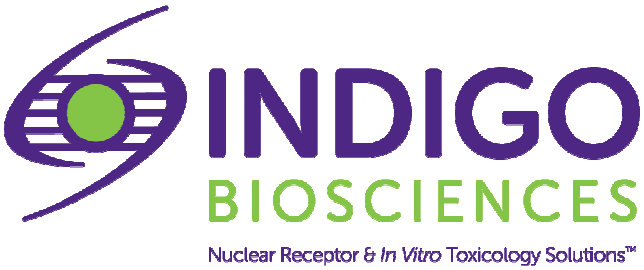

Human Aryl Hydrocarbon Receptor (AhR) Reporter Assay System

96-well Format Assays
Product # IB06001

▪

Technical Manual
(version 7.2i)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human 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. n

AhR is a xenobiotic-sensing receptor that is responsive to polycyclic aromatic hydrocarbons found in the environment as 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 the regulation of Cytochrome P450 gene expression.

The AhR is present in the cytosol of most cell types where, in the non-active state, it is complexed 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 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 encode 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 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, or cell titer adjustments 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, 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 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 ▪

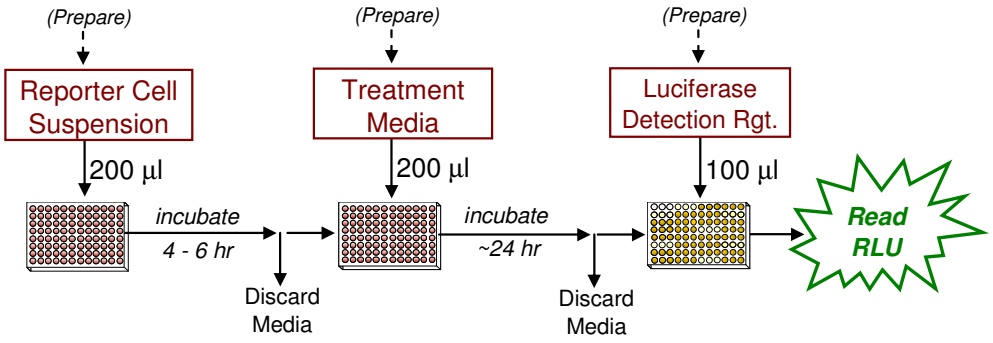
When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument plumbing; it will *not* be available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



