Human Aryl Hydrocarbon Receptor (AhR) Reporter Assay System

96-well Format Assays
Product # IB06001

Technical Manual
(version 6.0)

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Human AhR Reporter Assay System
96-well Format Assays

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I. Description

- Background -

Although technically not a member of the Nuclear Receptor superfamily, the AhR shares many of the same attributes. The AhR is a member of the basic helix-loop-helix, Per-Arnt-Sim (bHLH-PAS) family of transcription factors and is responsible for the toxicologic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, often referred to as simply “dioxin”) and several other related polycyclic aromatic hydrocarbons. The basic mechanism of action of dioxin and related compounds has been extensively studied, in particular as it relates to regulation of cytochrome P450 1A1 (CYP1A1). AhR is present in most cell types and in the non-active state is cytosolic and exists in a complex with chaperone proteins such as heat shock protein 90 (Hsp90). Binding of TCDD and related molecules to AhR leads to nuclear translocation and hetero-dimerization with its partner protein ARNT, another bHLH-PAS family member. The AhR-ARNT hetero-dimer binds to specific cognate DNA sequence elements known as dioxin/xenobiotic response elements (DRE/XRE) present in the regulatory region of specific genes such as CYP1A1. Binding of the AhR:ARNT heterodimer to these elements, and subsequent recruitment of transcription co-activator complexes, leads to increased transcription of the specific gene, known as “target genes”. There is a battery of genes affected in this manner and targets include certain xenobiotic-metabolizing enzymes, such as CYP1A1, CYP1A2, CYP2B1, and UGT1A6. In addition, genes affected directly and indirectly by the TCDD/AhR-complex code for both inhibitory and stimulatory growth factors and their gene products affect cellular growth and differentiation leading to tumor promotion and carcinogenicity as well as other forms of toxicity.

- The Assay System -

INDIGO’s 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 AhR activity. The principle application of this reporter assay system is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against human AhR.

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, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.

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

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using Compound Screening Medium (CSM), as described in Step 2 of the Assay Protocol. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 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 diluted in CSM 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.
### Considerations for Automated Dispensing

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will not be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (96-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reporter Cell Suspension</td>
<td>100 µl / well</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td></td>
<td>9.6 ml / plate</td>
<td></td>
</tr>
<tr>
<td>(12 ml, prepared from kit components)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDR</td>
<td>100 µl / well</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td></td>
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### Assay Scheme

**Figure 1.** Assay workflow. *In brief,* Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22 -24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.

1. **Reporter Cell Suspension (in CRM)**
2. **Test Compounds (2x-concentration in CSM)**
3. **Incubate ~24 hr**
4. **Discard Media**
5. **Luciferase Detection Reagent**
6. **Read RLU ≥ 5 min.**
**Assay Performance**

**Human AhR Agonist Assays**

- Pifithrin-α-hydrobromide
- β-naphthoflavone
- Omeprazole
- MeBio
- FICZ
- ITE

**Figure 2. Agonist dose-response analyses of Human AhR.**

Agonist analyses of Human AhR Reporter Cells were performed according to the protocol described in this Technical manual, using the reference agonists MeBIO (provided), FICZ (6-Formylindolo(3,2-b)carbazole; Enzo), ITE (2-(1H-indole-3-ylcarbonyl)-4-thiazolecarboxylic methyl ester; Tocris), b-Naphthoflavone (Sigma Aldrich), Omeprazole and Pifithrin-a-hydrobromide (each from Tocris). Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Fold-activation (i.e., S/B) and Z' values were calculated as described by Zhang, et al. (1999)¹. Non-linear regression and EC₅₀ analyses were performed using GraphPad Prism software.

The reference agonist MeBIO yielded a maximal fold-activation = 145, EC₅₀ = 4 nM, and a Z' value of 0.69. High values of fold-activation and Z' confirm the robust performance of this assay, and it's suitability for HTS¹.


\[
Z' = 1 - \frac{3 \times (SD_{\text{Control}} + SD_{\text{Background}})}{(RLU_{\text{Control}} - RLU_{\text{Background}})}
\]
Figure 3. Antagonist dose-response analyses of Human AhR.

Antagonist analyses of Human AhR Reporter Cells were performed according to the protocol described in this Technical manual, using the reference antagonists GNF351 (Calbiochem) and CH 223191 (Tocris).
II. Product Components & Storage Conditions

This Human AhR Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of AhR Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, Reporter Cells must be maintained at -80°C until immediately prior to use.

