

**Human RAR-related Orphan Receptor, Alpha
(NR1F1, RORA, ROR α)
Reporter Assay System**

96-well Format Assays
Product # IB04011

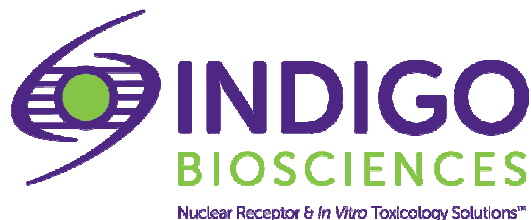
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Human ROR α Reporter Assay System 96-well Format Assays

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I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary non-human mammalian cells engineered to provide high-level expression of a hybrid form of the **Human RAR-related Orphan Receptor Alpha (NR1F1)**. The N-terminal DNA binding domains (DBD) of the native ROR α has been substituted with that of the yeast GAL4-DBD. The reporter gene is beetle luciferase functionally linked to the GAL4 upstream activation sequence (UAS).

As is true *in vivo*, these reporter cells express ROR α in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of ROR α in the absence of added ligand, and the dose-dependent *loss* of constitutive activity upon treatment with compounds exerting inverse-agonist activity. Conversely, **Figure 3** demonstrates that ligand interactions can also result in agonist responses, producing moderate increases in ROR α activity above its already high level of constitutive activity. Therefore, the principal applications of this assay are in the screening of test samples to quantify *inverse-agonist* and/or *agonist* activities that they may exert against human ROR α .

INDIGO's Nuclear Receptor Assay kits are all-inclusive cell-based assay systems. In addition to ROR α Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a buffer for rinsing assay wells, the reference inverse-agonist ATRA, reagents to prepare Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor assay kits 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 O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO₂, 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 ▪

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. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 7* and depicted in Appendix 1 for the reference inverse-agonist ATRA, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if test compound solubility is expected to be problematic,

2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should *never* exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 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 that they are considered to be 'single-use' reagents.

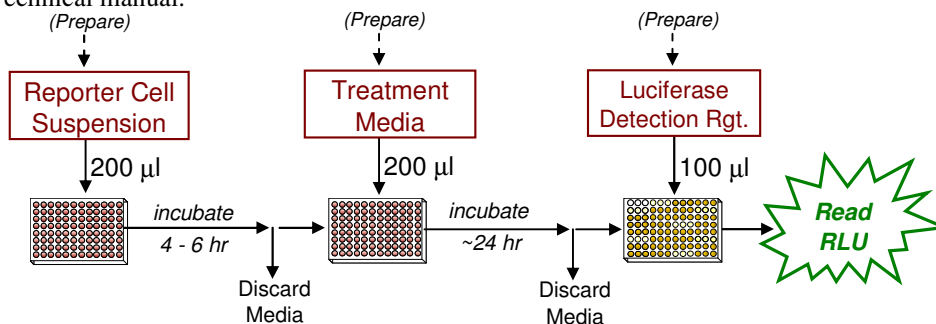
▪ Considerations for Automated Dispensing ▪

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 (96-well plate)	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 21 ml (prepared from kit components)	200 µl / well 19.2 ml / plate	~ 1.8 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 is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded, and the prepared treatment media are added. Following 22-24 hr incubation, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer. *Note:* If INDIGO's Live Cell Multiplex (LCM) Assay is to be incorporated, refer to the assay workflow schematic provided in the LCM Assay Technical manual.



