

**Rat Farnesoid X Receptor
(nr1h4, rFXR)
Reporter Assay System**

96-well Format Assays
Product # R00601

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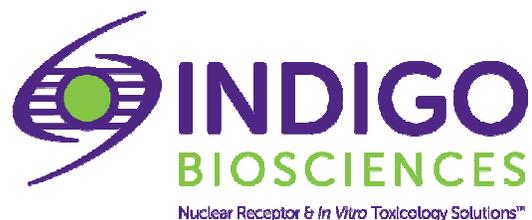
Technical Manual
(version 7.1d)

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Rat FXR Reporter Assay System 96-well Format Assays

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I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary non-human mammalian cells engineered to provide constitutive, high-level expression of **Rat Farnesoid X Receptor (nr1h4)**, a ligand-dependent transcription factor referred to herein as **rFXR**.

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to an FXR-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in FXR activity. The principal application of this assay is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against rat FXR.

FXR 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, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.

INDIGO Bioscience's Nuclear Receptor Assay kits are all-inclusive cell-based assays. In addition to rFXR 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 assays 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 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 Nuclear Receptor Assays 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 ▪

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **Compound Screening Medium (CSM)**, as described in *Step 2* of the **Assay Protocol**. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. 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.

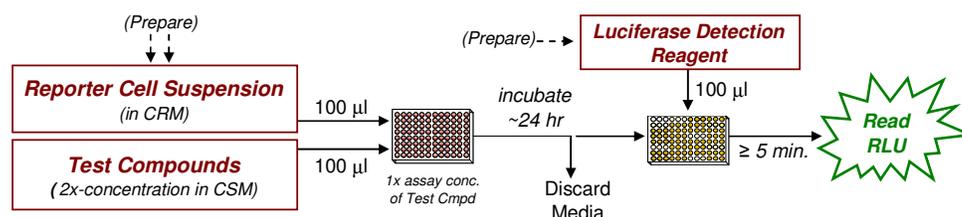
▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully 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.

Stock Reagent & Volume provided	Volume to be Dispensed (96-well plate)	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 12 ml (prepared from kit components)	100 µl / well 9.6 ml / plate	~ 2.4 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*, Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds. Following 22 -24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪

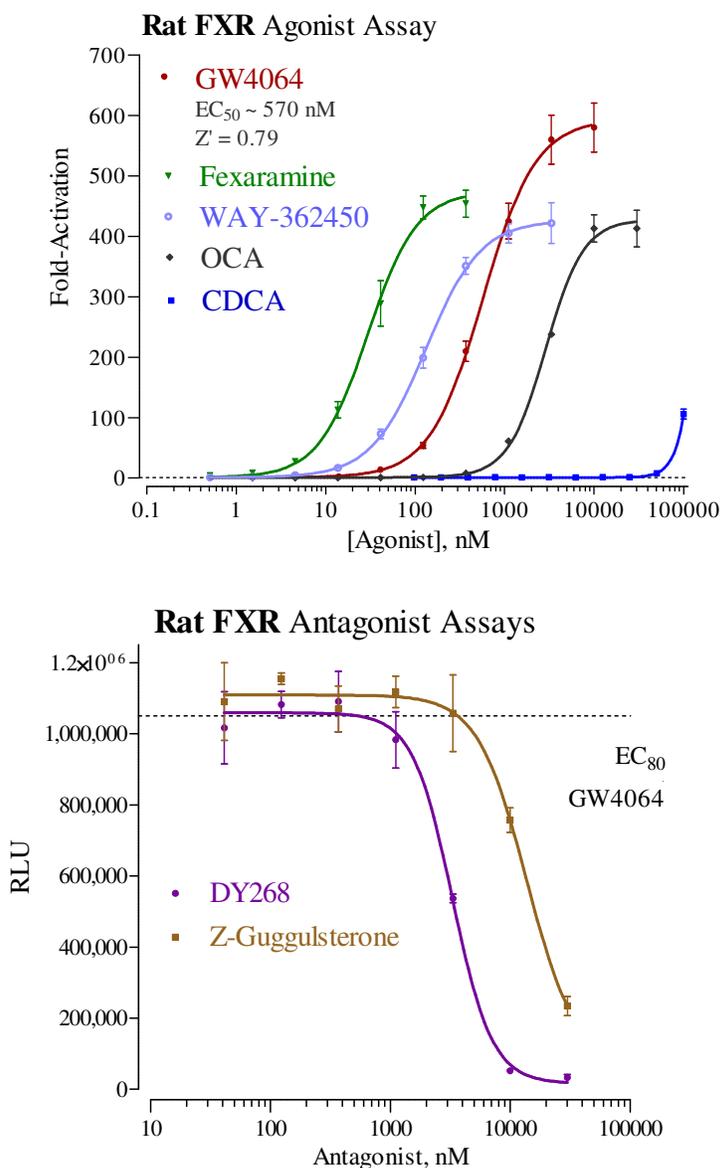


Figure 2. Agonist and Antagonist dose responses of the rat FXR Assay.

Dose-response assays were performed as described in this Technical Manual. FXR reference agonists GW4064 (provided), Fexaramine and CDCA (Cayman Chemical), Obeticholic acid (OCA) and WAY-362450 (Selleckchem) were analyzed.

For antagonist analyses the suspension of FXR reporter cells were pre-treated with a sub-maximal (~ EC₃₀) concentration of the agonist GW4064; prepared treatment media supplemented with the antagonist references Z-Guggulsterone (Cayman Chemical) and DY 268 (Tocris) were dispensed into assay wells.

Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration. Z' values were calculated as described by Zhang, *et al.* (1999)¹.

RESULTS: The large assay window and high Z' values confirm the robust performance of both agonist-mode and antagonist-mode setups for this rat FXR assay.

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.

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

II. Product Components & Storage Conditions

This Rat FXR assay kit contains materials to perform assays in a single 96-well assay plate.

The aliquot of rFXR 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ Rat FXR Reporter Cells	1 x 2.0 mL	-80°C
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ GW4064, 5.0 mM (in DMSO)* (reference agonist for FXR)	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, cell-culture ready)	1	ambient

* *NOTE:* On occasion, GW4064 has been observed to precipitate out of DMSO solution. Upon thawing, briefly spin the 5.0 mM GW4064 stock solution and inspect the bottom of the tube for the presence of a white micro-pellet. *If a precipitate is observed*, use a water bath to heat the solution to 45 – 55°C for up to 15 minutes. Vortex every 5 minutes. It is important to ensure complete dissolution of all flocculent material before preparing dilutions with CSM.

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 bucket (*Step 3*)
- cell culture-rated 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
- 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 1-8* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 9-14* 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 EC₅₀ – EC₈₅) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This FXR assay kit includes a 5.0 mM stock solution of GW4064, a potent agonist of FXR (Figure 2) that may be used to setup such receptor inhibition studies. 1.0 μM GW4064 typically corresponds to ~EC₈₀ in this cell-based assay. Hence, it presents a suitable concentration of agonist to be used when screening test compounds for inhibitory activity.

Adding the reference agonist to the bulk suspension of Reporter Cells (*i.e.*, prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 5b* of the following protocol. Note that, in *Step 6*, 100 μl of treatment media is combined with 100 μl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be useful when preparing the cell suspension supplemented with the desired 2x EC₈₀ concentration (2 μM) of the challenge agonist GW4064. (See the *NOTE on page 6 pertaining to GW4064 solubility.)

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

1.) Remove Cell Recovery Medium (CRM) and Compound Screening Medium (CSM) from freezer storage and thaw in a 37°C water bath.

2.) Prepare dilutions of treatment compounds (first see *Note 5.3*): Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens.

The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

Note that, in *Step 6*, 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 and reference material(s). Use **CSM** to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

Preparing the positive control: This FXR assay kit includes a 5.0 mM stock solution of **GW4064**, a potent reference agonist of FXR. The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a complete dose-response: 3000, 1000, 333, 111, 37.0, 12.3, and 4.12 nM (final assay concentrations), and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

3.) Rapid Thaw of the Reporter Cells: *First*, retrieve the tube of **CRM** from the 37°C water bath and sanitize the outside surface with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. When ready, perform a *rapid thaw* of the frozen cells by transferring a 10 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 12 ml.

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

5.) a. Agonist-mode assays. Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain a homogenous cell suspension. Without delay, dispense 100 μ l of cell suspension into each well of the assay plate.

~ or ~

b. Antagonist-mode assays. Gently invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain a homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x EC80-concentration of challenge agonist (refer to "*A word about antagonist-mode assay setup*", pg. 7). Dispense 100 μ l of cell suspension into each well of the assay plate.

NOTE 5.1: 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.

NOTE 5.2: 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 assay plate in identical manner to the white assay plate.

NOTE 5.3: For logistical reasons, some users find it more convenient to first plate the reporter cells and then prepare their test compound dilutions. That strategy works equally well. Once plated, cells may be placed in an incubator for several hours before proceeding to *Step 6*.

NOTE 5.4: 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-channel dispenser and dispense 100 μ l of sterile water into each of the seven inter-well spaces per column of wells.

6.) Dispense 100 μ l of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity ($\geq 85\%$) 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 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.

9.) 30 minutes before intending to quantify FXR 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.) 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*.

11.) *Immediately before proceeding to Step 12*, 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.

12.) Following the 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.

13.) Add 100 μ l of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes. Do not shake the assay plate during this period.

14.) Quantify luminescence.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Human FXR Assays	
IB00601-32	Human FXR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00601	Human FXR Reporter Assay System 1x 96-well format assay
IB00602	Human FXR Reporter Assay System 1x 384-well format assays
Mouse FXR Assays	
M00601-32	Mouse FXR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
M00601	Mouse FXR Reporter Assay System 1x 96-well format assay
Rat FXR Assays	
R00601-32	Rat FXR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
R00601	Rat FXR Reporter Assay System 1x 96-well format assay
Dog FXR Assay Products	
D00601-32	Dog FXR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
D00601	Dog FXR Reporter Assay System 1x 96-well format assay
Cynomolgus Monkey FXR Assays	
C00601-32	Monkey FXR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
C00601	Monkey FXR Reporter Assay System 1x 96-well format assay
Bulk volumes of Assay Reagents may be custom manufactured to Accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
<i>Product No.</i>	<i>Product Descriptions</i>
LCM-01	Reagent volumes to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plates
LCM-05	Reagent in 5x-bulk volume to perform 480 Live Cell Assays performed in 5x 96-well plates
LCM-10	Reagent in 10x-bulk volume to perform 960 Live Cell Assays performed in 10x 96-well plates

Please refer to INDIGO Biosciences website for additional product offerings.

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VI. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic or contact use in humans or animals.

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APPENDIX 1

Example scheme for the serial dilution of GW4064 reference agonist, and setup of the rat FXR dose-response assay.

