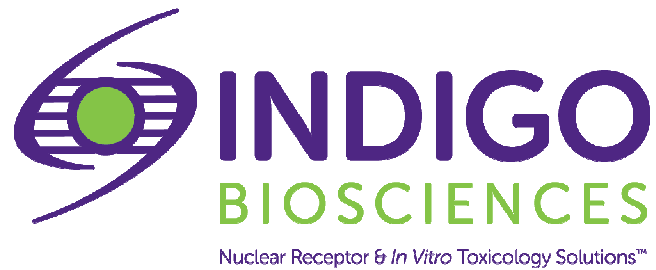

Human Retinoic Acid Receptor Reporter Assays

PANEL

RARα, RARβ, RARγ

32 Assays each in 96-well Format
Product #IB02301-32P

▪

Technical Manual
(version 7.1)

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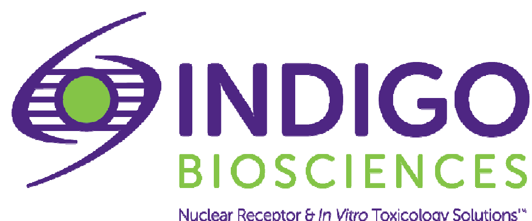

Human RAR Reporter Assays PANEL
RARα, RARβ, RARγ
32 Assays each in 96-well Format

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I. Description

▪ The Assay System ▪

INDIGO's **PANEL** of **RAR Assays** utilizes non-human mammalian cells engineered to individually express **Human Retinoic Acid Receptors, RARα** (NR1B1), **RARβ** (NR1B2), or **RARγ** (NR1B3).

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

RAR 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 RAR 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 assay kits 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.

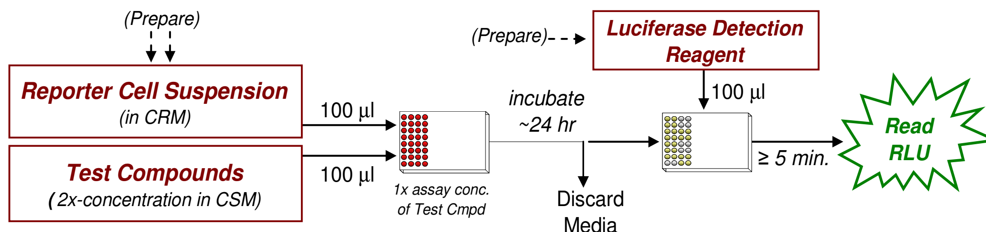
▪ Preparation of Test Compounds ▪

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*, RAR Reporter Cells are dispensed into 32 wells of the assay plate and then immediately dosed with the user's test compounds. Following 22 -24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each sample well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪

Figures 2, **3**, and **4** present agonist and antagonist performance data for the RARα, RARβ, and RARγ assays. All assays were performed using manual dispensing and following the protocol described in this Technical Manual. To assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, "mock" reporter cells, which contain only the luciferase vector, were treated with agonist, as noted in respective figures (mock reporter cells are not provided with assay kits). For each assay, luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ($n \geq 6$). Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software.

[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.