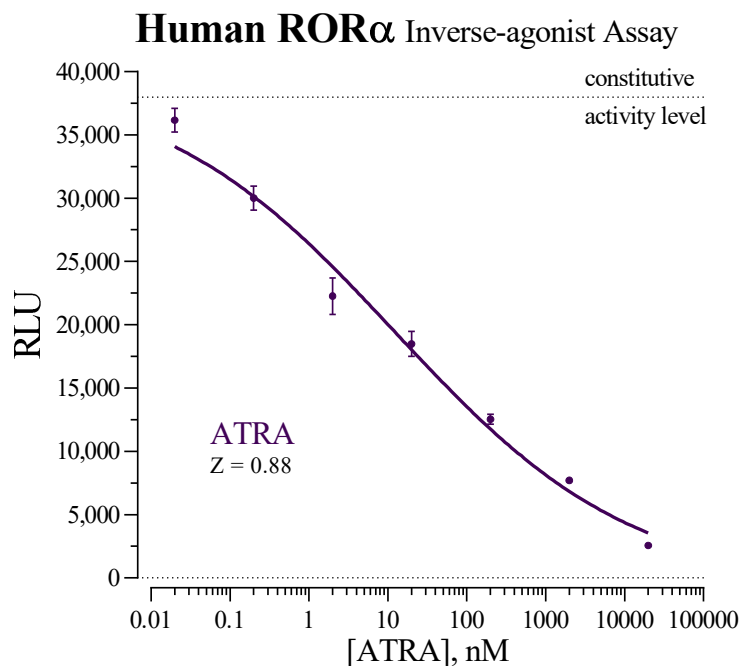


Figure 2. Inverse-agonist dose-response performance of the Human ROR α assay.

Inverse-agonist analyses of Human ROR α Reporter Cells using All *trans*-Retinoic Acid (ATRA; provided). 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 ($n \geq 6$). Z' value was calculated as described by Zhang, *et al.* (1999)¹. Non-linear regression was 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.

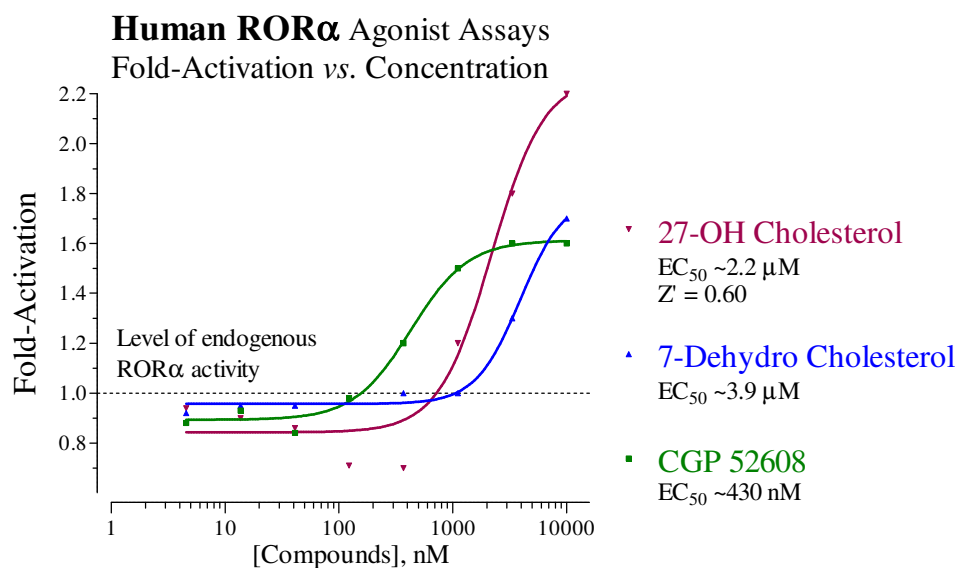


Figure 3. Agonist dose-response performance of the Human ROR α assay.

Agonist response of the Human ROR α Reporter Cells is demonstrated using 27-Hydroxy Cholesterol (27OHC; Cayman Chemical), 7-dehydro Cholesterol (Sigma), and CGP 52608 (Sigma).

Values of *Fold-Activation* are plotted against concentration. 27OHC provides greater than a 2-fold increase in ROR α activity above the already high endogenous activity level. Z' values confirm the robust performance of the agonist-mode ROR α assay.

When contemplating concentration ranges for screening test compounds of unknown bioactivity, it is important to note the great disparity in potencies between inverse-agonists and these agonist reference compounds: ATRA IC₅₀ < 1 nM, whereas 27OHC > 1 μ M.

II. Product Components & Storage Conditions

This Human ROR α Assay kit contains materials to perform assays in a single collagen-coated 96-well assay plate.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in Step 2 of 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 inspect and 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, reporter 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ ROR α Reporter Cells	1 x 2.0 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ all <i>trans</i> -Retinoic Acid, 10 mM (in DMSO) (reference inverse-agonist for ROR α)	1 x 30 μ L	-20°C
▪ Detection Substrate	1 x 6.0 mL	-80°C
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well assay plate (white, sterile, collagen-coated)	1	-20°C

NOTE: This assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or colder) until use.

