Zebrafish Aryl Hydrocarbon Receptor (zfAhR) Reporter Assay System

96-well Format Assays
Product # Z06001

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Technical Manual
(version 7.2)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Zebrafish AhR Reporter Assay System
96-well Format Assays

I. Description

▪ Background ▪

As its name implies, the Aryl Hydrocarbon Receptor (AhR) is a xenobiotic-sensing receptor. It is 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).

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.

The AhR is present in the cytosol 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 association 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 several cytochrome P450 genes. Other target genes of the TCDD/AhR-complex encode inhibitory or stimulatory growth factors that mediate cellular growth and differentiation.

▪ The Assay System ▪

INDIGO's **Zebrafish Aryl Hydrocarbon Receptor (zfAhR) Reporter Cells** are constructed in zebrafish ZF4 cells, a cell line derived from *Brachydanio rerio* embryos. This zebrafish cell line has been modified to contain the luciferase reporter gene functionally linked to tandem DRE/XRE genetic response elements and a minimal promoter sequence. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in zfAhR 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 the native zebrafish AhR.

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

▪ The Assay Chemistry ▪

INDIGO's cell-based assay format capitalizes 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 **Compound Screening Medium (CSM**; as described in *Step 7*) to achieve the desired assay concentrations.

> *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 regarded as 'single-use' reagents.

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

▪ Considerations for Automated Dispensing ▪

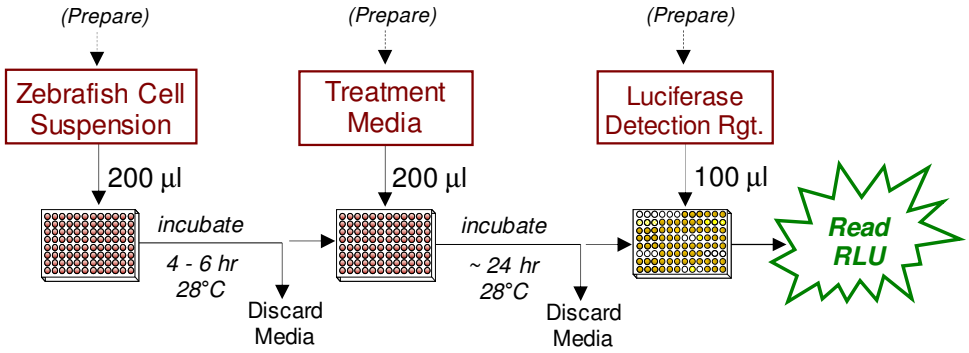
When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument plumbing; it will *not* be available 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.

In brief, 200 µl of reporter cell suspension is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Note that zebrafish cells are cultured at **28°C.** Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared treatment media are added. Following 22-24 hr incubation, treatment media are removed, 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.



▪ Assay Performance ▪

Figure 2. *(next page)*

A. Agonist dose-response analyses of Zebrafish AhR.
Agonist analyses of zfAhR Reporter Cells were performed according to the protocol described in this Technical manual, using the reference agonists MeBIO (provided), TCDD (2,3,7,8-Tetrachlorodibenzo-P-dioxin (6-Formylindolo(3,2-b)carbazole; Cambridge Isotope Laboratories), ITE (2-(1H-indole-3-ylcarbonyl)-4-thiazolecarboxylic methyl ester; Tocris), and Indirubin (Cayman). Luminescence was quantified and average relative light units (RLU), Fold-Activation, and corresponding values of standard deviation (SD) and coefficient of variation (%CV) were determined for each treatment concentration ($n \geq 3$). Z' values were calculated as described by Zhang, *et al*. (1999)[1].

B. Antagonist dose-response analyses of Zebrafish AhR.
Antagonist analyses of zfAhR Reporter Cells were performed according to the protocol described in this Technical manual, using the reference antagonists GNF351 (Calbiochem).

Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software.

[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.

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

Figure 2A. Agonist dose-response analyses of Zebrafish AhR.

