INDIGO Biosciences, Inc.

The Nuclear Receptor Company™

Human Androgen Receptor
(NR3C4, AR)
Reporter Assay System

96-well Format Assays
Product # IB03001

▪

Technical Manual
(version 5.0)

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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 principle 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 (optionally) in diluting the test samples, a reference agonist, Luciferase Detection Reagent, a cell culture-ready assay plate, and a detailed protocol.

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor reporter assay systems 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 Nuclear Receptor Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 15 and 90+ minutes after initiating the luciferase reaction (refer to APPENDIX 2 for more information). There is no requirement to sequentially process-and-read single 96-well format assay plates. However, when *manually* processing assay plates, or when batch processing larger numbers of assay plates using an auto-dispenser, incorporate a 15 minute reaction-rest period to attain signal stability before quantifying RLU values. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

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▪ Preparation of Test Compounds ▪

This kit offers two alternative assay protocols, denoted as "**Variation 1**" and "**Variation 2**". Follow the protocol variation that best suits the starting configuration of test compounds.

We find that test compounds are typically stored in one of two ways:

1.) Test compounds are solvated in DMSO at pre-established working stock concentrations. Often these are arrayed in library plates, and archived as ready-use test material for high-throughput screening initiatives. In this situation, follow **Protocol Variation 1**, as depicted in **Figure 1a**.

> NOTE: When test compounds are solvated in 100% DMSO, it is advised that no less than a "100x" stock concentration (*preferably higher*) of each test compound be applied to assay wells. The user should carefully assess the impact on assay performance when introducing ≥ 1% DMSO to any cell-based assay.

or,

2.) Test compounds are solvated in DMSO as very high-concentration stocks, and are stored as master stocks. These master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. In this instance, users may elect to dilute test compounds to "2x" stocks using **Compound Screening Medium (CSM-2)** as described in **Protocol Variation 2**, and depicted in **Figure 1b**. By this method, the potentially adverse effects of introducing high concentrations of DMSO into the assay are avoided.

> *NOTE:* Test compounds diluted in CSM2 should be prepared immediately prior to assay setup. Test compounds diluted in such media may lack long-term stability and, therefore, should be considered single-use preparations; excess volumes should be discarded after use.

▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully considered 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, when performing Protocol Variation 1 or 2 (refer to Assay Schemes, page 5).

Protocol Variation 1 (see Fig 1a)				***Protocol Variation 2*** (see Fig 1b)		
Reagent Stock & Volume	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. available for instrument dead volume**		**Reagent Stock & Volume**	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. available for instrument dead volume**
Reporter Cell Suspension 24 ml	200 µl / well — 19.2 ml / plate	**~ 4.8 ml**		Reporter Cell Suspension 12 ml	100 µl / well — 9.6 ml / plate	**~ 2.4 ml**
LDR 12 ml	100 µl / well — 9.6 ml / plate	**~ 2.4 ml**		LDR 12 ml	100 µl / well — 9.6 ml / plate	**~ 2.4 ml**

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▪ Assay Schemes ▪

In brief, Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds, according to either protocol ***Variation 1*** or ***Variation 2***. Following an overnight incubation, the treatment media are discarded and Luciferase Detection Reagent (LDR) is added. The intensity of light emission from each sample well is quantified using a plate-reading luminometer.

Due to the experiment-specific nature of these steps, this protocol and its attendant "*NOTES*" are intended as guidelines to assist researchers in formulating an assay design that is optimally suited to achieve their specific research goals.

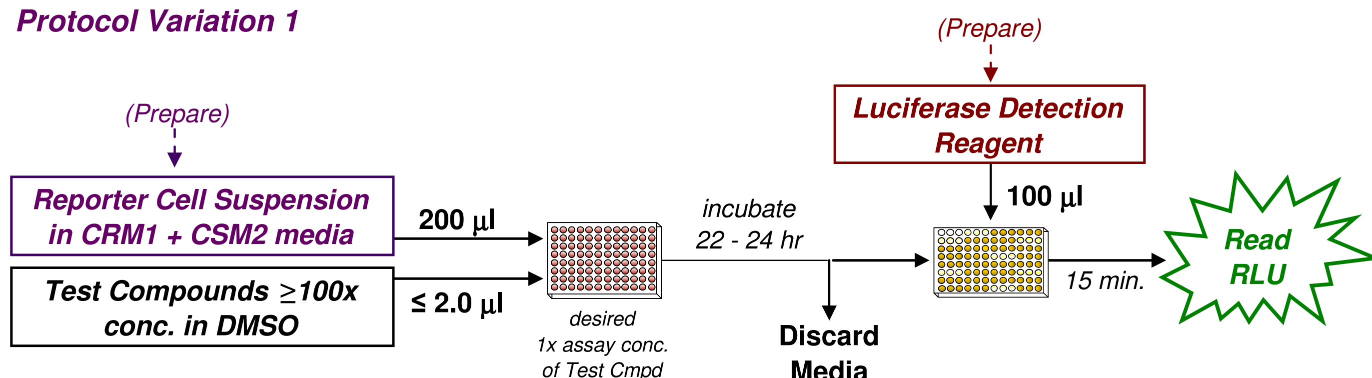

Figure 1a. ***Protocol Variation 1:*** Test compound dilutions are previously prepared in DMSO and archived. If the working stocks were prepared using 100% DMSO, test compound concentrations of *at least* "100x" should be used, thereby assuring that the final assay concentration of DMSO is *no greater than 1%.*

