Aryl Hydrocarbon Receptor (AhR) Bioassay

Environmental Monitoring for
Polycyclic Aromatic Hydrocarbon (PAH) and
Halogenated Aromatic Hydrocarbon (HAH)
Pollutants

96-well Format Assays
Product # EM06001

▪▪▪

Technical Manual
(version 7.2EM)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Aryl Hydrocarbon Reporter (AhR) Bioassay
96-well Format Assay

I. Description

▪ The Assay System ▪

The principal application of this Aryl Hydrocarbon Receptor bioassay is to screen processed environmental samples, with the aim of quantifying the aggregate bioactivities, if any, of contaminating biohazardous chemical entities. Contaminants of particular concern include the polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) such as dioxin, dioxin-like chemicals, and PCBs.

This assay utilizes proprietary mammalian reporter cells expressing the Aryl Hydrocarbon Receptor (AhR; AHR) and a luciferase reporter gene functionally linked to an AhR-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in AhR activity.

The AhR 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 treatment steps such as spin-and-rinse of cells, viability determinations, or cell titer adjustments prior to assay setup.

This AhR assay kit is an all-inclusive assay system. In addition to Reporter Cells, included are two optimized media for use during cell culture and in diluting the user's test samples, Luciferase Detection Reagent, and a cell culture-ready assay plate.

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▪ AhR Background ▪

The aryl hydrocarbon receptor (AhR) is a ligand-activated member of the basic helix-loop-helix- PER-ARNT-SIM (bHLH-PAS) family of transcription factors. It was initially characterized by its sensitivity to halogenated aromatic hydrocarbons (HAHs) including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin), and polycyclic hydrocarbons (PAHs) such as benzo[*a*]pyrene (BaP). Recent interests in AhR have focused on its diverse physiological functions in a variety of systems, including the liver, intestine, skin, and immune system.[1]

The prototypical signaling pathway of AhR is similar to that of the nuclear receptor superfamily. In the absence of ligand, AhR is located in the cytoplasm, bound to a chaperone complex including a dimer of Hsp90, AIP, and p23. When bound to a ligand, the AhR translocates into the nucleus, where it binds to its dimerization partner, ARNT. The AhR-ARNT complex then binds specific cognate DNA sequence elements known as dioxin/xenobiotic/AHR response elements (DRC/XRE/AHRE), located upstream of target genes. Notable among these are CYP1A1, CYP1A2, and CYP1B, all members of the cytochrome P450 family of phase I drug metabolizing enzymes.[2]

[1] Stockinger, B, *et al*., (2014) The Aryl Hydrocarbon Receptor: Multitasking in the Immune System. Annu. Rev. Immunol. 32:403-32.

[2] Beischlag, T, *et al*., (2008) The Aryl Hydrocarbon Receptor Complex and the Control of Gene Expression. Crit Rev Eukaryot Gene Expr. 18(3): 207-250.

▪ The Assay Chemistry ▪

INDIGO's various receptor 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 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, TCDD Standard Curve, Test-, and Control-Sample Treatment Media ▪

Preparation of 2,3,7,8-Tetrachlorobibenzo-P-Dioxin (2,3,7,8-TCDD) treatment media for Standard Curve Generation. Due to its biohazardous nature, 2,3,7,8-TCDD cannot be provided as a shipped component of this AhR assay kit. The user will procure this reference compound (*e.g.,* Cambridge Isotope Laboratories; cat# ED-901-B). Use DMSO to prepare a 225 nM stock of TCDD.

> ***CAUTION:*** TCDD is a significant biohazard. Use appropriate personal protective devices and a fume hood when working with TCDD stocks. All plasticware and assay reagents that come into contact with TCDD-containing solutions should be handled with great care and disposed of appropriately as biohazardous solid or liquid waste.

This stock is then used to generate a series of serial dilutions that will be used to generate a TCDD Standard Curve, thus allowing one to correlate measured Test Sample-induced AhR activity to a TCDD Bioequivalent (TCDD-BEq) concentration. Depending on the preference of the local regulatory authority, TCDD 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

(continued)

2. **'Method Spike'**: Ultra-pure water spiked with the reference analyte → SPE → concentrated DMSO stock. For the AhR assay, 2,3,7,8-TCDD (MW 321.97) is the reference analyte 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 prepared TCDD 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 AhR 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.

