Human Retinoid X Receptor Gamma
(NR2B3, RXRG, RXRγ)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # IB00821-32

Technical Manual
(version 7.1b)

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Human RXRγ Reporter Assay System
3x32 Assays in 96-well Format

I. Description
- The Assay System
- The Assay Chemistry
- Preparation of Test Compounds
- Assay Scheme
- Assay Performance

II. Product Components & Storage Conditions

III. Materials to be Supplied by the User

IV. Assay Protocol
- A word about Antagonist-mode assay setup
  - DAY 1 Assay Protocol
  - DAY 2 Assay Protocol

V. Related Products

VI. Limited Use Disclosures

APPENDIX 1: Example Scheme for Serial Dilution
I. Description

- The Assay System -
This nuclear receptor assay utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the Human Retinoid X Receptor Gamma (NR2B3), a ligand-dependent transcription factor commonly referred to as RXRG or RXRγ.

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

RXRγ 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 Assays are all-inclusive cell-based assay systems. In addition to RXRγ 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 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 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**

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. **NOTE:** The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

Immediately prior to setting up the assay plate(s) master stocks are serially diluted using **Compound Screening Medium (CSM)**; as described in *Step 2 of the Assay Protocol* to generate 2x-concentrated treatment media.

**NOTE:** CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of hydrophobic test compounds diluted in CSM will lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is advised 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 2. Agonist and Antagonist dose-response analyses of the Human RXRγ

A.) Analyses of RXRγ Reporter Cells using the reference agonists 9-cis-Retinoic Acid (provided). B.) Analyses of RXRβ antagonist dose-responses using HX531 and UVI3003 (Tocris). Assay setups and quantification of RXRγ activity were performed following the protocol provided in this Technical Manual. Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 4). Values of Fold-activation and Z’ were calculated as described by Zhang, et al. (1999)1. Non-linear regression and EC50 analyses were performed using GraphPad Prism software. High S/B and Z’ scores confirm the robust performance of this RXRγ Assay.


\[ Z' = 1 - \frac{3 \times (SD_{\text{Control}} + SD_{\text{Background}})}{(RLU_{\text{Control}} - RLU_{\text{Background}})} \]
II. Product Components & Storage Conditions

This Human RXRγ Assay kit 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.

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RXRγ Reporter Cells</td>
<td>3 x 0.60 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Cell Recovery Medium (CRM)</td>
<td>1 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>9-cis-Retinoic Acid, 10 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>(reference agonist for RXRγ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detection Substrate</td>
<td>3 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Detection Buffer</td>
<td>3 x 2.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Plate frame</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>Snap-in, 8-well strips</td>
<td>12</td>
<td>ambient</td>
</tr>
<tr>
<td>(white, sterile, cell-culture ready)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 sterilized 96 deep-well blocks (*e.g.*, Axygen Scientific, #P-2ML-SQ-C-S), or appropriate similar vessel for generating dilution series of reference 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<sub>50</sub> ~ EC<sub>85</sub>) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This RXRγ Assay kit includes a 10 mM stock solution of 9-cis-Retinoic Acid, an agonist of RXRγ that may be used to setup antagonist-mode assays. 88 nM 9-cis-Retinoic Acid typically approximates EC<sub>70-80</sub> in this cell-based assay. Hence, it presents a suitable assay 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 (~180 nM) of the reference agonist 9-cis RA. 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 5.4): 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 RXRβ Assay kit includes a 10 mM stock solution of 9-cis-Retinoic Acid, a reference agonist of RXRβ. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 2500, 500, 100, 20.0, 4.00, 0.800, and 0.160 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 and immerse them in dry ice for transport to the laminar-flow hood: retrieve 1 tube for 32 assay wells, 2 tubes for 64 assay wells, and 3 tubes for 96 assay wells. When ready, transfer the tube(s) of frozen cells into a rack and, without 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 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, during the 5 - 10 minutes incubation period, 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(s) with a 70% alcohol swab, then transfer it into the cell culture hood. If more than one tube cells were thawed, pool the individual tubes into a common reservoir.

5.) a. Agonist-mode assays. Gently invert the tube of Reporter Cells several times to gain a 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 a 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. 7). Dispense 100 µl of cell suspension into each well of the assay plate.

**NOTE 5.1:** If INDIGO’s Live Cell Multiplex Assay is to be incorporated, a minimum of 3 ‘cell blank’ wells (meaning cell-free but containing ‘Compound Screening Media’) must be included in the assay plate to allow quantification of plate-specific fluorescence background (refer to the LCMA Technical Manual).

