Human Retinoic Acid Receptor Beta
(NR1B2, RARB, RARβ)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # IB02101-32

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Technical Manual
(version 7.1)

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Human RARβ Reporter Assay System
3x 32 Assays in 96-well Format

I. Description

▪ The Assay System ▪

This nuclear receptor assay system utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the **Human Retinoic Acid Receptor, Beta** (**NR1B2**)**,** a ligand-dependent transcription factor commonly referred to as RARB or **RARβ**.

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a RARβ-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in RARβ 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 human RARβ.

RARβ 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 RARβ 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.

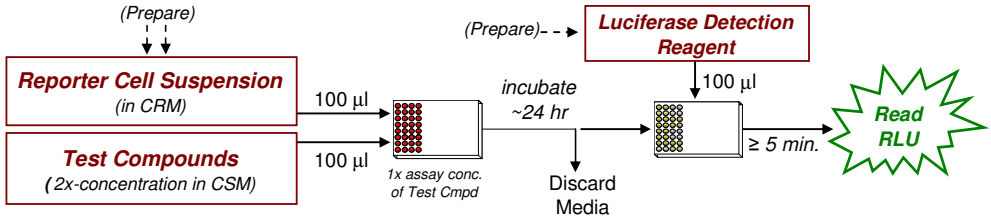
▪ Preparation of Test Compounds ▪

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 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.

▪ 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 ▪

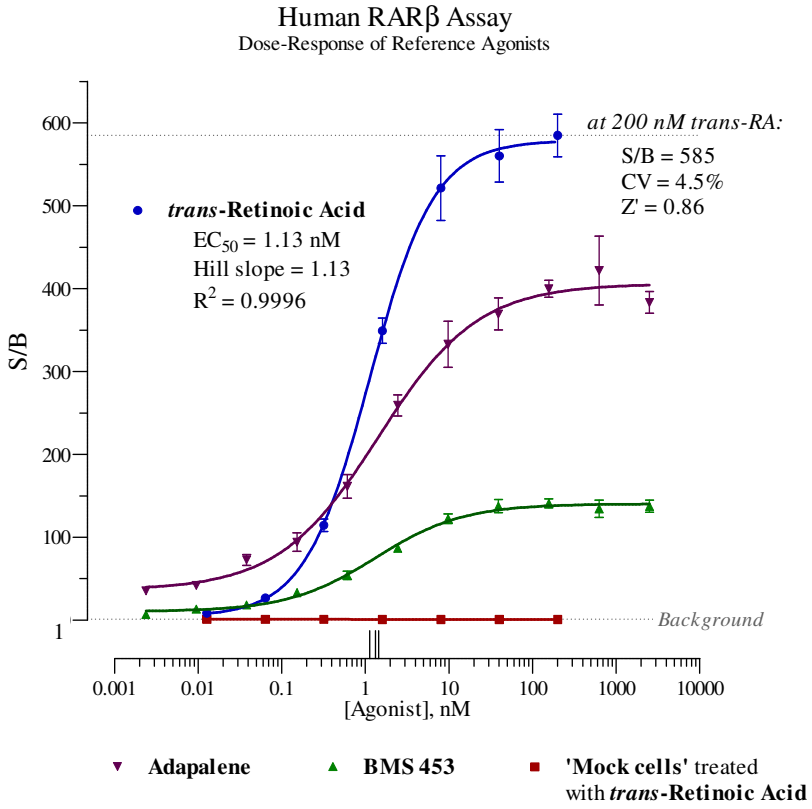


Figure 2a. Agonist dose-response analyses of the RARβ Assay.

