Human Retinoic Acid Receptor Gamma

(NR1B3, RARG, RARγ)

Reporter Assay System

3x 32 Assays in 96-well Format

Product # IB02001-32

▪

Technical Manual

(version 6.0)

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

I. Description

- The Assay System……………………….……………….……..……..….3
- The Assay Chemistry……………………….……………….……..……......3
- Preparation of Test Compounds……….……………….………..……….4
- Assay Scheme..............................…………….............………..……....4
- Assay Performance………………………..………………..……..……..…5

II. Product Components & Storage Conditions ……………………………….7

III. Materials to be Supplied by the User……………………...…...……...…8

IV. Assay Protocol

- A word about *Antagonist*-mode assay setup…………...…............…...…8
- *DAY 1 Assay Protocol*…………………………...…..…...……..…...…9
- *DAY 2 Assay Protocol*…………………………...…..…...……..…...…11

V. Related Products……………………………..………………….…..….…..12

VI. Limited Use Disclosures…………………………………….………...……...12

APPENDIX 1: Example Scheme for Serial Dilution..………...…………..……....13

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I. Description

▪ The Assay System ▪

This nuclear receptor assay system utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the **Human Retinoic Acid Receptor Gamma** (**NR1B3**)**,** a ligand-dependent transcription factor commonly referred to as RARG or **RARγ**.

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

RARγ Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields exceptional cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for cumbersome intermediate treatment steps such as spin-and-rinse of cells, viability determinations, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.
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INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to RARγ Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a reference agonist, Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor reporter assay systems capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg^{+2}-dependent reaction that consumes O_2 and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO_2, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU's).

INDIGO's Nuclear Receptor Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5 minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

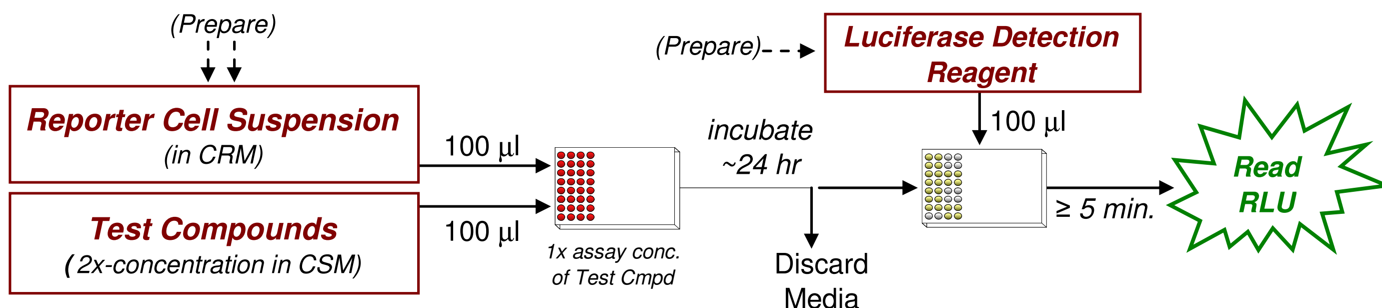
▪ Preparation of Test Compounds ▪

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **Compound Screening Medium (CSM)**, as described in *Step 2* of the **Assay Protocol**. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

> *NOTE:* CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup, and are considered to be 'single-use' reagents.

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds. Following 22 -24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.



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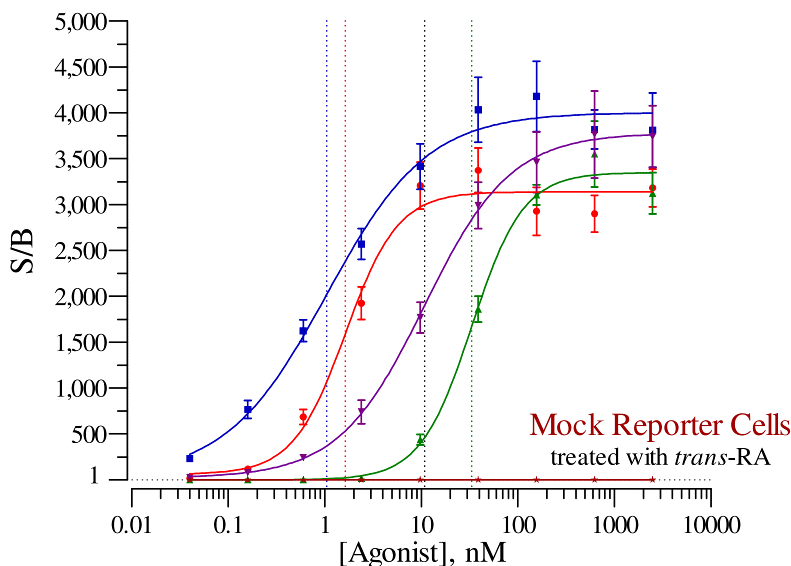
▪ Assay Performance ▪