**NOTE 5.2:** 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 5.3:** 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 5.4:** 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 cell culture incubator (37°C, humidified 5% CO₂) for 22 - 24 hours.

**NOTE:** Ensure a high-humidity (≥70%) environment within the cell culture incubator. This is critical to mitigate the onset of deleterious "edge-effects" in the assay plate.

8.) For greater convenience on Day 2, retrieve the appropriate number of vials 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 receptor activity, remove Detection Substrate from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature.

   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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00821-32</td>
<td>Human RXRγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
</tr>
<tr>
<td>IB00821</td>
<td>Human RXRγ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00822</td>
<td>Human RXRγ Reporter Assay System 1x 384-well format assays</td>
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</tbody>
</table>

Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCM-01</td>
<td>Reagents to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform 480 Live Cell Assays performed in 5x 96-well plates</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays performed in 10x 96-well plates</td>
</tr>
</tbody>
</table>

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, diagnostic or contact use in humans or animals.

“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.

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APPENDIX 1

Example scheme for the serial dilution of 9-cis-Retinoic Acid reference agonist, and the setup of an RXRγ 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.
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(NR2B3, RXRG, RXRγ)
Reporter Assay System

96-well Format Assays
Product # IB00821

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I. Description
- The Assay System .......................................................... 3
- The Assay Chemistry ...................................................... 3
- Preparation of Test Compounds ....................................... 4
- Considerations for Automated Dispensing ......................... 4
- Assay Scheme .................................................................. 4
- Assay Performance .......................................................... 5

II. Product Components & Storage Conditions ....................... 6

III. Materials to be Supplied by the User ............................... 7

IV. Assay Protocol
- A word about Antagonist-mode assay setup ....................... 8
  - DAY 1 Assay Protocol .................................................... 8
  - DAY 2 Assay Protocol .................................................... 9

V. Related Products ............................................................ 10

VI. Limited Use Disclosures ............................................... 10

APPENDIX 1: Example Scheme for Serial Dilution ................. 11
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  INDIGO’s Reporter Cells include the luciferase reporter gene functionally linked to a RXRγ-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in RXRγ 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 human RXRγ.

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**Preparation of Test Compounds**

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. NOTE: The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

Immediately prior to setting up the assay plate(s) master stocks are serially diluted using **Compound Screening Medium (CSM)**, as described in Step 2 of the Assay Protocol to generate 2x-concentrated treatment media.

NOTE: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of hydrophobic test compounds diluted in CSM will lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is advised 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 consider 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.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (96-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reporter Cell Suspension 12 ml</td>
<td>100 µl / well 9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td>(prepared from kit components)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDR 12 ml (prepared from kit components)</td>
<td>100 µl / well 9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
</tbody>
</table>

**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 Scheme Diagram]
**Assay Performance**

![Assay Performance Diagram]

**Figure 2. Agonist and Antagonist dose-response analyses of the Human RXRγ.**

A.) Analyses of RXRγ Reporter Cells using the reference agonists 9-cis-Retinoic Acid (provided). B.) Analyses of RXRγ antagonist dose-responses using HX531 and UVI3003 (Tocris). Assay setups and quantification of RXRγ activities were performed following the protocol provided in this Technical Manual. Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 4). Values of Fold-activation and Z’ were calculated as described by Zhang, et al. (1999)¹. Non-linear regression and EC₅₀ analyses were performed using GraphPad Prism software. High S/B and Z’ scores confirm the robust performance of this RXRγ Assay.


\[ Z’ = 1 - \frac{\text{SD}^{\text{Control}} + \text{SD}^{\text{Background}}}{\text{RLU}^{\text{Control}} - \text{RLU}^{\text{Background}}} \]

**II. Product Components & Storage Conditions**

This Human RXRγ Assay kit contains materials to perform assays in a single 96-well assay plate.

The aliquot of RXRγ Reporter Cells is provided as a single-use reagent. Once thawed, 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.