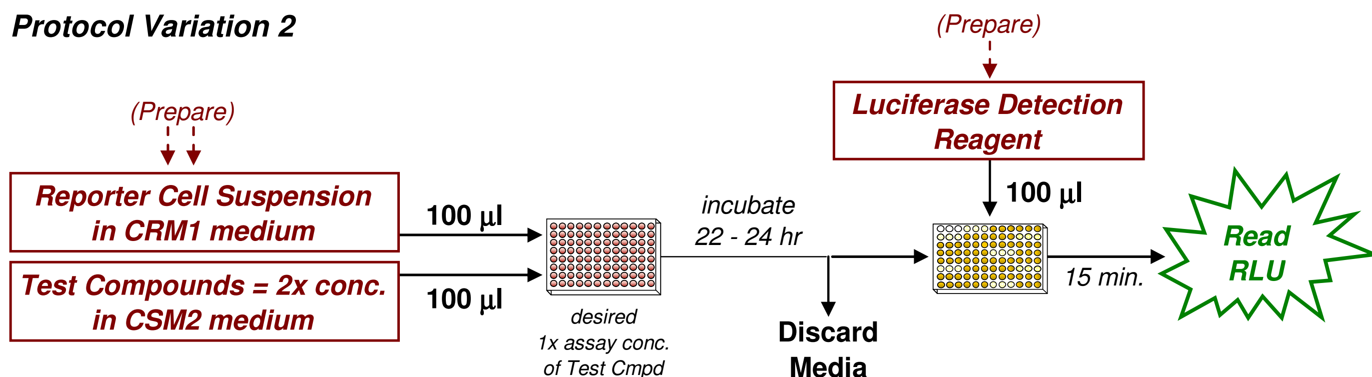

Figure 1b. ***Protocol Variation 2:*** Test compounds are diluted in Compound Screening Medium (CSM2) to generate "2x" concentrations.

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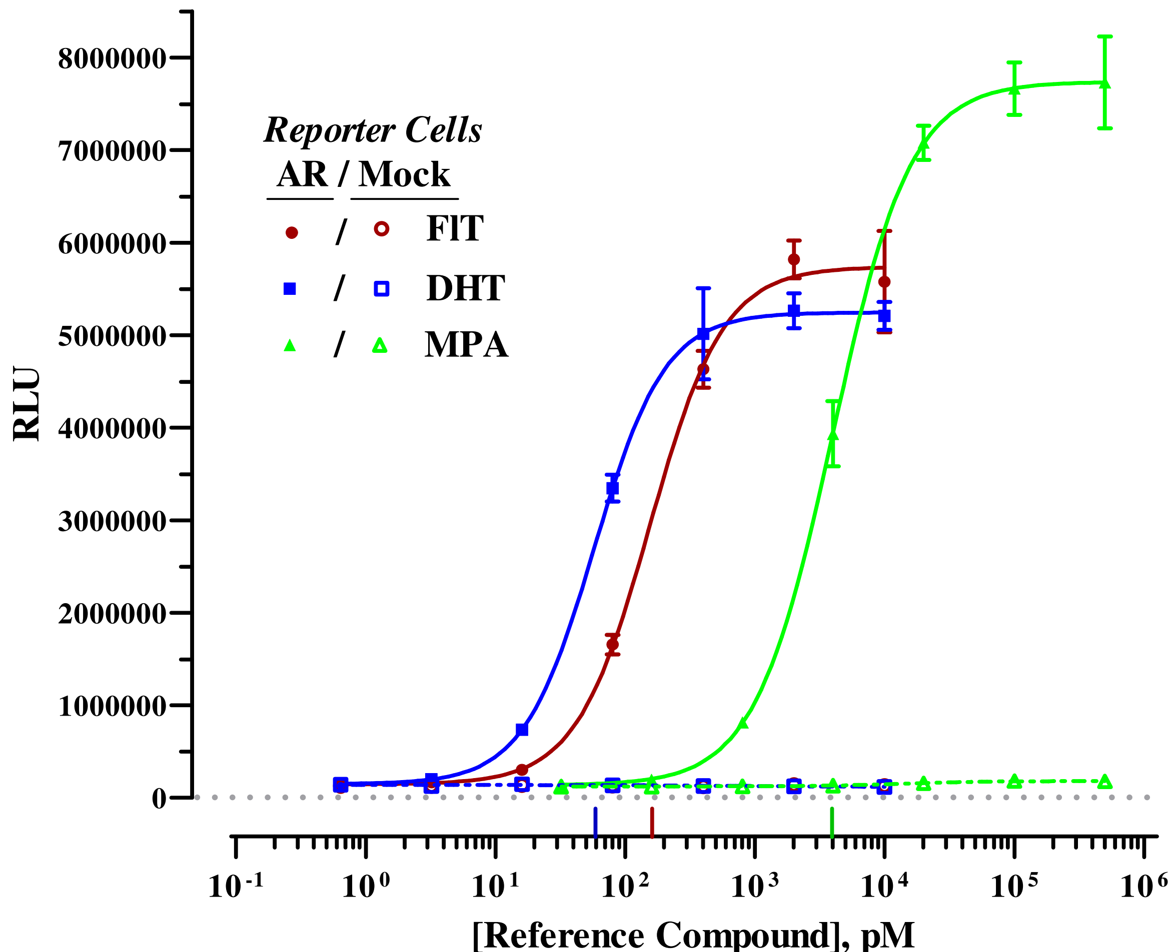

