



Mouse Aryl Hydrocarbon Receptor (mAhR) Reporter Assay System

3x32 Assays in 96-well Plate Format
Product # M06001-32

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Technical Manual *(version 7.2i)*

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I. Description

▪ 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 ability to sense xenobiotics, particularly environmental contaminants including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, also known as dioxin) and polycyclic hydrocarbons (PAHs), such as benzo[a]pyrene. Recent interest in AhR has also focused on its diverse physiological functions in a variety of systems, including but not limited to 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 heterodimerization 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 responsive genes, most notable of which are members of the cytochrome P450 family of phase I drug metabolizing enzymes (predominantly CYP1A1, CYP1A2, and CYP1B1).²

Differences in relative affinity for TCDD between species are known to exist. For example, the mouse AhR^{b-1} allele binds TCDD with 10-fold higher relative affinity than human AhR.³ For this reason, mouse AhR can be used as a sensitive tool for detecting the presence of dioxins and similar environmental pollutants.

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.

³ Ramadoss, P, *et al.*, (2004) Use of 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin as a probe to determine the relative ligand affinity of human versus mouse aryl hydrocarbon receptor in cultured cells. *Mol Pharmacol.* 66(1):129-36.

▪ The Assay System ▪

INDIGO's **Mouse Aryl Hydrocarbon Receptor (AhR) Reporter Cells** include the 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 mouse AhR activity. The principal application of this assay is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against mouse AhR.

Mouse 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.

INDIGO's Mouse AhR assay kit is an all-inclusive system. In addition to Mouse AhR Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, the reference agonist MeBIO, Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's cell-based 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 assay kits feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating 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 Test Compounds ▪

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 7* and depicted in Appendix 1 for the reference agonist MeBIO, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if test compound solubility is expected to be problematic,

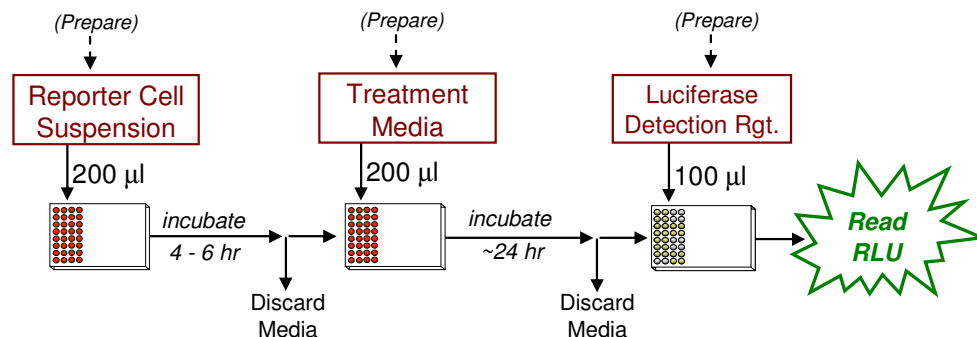
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 0.4%.

NOTE: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup and are considered to be 'single-use' reagents.

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 200 μ l 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 200 μ l/well of the prepared treatment media are added. Following 22 - 24 hours incubation, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in terms of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