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 2*)
- 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:* clear 96-well assay plate, sterile, *collagen-coated*, 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 1-11* are performed on **Day 1**, requiring less than 2 hours of bench work and a 4 hr incubation to complete. *Steps 12-17* are performed on **Day 2** and require less than 1 hour to complete.

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

1.) Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Rapid Thaw of the Reporter Cells: *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from **each of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **21 ml**.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) Gently invert the tube of Reporter Cells several times to gain a homogenous cell suspension, then transfer the cell suspension into a reservoir. Using an 8-channel pipette, dispense **200 µl / well** of cell suspension into the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

NOTE 4.3: 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 into a clear, *collagen-coated* 96-well assay plate. Continue to process the clear plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO₂) for 4 - 6 hours.

Near the end of the 4-6 hour pre-incubation period:

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

7.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations: Use CSM to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 9*, the prepared treatment media will be dispensed at 200 µl / well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

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

Prepare the treatment media at the desired final assay concentrations: This RORα Assay kit includes a 10 mM stock solution of ATRA, an inverse-agonist of RORα. The following 8-point treatment series, prepared in serial 10-fold decrements, provides a suitable dose-response range: 20000, 2000, 200, 20, 2.0, 0.20, 0.020 and 0.0020 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

8.) At the end of the 4-6 hr pre-culture period, discard the media. The preferred method is to use a 'wrist flick' to eject media 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.

9.) Dispense **200 µl / well** of each prepared treatment media into the assay plate.

NOTE: 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

10.) Transfer the assay plate into a cell culture incubator 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.

11.) 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 an open bench top.

12.) 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.

13.) 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*.

14.) *Immediately before proceeding to Step 15*, 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.

15.) Following 22 - 24 hours incubation in treatment media, 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.

16.) 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 following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Related Products

Product No.	Product Descriptions
Human RORα Assay Kit Products	
IB04011-32	Human ROR α Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
IB04011	Human ROR α Reporter Assay System 1x 96-well format assays
Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay Products	
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 contained in 5 x 96-well assay plates
LCM-10	Reagent in 10x bulk volume to perform 960 Live Cell Assays contained in 10 x 96-well assay plates

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, diagnostic, or contact use in humans or animals.