Figure 2. Agonist dose-response of the AR Reporter Assay.
Dose-response analyses of AR Reporter Cells were performed according to the protocol provided in this Technical Manual, using three common AR reference agonists: 6α-Fl Testosterone (FlT), Di-Hydroxy Testosterone (DHT) and Medroxy-Progesterone 17-Acetate (MPA)*. To assess the amount of background signal contributed by other factors that may cause gratuitous activation of the luciferase reporter gene, "mock" reporter cells were prepared to contain only the luciferase vector (*i.e.,* cells withOUT the AR expression vector. Mock reporter cells are not provided with assay kits). AR Reporter Cells and Mock Reporter Cells were treated with reference agonists at assay concentrations spanning approximately 0.01x EC_{50} to 100x EC_{50} for each compound. The assay concentration range for FlT and DHT were: 10000, 2000, 400, 80, 16, 3.2, 0.64 and 0 pM., and for MPA: 500, 100, 20, 4.0, 0.80, 0.16, 0.032 and 0 nM. Luminescence was quantified using a GloMax-Multi+ luminometer. Average relative light units (RLU) and corresponding standard deviation (SD) values were determined. Non-linear regression analyses were plotted using GraphPad Prism software. EC_{50} concentrations for each agonist were determined to be: FlT, 160 pM; DHT, 58 pM; and MPA, 4.0 nM.

* FlT is provided with this AR Reporter Assay System kit. DHT and MPA were obtained from Steraloids, Inc.

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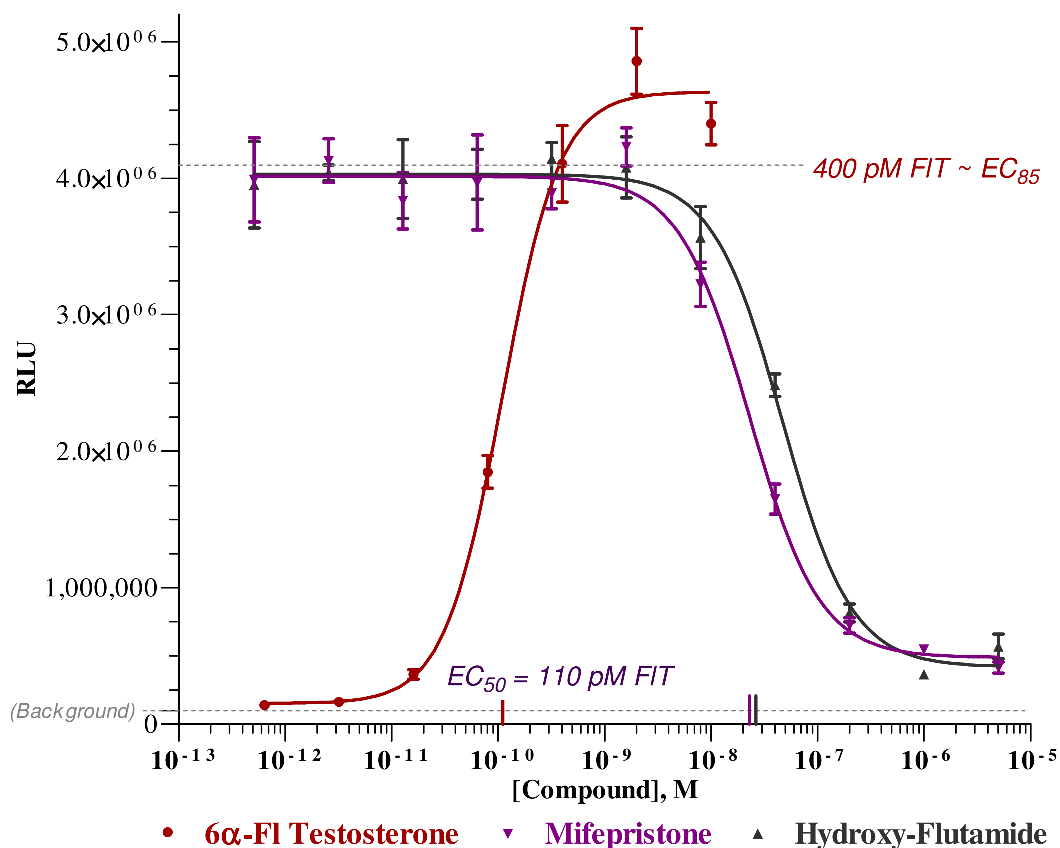

