INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human Liver X Receptors Reporter Assay PANEL

LXRα (NR1H3)
LXRβ (NR1H2)

48 Assays each in 96-well Format
Product #IB00321-48P

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Technical Manual
(version 7.1)

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Human LXR Assays PANEL
LXRα and LXRβ
48 Assays each in 96-well Format

I. Description

▪ The Assay System ▪

INDIGO's **PANEL** of **LXR Assays** utilizes non-human mammalian cells engineered to express Human Estrogen Receptors Alpha (NR1H3) and Beta (NR1H2), ligand-dependent transcription factors commonly referred to as **LXRα** and **LXRβ**.

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

LXR 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.

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INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to LXR 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 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 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.

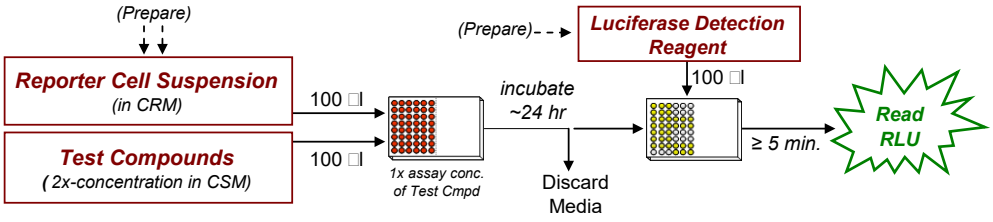
▪ Preparation of Test Compounds ▪

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%.

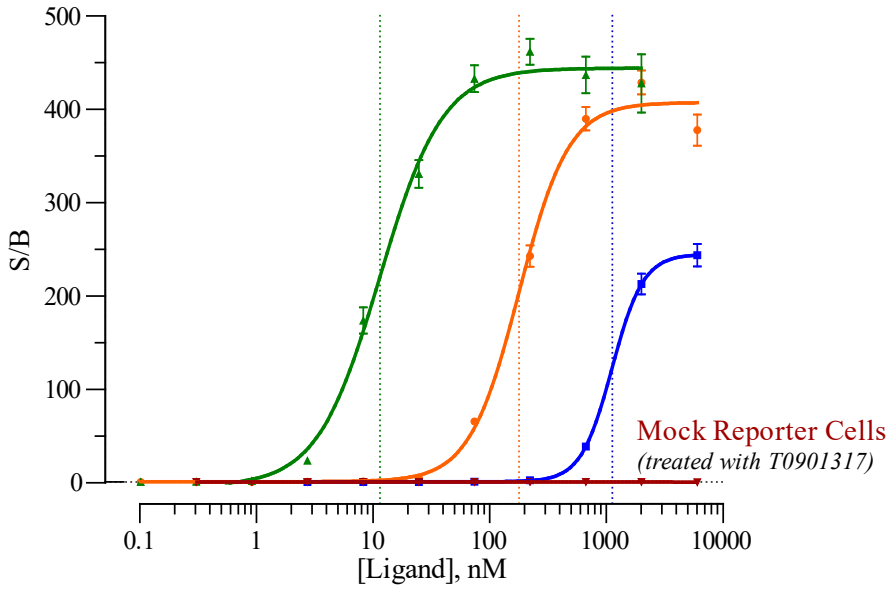
NOTE: CSM is specially formulated to stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. 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*, LXR Reporter Cells are dispensed into 48 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 sample well is quantified using a plate-reading luminometer.



▪ **Assay Performance**



▲ **APD**
EC_{50} = 11 nM
Hill slope = 1.622
R^2 = 0.9965
at 2.0 µM:
S/B = 430
% CV = 7.3
Z' = 0.78

● **T0901317**
EC_{50} = 180 nM
Hill slope = 2.048
R^2 = 0.9952
at 2.0 µM:
S/B = 430
% CV = 4.0
Z' = 0.88

▪ **GW3965**
EC_{50} = 1.1 µM
Hill slope = 3.266
R^2 = 1.000
at 6.0 µM:
S/B = 244
% CV = 4.9
Z' = 0.85