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

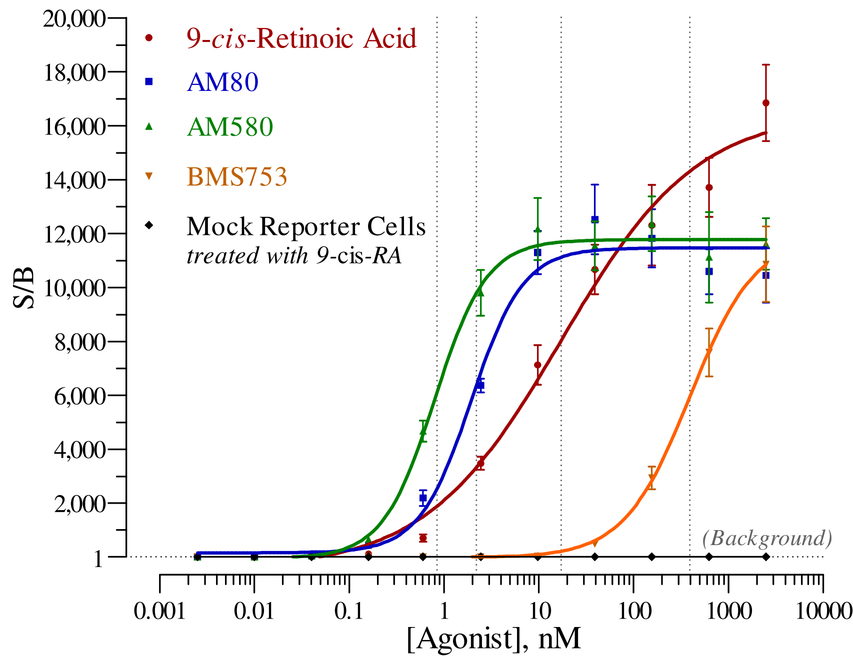


Figure 2a. Agonist dose-response analyses of the RARα Assay.
Validation of the RARα Assay was performed using reference agonists 9-*cis*-Retinoic Acid (9-*cis*-RA; provided), AM80 (Tocris), AM580 (Tocris) and BMS753 (Tocris). Final assay concentrations of agonist treatment media ranged between 2.5 pM and 2.5 µM, and included a 'no-treatment' control. APPENDIX 1 describes an abbreviated 7-point dilution scheme that we find suitable to produce a full dose-response curve.

RARα reporter cells treated with 2,500 nM 9-*cis*-RA yielded an average RLU value with CV=8.4%, S/B ~ 16,800, and a corresponding Z'= 0.75. Mock reporter cells treated with 9-*cis*-RA demonstrate no significant background luminescence (≤ 0.006% that of the reporter cells at EC_{Max}). Thus, luminescence results strictly through ligand-dependent activation of the human RARα expressed in these reporter cells.

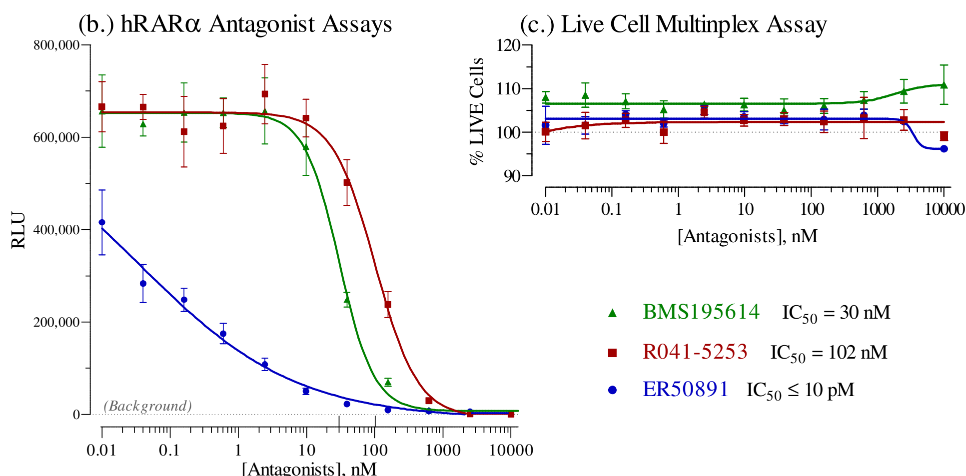


Figure 2b. Validation of RARα *antagonist* dose-responses performed in combination with INDIGO's Live Cell Multiplex Assay.

RARα antagonist assays were performed using BMS195614, R041-5253, and ER50891 (all from Tocris). To confirm that the observed drop in RLU values resulted from receptor inhibition, not induced cell death, the relative numbers of live cells in each assay well were determined at the end of the treatment period using INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01).

Final assay concentrations of the respective antagonists ranged between 10 µM and 10 pM, including a 'no antagonist' control. Each treatment also contained 15 nM (approximating EC_{50}) 9-*cis*-RA as challenge agonist. Assay plates were incubated for 23 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 RARα activity for each treatment condition.

BMS195614, R041-5253, and ER50891 all 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 10 µM. Hence, the observed reduction in RLU values can be attributed to dose-dependent inhibition of RARα activity, and *not* to induced cell death.

