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

384-well Format Assays
Product # IB00822

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Technical Manual
(version 7.1)

www.indigobiosciences.com

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

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

Technical Service:
814-234-1919
techserv@indigobiosciences.com

INDIGO BIOSCIENCES
The Nuclear Receptor Experts™

Human RXRγ Reporter Assay System
384-well Format Assays

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 principle 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 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.

▪ 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.

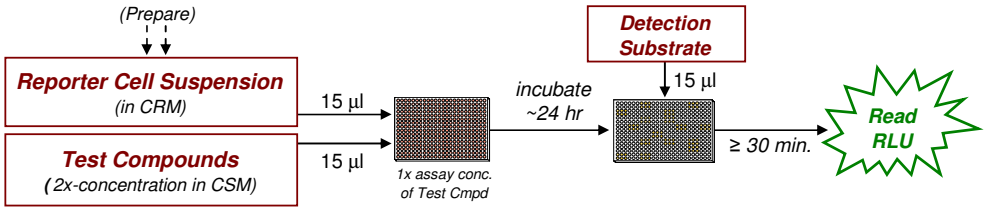
▪ Considerations for Automated Dispensing ▪

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.

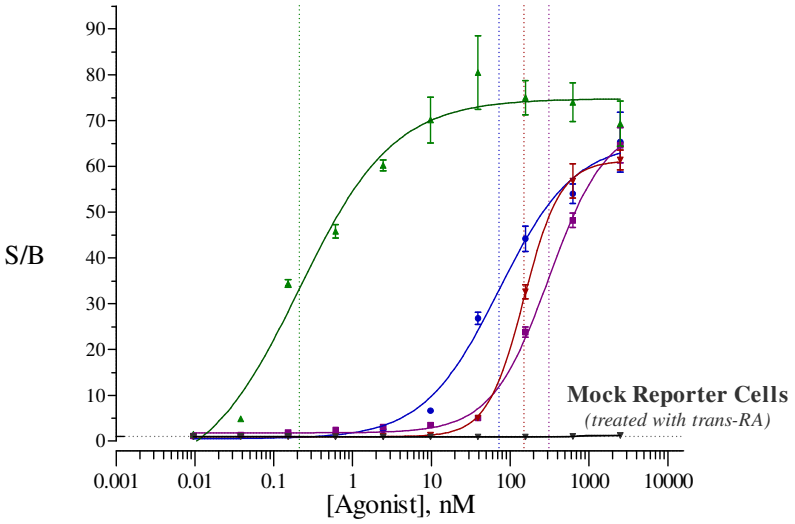
Stock Reagent & Volume provided	**Volume to be Dispensed** *(384-well plate)*	**Excess rgt. volume available for instrument dead volume**
Reporter Cell Suspension 7.5 ml *(prepared from kit components)*	15 µl / well 5.8 ml / plate	~ 1.7 ml
Detection Substrate 7.8 ml	15 µl / well 5.8 ml / plate	~ 2 ml

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, the prepared suspension of thawed 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 Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪



LG100268	9 *cis*-RA	SR11237	*trans*-RA
EC_{50} = 0.21 nM	EC_{50} = 72 nM	EC_{50} = 150 nM	EC_{50} = 310 nM
Hill slope = 0.728	Hill slope = 0.907	Hill slope = 1.9	Hill slope = 1.19
R^2 = 0.9777	R^2 = 0.9939	R^2 = 0.9998	R^2 = 0.9981
at 156 nM:	*at 2.5 µM:*	*at 2.5 µM:*	*at 2.5 µM:*
S/B = 75	S/B = 65	S/B = 61	S/B = 65
% CV = 5.7	% CV = 10	% CV = 4.0	% CV = 6.0
Z' = 0.82	**Z' = 0.67**	**Z' = 0.86**	**Z' = 0.82**