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</tr>
<tr>
<td>Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>9-cis-Retinoic Acid, 10 mM (in DMSO) (reference agonist for RXRγ)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
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

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 sterilized 96 deep-well blocks (e.g., Axygen Scientific, #P-2ML-SQ-C-S), or appropriate similar vessel for generating dilution series of reference 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<sub>50</sub> – EC<sub>85</sub>) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This RXRγ Assay kit includes a 10 mM stock solution of 9-cis-Retinoic Acid, an agonist of RXRγ that may be used to setup antagonist-mode assays. 100 nM 9-cis-Retinoic Acid typically approximates EC<sub>70-80</sub> in this cell-based assay. Hence, it is an appropriate assay 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 (~200 nM) of the reference agonist 9-cis RA. 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 5.4): 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 RXRγ Assay kit includes a 10 mM stock solution of 9-cis-Retinoic Acid, a reference agonist of RXRγ. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 2500, 500, 100, 20.0, 4.00, 0.800, and 0.160 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 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, transfer the tubes into a rack and (without delay) 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 gain a 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. 7). Dispense 100 µl of cell suspension into each well of the assay plate.

**NOTE 5.1:** If INDIGO’s Live Cell Multiplex Assay is to be incorporated, a minimum of 3 ‘cell blank’ wells (meaning cell-free but containing ‘Compound Screening Media’) must be included in the assay plate to allow quantification of plate-specific fluorescence background (refer to the LCMA Technical Manual).

**NOTE 5.2:** 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 5.3:** 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 5.4:** 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.

**NOTE 5.5:** 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 µl of sterile water into each of the seven inter-well spaces per column of wells.

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

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

**NOTE:** Ensure a high-humidity (≥70%) 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.
9.) 30 minutes before intending to quantify receptor 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.

    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 22 - 24 hours of incubation discard all media contents by ejecting it into an appropriate waste container. Gently tamp 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 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.
## Related Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00821-32</td>
<td>Human RXRγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
</tr>
<tr>
<td>IB00821</td>
<td>Human RXRγ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00822</td>
<td>Human RXRγ Reporter Assay System 1x 384-well format assays</td>
</tr>
</tbody>
</table>

Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

### LIVE Cell Multiplex (LCM) Assay

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCM-01</td>
<td>Reagents to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform 480 Live Cell Assays performed in 5x 96-well plates</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays performed in 10x 96-well plates</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.

[www.indigobiosciences.com](http://www.indigobiosciences.com)

## Limited Use Disclosures

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### APPENDIX 1

Example scheme for the serial dilution of 9-cis-Retinoic Acid reference agonist, and the setup of an RXRγ dose-response assay.

<table>
<thead>
<tr>
<th>Stepwise dilutions</th>
<th>2x conc.</th>
<th>Transfer</th>
<th>2.500 nM (0.025% DMSO)</th>
<th>1/20 x</th>
<th>1/100 x</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 nM Stock 9-cis RA</td>
<td>100 µl</td>
<td>100 µl</td>
<td>500 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>20.0 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>4.00 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>200 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>40.0 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>80.0 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>160.0 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>1.60 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>0.800 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>0.160 nM CSM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
<td>0.00 nM</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Discard 990 µl CSM

100 µl 100 µl 100 µl 100 µl 100 µl 100 µl 100 µl 100 µl 100 µl 100 µl 100 µl 100 µl

96-Well Assay Plate pre-loaded with 100µl per well of RXRγ Reporter Cells
Human Retinoid X Receptor Gamma
(NR2B3, RXRG, RXRγ)
Reporter Assay System

384-well Format Assays
Product # IB00822

Technical Manual
(version 8.0b)

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techserv@indigobiosciences.com
Human RXRγ Reporter Assay System
384-well Format Assays

I. Description
• The Assay System .......................................................... 3
• The Assay Chemistry ......................................................... 3
• Considerations for the Preparation and Automated Dispensing of Test Compounds ........................................... 4
• Considerations for Automated Dispensing of Other Assay Reagents ..... 4
• Assay Scheme ............................................................... 5
• Assay Performance ......................................................... 5

II. Product Components & Storage Conditions ......................... 6

III. Materials to be Supplied by the User ................................... 6

IV. Assay Protocol
• A word about Antagonist-mode assay setup ....................... 7
  • DAY 1 Assay Protocol .................................................. 7
  • DAY 2 Assay Protocol .................................................. 9

V. Related Products .................................................................. 10

VI. Limited Use Disclosures ..................................................... 10

APPENDIX 1a: Example Scheme for Serial Dilution when using tip-based dispensing of test compounds ........................................... 11

APPENDIX 1b: Example Scheme for Serial Dilutions when using acoustic dispensing of test compounds ................................... 12
I. Description

- The Assay System -
This nuclear receptor assay utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the **Human Retinoid X Receptor Gamma** (NR2B3), a ligand-dependent transcription factor commonly referred to as RXRG or RXRγ.