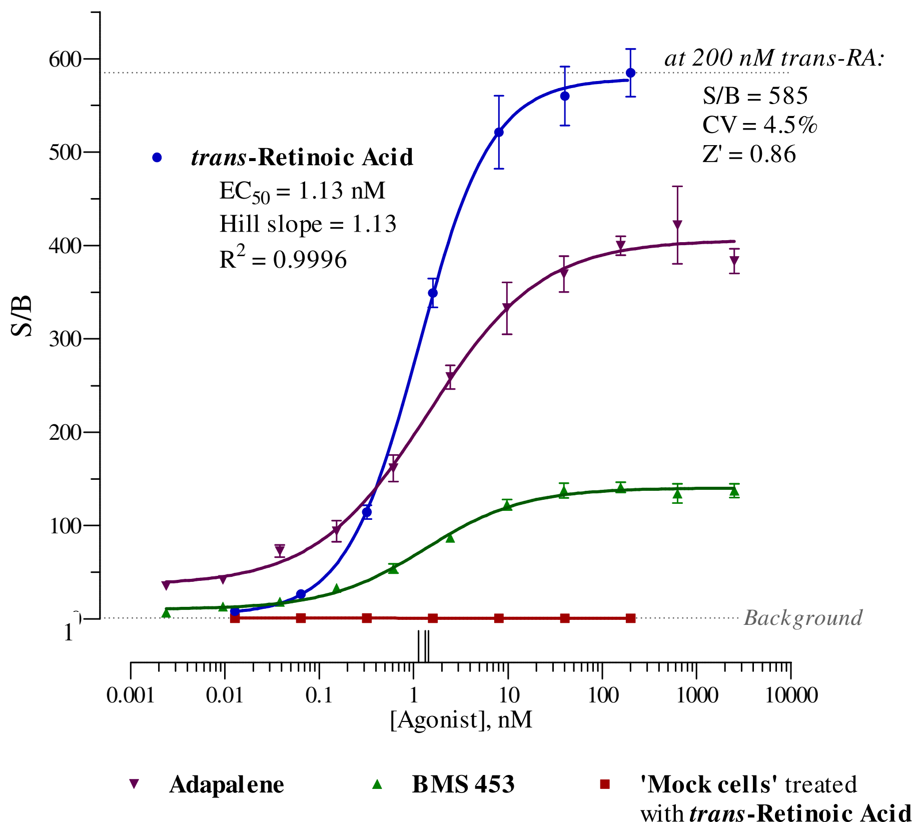


Figure 3a. Agonist dose-response analyses of the RARβ Assay.

Validation of the RARβ Assay was performed using the reference agonists all-*trans*-Retinoic Acid (provided), Adapalene (Tocris), and BMS 453 (Tocris). Final assay concentrations of agonist treatment media variously ranged between 2.4 pM and 2.5 µM, and included a 'no-treatment' control. APPENDIX 2 describes an abbreviated 7-point dilution scheme that we find suitable to produce a full dose-response curve.

Mock reporter cells treated with *trans*- retinoic acid demonstrate no significant background luminescence (≤ 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 assay, and demonstrate its suitability for use in HTS applications.

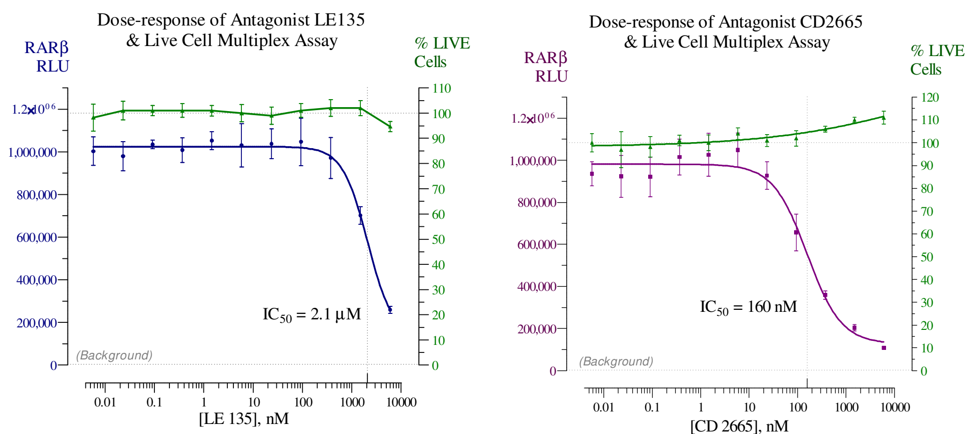
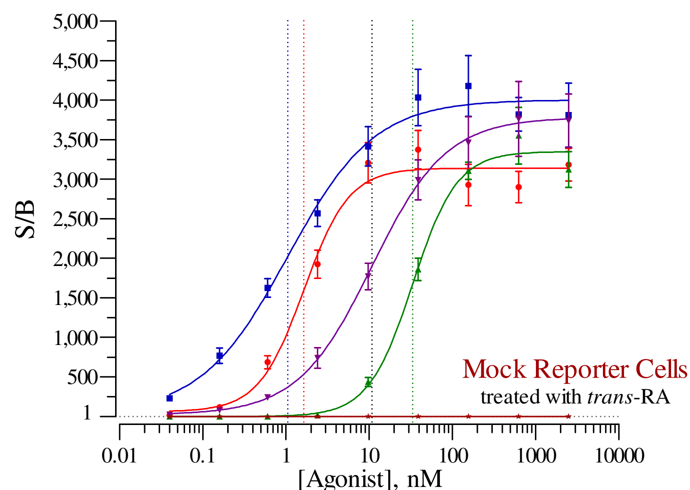


