solution of 17β-estradiol (E2), a potent physiological agonist of the estrogen receptors. Use a portion of this stock to generate an E2 Standard Curve, which will then be used to determine the E2 Bioequivalent (E2-BEq) activities of all test samples. As depicted in **APPENDIX 1**, begin by using DMSO to generate intermediate stocks that are 250x-concentrated relative to each final assay concentration. Next, perform a 125-fold dilution of these intermediate stocks by transferring a portion of each into CSM, thereby generating **2x-concentrated** Treatment Media, to be used in *Step 10*. As depicted in **APPENDIX 1**, the following 7-point treatment series (in terms of *final* assay concentrations), prepared in serial 3-fold decrements, is recommended for use in generating Standard Curve: 100, 33.3, 11.1, 3.70, 1.23, 0.412, and 0.137 pM 17β-estradiol. Always include a 'vehicle only' control, which will be 0.4% DMSO. A representative plot of a typical E2 Standard Curve is depicted in **Figure 2**.

b.) **Prepare 2x-concentrated Treatment Media containing the SPE-processed environmental samples.** Control and Test Sample stocks are derived from environmental test samples that have been previously processed by SPE and are now in the form of concentrated stocks in DMSO. So as to not exceed 0.4% DMSO carry-over into the assay wells, these SPE-processed stocks will be used as 250x-concentrations relative to the highest treatment concentration applied to the assay wells. As depicted in **APPENDIX 2**, use a portion of these primary stocks to make one (or two) subsequent **2-fold** dilutions using DMSO as the diluent. Next, perform a 125-fold dilution of the intermediate DMSO stocks by transferring a portion into CSM to generate **2x-concentrated** Treatment Media, to be used in *Step 10*.

8.) At the end of the 4-6 hours pre-culture period, discard the media. The preferred method is to use a 'wrist flick' to eject media into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

9.) Dispense **100 µl / well** of **CSM** into the assay plate.

10.) Dispense **100 µl** of each **2x-concentrated Treatment media** (prepared in *Steps 7a* and *7b*) into appropriate wells of the assay plate.

NOTE: If well-to-well variation due to 'edge-effects' is a concern, this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-channel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

11.) Transfer the assay plate back into the mammalian cell incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 70%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

12.) For greater convenience on *Day 2,* retrieve **Luciferase Detection Reagent (LDR)** from freezer storage and place it in a dark refrigerator (4°C) to thaw overnight.

DAY 2 Assay Protocol: Subsequent manipulations do *not* require special regard for aseptic technique and may be performed on a bench top.

13.) Approximately 30 minutes before intending to quantify receptor activity, remove **Luciferase Detection Reagent (LDR)** from the refrigerator and allow it to equilibrate to room temperature in a low-light area.

NOTE: Do NOT actively warm LDR above room temperature. If this reagent was not allowed to thaw overnight at 4°C, use a room temperature water bath to expedite thawing.

14.) Set the plate-reader to "luminescence" mode. Program the instrument to perform a single 5 second "plate shake" prior to reading the first well of the assay plate. Set the read time to 0.5 second (500 mSec) per well, *or less*.

15.) Immediately before proceeding to *Step 16*, pour out the entire 12 ml volume of **LDR** into a media basin; rock the basin briefly to mix the reagent.

16.) Following 22 - 24 hours incubation discard the treatment media contents of the assay wells by manually ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

17.) Add 100 µl of **LDR** to each assay well. Allow the plate to rest at room temperature for 5 - 10 minutes. Do not shake the assay plate during this period.

18.) Quantify luminescence.

19.) Data analyses. Many regulatory agencies have adopted acceptance criteria for functional bioassays based on units of *Percent Relative Activation* of the receptor. As an example, the representative E2 Standard Curve depicted in **Figure 2** is constructed in terms of those units. **APPENDIX 3** provides guidance for the mathematical manipulation of raw RLU measurements to achieve final calculations of '% Relative Activation of ER'. That series of calculations may be applied to all Control and Test samples.

