Rat Aryl Hydrocarbon Receptor (rAhR) Reporter Assay System

96-well Format Assays
Product # R06001

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Technical Manual
(version 7.2)

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

Rat AhR Reporter Assay System
96-well Format Assays

I. Description

▪ Background ▪

While technically not a nuclear receptor, the AhR is mechanistically and functionally similar to members of that super-family, being both a receptor and a ligand-activated transcription factor. More formally, the AhR is a member of the basic helix-loop-helix, Per-Arnt-Sim family of transcription factors. AhR is a xenobiotic-sensing receptor responsible for the adverse toxicologic effects elicited by various polycyclic aromatic hydrocarbon environmental and industrial pollutants, perhaps the most infamous being 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). 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).

The AhR is present in the cytosol of most cell types where, in the non-active state, it is in a complex with chaperone proteins such as Hsp90. Binding of a polycyclic aromatic hydrocarbon to AhR leads to nuclear translocation and hetero-dimerization with its partner protein ARNT. 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 a variety of target genes. Binding of AhR:ARNT to these elements, and subsequent recruitment of transcription co-activator complexes, induces the transcription of a battery of target genes, including 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 in addition to induced toxic responses.

▪ 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 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 rat 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, or cell titer adjustments prior to assay setup.

INDIGO's rat AhR assay kit is an all-inclusive system. In addition to Rat 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.

▪ The Assay Chemistry ▪

INDIGO's cell-based assay format 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 ▪

Test compounds are typically solvated at high-concentration in DMSO and stored frozen as master stocks. 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 *never* 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 diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that final treatment media are prepared 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 plumbing; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

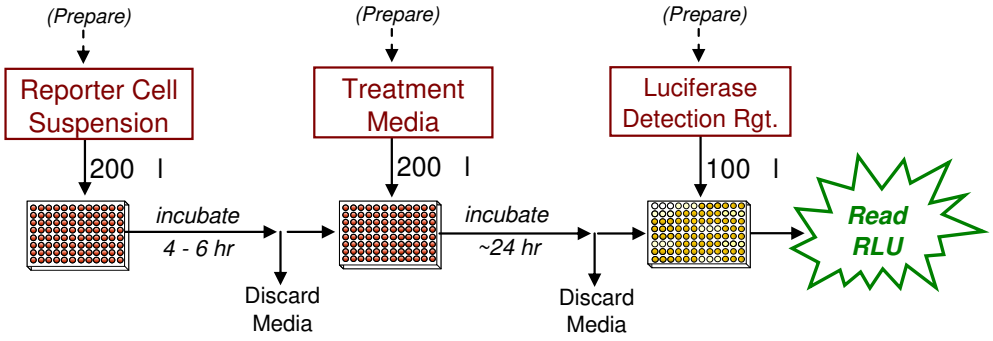
Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. volume available for instrument dead volume**
Reporter Cell Suspension 21 ml *(prepared from kit components)*	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ Assay Scheme ▪

Figure 1. Assay workflow.

NOTE: This AhR assay protocol includes Day 1 steps and dispensed volumes that are different from the conventional INDIGO assay protocol that users may be accustomed to when setting up INDIGO's other Nuclear Receptor Assays; please review the assay workflow, below.

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 200 µl/well of the prepared 1x-concentration treatment media are added. Following 22-24 hr incubation, treatment media are discarded and Luciferase Detection Reagent is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



