

**Dog Androgen Receptor
(NR3C4, AR)
Reporter Assay System**

3x 32 Assays in 96-well Format
Product # D03001-32

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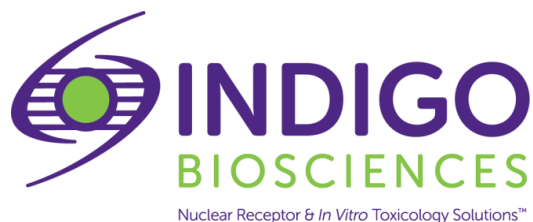
Technical Manual
(version 7.2)

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Dog AR Reporter Assay System 3x 32 Assays in 96-well Format

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I. Description

▪ The Assay System ▪

This AR assay utilizes proprietary cells engineered to provide constitutive, high-level expression of full length, unmodified **Dog 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 dog 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, the reference agonist 5 α Dihydro 11-keto Testosterone, 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 O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO₂, 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, **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.

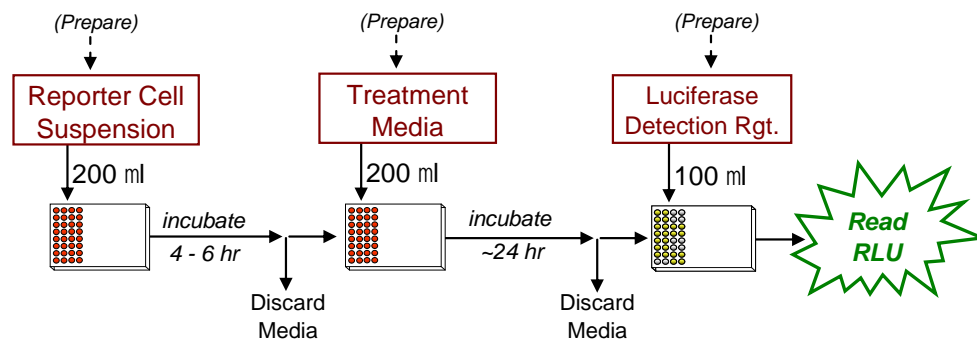
Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. DMSO-induced cytotoxicity can be expected above 0.4%.

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.

▪ Assay Scheme ▪

Figure 1. Assay workflow.

In brief, 200 μ l 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 μ l/well of the prepared treatment media are added. Following 22 - 24 hours 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.