V. Related Products for Environmental Monitoring Applications

Product No.	*Product Descriptions*
EM00431	**Estrogen Receptor** ER Bioassay for Environmental Monitoring 96-well format assay
EM06001	**Aryl Hydrocarbon Receptor** AhR Bioassay for Environmental Monitoring 96-well format assay
EM00201	**Glucocorticoid Receptor** GR Bioassay for Environmental Monitoring 96-well format assay
EM03001	**Androgen Receptor** AR Bioassay for Environmental Monitoring 96-well format assay
EM00501	**Mineralocorticoid Receptor** MR Bioassay for Environmental Monitoring 96-well format assay

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in **5x bulk volume** to perform **480** Live Cell Assays performed in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays performed in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

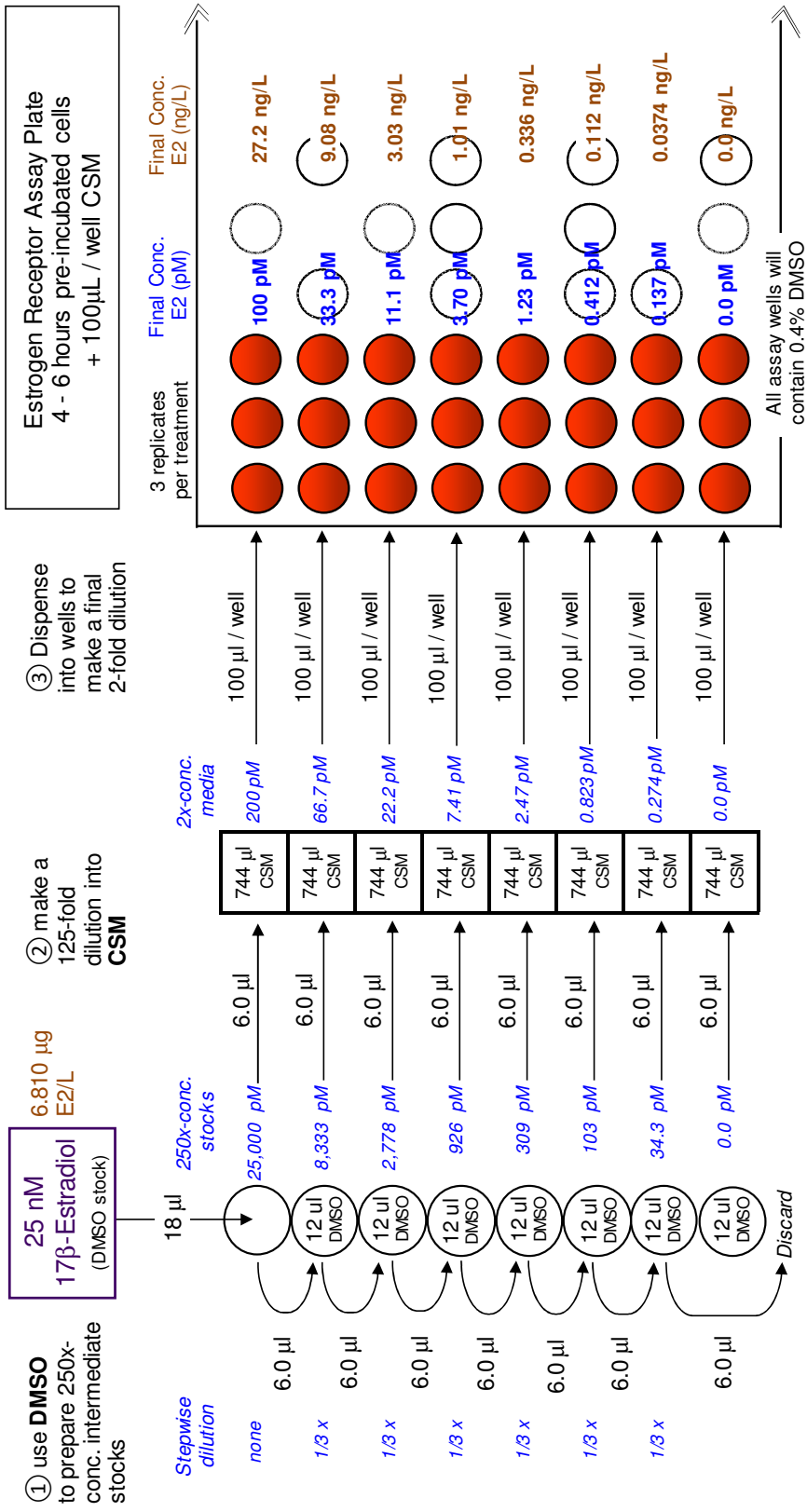
Products offered by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most recently updated version available.