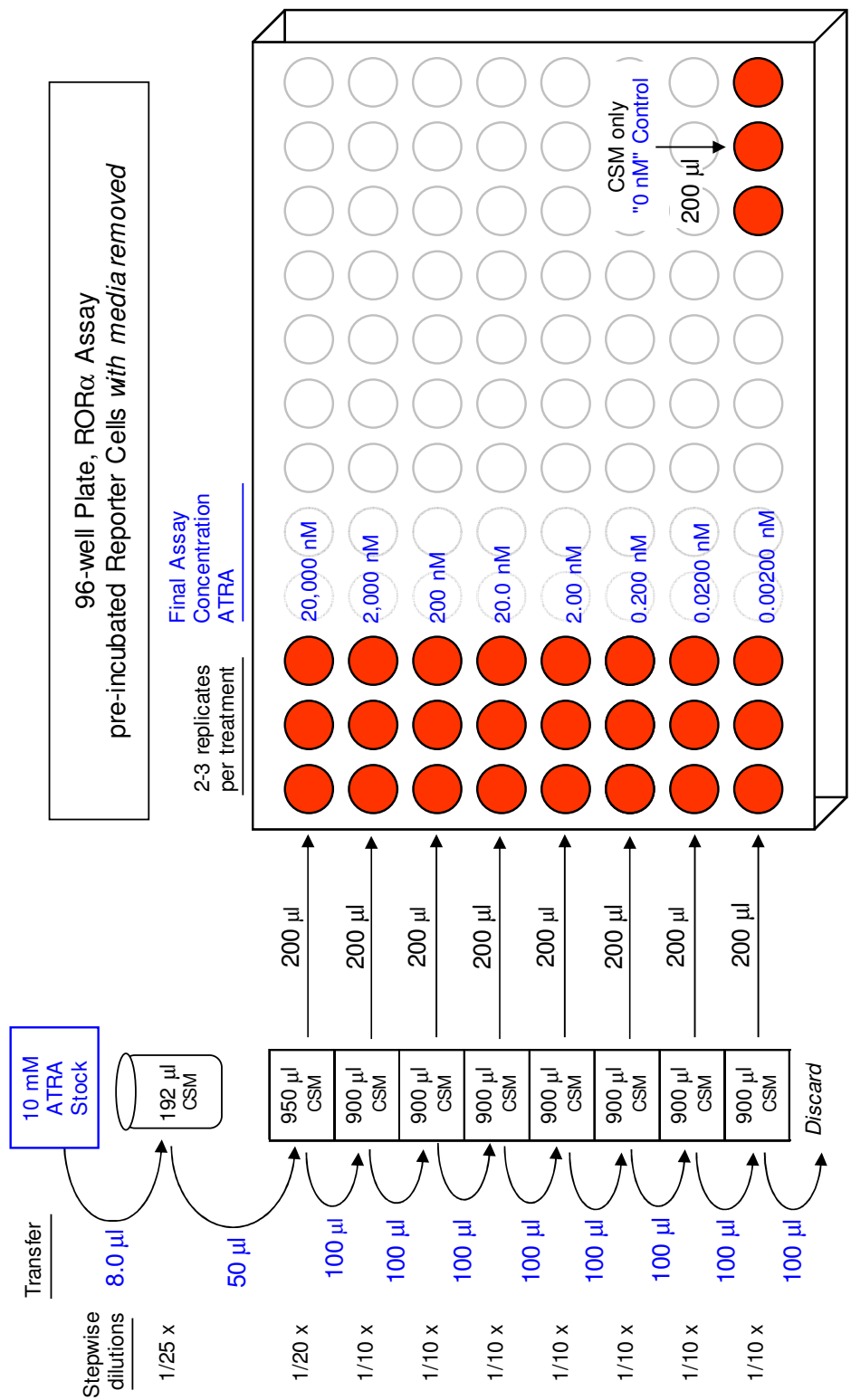
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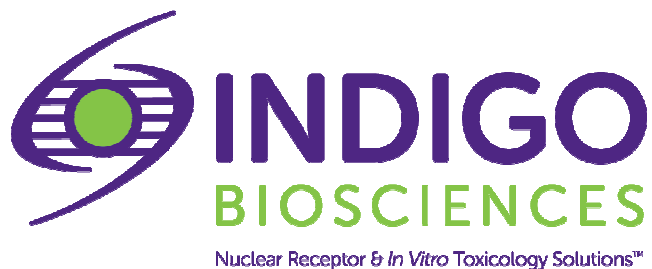
Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

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APPENDIX 1

Example scheme for the serial dilution of all *trans*-Retinoic Acid and the setup of an ROR α activation dose-response assay.





**Human RAR-related Orphan Receptor, Alpha
(NR1F1, RORA, ROR α)
Reporter Assay System**

3x 32 Assays in 96-well Format
Product # IB04011-32

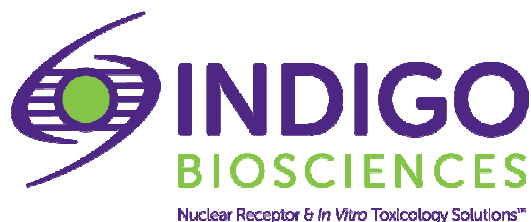
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Human ROR α Reporter Assay System 3x 32 Assays in 96-well Format

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Alternatively, if test compound solubility is expected to be problematic,