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

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Cell Recovery Medium (CRM)</td>
<td>1 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>MeBio, 1.0 mM (in DMSO) (reference activator of AhR)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Detection Substrate</td>
<td>1 x 6.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Detection Buffer</td>
<td>1 x 6.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>96-well assay plate (white, sterile, cell-culture ready)</td>
<td>1</td>
<td>ambient</td>
</tr>
</tbody>
</table>
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**
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-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 deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- antagonist reference compound (optional).

**DAY 2**
- plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. Steps 1-8 are performed on **Day 1**, requiring less than 2 hours to complete. Steps 9-15 are performed on **Day 2**, and require less than 1 hour to complete.

- **A word about Antagonist-mode assay setup**

Receptor inhibition 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 AhR Reporter Assay System kit includes a 1.0 mM stock solution of MeBio, an activator of AhR that may be used to setup antagonist-mode assays. 28 nM MeBio typically approximates EC₈₅ in this reporter assay (see Figure 1). Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that adding the reference agonist to the bulk suspension of Reporter Cells (i.e., prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in **Step 5b** of the following protocol. Note that, in **Step 6**, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.
DAY 1 Assay Protocol: All steps must be performed using proper aseptic technique.

1.) Remove Cell Recovery Medium (CRM) and Compound Screening Medium (CSM) from freezer storage and thaw.
   - CRM should be thawed and equilibrated to 37°C using a water bath. CRM pre-warmed to 37°C is required in Step 3.
   - CSM may be thawed in a 37°C water bath.

2.) Use CSM to prepare appropriate dilution series of Test Compound(s) and Reference Compound stocks to be screened for Agonist or Antagonist activities.

   The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

   Note that, in Step 6, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired final assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Plan dilution schemes carefully; this assay kit provides 35 ml of CSM.

   This AhR Reporter Assay System kit includes a 1.0 mM stock solution of MeBio, a potent activator of AhR. The following 7-point treatment series, prepared in serial 6-fold decrements, provides a suitable dose-response: 1000, 167, 27.8, 4.63, 0.772, 0.129, and 0.0214 nM (final assay concentrations), and including a 'no treatment' control. APPENDIX 1 provides an example for generating such a dilution series.

3.) First, retrieve the tube of CRM from the 37°C water bath and sanitize the outside surface with a 70% ethanol swab.

   Second, retrieve Reporter Cells from -80°C storage. Perform a rapid thaw of the frozen cells by transferring a 10 ml volume of 37°C CRM into the tube of frozen cells. Immediately place the tube in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 12 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface of the tube with a 70% alcohol swab.
5.)  

5.1. **Agonist-mode assays.** Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

~ or ~

5.2. **Antagonist-mode assays.** Invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 9). Dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

**NOTE 5.1:** Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate will cause well-to-well variation (= increased Standard Deviation) in the assay.

**NOTE 5.2:** Users sometimes prefer 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, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 100 µl of 2x-concentration treatment media into appropriate wells of the assay plate.

7.) Transfer the assay plate into a 37°C, humidified 5% CO2 incubator for 22 - 24 hours.

**NOTE:** Ensure a high-humidity (≥ 90%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

8.) For greater convenience on Day 2, retrieve **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.

9.) 30 minutes before intending to quantify 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. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

   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.

10.) Set the instrument to perform a single 5 second “plate shake” prior to reading the first assay well. Read time may be 0.5 second (500 mSec) per well, or less.

11.) Immediately before proceeding to Step 12, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of Luciferase Detection Reagent (LDR). Mix gently to avoid foaming.

12.) Following 22 - 24 hours of incubation discard all media contents by ejecting it 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.

13.) Add 100 µl per well of LDR to each well of the assay plate.

14.) Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

15.) Quantify luminescence between 5 - 90 minutes after the addition of LDR.
V. Related Products

<table>
<thead>
<tr>
<th>Human AhR Assay Kit Products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product No.</strong></td>
</tr>
<tr>
<td>IB06001-32</td>
</tr>
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<td>IB06001</td>
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Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

<table>
<thead>
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<tr>
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<td>LCM-05</td>
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<td>LCM-10</td>
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Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Limited Use Disclosures

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

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

Product prices, availability, specifications and claims are subject to change without prior notice.

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APPENDIX 1


1 For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.
Human Aryl Hydrocarbon Receptor (AhR) Reporter Assay System

3x 32 Assays in 96-well Format
Product # IB06001-32

Technical Manual
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• Assay Scheme •

Figure 1. Assay workflow. In brief, Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22 - 24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each sample well is quantified using a plate-reading luminometer.
Figure 2. Agonist dose-response analyses of Human AhR.