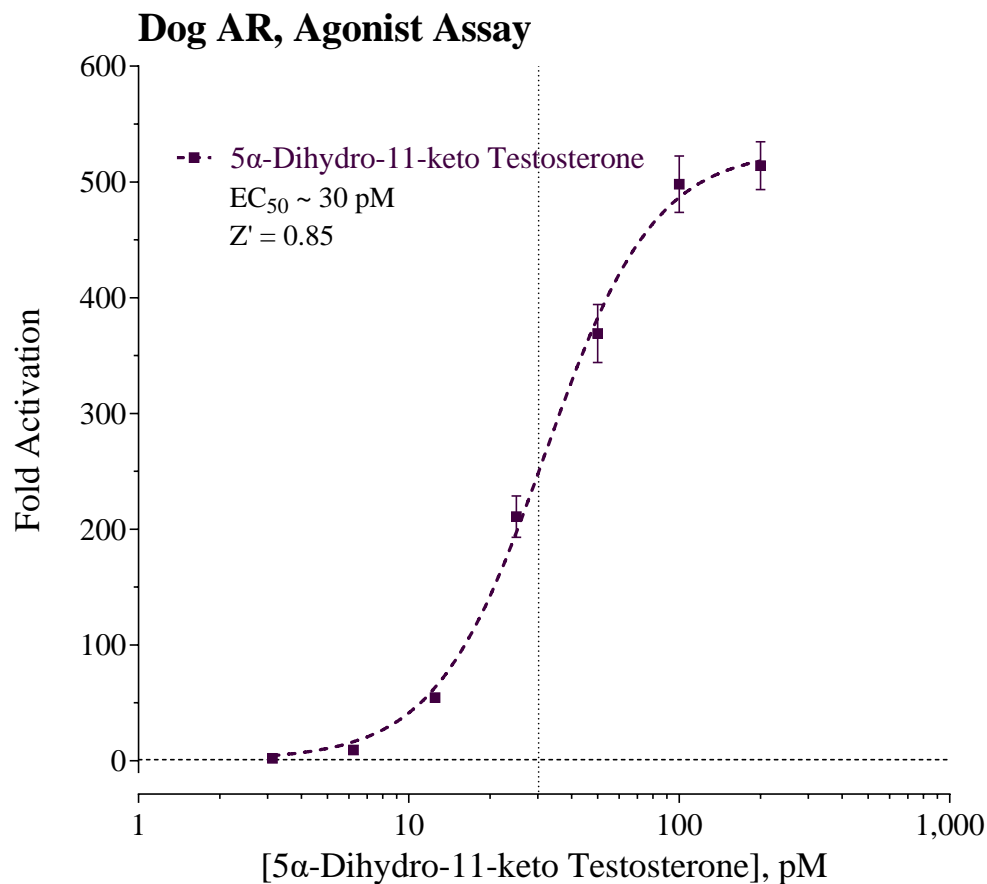


Figure 2. Agonist dose-response analyses.

Dog AR agonist dose-response assays were performed using the reference agonist 5α Dihydro 11-keto Testosterone (5αDH-11kT; provided), Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD), % coefficient of variation (%CV), and Fold-Activation were determined for each treatment concentration (n = 4). Z' values were calculated as described by Zhang, *et al.* (1999)¹. GraphPad Prism software was used to perform the least squares method of non-linear regression and to determine EC₅₀ values.

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.

$$Z' = 1 - [3 * (SD^{Ref EC100} + SD^{Vehicle}) / (RLU^{Ref EC100} - RLU^{Vehicle})]$$

II. Product Components & Storage Conditions

This Dog AR Assay kit contains materials to perform three distinct groups of assays in the format of a 96-well plate. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays

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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ Dog AR Reporter Cells	3 x 0.6 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ 5 α -Dihydro-11-keto Testosterone; ref. agonist (5 α DH-11-kT; 200 nM in DMSO)	1 x 30 μ L	-20°C
▪ Detection Substrate	3 x 2.0 mL	-80°C
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, collagen-coated wells)	12	-80°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% CO₂ incubator for mammalian cell culture
- 37°C water bath
- 70% alcohol wipes
- 8-channel electronic, repeat-dispensing pipettes & sterile tips appropriate for dispensing 200 μ l 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:* 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 and a 4-hour 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 AR assay kit includes a 200 nM stock solution of **5 α -Dihydro-11-keto Testosterone (5 α DH-11-kT)**, a potent agonist of AR (**Figure 2**) that may be used to setup such receptor inhibition studies. ~80 pM 5 α DH-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.

Add the challenge agonist (5 α DH-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 **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each 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 7.0 ml per tube.

Third, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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

4.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to disperse cell aggregates and gain a homogenous cell suspension. Dispense **200 μ l / 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 assay 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% CO₂) 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 200 nM 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 2-fold decrements, provides a complete dose-response: 200, 100, 50.0, 25.0, 12.5, 6.25, and 3.13 pM. Always include 'untreated' (or 'Vehicle only') control wells. **APPENDIX 1** provides guidance for generating this 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 EC₅₀ - EC₈₅ 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 final assay concentrations.

8.) At the end of the cell pre-incubation period: Discard the culture media. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using a single pipette tip or an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

9.) Dispense **200 µl / well** of each prepared treatment media into the assay plate.

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 the appropriate number of vials of **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.) Approximately 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.

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. Program 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:* To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

15.) Following 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 8*).

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.

18.) Data Analyses.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Dog AR Assay Products	
D03001-32	Dog AR Assay System; 3x 32 assays in 96-well format
D03001	Dog AR Assay System; 1x 96-well format assay
Human AR Assay Products	
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 performed in 5 x 96-well assay plates
LCM-10	Reagent in 10x bulk volume to perform 960 Live Cell Assays performed 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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