Figure 3b. Validation of RARβ *antagonist* dose-responses performed in combination with INDIGO's Live Cell Multiplex Assay.

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. 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.

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.



▪ Adapalene	• *trans*-Retinoic Acid	▴ BMS961	▾ CD1530
EC_{50} = 1.1 nM	EC_{50} = 1.6 nM	EC_{50} = 33 nM	EC_{50} = 11 nM
Hill slope = 0.84	Hill slope = 1.6	Hill slope = 1.6	Hill slope = 1.0
R^2 = 0.9896	R^2 = 0.9837	R^2 = 0.9945	R^2 = 0.9995
at 625 nM:	*at 625 nM:*	*at 625 nM:*	*at 625 nM:*
S/B = 3,820	S/B = 2,900	S/B = 3,550	S/B = 3,760
% CV = 5.6	% CV = 6.9	% CV = 10	% CV = 12%
Z' = 0.83	Z' = 0.80	Z' = 0.70	Z' = 0.62

Figure 4a. Agonist dose-response of the RARγ Assay.

Validation of the RARγ Assay was, using the reference agonists all-*trans*-Retinoic Acid (provided), Adapalene, BMS 961, and CD1530 (all from Tocris). Final assay concentrations of agonist treatment media variously ranged between 40 pM and 10 µM, and included a 'no-treatment' control. APPENDIX 2 describes an abbreviated 7-point dilution scheme that we find suitable to produce a full dose-response curve.

Mock reporter cells treated with *trans*-retinoic acid demonstrate no significant background luminescence (≤ 0.05% that of the reporter cells at EC_{Max}). Thus, luminescence results strictly through ligand-activation of the human RARγ expressed in these reporter cells. These data confirm the robust performance of this RARγ assay, and demonstrate its suitability for use in HTS applications.

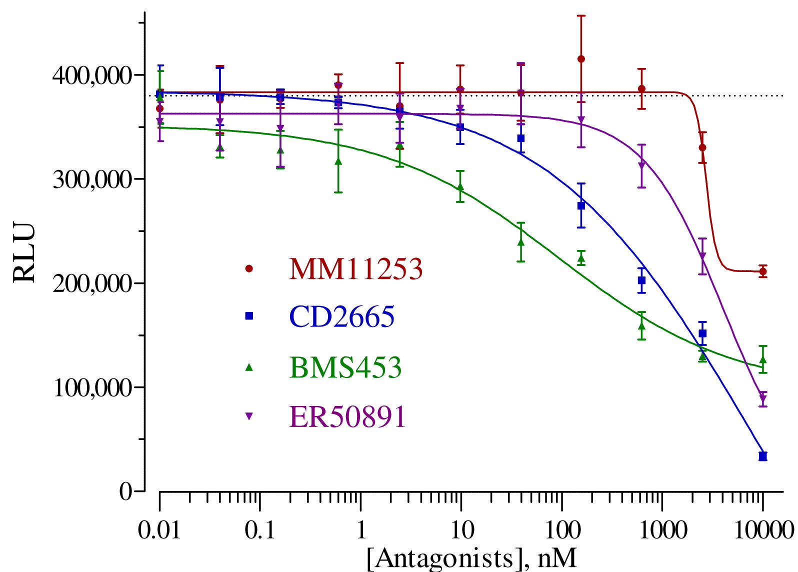


Figure 4b. Validation of RARγ Assay *antagonist* dose-responses.

RARγ antagonist assays were performed using MM11253, CD2665, BMS453 and ER50891 (all from Tocris). Final assay concentrations of the respective antagonists ranged between 10 µM and 10 pM, and included a 'no antagonist' control. Each treatment also contained 3.8 nM (~ EC_{80}) of *trans*-Retinoic Acid. Assay plates were incubated for ~24 hrs, then processed to quantify RARγ activity for each treatment condition.