Figure 2. Agonist dose-response analyses of the Human RXRγ assay.
Analyses of RXRγ Reporter Cells using 9-*cis*-Retinoic Acid (provided), SR11237 (Tocris), and *trans*-Retinoic Acid and LG100268 (Sigma-Aldrich). In addition, to assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, "mock" reporter cells, which contain only the luciferase vector, were treated with *trans*-Retinoic Acid (mock reporter cells are not provided with assay kits). Final assay concentrations for each agonist were: 2500, 625, 156, 39.1, 9.77, 2.44, 0.610, 0.153, 0.0381, 0.00954, and 0 nM. Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ($n \geq 6$). Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al.* (1999)[1]. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software. Mock reporter cells demonstrate no significant background, thus, luminescence results strictly through ligand-activation of RXRγ expressed in these reporter cells. Low %CV, and high S/B and Z' scores confirm the robust performance of this RXRγ Assay.

[1] Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4**(2), 67-73.

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

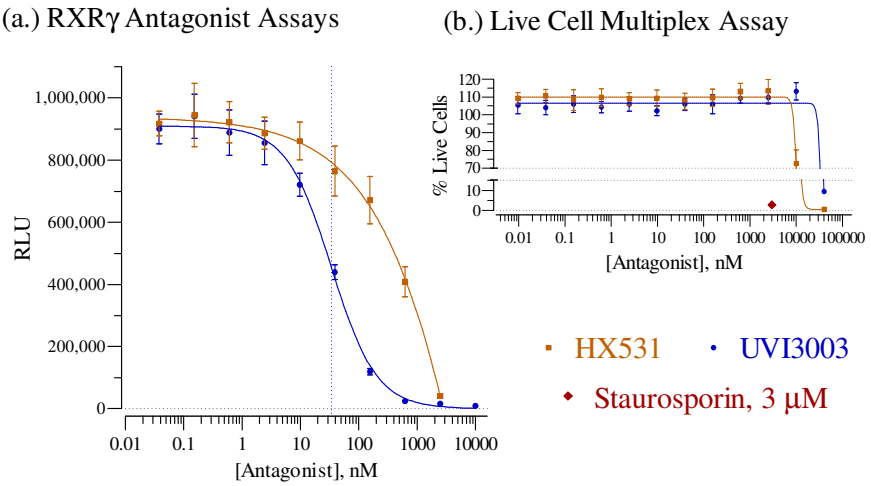


Figure 3. Validation of RXRγ Assay *antagonist* dose-responses (a) performed in combination with INDIGO's Live Cell Multiplex Assay (b).
RXRγ antagonist assays were performed using UVI3003 and HX531 (Tocris). Assay setup and quantification of RXRγ activity were performed following the protocol provided in this Technical Manual. 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 periods using INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01). Final assay concentrations of the respective antagonists ranged between 40 µM and 38 pM, and included a 'no antagonist' control ($n \geq 6$ per treatment). Each treatment also contained 700 nM (~ EC_{75}) of the alternative agonist *trans*-Retinoic Acid. 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. The assay plate was then further processed to quantify RXRγ activity for each treatment condition.

Results: LCM Assays revealed that RXRγ Reporter Cells treated with HX531 experienced emerging cell death at 10µM, and profound cell death at 40µM treatments. Cell death produces 'false-positive' antagonist data; accordingly, these data points were dropped from the HX531 antagonist dose-response plot. Cells treated with UVI3003 experienced emerging cell death at 40 µM treatment, and this data point was dropped from the UVI3003 antagonist dose-response plot. No significant variance in the numbers of live cells per assay well were observed for either reference antagonist at, or below, 2.5 µM treatment concentrations. Hence, the observed dose-dependent reductions in RLU values up to 10 µM UVI3003 and 2.5 µM HX531 are due to receptor inhibition, and *not* treatment-induced cell death.

II. Product Components & Storage Conditions

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

The aliquot of RXRγ Reporter Cells is provided as a single-use reagent. 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.***
▪ RXRγ Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 6 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ 9-*cis*-Retinoic Acid, 10 mM (in DMSO) (reference agonist for RXRγ)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 7.8 mL	**-80°C**
▪ 384-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

▪ 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 384-well 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 9-13* 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 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. 160 nM 9-*cis*-Retinoic Acid typically approximates EC_{70} 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*, 15 µl of treatment media is combined with 15 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: 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%.