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

RXRγ 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 Assays are all-inclusive cell-based assay systems. In addition to RXRγ 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 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 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 Assays feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction. Incorporating a 30-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.
Considerations for the Preparation and Automated Dispensing of Test compounds

Small molecule compounds are typically solvated at high concentration (ideally 1,000x-concentrated) in DMSO and stored frozen as master stocks. For 384-well format assays these master stocks will be diluted by one of two alternative methods, the selection of which will be dictated by the type of dispensing instrument that is to be used. This Technical Manual provides detailed protocols for each of these two alternative methods:

a.) Assay setups in which a conventional tip-based instrument is used to dispense test compounds into assay wells (in black text). Use Compound Screening Medium (CSM) to generate a series of 2x-concentration test compound treatment media, as described in Step 2a of the Assay Protocol. The final concentration of DMSO carried over into assay reactions should not exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO.

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.

and,

b.) Assay setups in which an acoustic transfer device is used to dispense test compounds into assay wells (text highlighted in blue). Use DMSO to make a series of 1,000x-concentrated test compound stocks that correspond to each desired final assay concentrations, as described in Step 2b of the Assay Protocol.

Considerations for Automated Dispensing of Other Assay Reagents

When dispensing into a small number of assay plates, first carefully consider the dead volume requirement of your tip-based 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 on a per kit basis. Always pool the individual reporter cell suspensions and all other respective assay kit reagents before processing multiple 384-well assay plates.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (384-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>when using tip dispensing of test cmpds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reporter Cell Suspension 7.5 ml</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 1.7 ml</td>
</tr>
<tr>
<td><strong>when using acoustic dispensing of test cmpds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reporter Cell Suspension 15 ml</td>
<td>30 µl / well 11.5 ml / plate</td>
<td>~ 3.4 ml</td>
</tr>
<tr>
<td>Detection Substrate 7.8 ml</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 2 ml</td>
</tr>
</tbody>
</table>
**Assay Scheme**

The Day 1 preparation, volumes, and chronology of dispensed cells and test compounds are different between assay setups using a *tip-based dispenser* (1a) and those using an *acoustic transfer device* (1b). Following 22-24 hr incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

**Figure 1a.** Assay workflow if using conventional *tip-based* dispensing of test compounds.

**Figure 1b.** Assay workflow if using *acoustic* dispensing of test compounds.

**Assay Performance**

**Figure 2.** Dose-response analyses of the Human RXRα assay.

*(A.)* Agonist assays. RXRβ Reporter Cells using 9-cis-Retinoic Acid (provided).

*(B.)* Antagonist assays. Reporter cells were co-treated with 100 nM (EC₅₀,80) of 9-cis-Retinoic Acid and varying concentrations of either UVI3003 or HX531 (Tocris).

Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 4). Z’ values were calculated as described by Zhang, *et al.* (1999)¹. Non-linear regression and EC₅₀ analyses were performed using GraphPad Prism software.

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


\[ Z' = 1 - \frac{3 \times (SD_{Ref} + SD_{Blank})}{(RLU_{Ref} - RLU_{Blank})} \]
**II. Product Components & Storage Conditions**

This assay kit contains materials to perform assays in a single 384-well assay plate.

Cryopreserved mammalian cells are temperature sensitive! To ensure maximal viability the tube of Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in this protocol.

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, cells can NOT be refrozen. Nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• RXRγ Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>1 x 7 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• 9-cis-Retinoic Acid, 10 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>(reference agonist for RXRγ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>1 x 7.8 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• 384-well assay plate</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>(white, sterile, cell-culture ready)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**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 container
• 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 & tips suitable for dispensing 15 µl.
• disposable media basins, sterile.
• sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or sterilized 96 deep-well blocks (e.g., Axygen Scientific, #P-2ML-SQ-C-S), or appropriate similar vessel for generating dilution series of reference and test compound(s).
• antagonist reference compound (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 9-13* 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 varying concentrations of the test compound(s) to be evaluated for antagonist activity. This RXRγ Reporter Assay kit includes a 10 mM stock solution of **9-cis-Retinoic Acid**, an agonist of RXRγ that may be used to setup antagonist-mode assays. 100 nM 9-cis-Retinoic Acid typically approximates EC$_{70-80}$ in this assay. Hence, it presents an appropriate assay 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 protocol when performing tip-based dispensing, and *Step 6b* of the protocol when using an acoustic transfer device to dispense test compounds.