II. Product Components & Storage Conditions

This Human RAR assays PANEL contains materials to perform 32 RARα assays, 32 RARβ assays, and 32 RARγ assays, all in a single 96-well plate format. All reagents are supplied with sufficient extra volume to accommodate the needs of performing 3 individual groups of assays.

The individual aliquots of RAR Reporter Cells and Detection Substrate and Detection Buffer are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, "Reporter Cells" must be maintained at -80°C until immediately prior to use.

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

Kit Components	*Amount*	*Storage Temp.*
▪ RARα Reporter Cells	1 x 0.60 mL	**-80°C**
▪ RARβ Reporter Cells	1 x 0.60 mL	**-80°C**
▪ RARγ Reporter Cells	1 x 0.60 mL	**-80°C**
▪ Cell Recovery Media (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Media (CSM)	1 x 35 mL	-20°C
▪ *RARα reference agonist:* 9 *cis*-Retinoic Acid, 10 mM	1 x 30 µL	-20°C
▪ *RARβ and RARγ reference agonist:* *trans*-Retinoic Acid, 10 mM	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ 96-well format plate frame	1	ambient
▪ snap-in, 8-well strips (white, sterile, cell culture treated)	12	ambient

III. Materials to be Supplied by the User

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

DAY 1

▪ cell culture-rated laminar flow hood.

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

▪ 37°C water bath.

▪ 70% alcohol wipes

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

▪ disposable media basins, sterile.

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

▪ antagonist reference compounds (optional).

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-8* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 9-15* are performed on **Day 2**, and require less than 1 hour to complete.

▪ A word about Antagonist-mode assay setup ▪

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically EC_{50} – EC_{85}) of a known *agonist* AND the test compound(s) to be evaluated for antagonist activity. We find that adding the reference agonist to the bulk suspension of Reporter Cells (*i.e.,* prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 5b* of the following protocol.

This RAR Assay Panel kit provides a commonly used reference agonist for each RAR assay; they may be used effectively to setup respective receptor inhibition assays.

▪ **RARα**: 9*cis*-Retinoic Acid is provided as a 10 mM stock in DMSO; it may be used as an agonist of RARα to set up antagonist-mode assays. 20 nM 9-*cis*-Retinoic Acid typically approximates EC_{50} in this reporter assay.

▪ **RARβ**: *trans*-Retinoic Acid is provided as a 10 mM stock in DMSO; it may be used as an agonist of RARβ to set up antagonist-mode assays. 3 nM *trans*-Retinoic Acid typically approximates EC_{75} in this assay.

▪ **RARγ**: *trans*-Retinoic Acid is provided as a 10 mM stock in DMSO; it may be used as an agonist of RARγ to set up antagonist-mode assays. 2 nM *trans*-Retinoic Acid typically approximates EC_{50} in this assay.

Note: In *Step 6*, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist.

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

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

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

Total DMSO carried over into assay reactions should never exceed 0.4%.

Note that, in *Step 6*, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

Preparing the positive control: This RAR Assay Panel kit provides a commonly used reference agonist for each RAR assay.

▪ **RARα.** 9 *cis*-Retinoic Acid is provided as a 10 mM stock in DMSO. We find the RARα assay exhibits a complete dose-response to 9 *cis*-Retinoic Acid using an *assay* concentration range of: 2500, 625, 156, 39.1, 9.77, 2.44, 0.610, and 0.153 nM, and including a 'no treatment' control.

▪ **RARβ** and **RARγ.** *trans*-Retinoic Acid is provided as a 10 mM stock in DMSO. We find that the RARβ and RARγ assays both exhibit complete dose-responses to all *trans*-Retinoic Acid using *assay* concentration ranges of: 200, 33.3, 5.56, 0.926, 0.154, 0.0257, and 0.00429 nM, and including a 'no treatment' control.

3.) Rapid Thaw of the Reporter Cells: *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. Perform a *rapid thaw* of the frozen cells by transferring a 3.0 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 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.

5.) ***a. Agonist*-mode assays**. Invert the tube of RAR Reporter Cells several times to disperse cell aggregates and gain a homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into respective strip-wells of the assay plate.