Validation of the RARβ Assay was performed using manual dispensing and following the protocol described in this Technical Manual, using the reference agonists all-*trans*-Retinoic Acid (provided), Adapalene (Tocris), and BMS 453 (Tocris). In addition, to assess the level of background signal contributed by non-specific factor(s) that may cause activation of the luciferase reporter gene, "mock" reporter cells were specially prepared to contain only the luciferase reporter vector (mock reporter cells are not provided with assay kits). RARβ Reporter Cells and Mock reporter cells were identically treated with *trans*-retinoic acid, as described in Appendix 1. Luminescence was quantified using a GloMax-Multi+ plate-reading luminometer (Promega Corp.). Average relative light units (RLU) and respective standard deviation (SD) and Signal-to-Background (S/B) values were determined for each treatment concentration ($n \geq 6$). Z' values were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression analyses were performed and EC_{50} values determined using GraphPad Prism software. Mock reporter cells treated with *trans*- retinoic acid demonstrate no significant background luminescence ($\leq 0.05\%$ that of the reporter cells at EC_{Max}). Thus, luminescence results strictly through ligand-activation of the human RARβ expressed in these reporter cells. These data confirm the robust performance of this RARβ 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.

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

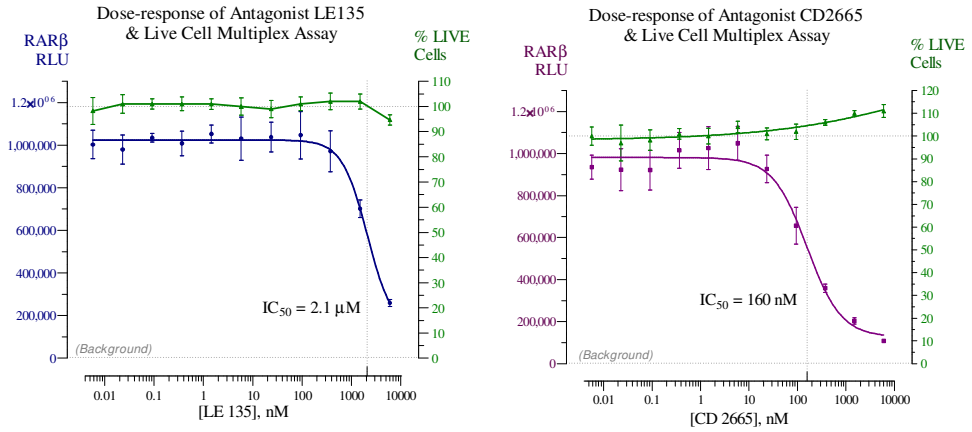


Figure 2b. Validation of RARβ *antagonist* dose-responses performed in combination with INDIGO's Live Cell Multiplex Assay.

RARβ antagonist assays were performed using LE 135 (Tocris), and CD 2665 (Tocris). Assay setup and quantification of RARβ activity were performed following the protocol described in this Technical Manual. To confirm that the observed drop in RLU values resulted from receptor inhibition, as opposed to induced cell death, the relative numbers of live cells in each assay well were determined using INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01).

Final assay concentrations of the respective antagonists ranged between 6 µM and 5.7 pM, including a 'no antagonist' control ($n \geq 6$ per treatment; highest [DMSO] ≤ 0.15% *f.c.*). Each treatment also contained 3 nM (approximating EC_{50}) *trans*-Retinoic Acid as challenge agonist. Assay plates were incubated for 24 hrs, then processed according to the LCM Assay protocol to quantify relative numbers of live cells per treatment condition. Plates were then further processed to quantify RARβ activity for each treatment condition.

Results: LE 135 and CD 2665 both caused dose-dependent reduction in RLU values. The LCM Assay reveals no significant variance in the numbers of live cells per assay well, up to the maximum treatment concentration of 6 µM. Hence, the observed reduction in RLU values can be attributed to dose-dependent inhibition of RARβ activity, and *not* to cell death.

II. Product Components & Storage Conditions

This Human RARβ Reporter Assay System contains materials to perform three distinct groups of assays in a 96-well plate format. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays.

The individual aliquots of Reporter Cells are provided as single-use reagents. 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.