Note that when using a **tip-based instrument** for the dispensing of 2x-concentrated test compounds the cell suspension must also be supplemented with a 2x-concentration of the challenge agonist 9-cis-retinoic acid.

When using an **acoustic transfer** device for the dispensing of 1,000x-concentrated test compounds the cell suspension should be supplemented with a 1x-concentration of the challenge agonist 9-cis-retinoic acid.

---

**DAY 1 Assay Protocol:**

All steps must be performed using proper 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. **NOTE** that test and reference compounds will be prepared differently when using tip-dispensing vs. **acoustic dispensing**. Regardless of the method, the total DMSO carried over into assay reactions should not exceed 0.4%.

   a. **Tip dispensing method:** In *Step 6*, 15 µl / well of the prepared treatment media is added to the assay that has been pre-dispensed with 15 µl /well 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 volumes carefully; this assay kit provides 35 ml of CSM.

   b. **Acoustic dispensing method:** In *Step 6*, 30 nl / well of 1,000x-concentrated test compound solutions (prepared in DMSO) are added to the assay plate using an acoustic transfer device.

**Preparing the positive control:** This RXRγ Assay kit includes a 10 mM stock solution of **9-cis-Retinoic Acid**, a reference agonist of RXRγ. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 2500, 500, 100, 20.0, 4.00, 0.800, and 0.160 nM, and including a ‘no treatment’ control.

**APPENDIX 1a** provides an example for generating such a dilution series to be used when **tip-dispensing** compound solutions prepared in CSM (15 µl / well).

**APPENDIX 1b** provides an example for generating such a series of 1,000x-concentrated solutions of compounds prepared in DMSO to be used when performing **acoustic dispensing** (30 nl / well).
When using **tip-based** instrumentation for dispensing test compounds …

3.) Prepare Reporter Cell suspension. *First*, retrieve the tube of CRM from the 37°C water bath, sanitize the outside 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. Perform a *rapid thaw* of the frozen cells by transferring a 5.5 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 ml.

4.) Retrieve the tube of 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.) Gently invert the tube of cells several times to gain a homogenous cell suspension.

* a. for **Agonist-mode assays**: Dispense **15 µl / well** of cell suspension into the Assay Plate.

   ~ or ~

* b. for **Antagonist-mode assays**: Supplement the bulk volume of Reporter Cells suspension with a 2x-concentration of the challenge agonist (refer to "A word about antagonist-mode assay setup", pg. 7). Dispense **15 µl / well** of cell suspension into the Assay Plate.

6.) Dispense **15 µl / well** of 2x-concentrated treatment media (from Step 2a) into the assay plate.

---

When using an **acoustic transfer device** for dispensing test compounds …

3.) Dispense **30 nl / well** of the 1,000x-concentrated compounds (in DMSO solutions, from Step 2b) into the assay plate.

4.) Prepare Reporter Cell suspension. *First*, retrieve the tube of CRM from the 37°C water bath, sanitize the outside 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. Transfer the tube of cells into a rack and, without delay, perform a *rapid thaw* of the cells by transferring a 5.5 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and place it in a 37°C water bath for 5 - 10 minutes.

5.) Retrieve the tube of cell suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab. Add an additional 7.5 ml of CSM to the tube. The resulting volume of cell suspension will be 15 ml.

6.) Gently invert the tube of cells several times to gain a homogenous cell suspension.

* a. for **Agonist-mode assays**: Dispense **30 µl / well** of cell suspension into the Assay Plate that has been pre-dispensed with test compounds.

   ~ or ~

* b. for **Antagonist-mode assays**: First supplement the bulk volume of Reporter Cells suspension with the challenge agonist **9-cis Retinoic Acid** to achieve an EC₃₀ – EC₈₀ concentration (refer to "A word about antagonist-mode assay setup", pg. 7). Then dispense **30 µl / well** of cell suspension into the Assay Plate that has been pre-dispensed with test compounds.

**NOTE:** 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:** Following the dispensing of Reporter Cells and test compounds INDIGO recommends performing a *low-speed* spin of the assay plate (with lid) for 1 minute using a room temperature centrifuge fitted with counter-balanced plate carriers.
7.) Transfer the assay plate into a cell culture incubator (37°C, humidified, 5% CO₂) for 22 - 24 hours.

   **NOTE:** Ensure a high-humidity (≥ 70%) 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** from freezer storage and place 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.) Approximately 30 minutes before intending to quantify receptor activity remove **Detection Substrate** 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 ensure a homogenous solution.