▪ 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 2. Agonist dose-response of the RARγ Assay.
Validation of the RARγ Assay was performed using manual dispensing and following the protocol described in this Technical Manual, using the reference agonists all-*trans*-Retinoic Acid (provided), Adapalene, BMS 961, and CD1530 (all from Tocris). In addition, to assess the level of background signal contributed by non-specific factor(s) that may cause activation of the luciferase reporter gene, "mock" reporter cells were specially prepared to contain only the luciferase reporter vector (mock reporter cells are not provided with assay kits). RARγ Reporter Cells and Mock reporter cells were identically treated with *trans*-retinoic acid. Luminescence was quantified using a GloMax-Multi+ plate-reading luminometer (Promega Corp.). Average relative light units (RLU) and respective standard deviation (SD) and Signal-to-Background (S/B) values were determined for each treatment concentration (n ≥ 6). Z' values were calculated as described by Zhang, *et al.* (1999)[1]. Non-linear regression analyses were performed and EC_{50} values determined using GraphPad Prism software.

Results: 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 Reporter Assay System, and demonstrate its suitability for use in HTS applications.[1]

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

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

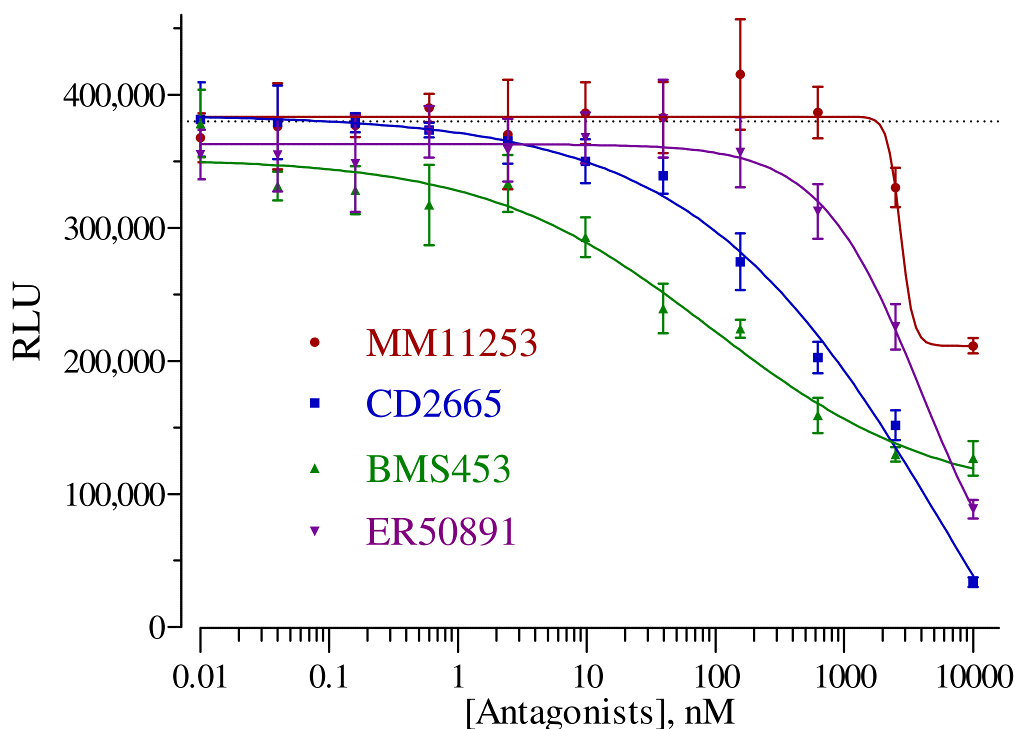


Figure 3. Validation of RARγ Assay *antagonist* dose-responses.