Figure 3. Antagonist dose-response of the AR Reporter Assay.

Analyses of AR Reporter Cells treated with 400 pM 6αFl-Testosterone (~EC_{85}) and challenged with 5-fold increasing concentrations of the reference antagonists Mifepristone (Cayman Chemical) or Hydroxy-flutaminde (Sigma), each at the following concentrations: 5.0 µM, 1.0 µM, 200 nM, 40 nM, 8.0 nM, 1.6 nM, 320 pM, 64 pM, 12.8 pM, 2.56 pM, 0.512 pM and 0 pM.

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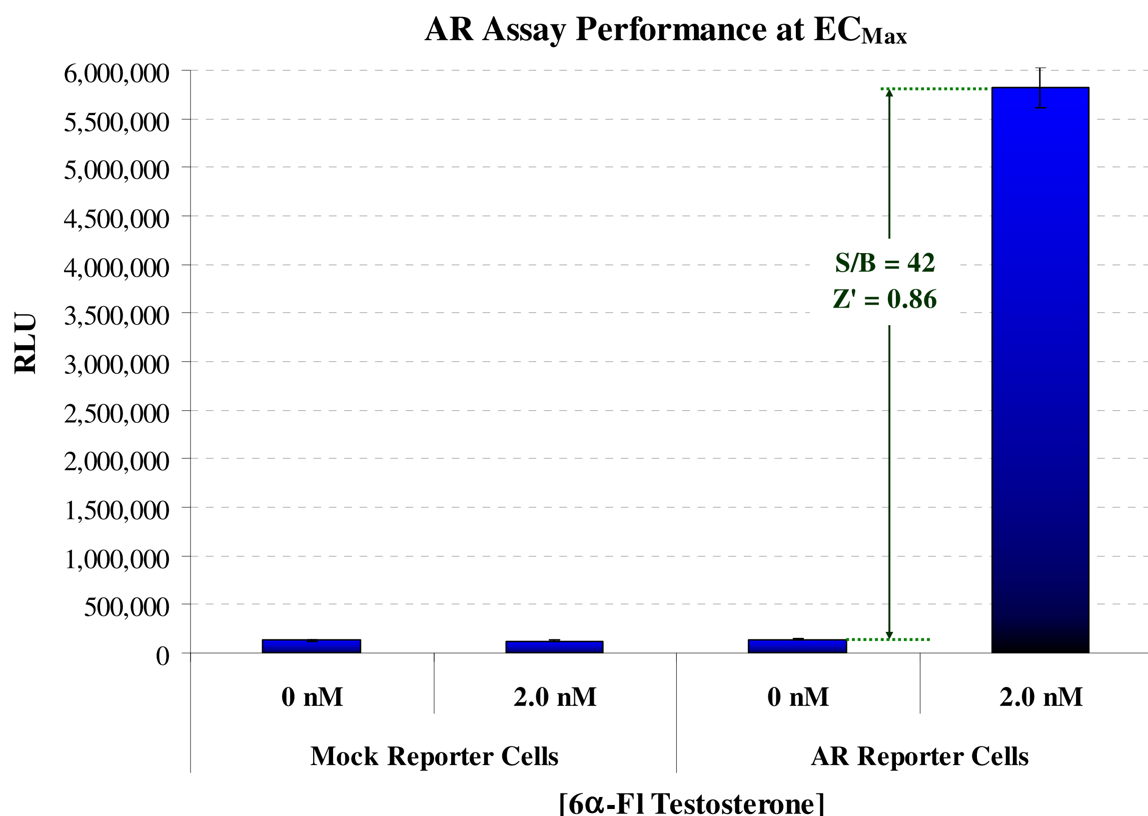

Figure 4. Validation of the AR reporter assay for screening applications.

AR reporter cells and Mock reporter cells were cultured, treated with 2 nM FlT ($n \geq 6$), and processed in identical manner. Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al*. (1999)[1].

> *NOTE:* RLU values will vary slightly between different production lots of reporter cells, and can vary *significantly* between different makes and models of luminometers.