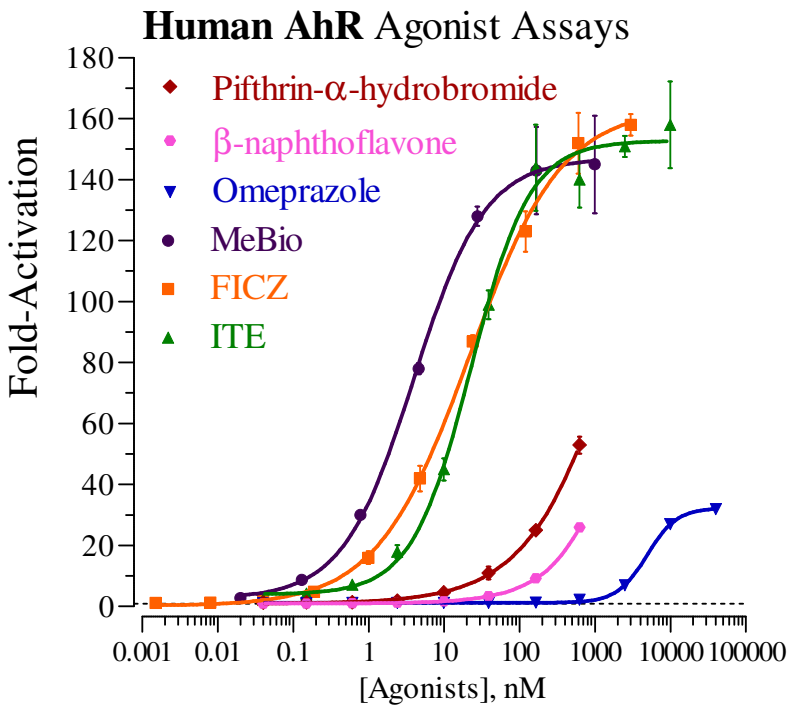


Figure 2. Agonist dose-response analyses of Human AhR.
Agonist analyses of Human AhR Reporter Cells were performed according to the protocol described in this Technical manual, using the reference agonists MeBIO (provided), FICZ (6-Formylindolo(3,2-b)carbazole; Enzo), ITE (2-(1H-indole-3-ylcarbonyl)-4-thiazolecarboxylic methyl ester; Tocris), β-Napthoflavone (Sigma), 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 ($n \geq 6$). Fold-activation (*i.e.,* S/B) and Z' values were calculated as described by Zhang, *et al.* (1999)[1]. Least squares fit non-linear regression and EC_{50} analyses were performed using GraphPad Prism software.

The reference agonist MeBIO yielded an EC_{50} = 4 nM, and a Z' value of 0.69, 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.

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

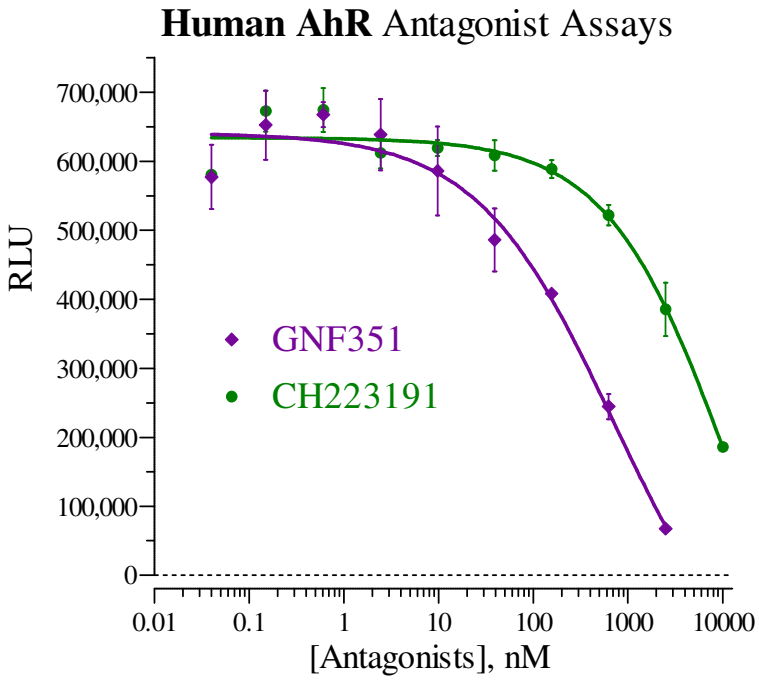


Figure 3. Antagonist dose-response analyses of Human AhR.
Antagonist analyses of Human AhR Reporter Cells were performed according to the protocol described in this Technical manual, using the reference antagonists GNF351 (Calbiochem) and CH 223191 (Tocris).

II. Product Components & Storage Conditions

This Human AhR 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 3* of this protocol.

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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

Kit Components	***Amount***	***Storage Temp.***
▪ AhR Reporter Cells	1 x 2.0 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ MeBIO, 1.0 mM (in DMSO) (positive control for AhR 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	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

▪ dry ice bucket (*Step 2*)
▪ cell culture-rated laminar flow hood.
▪ 37°C, humidified 5% CO_2 incubator for mammalian cell culture.
▪ 37°C water bath.
▪ 70% alcohol wipes
▪ 8-channel electronic, repeat-dispensing pipettes & sterile tips
▪ disposable media basins, sterile.
▪ 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 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 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 EC_{85} 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 inhibitory activity to AhR.

Add the challenge agonist (MeBIO) 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 the tube of **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from ***each* of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **21 ml**.

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

4.) Gently invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, dispense **200 µl / well** of cell suspension into the 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 a cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2) 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*, 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 not exceed 0.4%.