RARγ antagonist assays were performed using MM11253, CD2665, BMS453 and ER50891 (all from Tocris). Assay setup and quantification of RARγ activity were performed following the protocol described in this Technical Manual. Final assay concentrations of the respective antagonists ranged between 10 µM and 10 pM, and included a 'no antagonist' control (n ≥ 6 per treatment; highest [DMSO] ≤ 0.1% *f.c.*). 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 RARγ Reporter Assay System contains materials to perform three distinct groups of assays in a 96-well plate format. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays.

The individual aliquots of Reporter Cells are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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

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

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

Kit Components	*Amount*	*Storage Temp.*
▪ RARγ Reporter Cells	3 x 0.60 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ *trans*-Retinoic Acid, 10 mM (in DMSO) (reference agonist for RARγ)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	**-80°C**
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, cell-culture ready)	12	ambient

III. Materials to be Supplied by the User

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

DAY 1
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO_2 incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- *or* 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- 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 9-15* 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 between $EC_{50} – EC_{85}$) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This RARγ Reporter Assay System kit includes a 10 mM stock solution of ***trans*-Retinoic Acid**, an agonist of RARγ that may be used to setup antagonist-mode assays. 2 nM *trans*-Retinoic Acid typically approximates EC_{50} in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that adding the reference agonist to the bulk suspension of Reporter Cells (*i.e.,* prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 5b* of the following protocol. Note that, in *Step 6*, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.

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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.

- **CRM** should be thawed and equilibrated to 37°C using a water bath. CRM pre-warmed to 37°C is required in *Step 3*.
- **CSM** may be thawed in a 37°C water bath, but should then be allowed to equilibrate to room temperature.

☐ **2.)** Prepare Test Compound(s) and Reference Compound stocks to be screened for *Agonist* or *Antagonist* activities.

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. Plan dilution schemes carefully. This assay kit provides 35 ml of CSM.

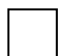
This RARγ Reporter Assay System kit includes a 10 mM stock solution of ***trans*-Retinoic Acid**, a reference agonist of RARγ. The following 8-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable dose-response: 100, 33.3, 11.1, 3.70, 1.23, 0.412, 0.137, and 0.0457 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

☐ **3.)** *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab, then place it in the cell-culture hood.

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 3 - 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, then transfer it into the cell culture hood.



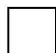
5.) ***a. Agonist*-mode assays**. Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

~ or ~

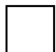
***b. Antagonist*-mode assays**. Invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "*A word about antagonist-mode assay setup*", pg. 8). Dispense 100 µl of cell suspension into each well of the 96-well 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 into a clear 96-well assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

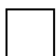


6.) Dispense 100 µl of 2x-concentration treatment media (prepared as described in *Step 2*) into appropriate wells of the assay plate.



7.) Replace the plate's lid and transfer it into a 37°C, humidified 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 90%) 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 -80°C 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 RARγ 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. 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 the assay plate from the incubator. Remove the plate's lid. 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 *via* a sweeping downward movement is NOT advised. 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 per well of **LDR** to each well of the assay plate.

☐ **14)** Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

☐ **15)** Between 5 - 90 minutes after adding LDR, place the assay plate in the luminometer and quantify luminescence.

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V. Related Products

RARγ Assay Products	
Product No.	***Product Descriptions***
IB02001-32	Human RARγ Reporter Assay System 3x 32 assays in 96-well format
IB02001	Human RARγ Reporter Assay System 1x 96-well format assay
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.	

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

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans. Other applications of this product may require licenses from others, including one or more of the institutions listed below.

