Zebrafish Androgen Receptor
(nr3c4, AR)
Reporter Assay System

96-well Format Assays
Product # Z03001

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Technical Manual
(version 7.2)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Zebrafish AR Reporter Assay System
96-well Format Assays

I. Description

▪ The Assay System ▪

This nuclear receptor assay system utilizes proprietary mammalian cells engineered to provide constitutive, high-level expression of the **Zebrafish (*Danio rerio*) Androgen Receptor** (nr3c4), a ligand-dependent transcription factor referred to herein as **zfAR.**

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to an AR-responsive promoter. Thus, quantifying changes in luciferase expression in the test sample treated reporter cells provides a sensitive surrogate measure of changes in zfAR activity.

The principal application of this assay is in the screening of test samples to quantify any functional bioactivity that they may exert against zebrafish AR. In particular, zebrafish reporter assays are frequently used in the monitoring of environmental samples for the presence of biohazardous chemical pollutants, such as endocrine disruptors.

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 Nuclear Receptor Assays are all-inclusive assay systems. In addition to zfAR 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 nuclear receptor assay kits 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).

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 compounds are typically solvated at high concentration (ideally 1,000x-concentrated) in DMSO and stored frozen as master stocks. 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. 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. Nonetheless, high concentrations of 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 then considered to be 'single-use' reagents.

▪ Considerations for Automated Dispensing ▪

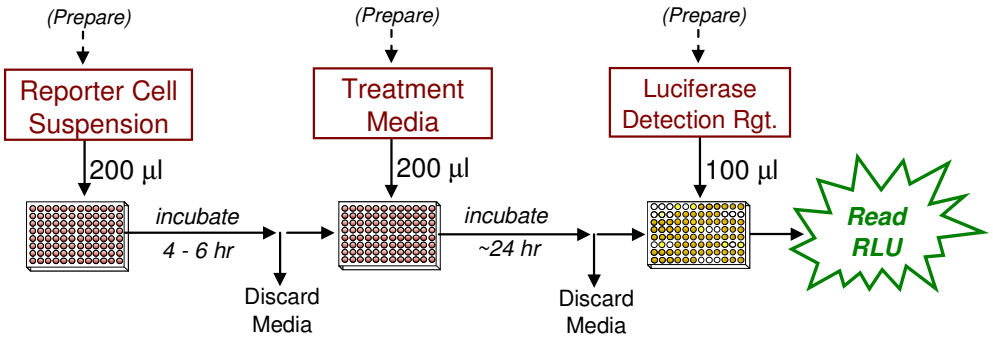
When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final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

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/well of Reporter Cells is dispensed into the assay plate, which is then 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, 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.