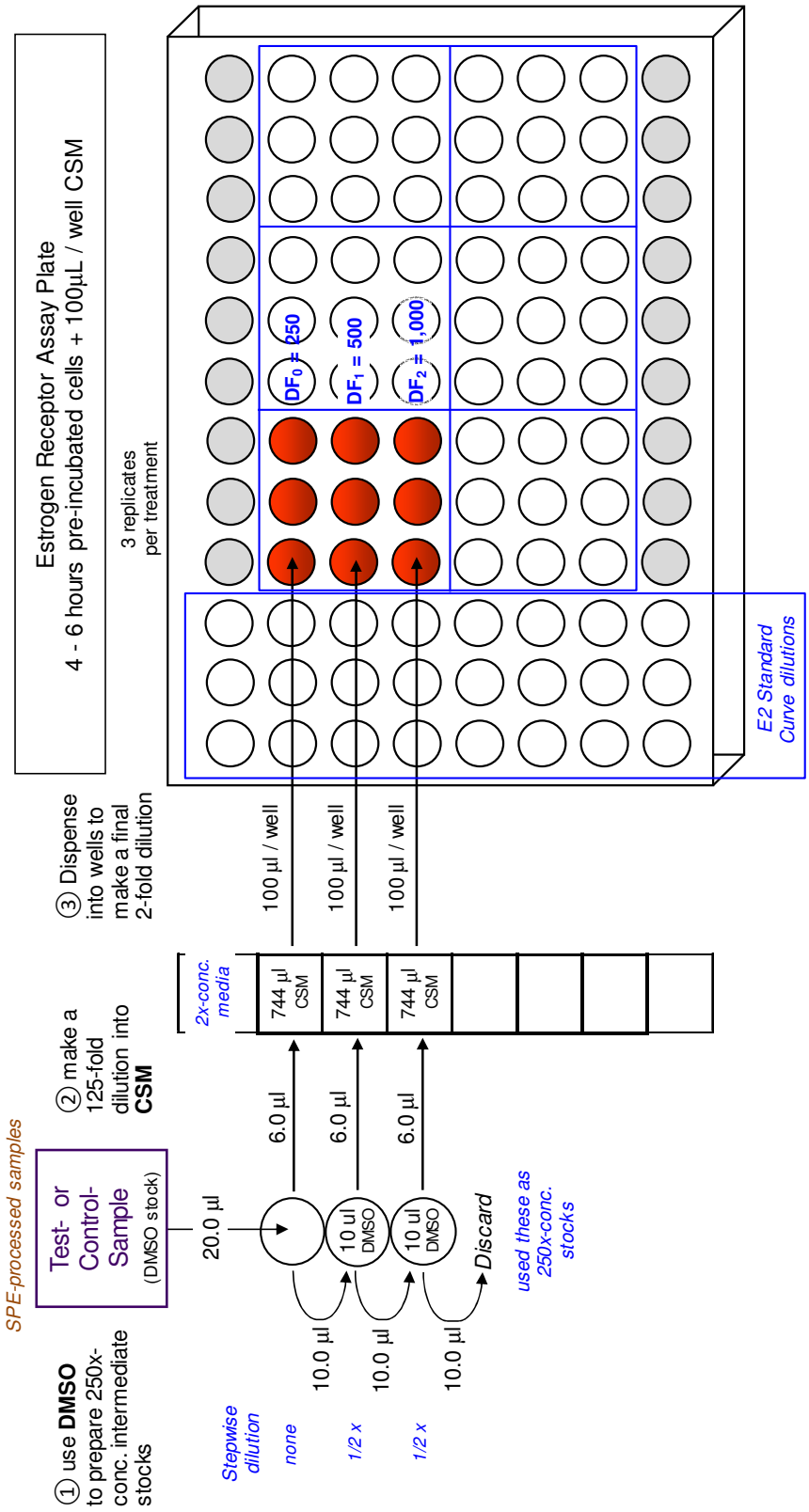
APPENDIX 1

Example scheme for the serial dilution of 17β-Estradiol to generate a Standard Curve.



APPENDIX 2

Example dilution scheme for the preparation of intermediate DMSO Stocks of SPE-processed Test and Control samples. A larger dilution increment, or greater numbers of dilutions may be warranted for some water samples. DF_x = Dilution Factor of respective sample dilutions in the assay plate. When feasible, avoid using edge wells (grey) for test sample treatments. These may be used for additional 'Vehicle only' background control wells, and for 'No Cells' control wells when incorporating the Live Cell Multiplex Assay.