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

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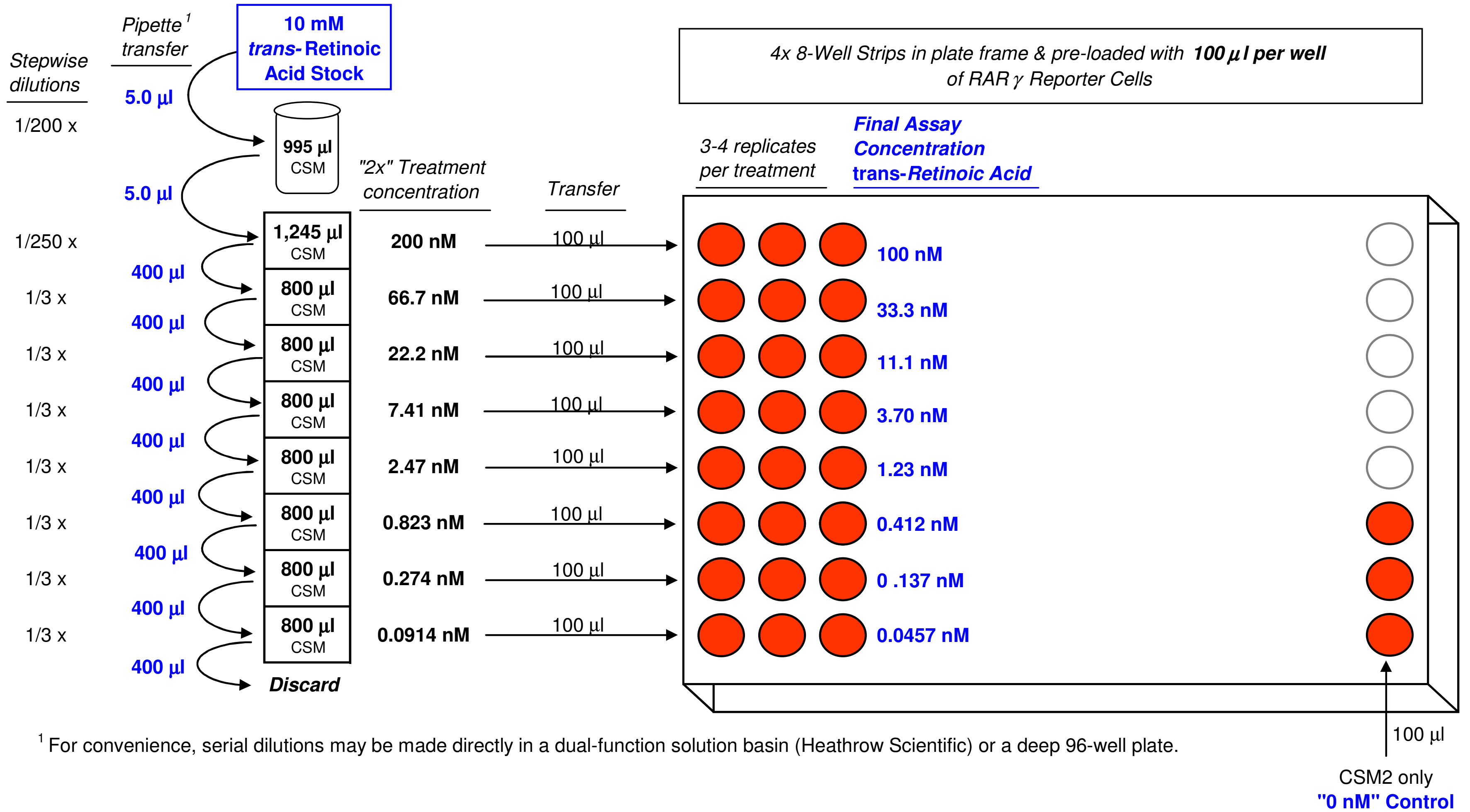
APPENDIX 1

Example scheme for the serial dilution of *trans*-Retinoic Acid reference agonist, and the setup of an RARγ 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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96-well Format Assays
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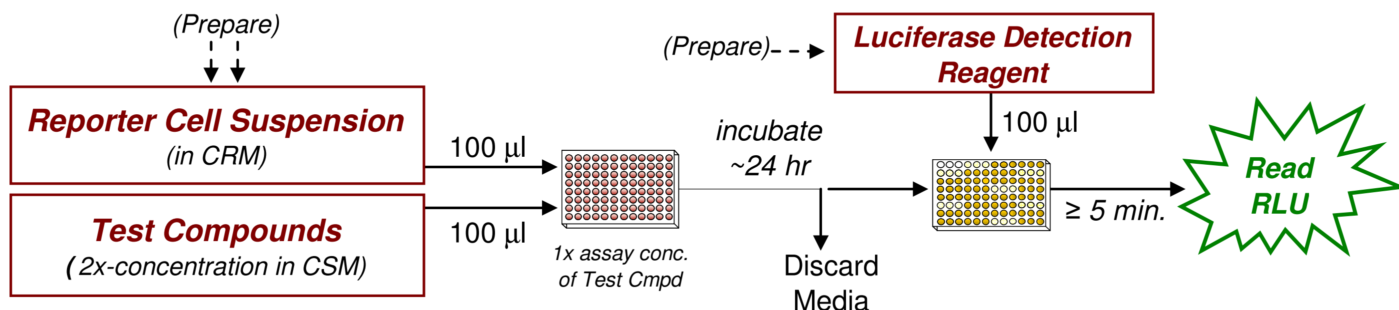
▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully considered 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 *(96-well plate)*	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 12 ml *(prepared from kit components)*	100 µl / well — 9.6 ml / plate	~ 2.4 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well — 9.6 ml / plate	~ 2.4 ml

▪ 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.