RESULTS & CONCLUSIONS: AR reporter cells treated with 2 nM FlT yielded S/B = 42 and a calculated Z' value of 0.86. Similarly treated mock reporter cells demonstrate very low-level background luminescence (≤ 2% that of the reporter cells at EC_{Max}). Thus, luminescence results through ligand-activation of the exogenous human AR expressed in these reporter cells. These data confirm the robust performance of this AR Reporter Assay System, and demonstrate its suitability for use in HTS applications.[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.

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

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II. Product Components & Storage Conditions

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

Aliquots of AR Reporter Cells are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen 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.

Kit Components	*Amount*	*Storage Temp.*
▪ AR Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium 1 (CRM-1)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium 2 (CSM-2)	1 x 35 mL	-20°C
▪ 6α-Fl Testosterone, 10 µM (in DMSO) (reference agonist for AR)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	**-80°C**
▪ 96-well assay plate (white, sterile, cell-culture ready)	1	ambient

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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

▪ cell culture-rated hood.

▪ 37°C, humidified 5% CO_2 incubator for mammalian cell culture.

▪ 37°C water bath.

▪ 70% alcohol wipes.

DAY 2

▪ plate-reading luminometer.

OPTIONAL

▪ 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).

▪ antagonist reference compound.

▪ auto-dispensing instrument(s) appropriate for sterile transfer of 200 µl and ≤ 2 µl volumes (*Protocol Variation 1*); *or*, 100 µl volumes (*Protocol Variation 2*).

~or ~

If manually dispensing reporter cells and treatment media:

▪ 8- *or* 12-channel electronic, repeat-dispensing pipettes & sterile tips

▪ disposable media basins (*aka* 'media reservoir'), sterile.

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IV. Assay Protocol

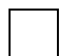
Review the entire assay protocol before starting.

Step 2 offers alternative assay setups: *Step 2A* outlines the setup of an *agonist* assay, whereas *Step 2B* outlines the setup of an assay to screen for *antagonist* activities.

Further, Steps 2, 5, and 6 offer Protocol **Variation 1** or **Variation 2** for the user to choose from. Refer to "Preparation of Test Compounds" on page 4.

Completing the assay requires an overnight incubation. *Steps 1-8* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 9-15* are performed on **Day 2**, and require less than 1 hour to complete.

DAY 1: All steps must be performed using proper aseptic technique.



1) Remove **Cell Recovery Medium 1 (CRM-1)** and **Compound Screening Medium 2 (CSM-2)** from freezer storage and thaw.

   - **CRM-1** should be thawed and equilibrated to 37°C using a water bath. CRM-1 pre-warmed to 37°C is required in *Step 3*.

   - **CSM-2** may be thawed in a 37°C water bath, then allowed to equilibrate to room temperature.

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2.) Preparing Test Compounds stocks to be screened for agonist activities (2A) or antagonist activities (2B).

☐

2A. Screening for *AGONIST* activities

This AR Reporter Assay System kit includes a 10 μM stock solution of **6α-Fl Testosterone**, a potent agonist of AR that may be used as a reference compound. We find the following assay concentration range provides a suitable dose-response: 10000, 2000, 400, 80.0, 16.0, 3.20, 0.64 and 0 picoMolar (pM; 10^{-12} Molar), as depicted in **Figure 2**. **APPENDIX 1** provides an example for generating such a dilution series.

Protocol Variation 1

Recommended for when Test Compounds are at pre-set working concentrations in DMSO.

2A.v1) Retrieve from freezer storage stock plates containing working concentrations of Test Compounds. Allow them to equilibrate to room temperature.

~ or ~

Protocol Variation 2

Recommended for when Test Compounds are to be diluted immediately prior to use
→ use CSM2 as diluent to generate **2x** concentrated stocks

NOTE: In *Step 6_Variation 2*, 100 μl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 μl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a "2x" concentration of the test compound(s) (*i.e.,* [CSM + 2x test cmpd]).

2A.v2) Use **CSM-2** to prepare the appropriate dilution series of 2x-concentrated reference agonist AND an appropriate dilution series of 2x-concentrated test compound(s) to be assayed. Plan dilution schemes carefully. This assay kit provides 35 ml of CSM-2.

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2B: Screening for *ANTAGONIST* activities

A common method of performing receptor inhibition studies is to prepare a co-mix of a known agonist (at a constant concentration typically between EC_{50} – EC_{85}) AND a dilution series of the test compound(s) to be evaluated for antagonist activity.

This AR Reporter Assay System kit includes a 10 µM stock solution of **6α-Fl Testosterone (FlT)**, a potent agonist of AR (**Figure 2**) that may be used to setup such receptor inhibition studies. 400 pM FlT 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 1** provides a guide for preparing CSM supplemented with an appropriate concentration of FlT.