Kit Components	***Amount***	***Storage Temp.***
▪ RARβ Reporter Cells	3 x 0.60 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ *trans*-Retinoic Acid, 10 mM (in DMSO) (reference agonist for RARβ)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, cell-culture ready)	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 (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 EC_{50} – EC_{85}) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This RARβ Reporter Assay System kit includes a 10 mM stock solution of ***trans*-Retinoic Acid**, an agonist of RARβ that may be used to setup antagonist-mode assays. 3 nM *trans*-Retinoic Acid typically approximates EC_{75} in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory 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. 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 used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of 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 (first see *Note 5.3*): 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 RARβ Reporter Assay System kit includes a 10 mM stock solution of ***trans*-Retinoic Acid**, a reference agonist of RARβ. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 200, 40.0, 8.00, 1.60, 0.320, 0.0640, 0.0128 nM, 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 with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, and 3 tubes for 96 assay wells. W*ithout delay*, Perform a rapid thaw of the frozen cells by transferring a 3.0 ml volume of 37°C CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. If only one tube of reporter cells is thawed (32 assays), the resulting volume of cell suspension will be 3.6 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. Sanitize the outside surface of the tube with a 70% alcohol swab, then transfer it into the cell culture hood.

5.) ***a. Agonist*-mode assays**. Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl 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 an homogenous cell suspension. Supplement the 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 each well 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, and/or lack of precision in dispensing uniform volumes across the assay plate *will* cause well-to-well variation (= increased Standard Deviation) in the assay.

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 (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM. Incubated overnight in identical manner to those reporter cells contained in 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 up to 3 hours before proceeding to *Step 6*.

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

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 **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 RARβ activity, remove **Detection Substrate** from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure an homogenous solution.

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*: To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 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

RARβ Assay Products	
Product No.	***Product Descriptions***
IB02101-32	Human RARβ Reporter Assay System 3x 32 assays in 96-well format
IB02101	Human RARβ Reporter Assay System 1x 96-well format assay
IB02102	Human RARβ 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.

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VI. Limited Use Disclosures

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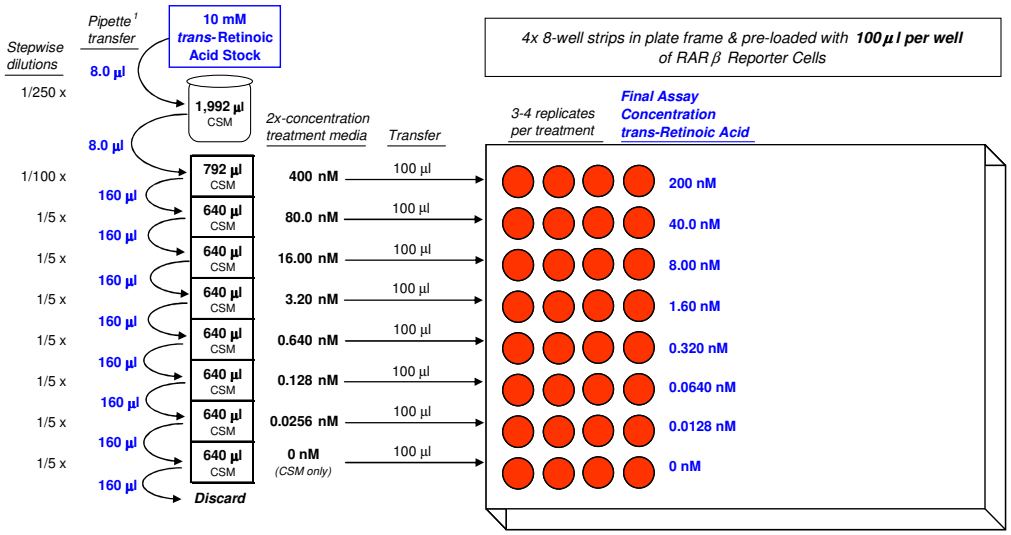
"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc.

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

APPENDIX 1

Example scheme for the serial dilution of *trans*-Retinoic Acid reference agonist, and the setup of an RARβ dose-response assay.



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