



INDIGO Biosciences, Inc.

The Nuclear Receptor Company™

**Human Liver X Receptor, Alpha
(LXR α , NR1H3)
Reporter Assay System**

**3x 32 Assays in 96-well Format
Product # IB00311-32**

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Technical Manual

www.indigobiosciences.com

1981 Pine Hall Road, State College, PA, 16801, USA

Customer Service:

814-234-1919; FAX 814-272-0152; customerserv@indigobiosciences.com

Technical Service:

814-234-1919; techserv@indigobiosciences.com



Human LXR α Nuclear Receptor Assay System 3x 32-well Format Assays

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I. Description

INDIGO Biosciences' Nuclear Receptor Reporter Assay products are optimized, all-inclusive, cell-based assay systems. They feature engineered reporter cells that contain a resident luciferase reporter gene, and express a specific human nuclear receptor within the cytoplasm and nuclear environments of fully functional, healthy, dividing mammalian cells. These assays are sensitive research tools intended for use in screening test compounds to quantify the functional activities they may exert against a given human nuclear receptor.

INDIGO's nuclear receptor assays incorporate a luciferase detection reagent formulated to provide stable light emission over at least 1 hour, thereby eliminating the need to sequentially process assay reactions *via* injectors. The stable, extended luminescence signal allows batch processing of assay plates, making it ideally suited for HTS applications.

INDIGO's Reporter cells are prepared using a proprietary cryo-preservation process called **CryoMite™**, which provides high cell viability post-thaw, and the convenience of immediate dispensing into assay plates without the need for intermediate processing steps or cell counts.

This **Liver X Receptor Alpha Assay System** contains reporter cells engineered to express human **NR1H3** protein, commonly referred to as **LXR α** . Following activation through ligand binding, LXR α induces expression of the luciferase reporter gene. The intensity of light emission from the subsequent luciferase reactions is quantified, and provides a direct correlation to the activation status of LXR α .

This kit is a complete assay system. In addition to LXR α Reporter Cells, it includes optimized media for diluting test compounds, the positive-control agonist T0901317, luciferase detection reagent, and a cell culture-ready assay plate. Kit components may be further stored at their indicated temperatures, making them ready for on-demand use.

II. Product Components & Storage Conditions

This Human LXR α Nuclear Receptor Assay System contains sufficient materials to perform three (3) distinct groups of assays in a 96-well plate format. Reagents are configured such that each group may comprise a maximum of 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assay. All reagents are supplied with sufficient extra volume to accommodate the needs of performing 3 individual groups of assays. The individual aliquots of LXR α Reporter Cells and Luciferase Detection Reagent (LDR) are single-use reagents. Extra volumes of these reagents should be discarded after each assay set-up.

This assay kit is shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels, or the entire kit may be further stored at -80°C. To ensure maximal viability, “LXR α Reporter Cells” must be maintained at -80°C until just prior to use.

The date of kit expiration is printed on the Product Qualification Insert.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp</u>
▪ LXR α Reporter Cells	3 x 0.70 mL	-80°C
▪ Cell Recovery Media 1 (CRM-1)	1 x 10 mL	-20°C
▪ Compound Screening Media 1 (CSM-1)	1 x 35 mL	-20°C
▪ T0901317, 10 mM (in DMSO)	1 x 30 μ L	-20°C
▪ Luc-Detection Reagent (LDR)	3 x 4.0 mL	-80°C
▪ 96-well format plate frame	1	ambient
▪ snap-in, 8-well strips (white, sterile, cell culture treated)	12	ambient

III. Alternative Applications for this Reporter Assay

This nuclear receptor reporter assay system is a sensitive and versatile research tool. As such, the user may configure the assay in one of several different ways to achieve their specific research objectives.

The “Assay Procedure” provided in Section V provides specific instructions for performing the LXR α reporter assay, including alternative set-ups at Step 2 for the user to choose from. For example, this assay may be configured to generate an agonist dose-response (Alternate 2A), an antagonist dose-response (Alternate 2C), or to perform high-throughput single-point screening of test compounds for agonist (Alternate 2B) or antagonist (Alternate 2D) activities. Due to the experiment-specific variables of such strategies, these Alternate Procedures (and their attendant “Notes”) are intended to serve as guidelines only. They are offered to assist researchers in formulating an assay design that is best suited to achieve their specific research goals.