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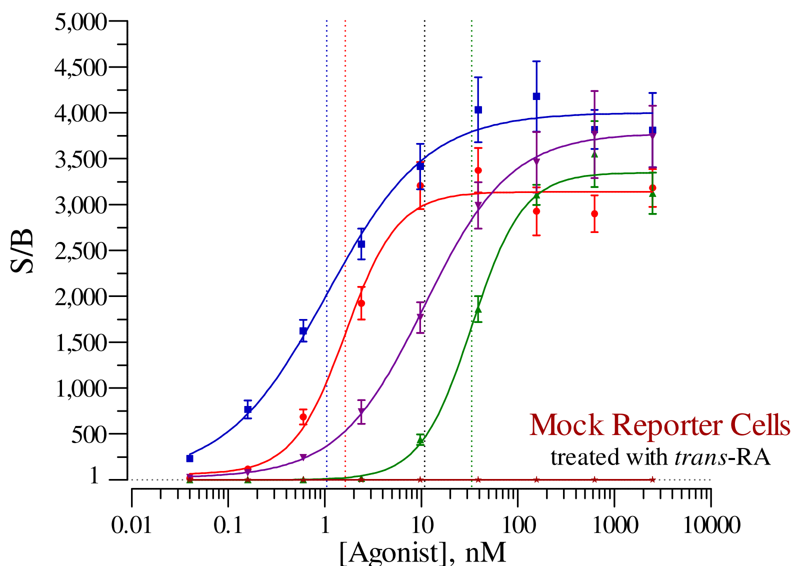
▪ Assay Performance ▪



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Z' = 0.83	Z' = 0.80	Z' = 0.70	Z' = 0.62

Figure 2. Agonist dose-response of the RARγ Assay.
Validation of the RARγ Assay was performed using manual dispensing and following the protocol described in this Technical Manual, using the reference agonists all-*trans*-Retinoic Acid (provided), Adapalene, BMS 961, and CD1530 (all from Tocris). In addition, to assess the level of background signal contributed by non-specific factor(s) that may cause activation of the luciferase reporter gene, "mock" reporter cells were specially prepared to contain only the luciferase reporter vector (mock reporter cells are not provided with assay kits). RARγ Reporter Cells and Mock reporter cells were identically treated with *trans*-retinoic acid. Luminescence was quantified using a GloMax-Multi+ plate-reading luminometer (Promega Corp.). Average relative light units (RLU) and respective standard deviation (SD) and Signal-to-Background (S/B) values were determined for each treatment concentration (n ≥ 6). Z' values were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression analyses were performed and EC_{50} values determined using GraphPad Prism software.

Results: 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 Reporter Assay System, and demonstrate its suitability for use in HTS applications.[1]