~ or ~

***b. Antagonist*-mode assays**. Invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain a homogenous cell suspension. Supplement the 3.6 ml bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "*A word about antagonist-mode assay setup*", pg. 12). Dispense 100 µl of cell suspension into respective strip-wells of the assay plate.

> *NOTE 5.1:* Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, 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 prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM (as in *Step 6*). Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 100 µl 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 RAR activity, remove the tubes of **Detection Substrate** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

10.) Turn on the luminometer. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be 0.5 second (500 mSec) per well, *or less*.

11.) *Immediately before proceeding to Step 12*: To read 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

RARα Assay Products	
Product No.	***Product Descriptions***
IB02201-32	Human RARα Reporter Assay System 3x 32 assays in 96-well format
IB02201	Human RARα Reporter Assay System 1x 96-well format assay
IB02202	Human RARα Reporter Assay System 1x 384-well format assays
RARβ Assay Products	
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
RARγ Assay Products	
IB02001-32	Human RARγ Reporter Assay System 3x 32 assays in 96-well format
IB02001	Human RARγ Reporter Assay System 1x 96-well format assays
IB02002	Human RARγ 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.	

Panel of RAR Assays	
Product No.	***Product Description***
IB00131-32P	Human RARγ, RARα and RARδ Reporter Assay PANEL 32 assays each in 1x 96-well plate

LIVE Cell Multiplex (LCM) Assay	
Product No.	***Product Descriptions***
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in 5x-bulk volume to perform **480** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Limited Use Disclosures

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

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

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

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APPENDIX 1

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

Stepwise dilutions | *Pipette transfer* | 10 mM 9 cis-Retinoic Acid

6.0 µl → 994 µl CSM (1/166.7 x)

60 µl → *2x-concentration treatment media* → *Transfer* → *Final Assay Concentration 9 cis-RA*

32 Wells pre-loaded with 100 µl RAR α Reporter Cells

Stepwise dilutions	Pipette transfer	Tube	2x-concentration treatment media	Transfer	Final Assay Concentration 9 cis-RA
1/10 x	60 µl	540 µl CSM	6,000 nM	100 µl	3,000 nM
1/6 x	100 µl	500 µl CSM	1,000 nM	100 µl	500 nM
1/6 x	100 µl	500 µl CSM	167 nM	100 µl	83.3 nM
1/6 x	100 µl	500 µl CSM	27.8 nM	100 µl	13.9nM
1/6 x	100 µl	500 µl CSM	4.63 nM	100 µl	2.31 nM
1/6 x	100 µl	500 µl CSM	0.772 nM	100 µl	0.386 nM
1/6 x	100 µl	500 µl CSM	0.129 nM	100 µl	0.0643 nM
		500 µl CSM	0 nM (CSM only)	100 µl	0 nM

100 µl → *Discard*

RARα Assay *2 - 4 replicates per treatment* | RARβ Assay | RARγ Assay

APPENDIX 2

Example scheme for the serial dilution of all *trans*-Retinoic Acid, and the setup of a **RARβ** and **RARγ** dose-response assays.

Stepwise dilutions | *Pipette transfer* | 10 mM *trans*-Retinoic Acid

4.0 µl → 996 µl CSM (1/250 x)

6.0 µl → *2x-concentration treatment media* → *Transfer* → *Final Assay Concentration trans-RA*

32 Wells pre-loaded with 100 µl RAR β Reporter Cells | *32 Wells pre-loaded with 100 µl RAR γ Reporter Cells*

Stepwise dilutions	Pipette transfer	Tube	2x-concentration treatment media	Transfer	Final Assay Concentration trans-RA
1/100x	6.0 µl	594 µl CSM	400 nM	100 µl	200 nM
1/6 x	100 µl	500 µl CSM	66.7 nM	100 µl	33.3 nM
1/6 x	100 µl	500 µl CSM	11.1 nM	100 µl	5.56 nM
1/6 x	100 µl	500 µl CSM	1.85 nM	100 µl	0.926 nM
1/6 x	100 µl	500 µl CSM	0.309 nM	100 µl	0.154nM
1/6 x	100 µl	500 µl CSM	0.0514 nM	100 µl	0.0257 nM
1/6 x	100 µl	500 µl CSM	0.00857 nM	100 µl	0.00429 nM
		500 µl CSM	0 nM (CSM only)	100 µl	0 nM

100 µl → *Discard*

RARα Assay | **RARβ Assay** *2 - 4 replicates per treatment* | **RARγ Assay** *2 - 4 replicates per treatment*