Figure 1
Human LXR α Reporter Assay
Agonist Dose-Response, Normalized

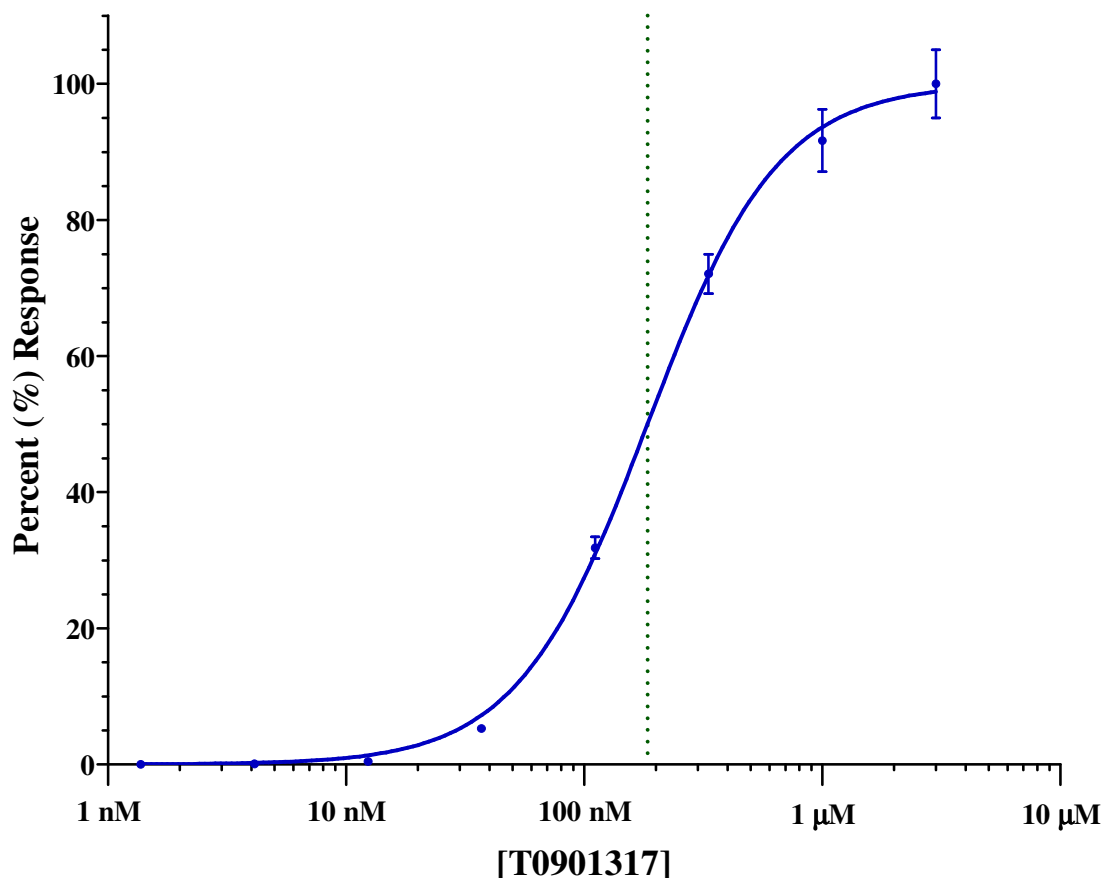


Figure 1. Representative agonist dose-response of the LXR α Reporter Assay System. LXR α dose-response assays were performed according to the protocol provided in this Technical Manual. Reporter cells were treated with T0901317 at the following final concentrations: 3000, 1000, 333, 111, 37.0, 12.3, 4.12, 1.37 and 0 nM. Treatment media were removed after 24 hr and LDR was applied directly to the cells. Luminescence was integrated over 1 second using a Tecan GENios Pro plate-reading luminometer. Average relative light unit (RLU) values and respective standard deviations were determined. Average values were normalized so that maximal RLU (EC_{Max}) = 100%. Agonist concentrations were transformed to log₁₀ nM. Agonist dose-responses were plotted by non-linear regression using GraphPad Prism software. The dotted line depicts the calculated EC₅₀ concentration of T0901317 in this particular assay.