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

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

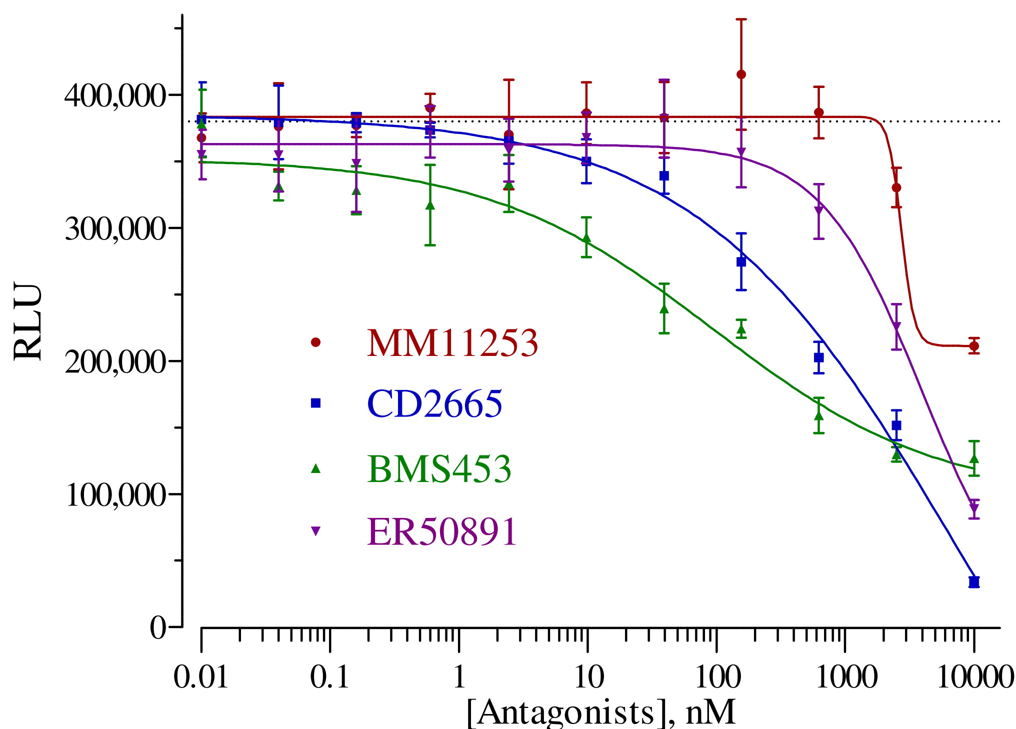

Figure 3. Validation of RARγ Assay *antagonist* dose-responses.

RARγ antagonist assays were performed using MM11253, CD2665, BMS453 and ER50891 (all from Tocris). Assay setup and quantification of RARγ activity were performed following the protocol described in this Technical Manual. Final assay concentrations of the respective antagonists ranged between 10 µM and 10 pM, and included a 'no antagonist' control (n ≥ 6 per treatment; highest [DMSO] ≤ 0.1% *f.c.*). 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.

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II. Product Components & Storage Conditions

This Human RARγ Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of RARγ 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.*
▪ RARγ Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ *trans*-Retinoic Acid, 10 mM (in DMSO) (reference agonist for RARγ)	1 x 30 μL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	**-80°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

- 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).
- 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 9-15* 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 between EC_{50} – EC_{85}) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This RARγ Reporter Assay System kit includes a 10 mM stock solution of ***trans*-Retinoic Acid**, an agonist of RARγ that may be used to setup antagonist-mode assays. 2 nM *trans*-Retinoic Acid typically approximates EC_{50} in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that adding the reference agonist to the bulk suspension of Reporter Cells (*i.e.,* prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 5b* of the following protocol. Note that, in *Step 6*, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.

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

☐ **1)** Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM)** from freezer storage and thaw.

- **CRM** should be thawed and equilibrated to 37°C using a water bath. CRM pre-warmed to 37°C is required in *Step 3*.
- **CSM** may be thawed in a 37°C water bath, but should then be allowed to equilibrate to room temperature.

☐ **2.)** Prepare Test Compound(s) and Reference Compound stocks to be screened for *Agonist* or *Antagonist* activities.

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. Plan dilution schemes carefully. This assay kit provides 35 ml of CSM.

This RARγ Reporter Assay System kit includes a 10 mM stock solution of ***trans-*** **Retinoic Acid**, a reference agonist of RARγ. The following 8-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable dose-response: 100, 33.3, 11.1, 3.70, 1.23, 0.412, 0.137, and 0.0457 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

☐ **3.)** *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab, then place it in the cell-culture hood.

Second, retrieve **Reporter Cells** from -80°C storage. 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 Reporter 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. Sanitize the outside surface of the tube with a 70% alcohol swab, then transfer it into the cell culture hood.

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☐ **5.)** ***a. Agonist*-mode assays**. Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

~ or ~

***b. Antagonist*-mode assays**. 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 96-well 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 into a clear 96-well assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

☐ **6.)** Dispense 100 µl of 2x-concentration treatment media (prepared as described in *Step 2*) into appropriate wells of the assay plate.

☐ **7.)** Replace the plate's lid and transfer it into a 37°C, humidified 5% CO_2 incubator for 22 - 24 hours.

> *NOTE:* Ensure a high-humidity (≥ 90%) 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 -80°C storage and place them in a dark refrigerator (4°C) to thaw overnight.

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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 RARγ 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. 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*, 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, retrieve the assay plate from the incubator. Remove the plate's lid and discard all media contents by ejecting it into an appropriate waste container. *Gently* tap 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.

☐ **14)** Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

☐ **15)** Between 5 - 90 minutes after adding LDR, place the assay plate in the luminometer and quantify luminescence.