***a. Agonist*-mode assays**. This AhR Assay kit includes a 1.0 mM stock solution of **MeBio**, a potent activator of AhR. The following 7-point treatment series, prepared in serial 5-fold decrements, provides a complete dose-response: 1000, 200, 40, 8.0, 1.6, 0.32, and 0.064 nM (final assay concentrations), 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 the challenge agonist to achieve the desired final assay-concentrations (refer to "*A word about antagonist-mode assay setup*", pg. 9). 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 4-6 hr pre-culture period, discard the media. The preferred method is to use a 'wrist flick' to eject media into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

9.) Dispense **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-channel 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 cell culture 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 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*, 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 by manually ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

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

17.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
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

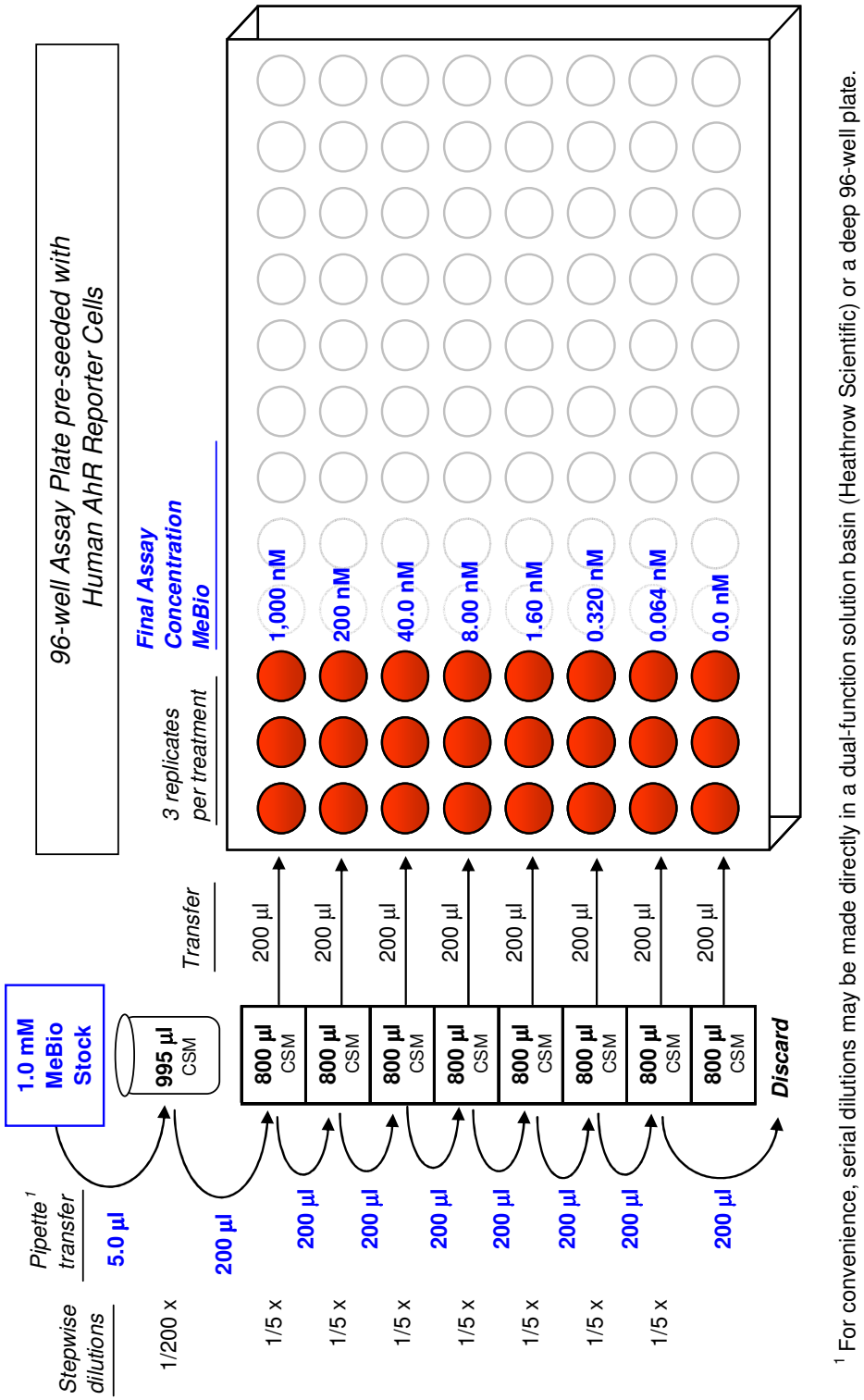
Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

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

Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

APPENDIX 1

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



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