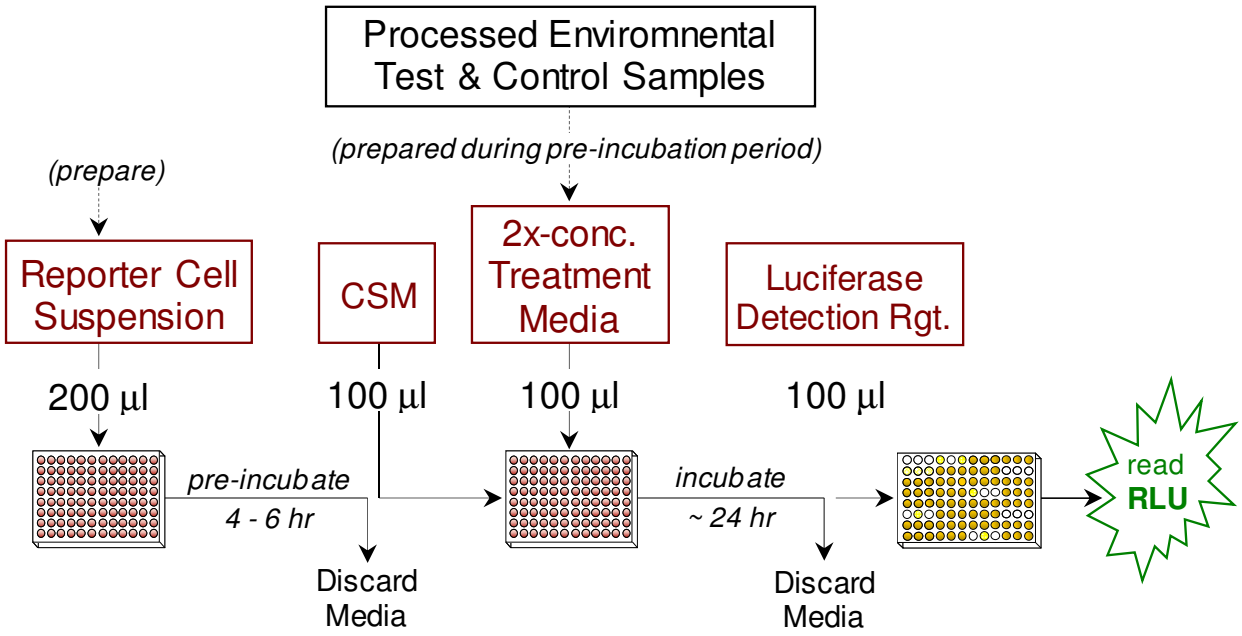


Figure 1. The AhR Assay Workflow*. Environmental 'Test' and 'Control' samples are processed and concentrated in advance of the AhR assay setup. Sample processing is most commonly performed *via* solid phase extraction (SPE), solvent 'blow-off', and a final solvent exchange into DMSO with a preferred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

In brief: 200 µl of Reporter Cells is 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.