Protocol Variation 1

Recommended for when Test Compounds are at pre-set working concentrations in DMSO.

2B.v1) Retrieve from freezer storage stock plates containing working concentrations of Test Compounds. Allow them to equilibrate to room temperature.

~ or ~

Protocol Variation 2

Recommended for when Test Compounds are to be diluted immediately prior to use
→ use CSM2 as diluent to generate **2x** concentrated stocks

NOTE: In *Step 6_Variation 2*, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a "2x" concentration of the reference agonist AND a "2x" concentration the test compound(s) (*i.e.,* [CSM + 2x FlT + 2x test cmpd]).

2B.v2) Supplement a portion of **CSM-2** with FlT to generate a sufficient stock volume of [CSM + 2x FlT]. Use this [CSM + 2x FlT] stock to then prepare a 2x-concentrated dilution series of each test compound to be evaluated for antagonist activity. If desired, prepare a dilution series of 2x-concentrated reference antagonist (supplied by the user). Plan dilution schemes carefully. This assay kit provides 35 ml of CSM-2.

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☐ **3)** Retrieve **AR Reporter Cells** from -80°C storage. Retrieve **CRM-1** from the 37°C water bath and sanitize the outside of the tube with a 70% ethanol swab. Transfer both tubes into a cell-culture hood. Perform a *rapid thaw* of the frozen cells by pipette-transferring 10.0 ml of the 37°C CRM-1 into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for *at least* 3 minutes.

☐ **4)** Retrieve the tube of Reporter Cell Suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab, then transfer it into the cell culture hood.

☐ **5.)** Invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension, then ...

Protocol Variation 1 Recommended for when test compounds are at pre-set working concentrations in DMSO.	***Protocol Variation 2*** Recommended for when Test Compounds are to be diluted immediately prior to use → use CSM2 as diluent to generate **2x** stocks
5a.v1) Transfer the entire volume of AR reporter cell suspension into a sterile vessel of ≥ 25 ml capacity. ***5b.v1)*** Add 12 ml of **CSM2** and mix gently to gain an homogenous cell suspension. ***5c.v1)*** If setting up an *Antagonist* assay, supplement the cell suspension with the desired final assay concentration of reference agonist (typically between EC_{50} – EC_{80}). ***5d.v1)*** Dispense 200 µl of cell suspension into each well of the 96-well Assay Plate.	***5.v2)*** Dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

NOTE 5.1: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate will cause well-to-well variation in the assay.

NOTE 5.2: Users often prefer the option of examining the reporter cells using a microscope. If so, the extra volume of cell suspension may be dispensed into a clear 96-well cell culture plate, treated +/- test compounds as desired, and processed in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense Test Compound stocks into assay wells.	
Protocol Variation 1 Recommended for when test compounds are at pre-set working concentrations in DMSO.	***Protocol Variation 2*** Recommended for when Test Compounds are to be diluted immediately prior to use → use CSM2 as diluent to generate **2x** stocks
6.v1) Dispense X µl* of Test Compound into each assay well. * The volume of test compound to be dispensed per assay well will depend on the concentration of the stock (*e.g.,* if using test compound stocks prepared at "200x" concentration, dispense 1.0 µl per well.) NOTE: When using test compounds solvated in 100% DMSO, it is advised that minimum concentrations of "100x" (*preferably higher)* are used. Carefully assess the impact on assay performance when introducing ≥ 1% DMSO into the assay.	***6.v2)*** Add 100 µl of "2x"-concentrated treatment media (prepared as described in *Variation 2* of Step 2A *or* 2B) to appropriate wells of the assay plate.

7) Replace the plate's lid and transfer it into a 37°C, humidified 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 90%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

8) For greater convenience on Day 2, retrieve **Detection Substrate** *and* **Detection Buffer** from -80°C storage and place them in a dark refrigerator (4°C) to thaw overnight.

(Continue on DAY 2)

DAY 2: Subsequent manipulations do *not* require special regard for aseptic technique, and may be performed on a bench top.

☐ **9)** 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.

☐ **10)** Turn on the luminometer. Set the instrument to perform a single 5 second "plate shake" prior to reading the assay plate. Read time may be 0.5 second (500 mSec) per well, or less.

☐ **11)** *Immediately before proceeding to Step 12*, combine the entire volumes of Detection Substrate and Detection Buffer to generate **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

☐ **12)** Following 22 - 24 hours of incubation, retrieve the assay plate from the incubator. Remove the plate's lid and discard all 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.

☐ **13)** Add 100 µl of room temperature **LDR** to each well of the assay plate.

> *NOTE:* When performing manual pipetting, avoid bubble formation. Scattered micro-bubbles will not pose a problem. However, bubbles covering the surface of the reaction mix, or large bubbles clinging to the sides of the assay well, will cause lens-effects that may significantly degrade the accuracy and precision of the assay data. In the event of excessive bubble formation during processing, spin the assay plate (with lid) at *low speed* for 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.

