Human Androgen Receptor
(NR3C4, AR)
Reporter Assay System

96-well Format Assays
Product # IB03001

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Technical Manual
(version 7.2d)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human AR Reporter Assay System

96-well Format Assays

I. Description

▪ The Assay System ▪

This nuclear receptor assay system utilizes proprietary non-human mammalian cells engineered to provide constitutive, high-level expression of full length, unmodified **Human Androgen Receptor** (NR3C4), a ligand-dependent transcription factor commonly referred to as **AR**.

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a *bona fide* AR-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in AR activity. Luciferase gene expression occurs after ligand-bound AR undergoes nuclear translocation, DNA binding, recruitment and assembly of the co-activators and accessory factors required to form a functional transcription complex, culminating in expression of the target gene. Unlike some other cell-based assay strategies, the readout from INDIGO's reporter cells demands the same orchestration of all intracellular molecular interactions and events that can be expected to occur *in vivo*.

AR Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields high cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for intermediate spin-and-wash steps, viability determinations, or cell titer adjustments.

The principal application of this assay product is in the screening of test samples to quantify functional activities, either agonist or antagonist, that they may exert against the human androgen receptor. It is an all-inclusive assay system that includes, in addition to AR Reporter Cells, two optimized media for use during cell culture and for preparing dilutions of test samples, a reference agonist, Luciferase Detection Reagent, a cell culture-ready assay plate, and a detailed protocol.

▪ 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%.

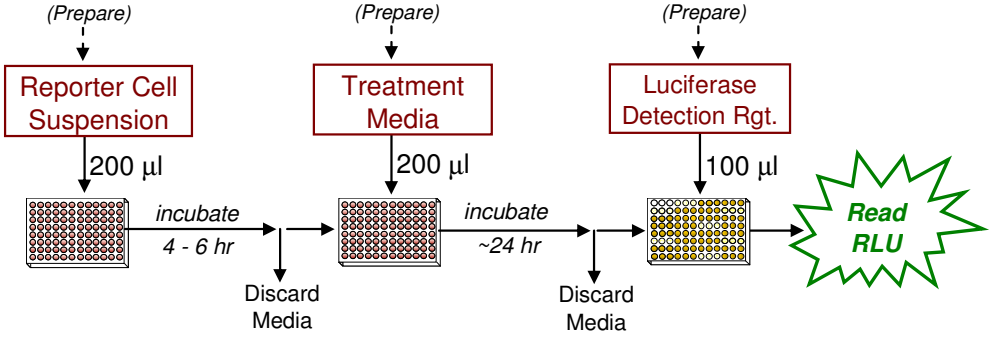
▪ Considerations for Automated Dispensing ▪

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 overview. 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 treatment media are added. Following 22-24 hr incubation, discard the treatment media and add Luciferase Detection Reagent. Photon emission (relative light units; RLU) from each assay well is quantified using a plate-reading luminometer.