APPENDIX 3

Guidance for Data Analyses. This method normalizes all data in terms of Relative Percent Activation; other methods may be applicable. The following formulae are constructed to follow the format used by Microsoft Excel software.

a. Begin by calculating the **Average RLU (aRLU)** value from the replicate wells of the various test samples, reference sample, and untreated control wells:

=AVERAGE(RLU1, RLU2, RLU3)

b. For each aRLU value calculate the corresponding value of **Standard Deviation (SD)**:

=STDEV(RLU1, RLU2, RLU3)

c. For each aRLU value calculate the corresponding value of **Percent Coefficient of Variation (%CV)**; this is sometimes referred to as Relative Standard Deviation (RSD):

= (SD/aRLU)*100

d. For each aRLU value calculate **Background-Subtracted aRLU ($aRLU^{-Bkg}$)**. "Background" is defined as the aRLU of the "Vehicle-only" (0.4% DMSO) treated reporter cells. Background subtraction of $aRLU^{Vehic}$, itself, will always **= 0**

Calculation of Background-Subtracted aRLU values		
Background subtract the Vehicle Control aRLU	$=aRLU^{Vehic} - aRLU^{Vehic}$	**= 0**
Background-subtracted aRLU values for all E2 Standards and SPE-processed Test Samples	$=aRLU^{Test} - aRLU^{Vehic}$	$= aRLU^{Test-Bkg}$

e. Calculate % Relative Activation (%RA). This manipulation normalizes all $aRLU^{-Bkg}$ values. Divide each $aRLU^{-Bkg}$ value by the *highest* $aRLU^{-Bkg}$ value from the E2 standard curve ($aRLU^{E2max-Bkg}$); typically, this corresponds to the 100 pM *or* 33.3 pM E2 treatment). Multiplying by 100 converts to units of percent.

Note: *i.)* normalized $aRLU^{E2max-Bkg}$ will always **= 100% Activation** of the ER.

ii.) normalized $aRLU^{Vehic-Bkg}$ will always **= 0% Activation** of the ER.

Calculation of % Relative Activation of ER	
%RA of E2 Standard Curve	$=(aRLU^{E2-Bkg} / aRLU^{E2max-Bkg})*100$
%RA all Test & Control samples	$=(aRLU^{Test-Bkg} / aRLU^{E2max-Bkg})*100$
%RA Vehicle Control	$=(0 / aRLU^{E2max-Bkg})*100$ *(= 0% RA)*

f. **Plot the E2 Standard Curve.** Use curve-fitting software (*e.g.,* GraphPad Prism) to perform the 4-parameter variable slope, least squares method of non-linear regression to plot '%RA +/- %CV *vs.* Log_{10}[E2]'. Constrain the bottom plateau of the sigmoidal curve to "0". The EC_{50} value of the E2 Standard Curve will auto-calculate.

g. **Determine Assay E2 Bioequivalence (Assay E2-BEq) concentrations.** Map the calculated %RA values of each test sample to the E2 Standard Curve to extrapolate their respective assay E2 bio-equivalent concentrations. Some agencies caution that a dilution of a test sample that yields a %RA value less than 10%, or greater than 90%, may be unreliable.

(continued)

h. **From each 'Assay E2-BEq' value, back-calculate to determine the respective E2-BEq concentration of bioactive endocrine disrupting chemical (EDC) pollutants in the o*riginal water sample*.** To calculate the final E2-BEq concentration in the original water sample, one must account for:

1.) the SPE-processed sample's **Enrichment Factor (EF)** relative to the original volume of water sample collected,
and
2.) the respective **dilution factors (DF_x)** used when preparing the series of DMSO intermediate stocks for the assay plate (from *Step 7b*).

	Intermediate DMSO stocks	*Calculation of E2-BEq in the Original Water Sample Collected*	
Water Sample A *(example)* **EF ≥ 5,000**	DMSO sample **DF_0 = 250x**	**=(Assay E2 BEq_0/EF)*DF_0**	*original sample* = E2-BEq
	first 2-fold DMSO dilution **DF_1 = 500x**	**=(Assay E2 BEq_1/EF)*DF_1**	*original sample* = E2-BEq
	second 2-fold DMSO dilution **DF2 = 1,000x**	**=(Assay E2 BEq_2/EF)*DF_2**	*original sample* = E2-BEq

i. Use the independent determinations of E2-BEq in the original water sample to calculate an **Average E2-Beq** value and its corresponding **Standard Error of Mean**:

Average	=AVERAGE(BEq_0,BEq_1,BEq_2)
SEM	=STDEV(BEq_0,BEq_1,BEq_2)/SQRT(COUNT(BEq_0,BEq_1,BEq_2))

j. Consult local water authority policies for guidance in assessing whether the calculated E2-BEq levels of contaminating EDC's exceed the level of concern.