Figure 2. Agonist dose-response analyses of the Human LXRα assay.
Analyses of LXRα Reporter Cells using T0901317 (provided), GW3965 (Tocris), and Acetyl Podocarpic Acid (ADP; Cayman Chemical). In addition, to assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, "mock" reporter cells, which contain only the luciferase vector, were treated with T0901317 (mock reporter cells are not provided with assay kits). Concentrated stocks of agonists were prepared in DMSO, then serially diluted using CSM. Final assay concentrations for agonists ranged between 6 µM and 0.1 nM, including a 'no treatment' control. 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 ($n \geq 6$). Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software. Mock reporter cells demonstrate no significant background, thus, luminescence results strictly through ligand-activation of LXRα expressed in these reporter cells. Low %CV, and high S/B and Z' scores confirm the robust performance of the LXRα 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.

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

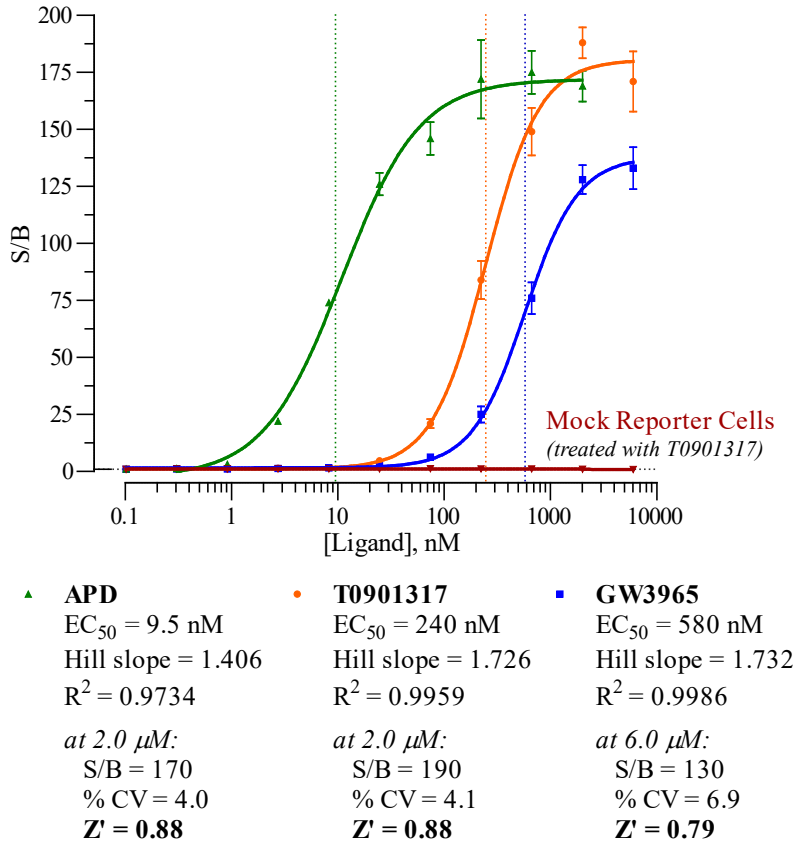


Figure 3. Agonist dose-response analyses of the Human LXRβ assay.
Analyses of LXRβ Reporter Cells using T0901317 (provided), GW3965 (Tocris), and Acetyl Podocarpic Acid (ADP; Cayman Chemical). In addition, to assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, "mock" reporter cells, which contain only the luciferase vector, were treated with T0901317 (mock reporter cells are not provided with assay kits). Concentrated stocks of agonists were prepared in DMSO, then serially diluted. Final assay concentrations for agonists ranged between 6 µM and 0.1 nM, including a 'no treatment' control. 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 ($n \geq 6$). Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al.* (1999)[1]. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software. Mock reporter cells demonstrate no significant background, thus, luminescence results strictly through ligand-activation of LXRβ expressed in these reporter cells. Low %CV, and high S/B and Z' scores confirm the robust performance of the LXRβ Assay.

II. Product Components & Storage Conditions

This Human LXR Reporter Assays PANEL contains materials to perform 48 LXRα assays and 48 LXRβ assays, all in a single 96-well plate format. All reagents are supplied with sufficient extra volume to accommodate the needs of performing 2 individual groups of assays.