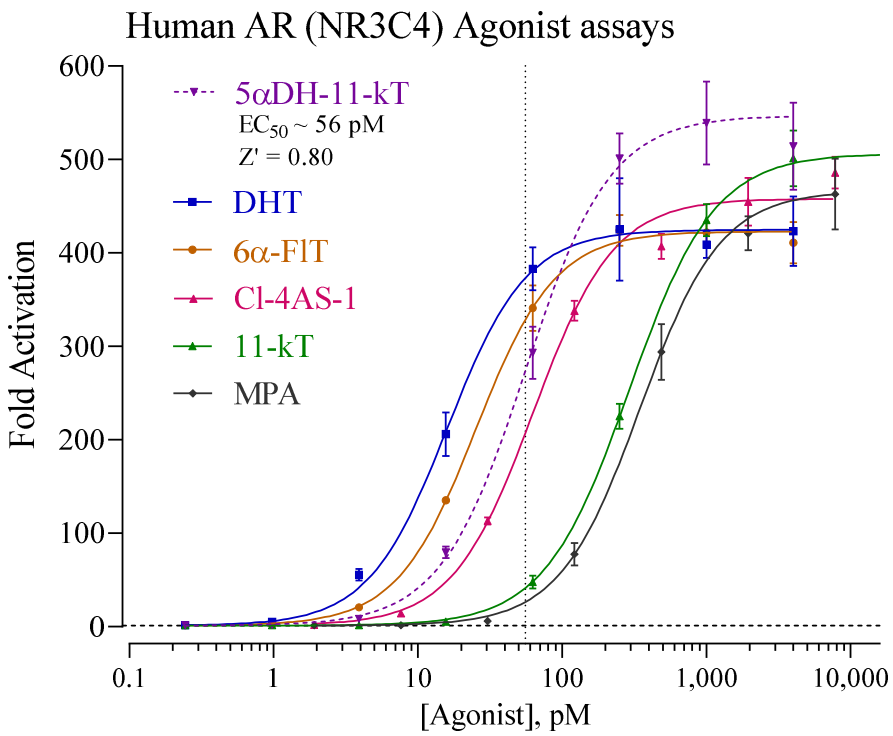


Figure 2. Agonist dose-response analyses.

Human AR agonist dose-response assays were performed using the reference agonists 5αDihydro 11-keto Testosterone (5αDH-11kT; provided), Dihydro Testosterone (DHT; Steraloids Inc.), 6α-Fl Testosterone (6αFlT; Enzo Life Sci), Cl-4As-1, 11-keto Testosterone (11-kT; Sigma-Aldrich) and Medroxy-Progesterone 17-Acetate (MPA; Steraloids, Inc.). 'Untreated' controls consisted of vehicle (0.1% DMSO) only. Luminescence was quantified and values of average relative light units (RLU), corresponding standard deviation (SD), Fold-Activation and Z'[1] were determined. Non-linear regression analyses of Fold-Activation *vs.* Log_{10} transformed concentration were plotted and EC_{50} values determined 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.

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

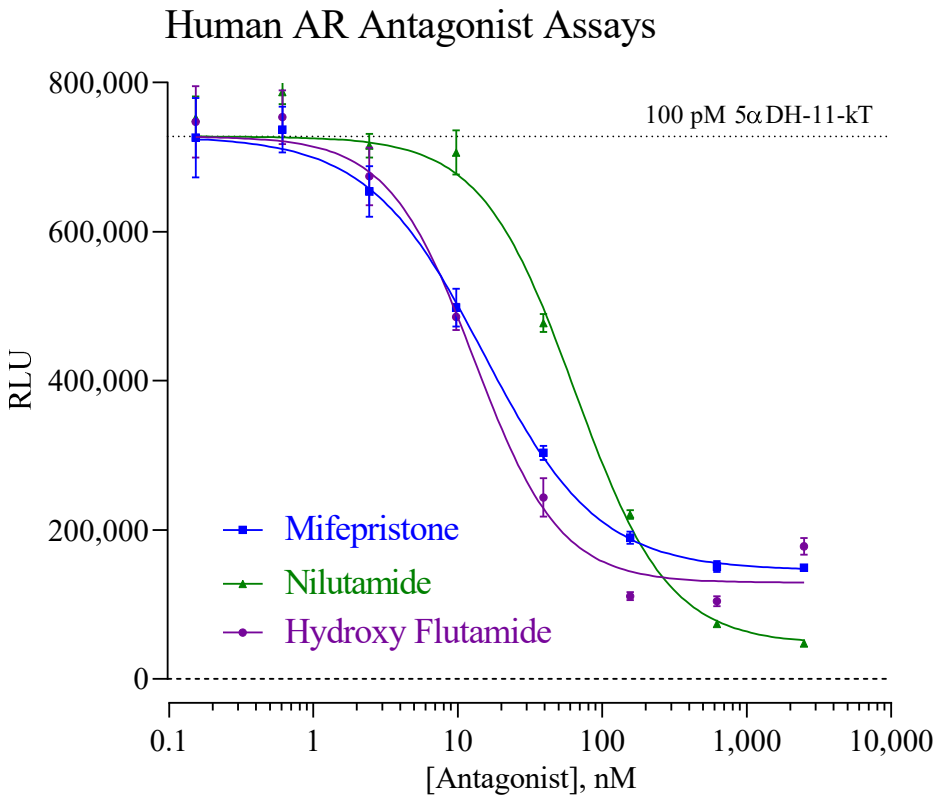


Figure 3. Antagonist dose-response of the AR Reporter Assay.