☐ **14)** Allow the assay plate to rest at room temperature for at least 15 minutes following the addition of LDR. Do not shake the assay plate during this period.

> *NOTE:* See **APPENDIX 2** for information regarding signal stability.

☐ **15)** Between 15 - 90 minutes after adding LDR, place the assay plate in the luminometer and quantify luminescence using the instrument parameters described in *Step 10*.

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V. Related Products

AR Family of Assay Products	
Product No.	***Product Descriptions***
IB03001-32	Human AR Reporter Assay System 3x 32 assays in 96-well format
IB03001	Human AR Reporter Assay System 1x 96-well format assay
IB03001-B10	Human AR Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 96-well plates
IB03002	Human AR Reporter Assay System 1x 384-well format assays
IB03002-B10	Human AR Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 384-well plates
Alternative volumes of Bulk Assay Reagents may be custom manufactured to better accommodate Customer needs. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
Product No.	***Product Descriptions***
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 in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans. Other applications of this product may require licenses from others, including one or more of the institutions listed below.

The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Patent 5,583,024. INDIGO Biosciences, Inc. has entered into a license agreement with The Regents of the University of California (Oakland, CA) for commercial application of the cDNA encoding the native luciferase of *Photinus pyrallis*.

"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc.

Product prices, availability, specifications and claims are subject to change without prior notice.

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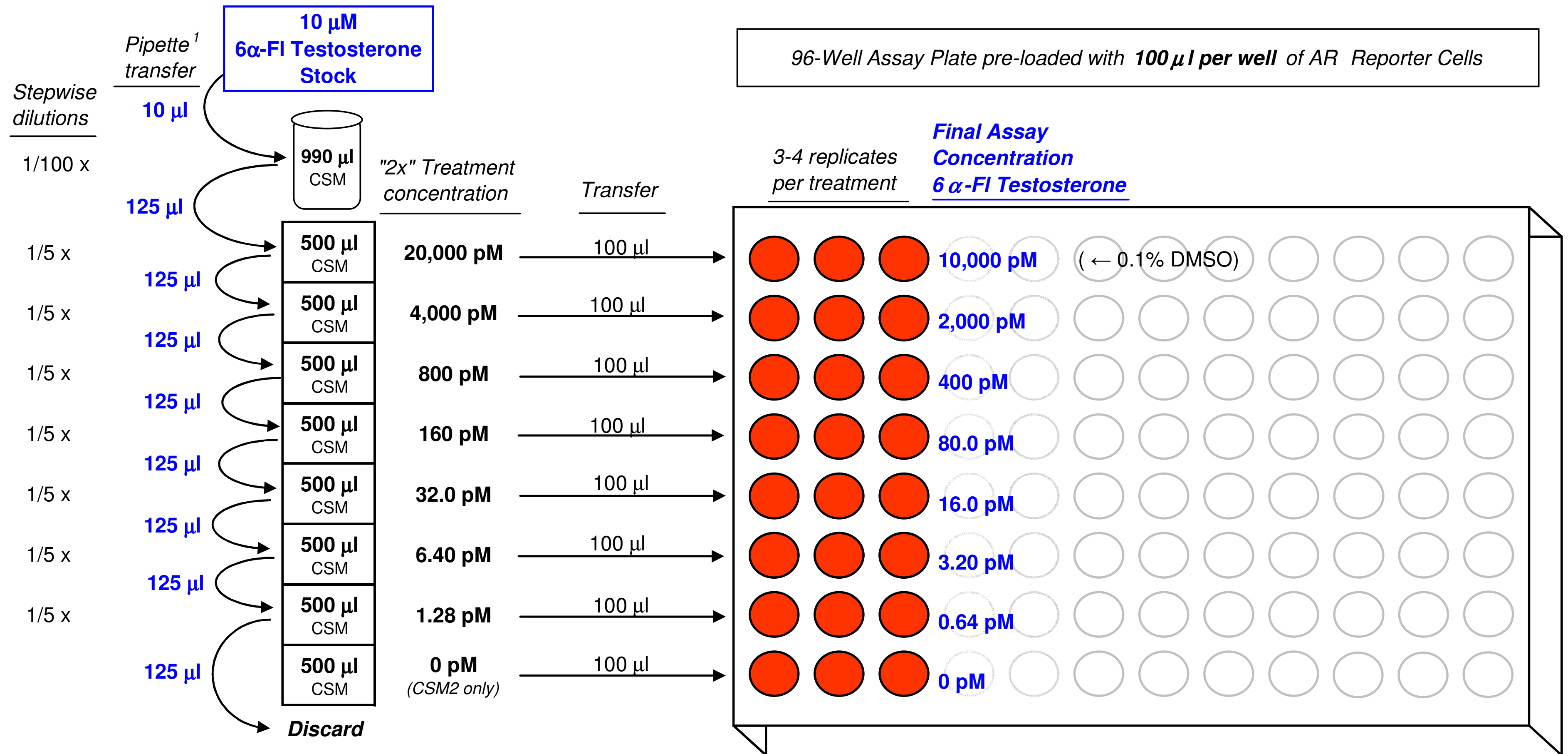
APPENDIX 1

Example scheme for the serial dilution of 6α-Fl Testosterone reference agonist, and the setup of an AR dose-response assay when following *Protocol Variation 2* (Steps 2, 5 & 6).