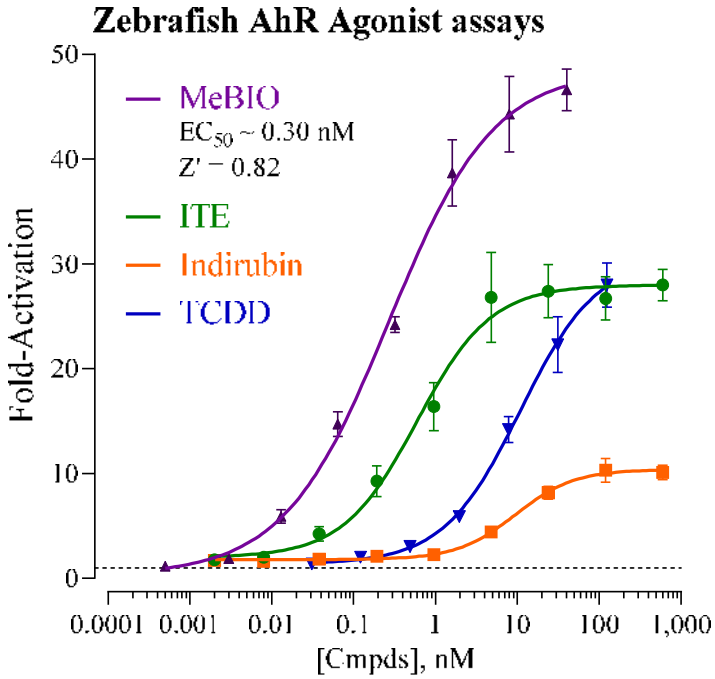
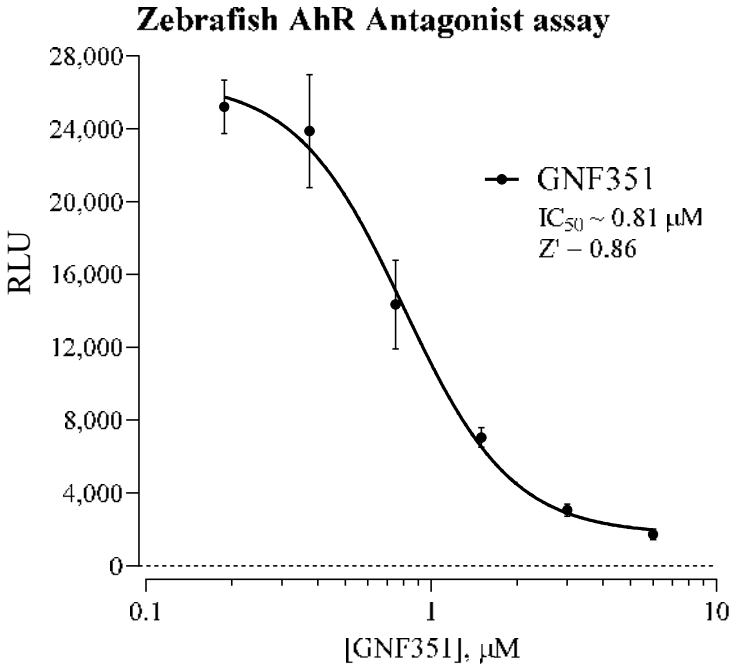


Figure 2B. Antagonist dose-response analyses of Zebrafish AhR.



II. Product Components & Storage Conditions

This Zebrafish AhR Assay kit contains materials to perform assays in 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 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 the tube of reporter cells into 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.*
▪ zfAhR 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, 100 µM (in DMSO) (positive control for zfAhR activation)	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	**-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 colder) until use.

III. Materials to be Supplied by the User

The materials below must be provided by the user. Note that culturing Zebrafish cells requires the use of a **28°C** incubator. Attempting to culture the reporter cells at either 37°C or room temperature (~ 22°C) will significantly compromise assay performance.

DAY 1

▪ dry ice bucket (*Step 2*)
▪ cell culture-rated laminar flow hood.
▪ **28°C**, humidified 5% CO_2 incubator for culturing Zebrafish cells.
▪ 37°C *and* 28°C water baths (*Steps 1 & 2*).
▪ 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:* antagonist reference compound
▪ *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 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_{80}) of an agonist AND the test compound(s) to be evaluated for antagonist activity. This zfAhR Assay kit includes a 100 µM stock solution of **MeBIO**, an activator of zfAhR that may be used to setup antagonist-mode assays. 3.0 nM MeBIO typically approximates EC_{80} in this cell-based assay (see **Figure 2**). Hence, it presents a suitable concentration of agonist to be used when screening test compounds for inhibitory activity to zfAhR.

Add the challenge agonist, MeBIO, to a bulk volume of **CSM** at an EC_{50} – EC_{80} 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 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 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 **28°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 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 96-well 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 identical manner to the white assay plate.

5.) Pre-incubate reporter cells: Place the assay plate into an incubator dedicated to Zebrafish cell culture (**28°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.) 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 assay kit includes a 100 µM stock solution of **MeBIO**, a potent activator of zfAhR. The following 8-point treatment series, prepared in serial 5-fold decrements, provides a complete dose-response: 100, 20, 4.0, 0.80, 0.16, 0.032, 0.0064 and 0.0013 nM, and including a 'no treatment' control wells. **APPENDIX 1** provides guidance for generating this a dilution series.

~ or ~

***b. Antagonist*-mode assays:** When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist MeBIO to achieve an EC_{80} concentration (~ 3 nM; refer to "*A word about antagonist-mode assay setup*", pg. 8). The agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired final assay concentrations.

8.) ***At the end of the 4 – 6 hr 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 / well** of each prepared treatment media into 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-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

10.) Transfer the assay plate into a **28°C**, humidified 5% CO_2 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 **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 zfAhR 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, discard the media contents of the assay plate 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

Zebrafish AhR Assay Kit Products	
Product No.	*Product Descriptions*
Z06001-32	3x 32 zfAhR assays; strip-wells in 96-well plate frame
Z06001	1x 96-well format zfAhR assays
Rat AhR Assay Kit Products	
R06001-32	3x 32 rAhR assays; strip-wells in 96-well plate frame
R06001	1x 96-well format rAhR assays
Human AhR Assay Kit Products	
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
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays

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 and the setup of a zfAhR dose-response assay.