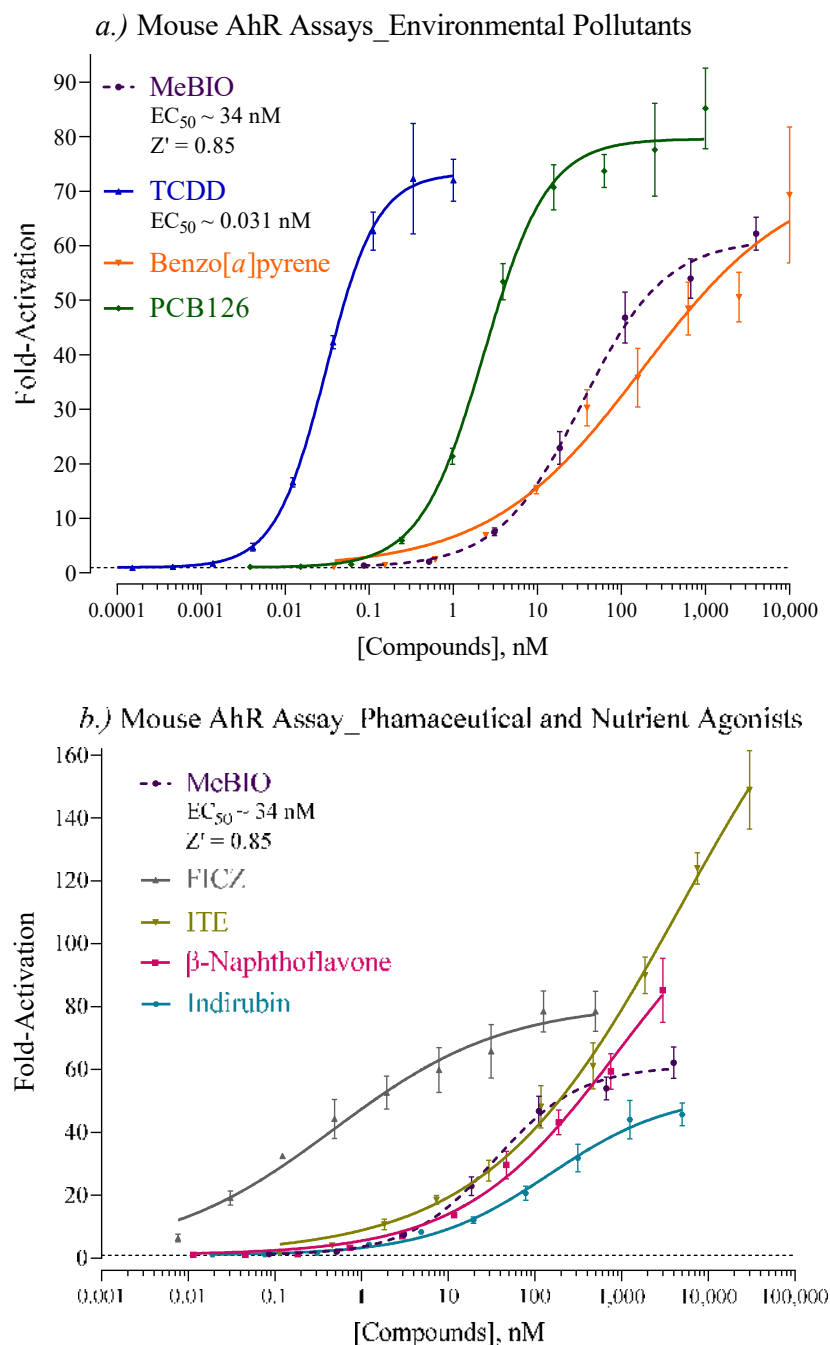


Figure 2. Agonist dose-response analyses of Mouse AhR. Agonist analyses of Mouse AhR Reporter Cells were performed using the reference agonist MeBIO (provided) and a.) examples of environmental contaminants of concern: TCDD (2,3,7,8-Tetrachlorodibenzo-p-dioxin; Cambridge Isotope Laboratories, Inc.), Benzo[a]pyrene (Sigma-Aldrich), and PCB126, or b.) prototypical activators of AhR that are of nutritional or pharmaceutical relevance: FICZ (6-Formylindolo[3,2-b]carbazole; Cayman Chemical), ITE (2-(1H-indol-3-ylcarbonyl)-4-thiazolecarboxylic methyl ester; Cayman Chemical), β -Naphthoflavone (Sigma-Aldrich), and Indirubin. Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ($n = 3$ / conc.). Values of Fold-activation and Z' were calculated as described by Zhang, *et al.* (1999)⁴. Least squares fit non-linear regression and EC_{50} analyses were performed using GraphPad Prism software. The reference agonist MeBIO yielded an $EC_{50} \sim 34$ nM, and a Z' value of 0.85, confirming the robust performance of this assay, and its suitability for HTS.⁴

⁴ Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen.* 4(2), 67-73.

$$Z' = 1 - [3 * (SD_{Control} + SD_{Background}) / (RLU_{Control} - RLU_{Background})]$$

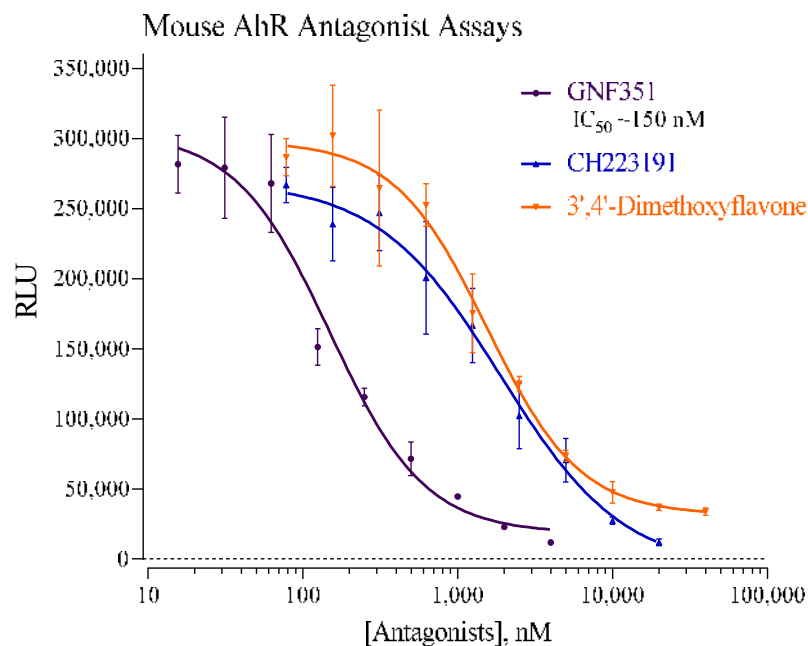


Figure 3. Mouse AhR Antagonist dose-response analyses. Antagonist analyses of Mouse AhR Reporter Cells were performed according to the protocol described in this Technical Manual. Briefly, Mouse AhR Reporter Cells were co-treated with an EC₈₀ concentration of the reference agonist MeBIO (provided) and varying concentrations of the AhR antagonists GNF351 (Calbiochem), CH223191 (Tocris), and 3',4'-Dimethoxyflavone (Sigma). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown). Non-linear regression analyses of RLU vs. Log₁₀[Antagonists, nM] were plotted and IC₅₀ determination made for GNF351 using GraphPad Prism software.