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

APPENDIX 2
Signal Stability of the Nuclear Receptor Reporter Assay

The ERβ Reporter Assay is used here to demonstrate the light emission profile of INDIGO's nuclear receptor assay system products.

As seen in **Figure 5**, between 5 and 15 minutes after adding LDR to assay wells the luminescence intensity of the reaction decays by ~12%. However, luminescence signal stabilizes and remains essentially constant over the ensuing 75 minute reaction period. From T=15 minute to T=90^+ minutes, average luminescence measured from the same set of assay wells deviate by *less* than 5%

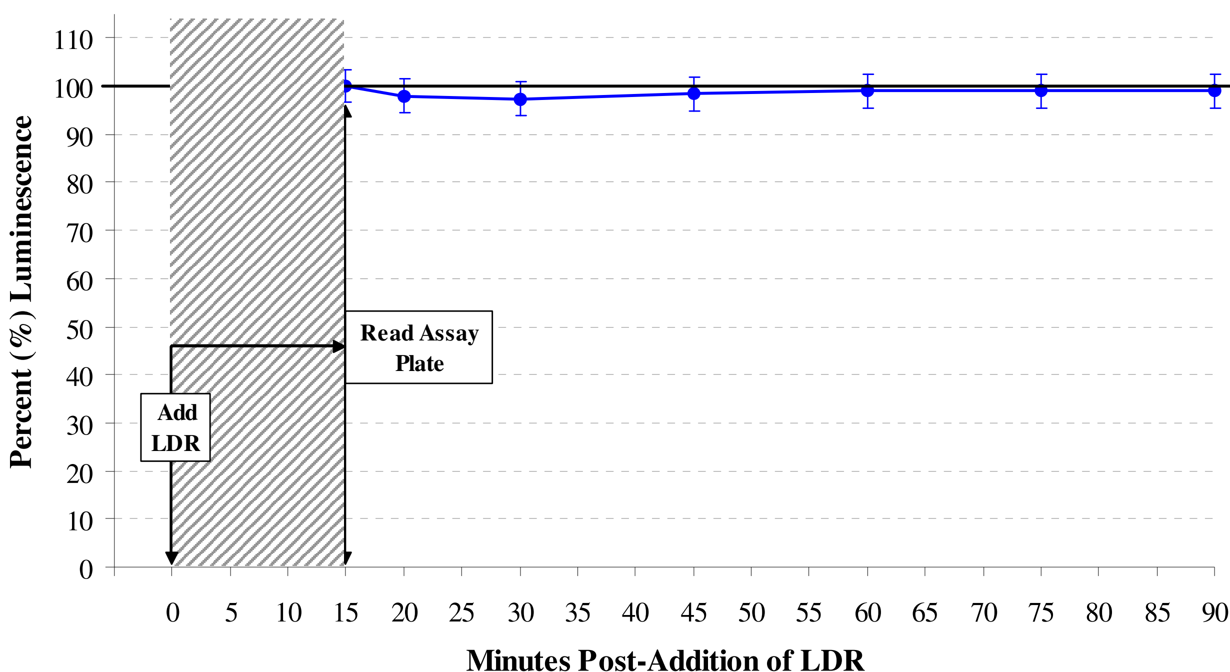


Figure 5. Stability of the luminescence signal. ERβ reporter cells were cultured in 8 replicate wells of a 96-well assay plate, treated with 1 nM 17-β-estradiol for 24 hr, and media were replaced with LDR according to the protocol provided in Technical Manual #00411. Following an initial 5 minute rest period at room temperature, luminescence intensities were quantified by integrating light emission over 500 mSec. After the initial 5 minute time point, the assay plate was re-read at 10, 15, 20, 30, 45, 60, 75 and 90 minutes post-addition of LDR. Average RLU and respective standard deviation values were calculated, then normalized so that the luminescence signal at 15 minutes = 100%.

Allowing a rest period of 15 minutes after the addition of LDR is important for users who elect to manually process plates, or HTS users who batch-process large numbers of assay plates. In such instances, a significant time differential may occur between processing the first and last assay plates. Nonetheless, due to the stable emission profile of the luciferase reaction between 15 - 90 minutes, one may be confident in comparing signal output from test samples in the first assay plate to those in the last plate in the stack.