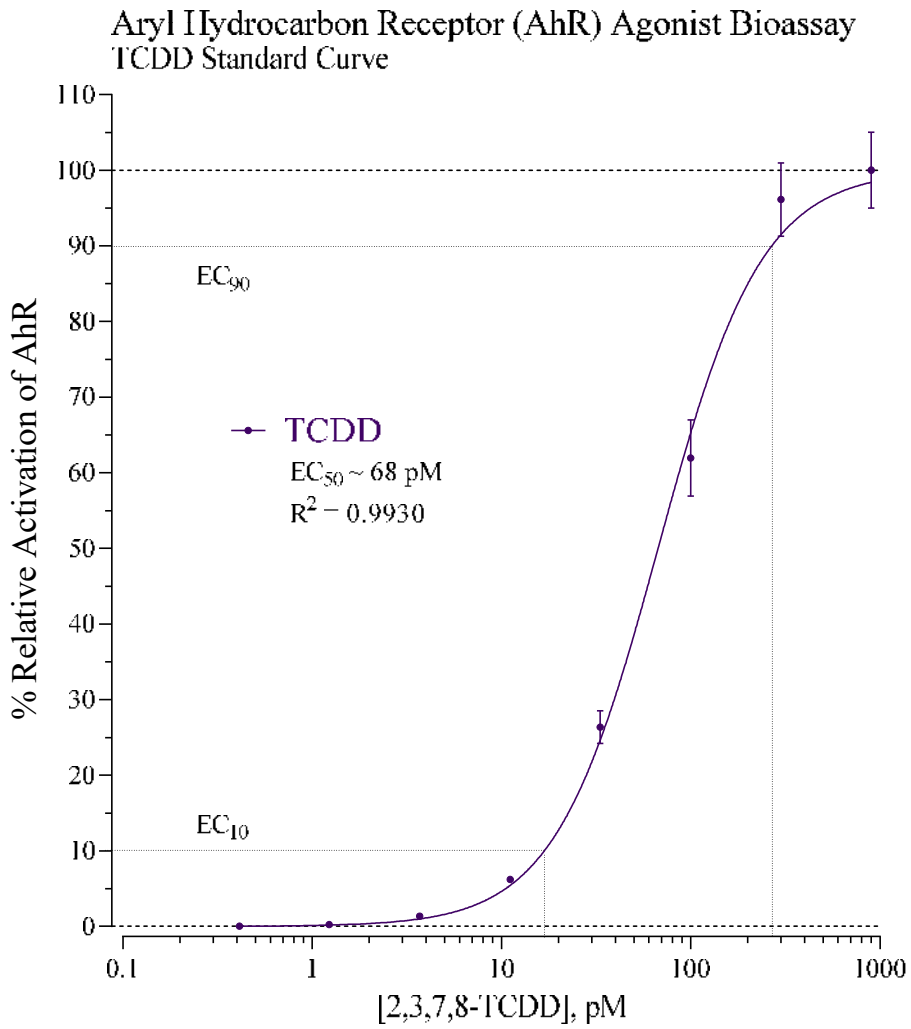


Figure 2. Generating a Standard Curve of TCDD activity.

The reference agonist 2,3,7,8-TCDD (Cambridge Isotope Laboratories; cat# ED-901-B) was 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 Relative Activation (meaning, relative to the EC_{100} concentration of TCDD) were calculated for each treatment concentration of TCDD, as described in APPENDIX 3. GraphPad Prism software was used to plot % Relative Activation *vs.* Log_{10}[TCDD, 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 AhR 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.*
▪ AhR 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
▪ 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

▪ 2,3,7,8-TCDD, 225 nM (72.4 µg TCDD / L). Reference analyte.

▪ 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 TCDD, 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, *collagen-coated*, for viewing cells on *Day 2*

▪ *Optional:* AhR 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). 2,3,7,8-TCDD (to be procured) is a potent agonist of AhR and may be used to set up receptor inhibition studies. 160 pM TCDD 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 AhR.

Add the challenge agonist, TCDD, to a bulk volume of **CSM** at a **2x-EC_{80}** concentration (*i.e.,* ~320 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 TCDD 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 TCDD Standard Curve.** Procure TCDD (see *CAUTION*, pg. 4). Use DMSO to prepare a 225 nM stock. Use a portion of this stock to generate a TCDD Standard Curve, which will then be used to determine the TCDD Bioequivalent (TCDD-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 8-point treatment series (in terms of *final* assay concentrations), prepared in serial 3-fold decrements, is recommended for use in generating the Standard Curve: 900, 300, 100, 33.3, 11.1, 3.70, 1.23, and 0.412 pM TCDD. Always include a 'vehicle only' control, which will be 0.4% DMSO. A representative plot of a typical TCDD 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 TCDD 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 AhR'. 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.