Analyses of AR Reporter Cells treated with 100 pM 5αDihydro-11-keto Testosterone (~EC_{70}) and challenged with the AR reference antagonists Mifepristone (Enzo Biochem) Nilutamide or Hydroxy-Flutamide (Sigma). No compound-induced cytotoxicity was detected.

II. Product Components & Storage Conditions

This Human AR 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 Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 2* of this protocol.

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first 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 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.

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

Kit Components	*Amount*	*Storage Temp.*
▪ AR 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
▪ 5α-Dihydro-11-keto Testosterone; ref. agonist (5αDH-11-kT; 2.0 µM in DMSO)	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, collagen-coated)	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 following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

DAY 1

▪ dry ice *(Step 2)*
▪ 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 appropriate for dispensing 200 µl volumes.
▪ 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, 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 actual 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 AR Reporter Assay System kit includes a 2.0 µM stock solution of **5α-Dihydro-11-keto Testosterone (5αDH-11-kT)**, a potent agonist of AR (**Figure 2**) that may be used to setup such receptor inhibition studies. 100 pM 5αDH-11-kT typically corresponds to ~EC_{70} in this reporter assay. Hence, it presents a suitable assay concentration of agonist to be used when screening for inhibitory compounds. **APPENDIX 1** is a guide for preparing CSM supplemented with appropriate concentrations of 5αDH-11-kT.

Add the challenge agonist (5αDH-11-kT) to a bulk volume of **CSM** at the desired EC_{50} – EC_{85} concentration. This medium is then used to prepare serial dilutions of test compounds to achieve their 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 **AR Reporter Cells** from -80°C storage, immerse the tube in dry ice and transport it to a laminar flow hood. When ready to proceed, place the tube of cells in a rack and, *without delay*, perform a rapid thaw of the frozen 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 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 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 (see 7a.) 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 AR Assay kit includes a 2.0 µM stock of **5α-Dihydro-11-keto Testosterone (5αDH-11-kT)**, a potent agonist of AR. The following 7-point treatment series, with concentrations presented in 4-fold decrements, provides a complete dose-response: 2000, 500, 125, 31.3, 7.81, 1.95, and 0.488 pM. Always include 'untreated' (or 'Vehicle only') control wells. **APPENDIX 1** provides guidance for generating this a dilution series.

~ or ~

b. ***Antagonist*-mode assays**. When setting antagonist assays, first supplement a bulk volume of CSM with the challenge agonist **5αDH-11-kT** to an EC_{50} - EC_{85} concentration (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 cell pre-incubation period, discard the culture media by manually 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 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, therefore, may be performed on a bench top.

12.) 30 minutes before intending to quantify AR 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*, gently invert the tubes of Detection Substrate and Detection Buffer several times to ensure homogenous solutions, then 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 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

Human AR Assay Products	
Product No.	***Product Descriptions***
IB03001-32	Human AR Assay System; 3x 32 assays in 96-well format
IB03001	Human AR Assay System; 1x 96-well format assay
IB03002	Human AR Assay System; 1x 384-well format assays
Rat AR Assay Products	
R03001-32	Rat AR Assay System; 3x 32 assays in 96-well format
R03001	Rat AR Assay System; 1x 96-well format assay
Zebrafish AR Assay Products	
Z03001-32	Zebrafish AR Assay System; 3x 32 assays in 96-well format
Z03001	Zebrafish AR Assay System; 1x 96-well format assay
LIVE Cell Multiplex (LCM) Assay	
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

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

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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 5α-Dihydro-11-keto Testosterone reference agonist, and the setup of a Human AR dose-response assay.