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V. Related Products

RARγ Assay Products	
Product No.	***Product Descriptions***
IB02001-32	Human RARγ Reporter Assay System 3x 32 assays in 96-well format
IB02001	Human RARγ Reporter Assay System 1x 96-well format assay
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.	

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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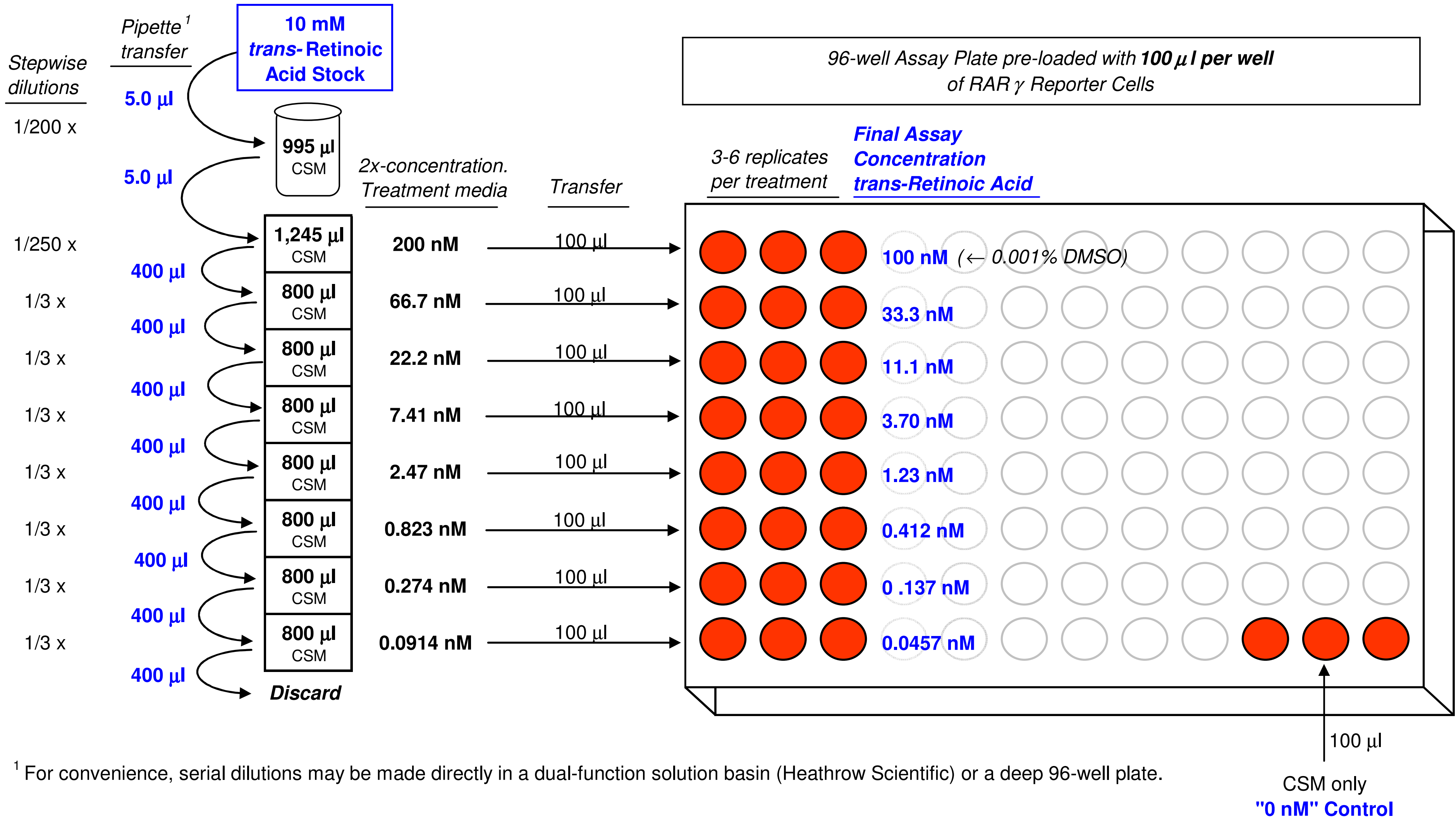
APPENDIX 1

Example scheme for the serial dilution of *trans*-Retinoic Acid reference agonist, and the setup of an RARγ 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.