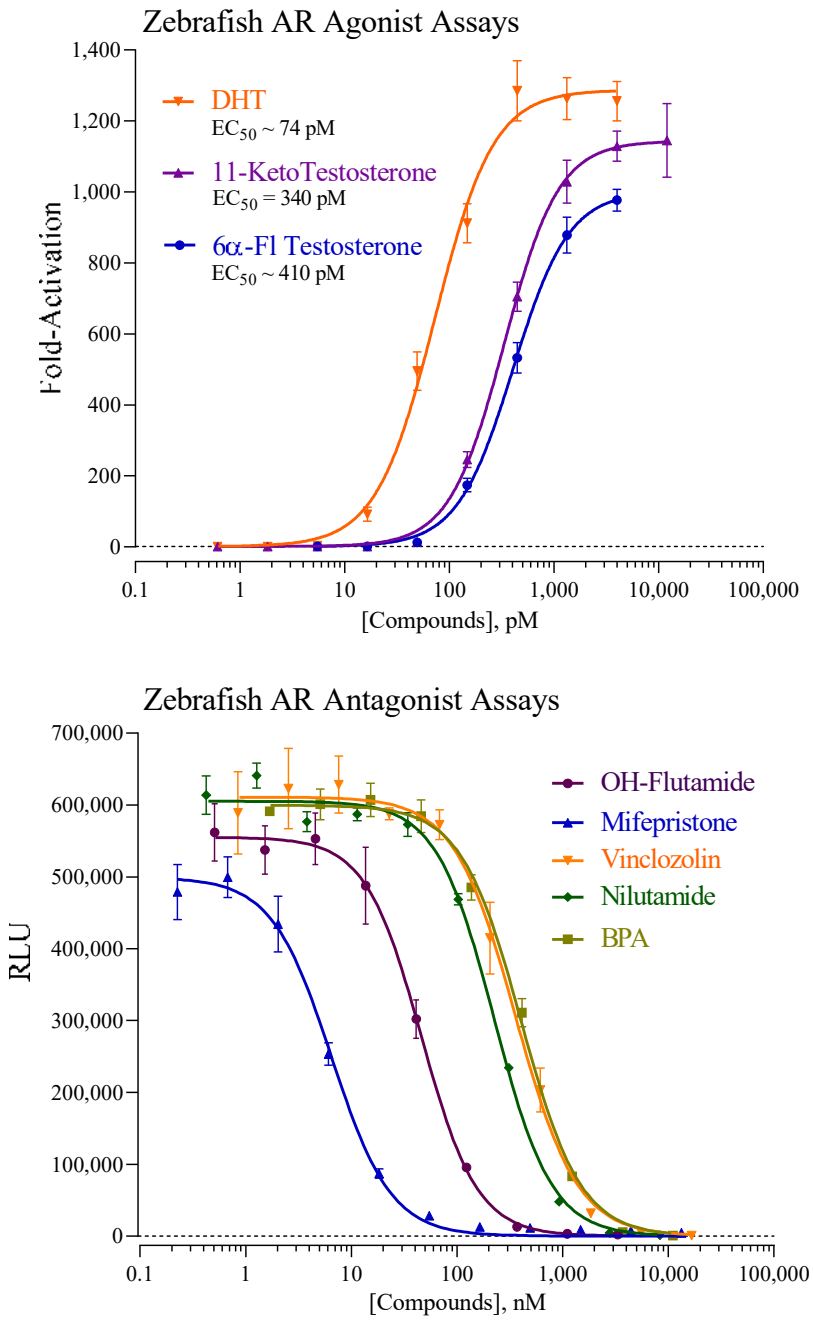


Figure 2. Agonist and Antagonist dose-responses of Zebrafish AR.
Agonist assays: zfAR Reporter Cells were treated with the reference agonists 11-KetoTestosterone (provided), DihydroTestosterone (DHT) and 6α-FluoroTestosterone. *Antagonist assays:* zfAR Reporter Cells were co-treated with a fixed EC_{80} concentration of the reference agonist 11-Ketotestosterone and increasing concentrations of the antagonists Hydroxy flutamide, Mifepristone, Vinclozolin, Nilutamide and bis-Phenol A (n = 3). Luminescence/well was quantified and values of average relative light units (RLU), standard deviation (SD), percent coefficient of variation (%CV) and fold-activation were determined for each treatment concentration. Values of Fold-activation (agonist assays) or average RLU (antagonist assays) +/- %CV were plotted against their corresponding drug treatment concentrations, Log_{10} (pM), using GraphPad Prism software. Z' values were calculated as described by Zhang, *et al*. (1999)[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.

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

II. Product Components & Storage Conditions

This Zebrafish AR Assay kit contains materials to perform assays in a single collagen-coated 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 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.

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

Kit Components	*Amount*	*minimum Storage Temp.*
▪ zfAR 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
▪ 11-Ketotestosterone, 12 µM (in DMSO) (reference agonist for zfAR)	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, *collagen-coated* assay plate (white, sterile, cell-culture ready)	1	**-20°C**

NOTE: This Assay kit contains an assay plate with wells that have been collagen-coated and dried; this 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

▪ container of dry ice (used in *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* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
▪ *Optional:* clear 96-well assay plate, 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 fixed, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known agonist AND varying concentrations of the test compound(s) to be evaluated for antagonist activity. This assay kit includes a 12 µM stock solution of **11-Ketotestosterone**, a reference agonist of zfAR that may be used to setup antagonist-mode assays. 700 pM 11-Ketotestosterone typically approximates ~EC_{70} in this assay. Hence, it presents a suitable concentration to be used when screening test compounds for inhibitory activity.

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. 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 **zfAR Reporter Cells** from -80°C storage, place it directly into dry ice and transport the cells to the laminar flow hood. When ready to begin, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by dispensing 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 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 electronic, repeat-dispensing 8-chanel pipette, dispense **200 µl / well** of cell suspension into all wells of 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 (preferably collagen-coated) 96-well assay plate. Continue processing the clear 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.

6.) ***Near the end of the 4-6 hour pre-culture 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 all treatment media at the desired final assay concentrations. In *Step 9*, the prepared treatment media will be 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 zfAR Assay kit includes a 12 µM stock solution of the reference agonist **11-ketotestosterone**. The following 7-point treatment series, with concentrations prepared in 4-fold decrements, provides a complete dose-response: 12000, 3000, 750, 188, 47, 12, and 3.0 pM. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') control wells.

~ or ~

***b. Antagonist*-mode assays**. When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist **11-ketotestosterone** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). The agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired series of treatment 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 / 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 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 receptor 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 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	
Product No.	***Product Descriptions***
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in all 96-well format assay formats
LCM-05	Reagent in 5x-bulk volume to perform **480** Live Cell Assays in all 96-well format assay formats
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays in all 96-well format assay 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 11-Ketotestosterone reference agonist, and the setup of a zfAR dose-response assay.