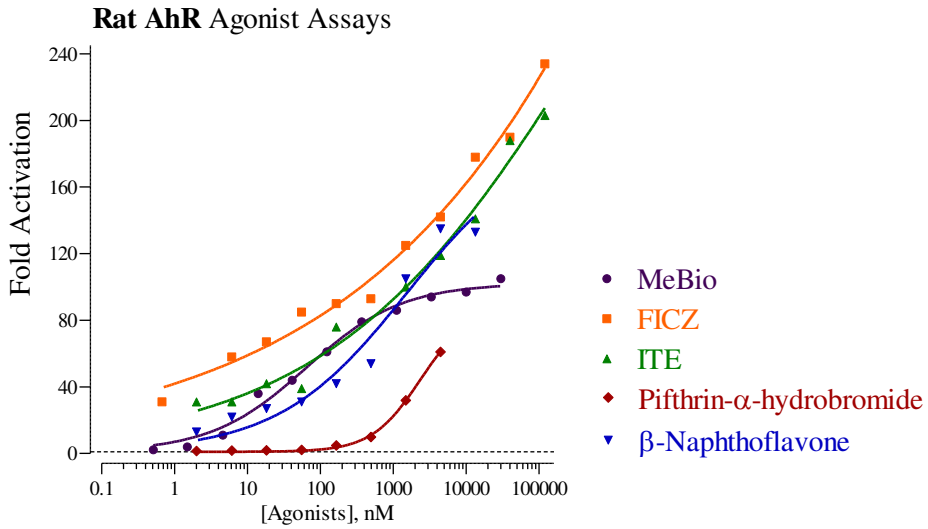


Figure 2. Agonist dose-response analyses of Rat AhR.
Agonist analyses of rat AhR Reporter Cells were performed according to the protocol described in this Technical manual, using the reference agonists MeBIO (provided), FICZ (6-Formylindolo(3,2-b)carbazole; Enzo), ITE (2-(1H-indole-3-ylcarbonyl)-4-thiazolecarboxylic methyl ester; Tocris), β-Napthoflavone (Sigma), Pifthrin-α-hydrobromide (Tocris).

Luminescence was quantified from the assay wells and average Relative Light Units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ($n \geq 6$). Fold-activation (*i.e.,* S/B) and Z' values were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software.

The reference agonist MeBIO yielded an EC_{50} = 65 nM, and a Z' value of 0.70, confirming the robust performance of this assay, and it's suitability for HTS[1].

[1] 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.

$$Z' = 1 - [3*(SD^{Control} + SD^{Background}) / (RLU^{Control} - RLU^{Background})]$$

II. Product Components & Storage Conditions

This rat AhR Assay kit 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.

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
▪ MeBio, 20 mM (in DMSO) (reference activator for rAhR)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well assay plate (white, sterile, cell-culture ready)	1	ambient

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% CO_2 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).
▪ *Optional:* antagonist reference compound.
▪ *Optional:* clear 96-well assay plate, sterile, cell culture treated, 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 bench work and a 4 hr incubation step to complete. *Steps 12-17* 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 EC_{50} – EC_{85}) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This AhR Assay kit includes a 20 mM stock solution of **MeBio**, an activator of rat AhR that may be used to setup antagonist-mode assays. 200 nM MeBio typically approximates EC_{80} in this cell-based assay (see **Figure 2**). Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity to rat AhR.

Add the challenge agonist to a bulk volume of **CSM** at an EC_{50} – EC_{85} concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. We find that this is an efficient and precise method of setting up 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 **Reporter Cells** from -80°C storage and, *without delay*, perform a rapid thaw of the frozen cells by transferring 9.5 ml from ***each* of the 2 tubes** of pre-warmed CRM into the 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 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 disperse cell aggregates and gain an homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, dispense **200 µl / well** of cell suspension into the 96-well Assay Plate.

NOTE 4.1: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling during the dispensing period. Likewise, take care to dispense uniform volumes across the assay plate.

NOTE 4.2: 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 cell culture treated assay plate. Continue to process the assay plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells: Place the assay plate into a 37°C, ≥ 85% humidity, 5% CO_2 incubator for 4 - 6 hours.

Near the end of the 4-6 hour pre-incubation period:

6.) 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*, the prepared treatment media are dispensed at **200 µl / well** into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should never exceed 0.4%.

***a. Agonist*-mode assays**. This AhR Assay kit includes a 20 mM stock solution of **MeBio**, a potent activator of AhR. The following 8-point treatment series, prepared in serial 5-fold decrements, provides a suitable dose-response: 20000, 4000, 800, 160, 32.0, 6.40, 1.28 and 0.256 nM, and including a 'no treatment' control. **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 an ~EC_{80} concentration of the challenge agonist (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** 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.

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% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 85%) 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 **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 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.

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*, 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.

15.) Following 22 - 24 hours incubation in treatment media, retrieve the assay plate from the incubator. 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.

16.) Add 100 µl of **LDR** to each well of the assay plate. 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.

17.) Quantify luminescence.

V. Related Products

Rat AhR Assay Kit Products	
Product No.	*Product Descriptions*
R06001-32	3x 32 rat AhR assays; strip-wells in 96-well plate frame
R06001	1x 96-well format rat AhR assays

Human AhR Assay Kit Products	
Product No.	*Product Descriptions*
IB06001-32	3x 32 AhR assays; strip-wells in 96-well plate frame
IB06001	1x 96-well format AhR assays
Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay Products	
Product No.	*Product Descriptions*
LCM-01	Reagents to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagents in 5x-bulk volume to perform **480** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

Please refer to INDIGO Biosciences website for updated product offerings.

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

APPENDIX 1

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