www.indigobiosciences.com

VI. Limited Use Disclosures

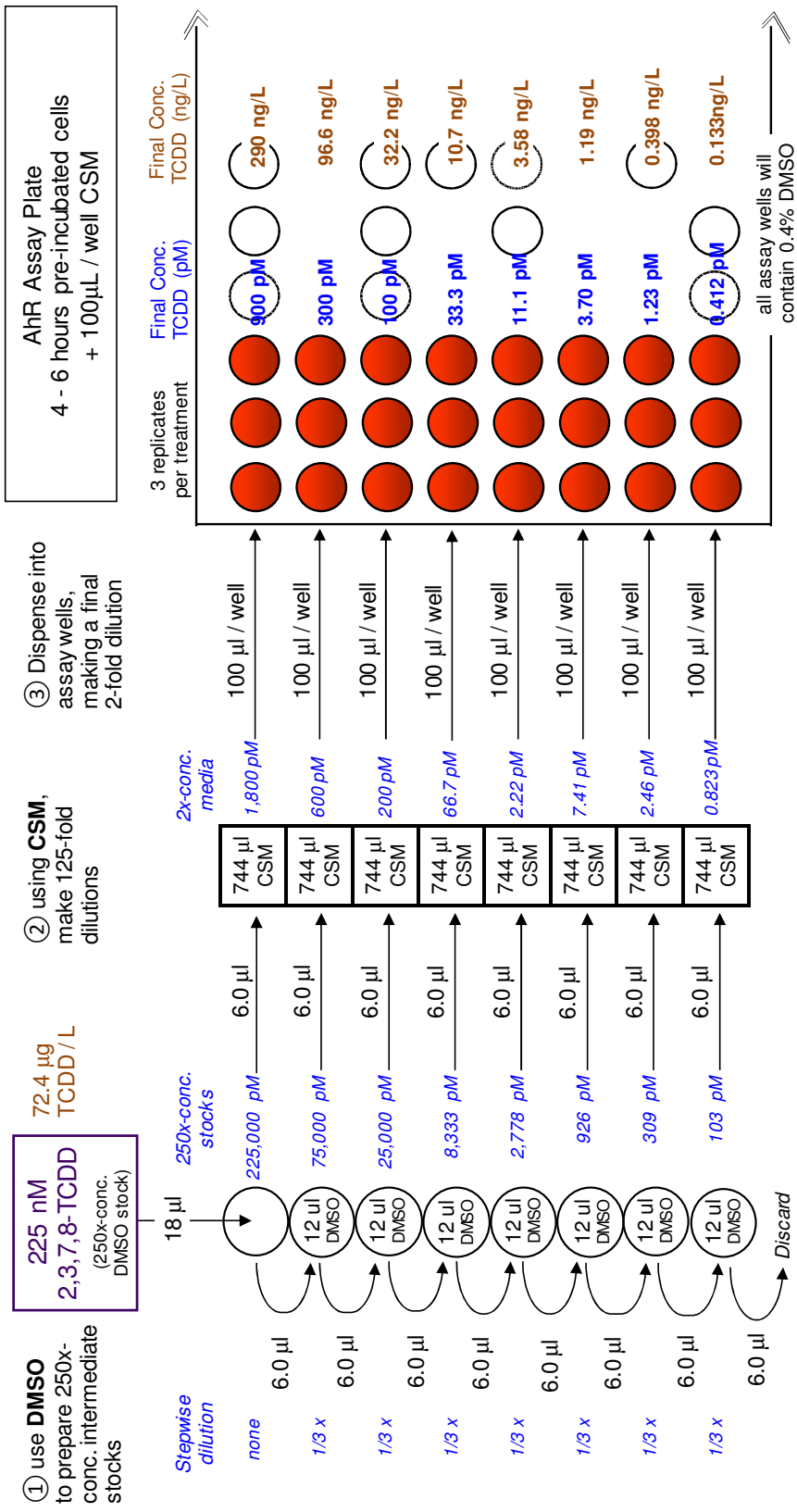
Products offered by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

"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.