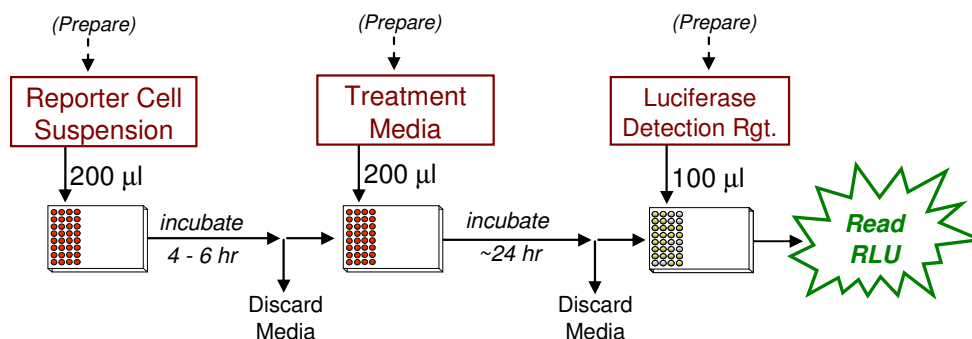
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▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, Reporter Cells is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded, and the prepared treatment media are added. Following 22-24 hr incubation, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



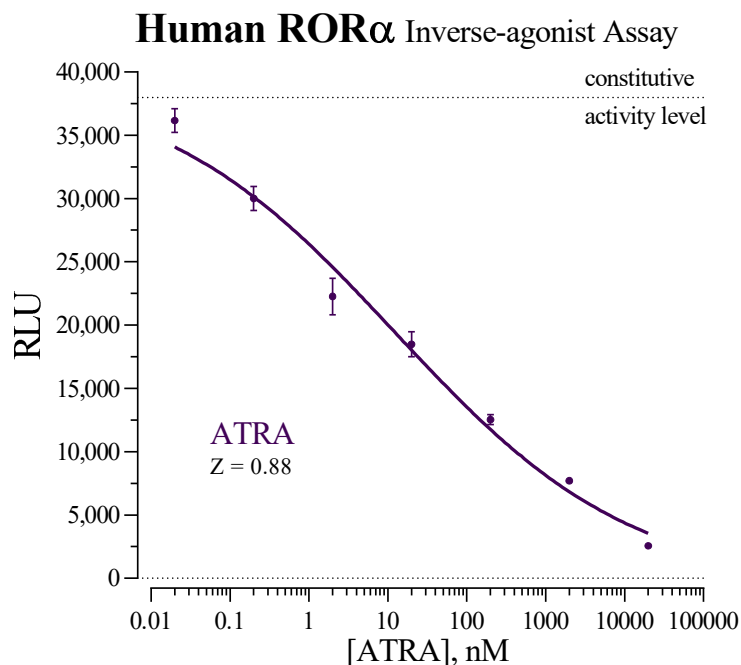


Figure 2. Inverse-agonist dose-response performance of the Human ROR α assay.

Inverse-agonist analyses of Human ROR α Reporter Cells using All *trans*-Retinoic Acid (ATRA; provided). 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 ($n \geq 6$). Z' value was calculated as described by Zhang, *et al.* (1999)¹. Non-linear regression was performed using GraphPad Prism software.

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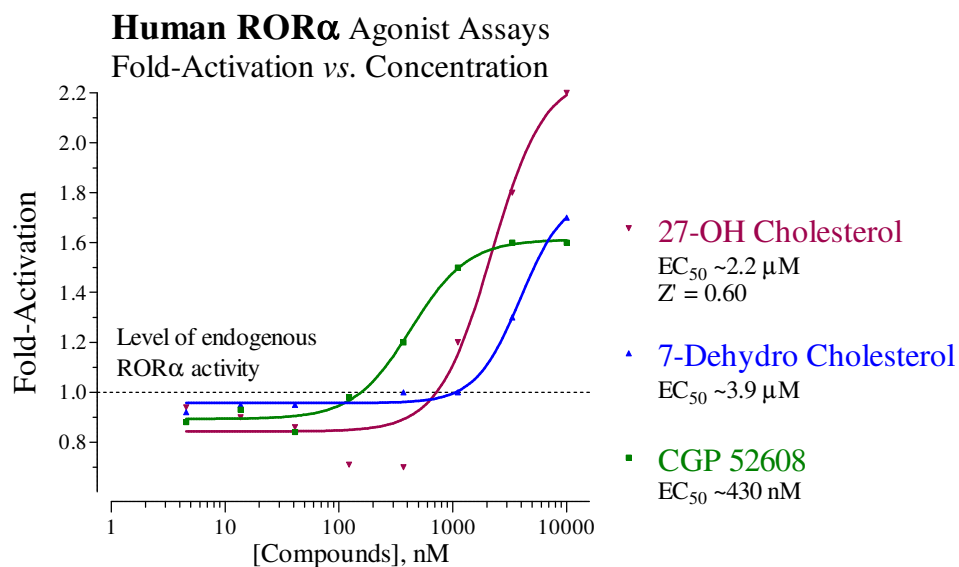


Figure 3. Agonist dose-response performance of the Human ROR α assay.