II. Product Components & Storage Conditions

This Mouse AhR Assay kit contains materials to perform three distinct groups of assays in a 96-well plate format. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in Step 2 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 tubes 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ Mouse AhR Reporter Cells	3 x 0.6 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ MeBIO, 4.0 mM (in DMSO) (positive control for mouse AhR activation)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	-80°C
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile)	12	-20°C

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 2*)
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- 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 dilution series of reference and test compound(s).
- *Optional*: reference antagonist (see Figure 3).
- *Optional*: clear 96-well assay plate, sterile, *collagen-coated*, for viewing cells on Day 2.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on **Day 1**, requiring less than 2 hours of actual bench work plus a 4 hr pre-incubation step. *Steps 12-17* are performed on **Day 2** and require less than 1 hour to complete.

▪ A word about Antagonist-mode assay setup ▪

Receptor antagonist assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC₅₀ – EC₈₅) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This Mouse AhR Assay kit includes a 4.0 mM stock solution of **MeBIO**, an activator of AhR that may be used to setup antagonist-mode assays. 66 nM MeBIO typically approximates EC₈₀ in this cell-based assay (see **Figure 2**). Hence, it provides a suitable assay concentration of agonist to be used when screening test compounds for antagonist activity to Mouse AhR.

Add the challenge agonist to a bulk volume of **CSM** at an EC₅₀ – EC₈₅ concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. This is an efficient and precise method of setting up Mouse AhR antagonist assays, and it is the method presented in *Step 7b* of this protocol.

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 tubes of **Reporter Cells** from -80°C storage and place them directly into dry ice for transport to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **7.0 ml** per tube.

Third, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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

4.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to gain a homogenous cell suspension. 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, 96-well assay plate. Continue to process the clear plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO₂) for 4 - 6 hours.

6.) Near the end of the pre-incubation period remove Compound Screening Medium (CSM) from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations: Use CSM to prepare an appropriate dilution series of the reference and test compound stocks. Prepare treatment media at the desired **final assay concentrations**. In *Step 9*, **200 µl / well** of the prepared treatment media are dispensed into the strip-wells of the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should not exceed 0.4%.

a. Agonist-mode assays. This Mouse AhR Assay kit includes a 4.0 mM stock solution of **MeBIO**, a potent activator of AhR. The following 7-point treatment series, prepared in serial 6-fold decrements, provides a complete dose-response: 4000, 667, 111, 18.5, 3.09, 0.514, and 0.086 nM (final assay concentrations). Always include 'no treatment', or 'vehicle only' control wells. **APPENDIX 1** provides an example for generating such a dilution series.

~ or ~

b. Antagonist-mode assays. When setting antagonist assays, first supplement a bulk volume of CSM with the challenge agonist to achieve the desired final assay-concentrations (refer to "*A word about antagonist-mode assay setup*", pg. 8). The agonist-supplemented CSM is then used to generate dilutions of test compound samples to achieve the desired final assay concentrations.

8.) At the end of the cell pre-incubation period, discard the culture media. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using either a single-tip, or an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

9.) Dispense **200 µl** of each treatment media into appropriate wells of the assay plate.

10.) Transfer the assay plate into a 37°C, humidified 5% CO₂ 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.

11.) For greater convenience on *Day 2*, retrieve the appropriate number of vials of **Detection Substrate and Detection Buffer** from freezer storage and place them 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.

12.) 30 minutes before intending to quantify mouse AhR activity, remove **Detection Substrate** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

13.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

14.) *Immediately before proceeding to Step 15:* To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

15.) Following 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 8*).

16.) Use an 8-channel pipette to dispense 100 µl of **LDR** to each well of the assay plate. Allow the plate to rest at room temperature for 5-10 minutes following the addition of LDR. Do not shake the plate during this period.

17.) Quantify luminescence.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Human AhR Assay Kit Products	
IB06001-32	Human AhR Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
IB06001	Human AhR Reporter Assay System 1x 96-well format assay
IB06002	Human AhR Reporter Assay System 1x 384-well format assay
Mouse AhR Assay Kit Products	
M06001-32	Mouse AhR Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
M06001	Mouse AhR Reporter Assay System 1x 96-well format assay
Rat AhR Assay Kit Products	
R06001-32	Rat AhR Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
R06001	Rat AhR Reporter Assay System 1x 96-well format assay
Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	
LIVE Cell Multiplex (LCM) Assay Products	
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 contained in 5 x 96-well assay plates
LCM-10	Reagent in 10x bulk volume to perform 960 Live Cell Assays contained 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 commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

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

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APPENDIX 1

Example scheme for the serial dilution of MeBIO reference agonist using CSM, and the setup of a Mouse AhR dose-response assay.