Figure 2

LXR α Assay Performance at EC_{Max}

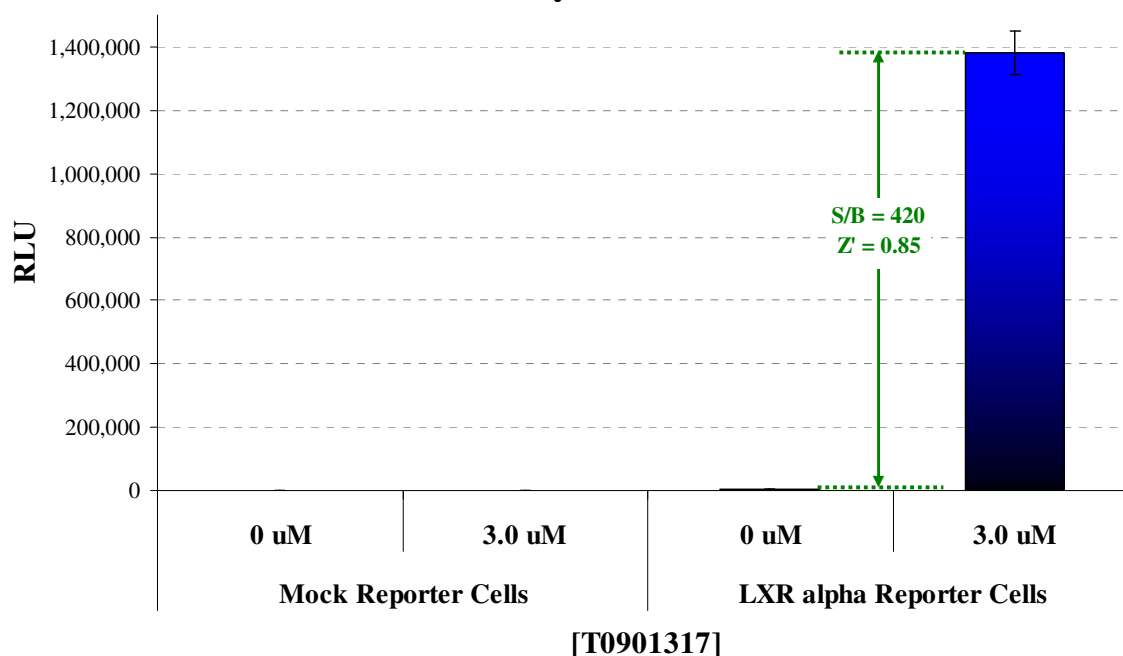


Figure 2. Validation of the LXR α reporter assay for screening applications.

LXR α reporter cells were treated with media alone, or media supplemented with 3.0 μ M T0901317. To assess the amount of background signal contributed from any other factor(s) causing gratuitous activation of the luciferase reporter gene, “mock” reporter cells were specially prepared to contain only the luciferase vector (*i.e.*, cells withOUT the LXR α expression vector; mock reporter cells not included with assay kits). Mock reporter cells and the LXR α reporter cells were cultured, treated with T0901317, and processed in identical manner. A minimum of six replicate assay wells were processed for each treatment condition. Luminescence was quantified as described in Figure 1. Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al.* (1999)¹.

RESULTS & CONCLUSIONS: In this particular experiment, LXR α reporter cells treated with 3.0 μ M T0901317 yielded a S/B = 420 and a calculated Z' value of 0.85. Similarly treated mock reporter cells demonstrated no significant background luminescence ($\leq 0.1\%$ that of the reporter cells at EC_{Max}). Thus, ligand-induced luminescence results through the activation of the exogenous human LXR α protein expressed in these reporter cells. These data confirm the robust performance of this LXR α Reporter Assay System, and demonstrate its suitability for use in high-throughput screening 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.

IV. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made available prior to initiating the assay procedure:

DAY 1

- cell culture rated hood.
- 37°C/humidified/5% CO₂ incubator for mammalian cell culture.
- 37°C water bath (Step 1 [optional] & Step 3 [required]).
- 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 control ligand(s) and user's test compound(s) (Step 2).
- *optional*: antagonist control compound (Step 2, Alternative Protocols C & D).
- 70% alcohol wipes (Step 4).
- pipette & sterile tips appropriate for the transfer of 100 μ l volumes (Steps 5 & 6). The use of a single-channel electronic pipette capable of repeat-dispensing is recommended.