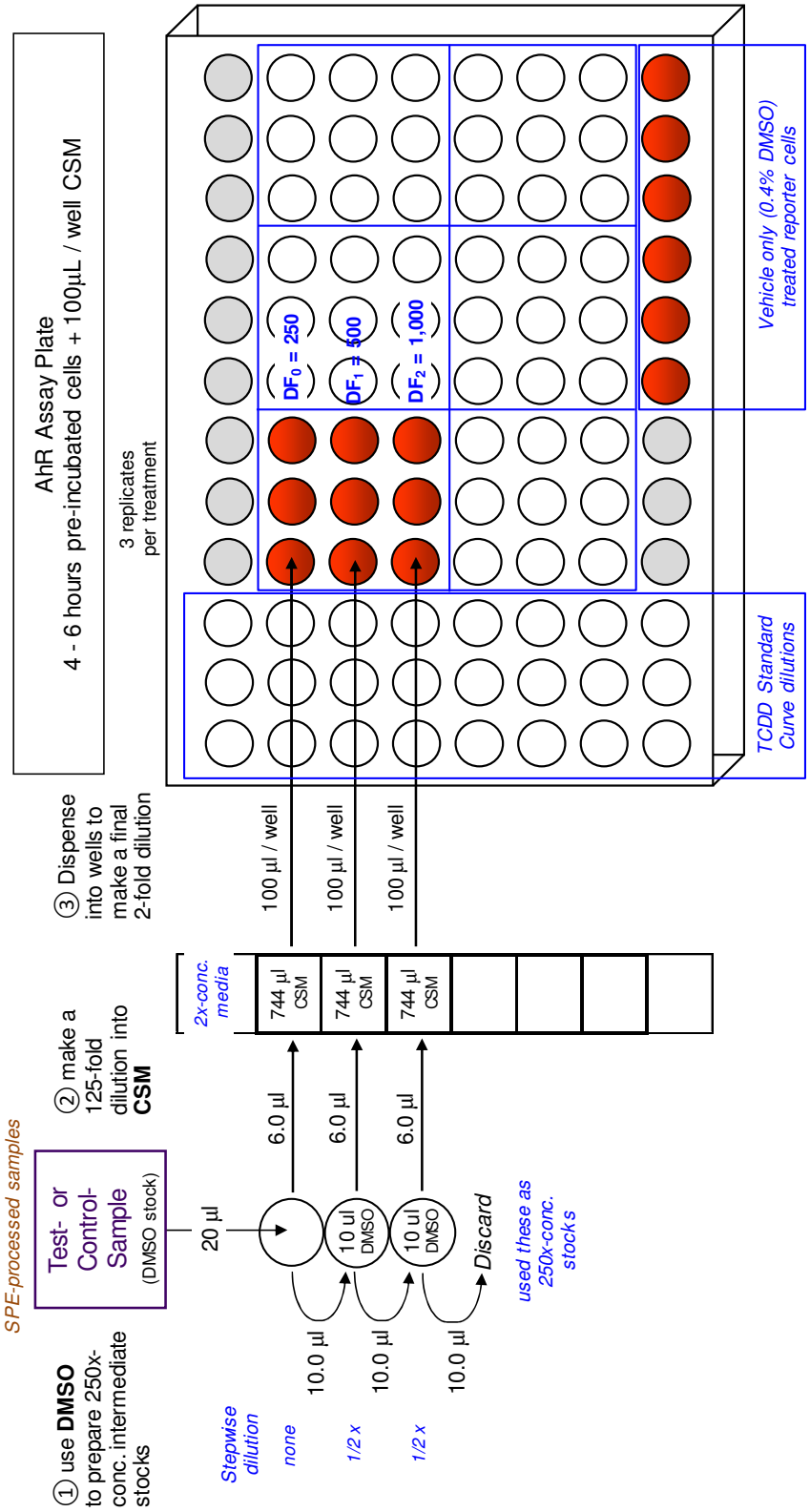
APPENDIX 1

Example scheme for the serial dilution of TCDD to generate a Standard Curve.
Include 'Vehicle only' (0.4% DMSO) assay wells on the assay plate (see APPENDIX 2)



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/or 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 TCDD 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 TCDD standard curve ($aRLU^{TCDDmax-Bkg}$); typically, this corresponds to the 900 pM *or* 300 pM TCDD treatment). Multiplying by 100 converts to units of percent.

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

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

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

f. **Plot the TCDD 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}[TCDD]'. Constrain the bottom plateau of the sigmoidal curve to "0". The EC_{50} value of the TCDD Standard Curve will auto-calculate.

g. **Determine Assay TCDD Bioequivalence (Assay TCDD-BEq) concentrations.** Map the calculated %RA values of each test sample to the TCDD Standard Curve to extrapolate their respective assay TCDD 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 TCDD-BEq' value, back-calculate to determine the respective TCDD-BEq concentration of bioactive polycyclic aromatic hydrocarbon (PAH) pollutants in the o*riginal water sample*.** To calculate the final TCDD-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 TCDD-BEq in the Original Water Sample Collected*	
Water Sample A *(example)* **EF ≥ 5,000**	DMSO sample **DF_0 = 250x**	**=(Assay TCDD BEq_0/EF)*DF_0**	*original sample* = TCDD-BEq
	first 2-fold DMSO dilution **DF_1 = 500x**	**=(Assay TCDD BEq_1/EF)*DF_1**	*original sample* = TCDD-BEq
	second 2-fold DMSO dilution **DF2 = 1,000x**	**=(Assay TCDD-BEq_2/EF)*DF_2**	*original sample* = TCDD-BEq

i. Use the independent determinations of TCDD-BEq in the original water sample to calculate an **Average TCDD-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 TCDD-BEq levels of contaminating PAH's exceed the level of concern.