Agonist analyses of Human AhR Reporter Cells were performed according to the protocol described in this Technical manual, using the reference agonists MeBIO (provided), FICZ (6-Formylindolo(3,2-b)carbazole; Enzo), ITE (2-(1H-indole-3-ylcarbonyl)-4-thiazolecarboxylic methyl ester; Tocris), b-Napthoflavone (Sigma Aldrich), Omeprazole and Pifthrin-a-hydrobromide (each from Tocris). Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Fold-activation (i.e., S/B) and Z’ values were calculated as described by Zhang, et al. (1999). Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software.

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II. Product Components & Storage Conditions

This Human AhR Reporter Assay System 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.

The individual aliquots of Reporter Cells are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>Detection Substrate</td>
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<td>-80°C</td>
</tr>
<tr>
<td>Detection Buffer</td>
<td>3 x 2.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Plate frame</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>Snap-in, 8-well strips (white, sterile, cell-culture ready)</td>
<td>12</td>
<td>ambient</td>
</tr>
</tbody>
</table>
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**
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-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 deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- antagonist reference compound (optional).

**DAY 2**
- plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. Steps 1-8 are performed on **Day 1**, requiring less than 2 hours to complete. Steps 9-15 are performed on **Day 2**, and require less than 1 hour to complete.

- A word about Antagonist-mode assay setup -

Receptor inhibition 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 AhR Reporter Assay System kit includes a 1.0 mM stock solution of MeBio, an activator of AhR that may be used to setup antagonist-mode assays. 28 nM MeBio typically approximates EC₈₅ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that adding the reference agonist to the bulk suspension of Reporter Cells (i.e., prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in Step 5b of the following protocol. Note that, in Step 6, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.
DAY 1 Assay Protocol: All steps must be performed using proper aseptic technique.

1.) Remove Cell Recovery Medium (CRM) and Compound Screening Medium (CSM) from freezer storage and thaw.
   - CRM should be thawed and equilibrated to 37°C using a water bath. CRM pre-warmed to 37°C is required in Step 3.
   - CSM may be thawed in a 37°C water bath.

2.) Use CSM to prepare appropriate dilution series of Test Compound(s) and Reference Compound stocks to be screened for Agonist or Antagonist activities.

   The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

   Note that, in Step 6, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired final assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Plan dilution schemes carefully; this assay kit provides 35 ml of CSM.

   This AhR Reporter Assay System kit includes a 1.0 mM stock solution of MeBio, a potent activator of AhR. The following 7-point treatment series, prepared in serial 6-fold decrements, provides a suitable dose-response: 1000, 167, 27.8, 4.63, 0.772, 0.129, and 0.0214 nM (final assay concentrations), and including a 'no treatment' control. APPENDIX 1 provides an example for generating such a dilution series.

3.) First, retrieve the tube of CRM from the 37°C water bath and sanitize the outside surface with a 70% ethanol swab.

   Second, retrieve Reporter Cells from -80°C storage. Perform a rapid thaw of the frozen cells by transferring a 3.0 ml volume of 37°C CRM into the tube of frozen cells. Immediately place the tube in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 3.6 ml.

   Third, 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.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface of the tube with a 70% alcohol swab.
5.) **a. Agonist-mode assays.** Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

~ or ~

**b. Antagonist-mode assays.** Invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 8). Dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

**NOTE 5.1:** Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate will cause well-to-well variation (= increased Standard Deviation) in the assay.

**NOTE 5.2:** Users sometimes prefer 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, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 100 µl of 2x-concentration treatment media into appropriate wells of the assay plate.

7.) Transfer the assay plate into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.

**NOTE:** Ensure a high-humidity (≥ 90%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

8.) For greater convenience on Day 2, retrieve **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.

9.) 30 minutes before intending to quantify 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. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

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.

10.) Set the instrument to perform a single 5 second “plate shake” prior to reading the first assay well. Read time may be 0.5 second (500 mSec) per well, or less.

11.) Immediately before proceeding to Step 12: 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.

12.) After 22-24 hours of incubation remove media contents from each well.

NOTE: Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media via a sweeping downward movement is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (e.g., Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus.

13.) Add 100 µl per well of LDR to each well of the assay plate.

14.) Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

15.) Quantify luminescence between 5 - 90 minutes after the addition of LDR.
**V. Related Products**

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Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

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Please refer to INDIGO Biosciences website for updated product offerings.

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APPENDIX 1


For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.