   **NOTE:** Do NOT actively warm Detection Substrate above room temperature. If this solution was 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.) Following 22 - 24 hours of incubation dispense **15 µl / well** of **Detection Substrate** to the assay plate.

   **NOTE:** Perform this reagent transfer carefully to 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 side walls of the well, will cause lens-effects that will degrade the accuracy and precision of the assay data. INDIGO recommends performing a final low-speed spin of the assay plate (with lid) for 1 minute using a room temperature centrifuge fitted with counter-balanced plate carriers.

12.) Allow the plate(s) to rest at room temperature for 30 minutes. Do not shake the assay plate(s) during this period.

   **NOTE:** the luminescent signal is unstable during the first 30 minutes of the luciferase reaction, however, after the initial 30-minute reaction period the luminescence signal achieves a stable emission output.

13.) Quantify luminescence.
### V. Related Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
</table>
| IB00821-32  | Human RXRγ Reporter Assay System  
3x 32 assays in 96-well format |
| IB00821     | Human RXRγ Reporter Assay System  
1x 96-well format assay |
| IB00822     | Human RXRγ 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.

Please refer to INDIGO Biosciences website for updated product offerings.  
[www.indigobiosciences.com](http://www.indigobiosciences.com)

### 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.

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

Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most current version available.

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APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference agonist 9-cis Retinoic Acid into CSM to generate 2x-concentrated treatment media. 15 µl / well are dispensed into assay plates using a tip-based instrument.

<table>
<thead>
<tr>
<th>Final Assay Concentration 9-cis RA</th>
<th>4-6 replicates per treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,500 nM</td>
<td></td>
</tr>
<tr>
<td>500 nM</td>
<td></td>
</tr>
<tr>
<td>100 nM</td>
<td></td>
</tr>
<tr>
<td>50 nM</td>
<td></td>
</tr>
<tr>
<td>20 nM</td>
<td></td>
</tr>
<tr>
<td>10 nM</td>
<td></td>
</tr>
<tr>
<td>5 nM</td>
<td></td>
</tr>
<tr>
<td>2 nM</td>
<td></td>
</tr>
<tr>
<td>1 nM</td>
<td></td>
</tr>
<tr>
<td>0.5 nM</td>
<td></td>
</tr>
<tr>
<td>0.25 nM</td>
<td></td>
</tr>
<tr>
<td>0.125 nM</td>
<td></td>
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<tr>
<td>0.0625 nM</td>
<td></td>
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<tr>
<td>0.03125 nM</td>
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</table>

Tip-based dispensing into a 384-Well Assay Plate pre-loaded with 15 µl per well of Human RXR γ Reporter Cells preparation of 2x-concentrated stocks in CSM. 10 mM Stock, 9-cis Retinoic Acid Final Assay Concentration 9-cis RA 4-6 replicates per treatment Transfer 2x conc.

<table>
<thead>
<tr>
<th>Stepwise dilutions</th>
<th>1/20 x</th>
<th>1/10 x</th>
<th>1/5 x</th>
<th>1/2 x</th>
<th>1 x</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990 µl CSM</td>
<td>950 µl CSM</td>
<td>800 µl CSM</td>
<td>800 µl CSM</td>
<td>800 µl CSM</td>
<td>800 µl CSM</td>
</tr>
<tr>
<td>990 µl CSM</td>
<td>475 µl CSM</td>
<td>390 µl CSM</td>
<td>390 µl CSM</td>
<td>390 µl CSM</td>
<td>390 µl CSM</td>
</tr>
<tr>
<td>495 µl CSM</td>
<td>247.5 µl CSM</td>
<td>207.5 µl CSM</td>
<td>207.5 µl CSM</td>
<td>207.5 µl CSM</td>
<td>207.5 µl CSM</td>
</tr>
<tr>
<td>247.5 µl CSM</td>
<td>123.75 µl CSM</td>
<td>103.75 µl CSM</td>
<td>103.75 µl CSM</td>
<td>103.75 µl CSM</td>
<td>103.75 µl CSM</td>
</tr>
<tr>
<td>123.75 µl CSM</td>
<td>61.875 µl CSM</td>
<td>51.5625 µl CSM</td>
<td>51.5625 µl CSM</td>
<td>51.5625 µl CSM</td>
<td>51.5625 µl CSM</td>
</tr>
</tbody>
</table>
APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the reference agonist 9-cis Retinoic Acid into DMSO to generate 1,000x-concentrated stocks. 30 nl / well are pre-dispensed into assay plates using an acoustic transfer device.