DAY 2

- pipette and tips appropriate for dispensing 100 μ l volumes (Step 10). The use of an 8- or 12- channel electronic pipette capable of repeat-dispensing 100 μ l volumes of LDR is recommended.
- platform shaker/vibrator for micro-titer plates (Step 11).
- 96-well plate-reading luminometer (Steps 12).

V. Assay Procedure

Review the entire protocol before starting. Completing the assay requires an overnight incubation. Steps 1-8 are performed on Day 1, requiring 1 – 2 hours to complete. Steps 9-14 are performed on Day 2, requiring ≤ 1.5 hours to complete.

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



- 1) Remove **Cell Recovery Media 1 (CRM-1)** and **Compound Screening Media 1 (CSM-1)** from freezer storage and thaw.
 - Room temperature CSM-1 is used in the next step. A water bath may be used to facilitate rapid thawing of CSM-1.
 - Thaw CRM-1 and warm to 37°C using a water bath or incubator; pre-warmed CRM-1 is required in Step 3.

ALTERNATIVE 2A: Agonist Dose-Response Assays (e.g., as depicted in Figure 1)

Use **CSM-1** to prepare a dilution series of 2x-concentrated control agonist (T0901317) AND an appropriate dilution series of 2x-concentrated test compound(s) to be assayed.

NOTE 2.0 In Step 6, 100 µl of the prepared [CSM+Cmpd] is added into an assay well already containing 100 µl of Reporter Cells. Hence, to achieve the desired *final* concentration of test compound in the assay one must prepare and dispense a “2x-concentration” of the test compound(s) (i.e., [CSM + 2x test cmpd]).

NOTE 2.1 When generating dose-response curves, it is recommended to perform all measurements in triplicate. In Step 6, 100 µl of [CSM+2x test cmpd] will be added per well of the assay plate. Therefore, devise an appropriate compound dilution scheme to yield a final volume of [CSM+2x test cmpd] that is slightly greater than 300 µl, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate.

NOTE 2.2 When using T0901317 as the positive-control agonist, we find the following assay concentration range provides a complete dose-response: 3000, 1000, 333, 111, 37.0, 12.3, 4.12, 1.37 and 0 nanoMolar (nM; 10⁻⁹ Molar), as depicted in Figure 1. However, as explained in *Note 2.0*, a 2x-concentrated dilution series of both the positive-control and test compounds are required.

[*Hint:* Generating this dilution series for T0901317 may be achieved by following the example presented in **APPENDIX 1**. In brief, use a portion of CSM to first perform a 100-fold dilution of the provided 10 mM T0901317 stock (e.g., mix 10 µl T0901317 stock with 990 µl of CSM). Use the resulting solution to then perform one 16.66-fold dilution to achieve the first desired 2x-concentration of 6000 nM. Continue by using a portion of the 6000 nM solution to perform seven sequential 3-fold dilutions to produce 2000, 667, 222, 74.1, 24.7, 8.23, and 2.74 nM 2x-concentrated stocks. Neat CSM is used to provide the “0 nM” control media.]

NOTE 2.3 If an organic solvent is the diluent used for test compound(s), devise an appropriate dilution scheme so that the concentration of solvent in [CSM + 2x test cmpd] does not exceed 0.2%.

ALTERNATIVE 2B: Screening for Agonist Activities via Single-Point Assay.

Use **CSM-1** to make an appropriate dilution of each test sample in the compound library. Also, prepare CSM-1 containing the desired positive-control reference agonist.

This LXR α Reporter Assay System kit includes a 10 mM stock solution of T0901317, a potent agonist of LXR α that may be used as a positive-control. An assay concentration of 3.0 μ M T0901317 typically provides $\geq 95\%$ activation of LXR α (Figure 1). Hence, 6.0 μ M T0901317 will provide a 2x-concentrated agonist that is a suitable positive-control reference. Refer to *Note 2.2* for a recommended dilution scheme to prepare the appropriate 2x-concentrated T0901317 solution.

