Human Androgen Receptor
(NR3C4, AR)
Reporter Assay System

96-well Format Assays
Product # IB03001

Technical Manual
(version 7.2b)

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Human AR Reporter Assay System
96-well Format Assays

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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**

Test compounds are typically solvated at high-concentration 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 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.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (96-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reporter Cell Suspension 21 ml (prepared from kit components)</td>
<td>200 µl / well 19.2 ml / plate</td>
<td>~ 1.8 ml</td>
</tr>
<tr>
<td>LDR 12 ml (prepared from kit components)</td>
<td>100 µl / well 9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
</tbody>
</table>

**Assay Scheme**

*NOTE:* This new version (v7.2) AR Assay protocol includes Day 1 steps and dispensed volumes that differ from the previous protocol that some users may be accustomed to. Please review the assay workflow, below.

**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.
**Assay Performance**

![Human AR Agonist Assay: Agonist dose-responses](image)

**Figure 2. Agonist dose-response of the AR Reporter Assay.**

Dose-response analyses of AR Reporter Cells were performed according to the protocol provided in this Technical Manual, using three common AR reference agonists: 6α-Fl Testosterone (6αFIT; provided), Di-Hydroxy Testosterone (DHT; Steraloids Inc.) and Medroxy-Progesterone 17-Acetate (MPA; Steraloids, Inc.). Reporter Cells were treated with reference agonists 6αFIT and DHT starting at 4 nM and MPA starting at 10 nM concentrations. Subsequent treatment concentrations were presented in serial 4-fold decrements. ‘Untreated’ control treatments consisted of vehicle (0.1% DMSO) only. Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined. Non-linear regression analyses were plotted using GraphPad Prism software. EC50 concentrations for each agonist were determined to be: DHT, 22 pM; MPA, 66 nM; and 6αFIT, 160 pM.


\[ Z' = 1 - \left[ 3 \times \left( \frac{SD_{Reference} + SD_{Bkg.}}{RLU_{Reference} - RLU_{Bkg.}} \right) \right] \]
Figure 3. Antagonist dose-response of the AR Reporter Assay.
Analyses of AR Reporter Cells treated with 670 pM 6αFl-Testosterone (~EC₅₀) and challenged with the reference antagonists Mifepristone (Enzo Biochem) or Hydroxy-Flutamide (Sigma), beginning at 2.5 μM and continuing with eight additional treatment concentrations presented in serial 4-fold decrements.
II. Product Components & Storage Conditions

This Human AR Assay kit contains materials to perform assays in a single 96-well assay plate.

The aliquot of AR Reporter Cells is provided as a 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.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, Reporter Cells must be maintained at -80°C until immediately prior to use.

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

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• AR Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>2 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 45 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• 6α-Fl Testosterone, 4.0 μM (in DMSO) (reference agonist for AR)</td>
<td>1 x 30 μL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>1 x 6.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Detection Buffer</td>
<td>1 x 6.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• 96-well assay plate (white, sterile, collagen-coated)</td>
<td>1</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

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 μl volumes.
• 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: 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 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 4.0 µM stock solution of **6αFl Testosterone (6αFIT)**, a potent agonist of AR (Figure 2) that may be used to setup such receptor inhibition studies. 600 pM 6αFIT typically corresponds to ~EC$_{80}$ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening for inhibitory compounds. **APPENDIX I** is a guide for preparing CSM supplemented with appropriate concentrations of 6αFIT. Add the challenge agonist (6αFIT) 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. We find that 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. Dispense **200 µl / well** of cell suspension into the assay plate.

   **NOTE 4.1:** Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling during the dispensing period. Likewise, take care to dispense uniform volumes across the assay plate. We recommend the use of electronic, repeat-dispensing multi-channel pipettes that are routinely calibrated.

   **NOTE 4.2:** 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 96-well cell culture treated assay plate. Continue to process the clear assay 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.
Near the end of the 4-6 hour pre-incubation period:

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 Compound Screening Medium (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 4.0 µM stock of 6α-Fl Testosterone, a potent reference agonist of AR. The following 7-point treatment series, with concentrations presented in 4-fold decrements, provides a complete dose-response: 4000, 1000, 250, 62.5, 15.6, 3.91, and 0.977 pM, and including a 'no treatment' control. APPENDIX 1 depicts a scheme 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 6α-Fl Testosterone to achieve the desired final assay-concentration in the range of EC50 - EC85 (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 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 of each “untreated Control” medium, and prepared “reference agonist Control” and “Test compound treatment” media into the assay plate.

10.) Transfer the assay plate into a 37°C, humidified 5% CO2 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, 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. 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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB03001-32</td>
<td>Human AR Assay System; 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>IB03001</td>
<td>Human AR Assay System; 1x 96-well format assay</td>
</tr>
<tr>
<td>IB03002</td>
<td>Human AR Assay System; 1x 384-well format assays</td>
</tr>
</tbody>
</table>

#### Rat AR Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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<tbody>
<tr>
<td>R03001-32</td>
<td>Rat AR Assay System; 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>R03001</td>
<td>Rat AR Assay System; 1x 96-well format assay</td>
</tr>
</tbody>
</table>

#### LIVE Cell Multiplex (LCM) Assay

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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</thead>
<tbody>
<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform <strong>96</strong> Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform <strong>480</strong> Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform <strong>960</strong> Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.

[www.indigobiosciences.com](http://www.indigobiosciences.com)

### VI. Limited Use Disclosures

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APPENDIX 1

Example scheme for the serial dilution of 6α-Fl Testosterone reference agonist, and the setup of a Human AR dose-response assay.