The individual aliquots of LXR Reporter Cells and Detection Substrate and Detection Buffer 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.***
▪ LXRα Reporter Cells	1 x 1.0 mL	**-80°C**
▪ LXRβ Reporter Cells	1 x 1.0 mL	**-80°C**
▪ Cell Recovery Media (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Media (CSM)	1 x 35 mL	-20°C
▪ TO901317, 10 mM (in DMSO) (reference agonist for LXRα and LXRβ)	1 x 30 µL	-20°C
▪ Detection Substrate	2 x 3.0 mL	**-80°C**
▪ Detection Buffer	2 x 3.0 mL	-20°C
▪ Plate frame	1	ambient
▪ snap-in, 8-well strips (white, sterile, cell culture treated)	12	ambient

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 laminar flow hood.

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

▪ 37°C water bath.

▪ 70% alcohol wipes

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

▪ disposable media basins, sterile.

▪ sterile multi-channel media basins *or* deep-well plates, *or* appropriate similar vessel for generating serial dilutions of test & reference compound(s).

▪ antagonist reference compounds (optional).

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 9-15* 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 EC_{50} – EC_{85}) of a known *agonist* AND the test compound(s) to be evaluated for antagonist activity. We find that 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.

This LXR Assay Panel kit includes a 10 mM stock solution of TO90117, a commonly used reference agonist for both LXRα and LXRβ (**Figure 2**) that may be used effectively to setup receptor inhibition studies.

▪ **LXRα**: We find that 222 nM TO901317 typically approximates EC_{50} in this reporter assay.

▪ **LXRβ**: We find that 667 nM TO901317 typically approximates EC_{75} in this reporter assay.

APPENDIX 1 provides a guide for preparing CSM supplemented with TO901317.

Note: 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.

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: Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens.

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 LXR Assay Panel kit includes a 10 mM stock solution of **TO901317**, a potent reference agonist for both LXRα and LXRβ. The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable dose-response: 6000, 2000, 667, 222, 74.1, 24.7, 8.23 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series

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. Plan dilution schemes carefully. This assay kit provides 35 ml of CSM.

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

Second, retrieve **Reporter Cells** from -80°C storage. Perform a *rapid thaw* of the frozen cells by transferring a 5.0 ml volume of 37°C CRM into the 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 6.0 ml.

Third, 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.

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 LXR Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into respective strip-wells 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 an homogenous cell suspension. Supplement the 6 ml bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "*A word about antagonist-mode assay setup*", pg. 8). Dispense 100 µl of cell suspension into respective strip-wells of the 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 *will* cause well-to-well variation (= increased Standard Deviation) in the assay.

NOTE 5.2: Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM (as in *Step 6*). Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 100 µl per well of 2x-concentration treatment media into appropriate wells of the assay plate.

7.) 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.

8.) For greater convenience on Day 2, retrieve the appropriate number of tubes 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 may be performed on a bench top.

9.) 30 minutes before intending to quantify LXR activity, remove the tubes of **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 luminometer to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be 0.5 second (500 mSec) per well, *or less*.

11.) *Immediately before proceeding to Step 12*: To read 48 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 6 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

12.) After 22-24 hours of incubation, remove media contents from each well.

NOTE: Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. 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 reporter cells and greatly increased well-to-well variability. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (*e.g.,* Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus.

13.) 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. Do not shake the assay plate during this period.

14.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
LXRα Assay Products	
IB00311-32	Human LXRα Reporter Assay System 3x 32 assays in 96-well format
IB00311	Human LXRα Reporter Assay System 1x 96-well format assay
IB00312	Human LXRα Reporter Assay System 1x 384-well format assays
LXRβ Assay Products	
IB00301	Human LXRβ Reporter Assay System 1x 96-well format assay
IB00301-32	Human LXRβ Reporter Assay System 3x 32 assays in 96-well format
IB00302	Human LXRβ Reporter Assay System 1x 384-well format assays
Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. 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.

"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

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

APPENDIX 1

Example scheme for the serial dilution of TO901317 reference agonist, and the setup of LXR dose-response assays.