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 Reporter 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, *without delay*, perform a rapid thaw of the frozen cells by transferring a 5.5 ml volume of the pre-warmed 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 7.5 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 disperse cell aggregates and gain a homogenous cell suspension. Without delay, dispense 15 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 15 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 (15 µl/well) into a clear 384-well cell culture treated assay plate, followed by 15 µl/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 15 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** 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.) 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. 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.) Following 22 - 24 hours of incubation add 15 µl of **Detection Substrate** to each well of the assay plate.

NOTE: Perform manual reagent transfers 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 may significantly degrade the accuracy and precision of the assay data. In the event of excessive bubble formation during manual processing, spin the assay plate (with lid) at *low speed* for 1-2 minutes 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

RXRγ Assay Products	
Product No.	***Product Descriptions***
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.	

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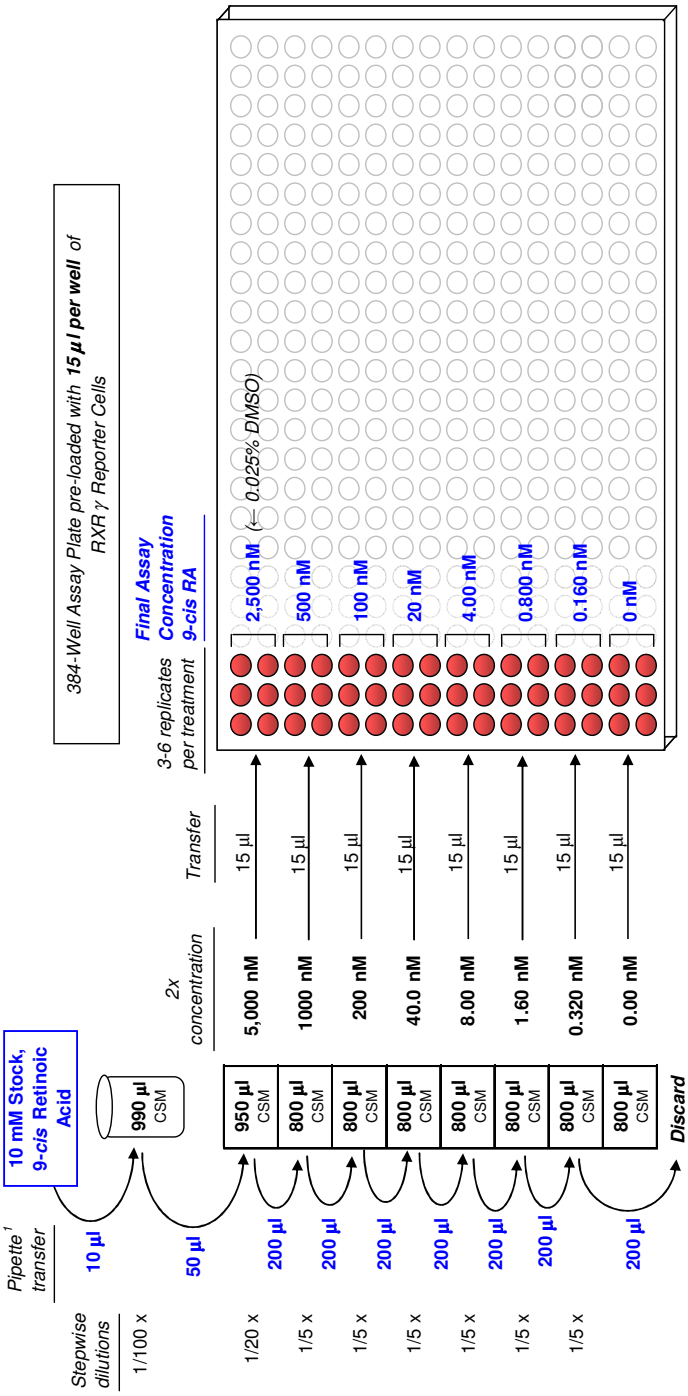
Product prices, availability, specifications and claims are subject to change without prior notice.

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.