NOTE 2.4 In Step 6, 100 μ l of the prepared [CSM+2x test cmpd] will be added per well of the assay plate. If single-point assays are to be made, prepare a volume of each test compound media that is slightly greater than 100 μ l, thus allowing accurate volume transfers into respective assay wells. This assay kit provides 35 ml of CSM. Therefore, plan dilution schemes carefully so that the total volume of CSM required to perform dilutions of the positive- and negative-controls AND all test compounds does not exceed 35 ml. If test compound stocks require extreme dilution to achieve the desired 2x-concentrations, use the original solvent to make one or two preliminary dilutions, followed by final dilutions in CSM. If an organic solvent is the diluent used for test compound(s), devise an appropriate dilution scheme so that the final assay concentration of solvent in [CSM + 2x test cmpd] does not exceed 0.2%.

ALTERNATIVE 2C: Antagonist Dose-Response Assays.

A common method of performing receptor inhibition studies is to prepare a co-mix of a known agonist (at a constant concentration typically between EC₅₀ – EC₈₅) AND a dilution series of the test antagonist compound to be evaluated. This LXR α Reporter Assay System kit includes a 10 mM stock solution of T0901317, a potent agonist of LXR α (Figure 1) that may be used effectively to setup receptor inhibition studies.

See *NOTE 2.0*, above.

Supplement a portion of **CSM-1** with an appropriate volume of T0901317 to generate a sufficient stock volume of [CSM+2x T0901317]. Use this [CSM+2x T0901317] stock to then prepare a 2x-concentrated dilution series of each test compound to be evaluated. If desired, also prepare a dilution series of 2x-concentrated positive-control antagonist (supplied by the user).

NOTE 2.5 667 nM T0901317 typically corresponds to ~EC₇₅ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used in setting up inhibition studies. Refer to *NOTE 2.2* for a suggested dilution scheme to prepare [CSM + 2x T0901317].

NOTE 2.6 When generating antagonist dose-response curves, it is recommended to perform all measurements in triplicate. In Step 6, 100 μ l of [CSM + 2x T0901317 + 2x test cmpd] will be added per well of the assay plate. Therefore, devise an appropriate compound dilution scheme to yield a final volume of [CSM + 2x T0901317 + 2x test cmpd] that is slightly greater than 300 μ l, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate. If test compound stocks require extreme dilution to achieve the desired 2x-concentrations, use the original solvent to make one or two preliminary dilutions, followed by final dilutions in [CSM + 2x T0901317 + 2x test cmpd]. If an organic solvent is the diluent used for test compounds, devise an appropriate dilution scheme so that the concentration of solvent in [CSM + 2x T0901317 + 2x test cmpd] does not exceed 0.2%.

ALTERNATE 2D: Screening for Antagonist Activities via Single-Point Assay.

A common method of performing receptor inhibition studies is to prepare a co-mix of a known agonist (at a concentration typically between EC_{50} – EC_{85}) AND a test concentration of the candidate antagonist compounds to be evaluated. This LXR α Reporter Assay System kit includes a 10 mM stock solution of T0901317, a potent agonist of LXR α (Figure 1) that may be used effectively to setup receptor inhibition studies.

See *NOTE 2.5*.

Supplement a portion of **CSM-1** with an appropriate volume of T0901317 to generate an appropriate stock volume of [CSM+2x T0901317]. Refer to *NOTE 2.1* for a recommended dilution scheme to prepare [CSM + 2x T0901317]. Use this stock of [CSM+2x T0901317] to then prepare a 2x-concentrated dilution of each test sample to be screened. If desired, also prepare an appropriate dilution of 2x-concentrated positive-control antagonist (supplied by the user).

NOTE 2.7 In Step 6, 100 μ l of the prepared [CSM + 2x T0901317 + 2x test compd] will be added per well of the assay plate. If single-point assays are to be made, prepare a volume of each test compound media that is slightly greater than 100 μ l, thus allowing accurate volume transfers into respective assay wells. This assay kit provides 35 ml of CSM. Therefore, plan dilution schemes carefully so that the total volume of [CSM + 2x T0901317 + 2x test compd] required to complete dilutions of the antagonist reference AND all test compounds does not exceed 35 ml. If test compound stocks require extreme dilution to achieve the desired 2x-concentrations, use the original solvent to make one or two preliminary dilutions, followed by final dilutions in [CSM + 2x T0901317 + 2x test compd]. If an organic solvent is the diluent used for test compounds, devise an appropriate dilution scheme so that the concentration of solvent in [CSM + 2x T0901317 + 2x test compd] does not exceed 0.2%.

- 3) Confirm that **CRM-1** has equilibrated to 37°C. Then, to perform 32 LXR α assay reactions, remove 1 tube of LXR α **Reporter Cells** from -80°C storage. Perform a *rapid thaw* of the frozen cells by transferring 3.0 ml of pre-warmed CRM-1 into the tube of frozen cells. Cap the tube and immediately place it in a 37°C water bath for an additional 2-3 minutes to ensure that the entire cell suspension rapidly equilibrates to 37°C.

NOTE: At this time, work in an aseptic hood to *carefully* mount four of the sterile 8-well strips (32 reactions) into the blank plate frame. Note that the strip-wells have *keyed ends* (square and round), hence, they will fit into the frame in only one orientation.

- 4) Invert the tube of reporter cells 6 times to disperse aggregates and achieve a homogenous cell suspension. Sanitize the outside surface of the tube with a 70% alcohol wipe, then transfer the tube of reporter cells into a cell culture hood.

- 5) Dispense 100 μ l of cell suspension into each of the 32 wells mounted in the **Plate Frame**.

NOTE: Take care to prevent cells from settling during the dispensing period. Lack of precision in transferring uniform volumes across the assay plate, and/or allowing cells to settle during the dispensing process, will cause well-to-well variation in the assay. For improved speed, precision, and ergonomic comfort, the use of an electronic repeat-dispensing pipette is recommended.

- 6) Add 100 μ l of 2x-concentrated treatment media (as prepared in Step 2) to appropriate wells of the assay plate.

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

- 8) For convenience on Day 2, retrieve **Luc-Detection Reagent (LDR)** from -80°C storage and place it in a dark refrigerator (+4°C) to allow it to thaw overnight.

(Continue on DAY 2)

DAY 2: Subsequent manipulations do not require special regard for aseptic technique.

- 9) 30 – 60 minutes before intending to quantify LXR α activity, remove **LDR** from the refrigerator and place it in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to achieve a homogenous solution free of flocculent material.

NOTE: Do not actively warm LDR above room temperature. Mix gently to avoid foaming.

- 10) Following 22 - 24 hours of incubation, retrieve the assay plate from the incubator. Remove the plate's lid. Remove and discard media from each well.

NOTE: This is most efficiently performed by tilting the plate on edge and using a pipette tip affixed to an aspiration apparatus. Because these plates are composed of a frame with snap-in strip wells, the practice of physically ejecting media *via* a sweeping downward movement is NOT advised.

- 11) Add 100 μ l of the *room temperature* LDR to each well of the assay plate. Pipette carefully to avoid bubble formation. Replace the plate lid.

- 12) Allow plates to incubate at room temperature for 5-10 minutes. Affix the plate to a micro-plate platform shaker or vibrator for the duration of the incubation period, thereby ensuring the formation of a complete and homogenous lysate.

- 13) Program the plate-reading luminometer. Injectors and read-delays should be turned off. Modern plate-reading luminometers are capable of efficiently integrating luciferase light emission over a read-time of 1 second per well, *or less*. (Consult the specific recommendations of the instrument manufacturer.) If a platform shaker/vibrator was NOT used in Step 12, program the luminometer to incorporate a single 5 second "plate shake" to ensure an homogenous lysate prior to commencing plate reading.

- 14) Read the assay plate anytime between 5-60 minutes after adding LDR.

VI. Related Products

LXRα Family of Assay Products	
<i>Product No.</i>	<i>Product Descriptions</i>
IB00311-32	Human LXR α Reporter Assay System 3x 32 assays in 96-well format
IB00311	Human LXR α Reporter Assay System 1x 96-well format assay
IB00311B	Human LXR α Reporter Assay System (Bulk Pac) 960 Assay Bulk Reagent Pack for 10x 96-well plates
IB00312	Human LXR α Reporter Assay System 1x 384-well format assays
IB00312B	Human LXR α Reporter Assay System (Bulk Pac) 3,840 Assay Bulk Reagent Pack for 10x 384-well plates
Alternative volumes of LXR α Assay Bulk Reagents can be custom manufactured. Please Inquire.	

PANEL of LXR Assays	
<i>Product No.</i>	<i>Product Description</i>
IB00321-48	Human LXR α and LXR β Reporter Assay PANEL 48 assays each in 1x 96-well plate

Please refer to INDIGO Biosciences website for updated product offerings.
www.indigobiosciences.com

VII. 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 pyralis*.

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

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