Agonist response of the Human ROR α Reporter Cells is demonstrated using 27-Hydroxy Cholesterol (27OHC; Cayman Chemical), 7-dehydro Cholesterol (Sigma), and CGP 52608 (Sigma).

Values of *Fold-Activation* are plotted against concentration. 27OHC provides greater than a 2-fold increase in ROR α activity above the already high endogenous activity level. Z' values confirm the robust performance of the agonist-mode ROR α assay.

When contemplating concentration ranges for screening test compounds of unknown bioactivity, it is important to note the great disparity in potencies between inverse-agonists and these agonist reference compounds: ATRA IC₅₀ < 1 nM, whereas 27OHC > 1 μ M.

II. Product Components & Storage Conditions

This Human ROR α Assay kit contains materials to perform three distinct groups of assays in the format of a 96-well plate. 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.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in Step 2 of 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 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.

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

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ ROR α Reporter Cells	3 x 0.6 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ all <i>trans</i> -Retinoic Acid, 10 mM (in DMSO) (reference inverse-agonist for ROR α)	1 x 30 μ L	-20°C
▪ Detection Substrate	3 x 2.0 mL	-80°C
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, collagen-coated wells)	12	-80°C

NOTE: This Assay kit contains 8-well strips that have been collagen-coated and dried; these strip wells should be stored frozen (-20°C or colder) until use.

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 2*)
- 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:* clear 96-well assay plate, sterile, *collagen-coated*, 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 1-11* are performed on **Day 1**, requiring less than 2 hours of actual bench work plus a 4 hr pre-incubation step. *Steps 12-17* are performed on **Day 2** and require less than 1 hour to complete.

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

1.) Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Rapid Thaw of the Reporter Cells: *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **7.0 ml** per tube.

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.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to disperse cell aggregates and gain a homogenous cell suspension. Dispense **200 µl / well** of cell suspension into the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

NOTE 4.3: 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 into a clear, *collagen-coated* 96-well assay plate. Continue to process the assay plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells: Place the assay plate into a mammalian cell incubator (37°C, ≥ 70% humidity, 5% CO₂) for 4 - 6 hours.

6.) Near the end of the pre-incubation period remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations: Use CSM to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 9*, the prepared treatment media will be dispensed at 200 µl / well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

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

Prepare the treatment media at the desired final assay concentrations: This ROR α Assay kit includes a 10 mM stock solution of ATRA, a potent inverse-agonist of ROR α . The following 8-point treatment series, prepared in serial 10-fold decrements, provides a suitable dose-response range: 20000, 2000, 200, 20, 2.0, 0.20, 0.020 and 0.0020 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

8.) At the end of the 4-6 hr pre-culture period, discard the pre-culture media. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media 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. Do *not* touch the well bottoms or run the tip of the aspiration device around the bottom circumference of the assay wells. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

9.) Dispense **200 µl / well** of each prepared treatment media into the assay plate.

10.) Transfer the assay plate into a cell culture incubator for 22 - 24 hours.

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

11.) 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 an open bench top.

12.) 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.

13.) 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*.

14.) *Immediately before proceeding to Step 15:* 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.

15.) Following 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 8*).

16.) 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 following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Related Products

Product No.	Product Descriptions
Human RORα Assay Kit Products	
IB04011-32	Human ROR α Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
IB04011	Human ROR α Reporter Assay System 1x 96-well format assays
Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay Products	
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 contained in 5 x 96-well assay plates
LCM-10	Reagent in 10x bulk volume to perform 960 Live Cell Assays contained in 10 x 96-well assay plates

Please refer to INDIGO Biosciences website for updated product offerings.

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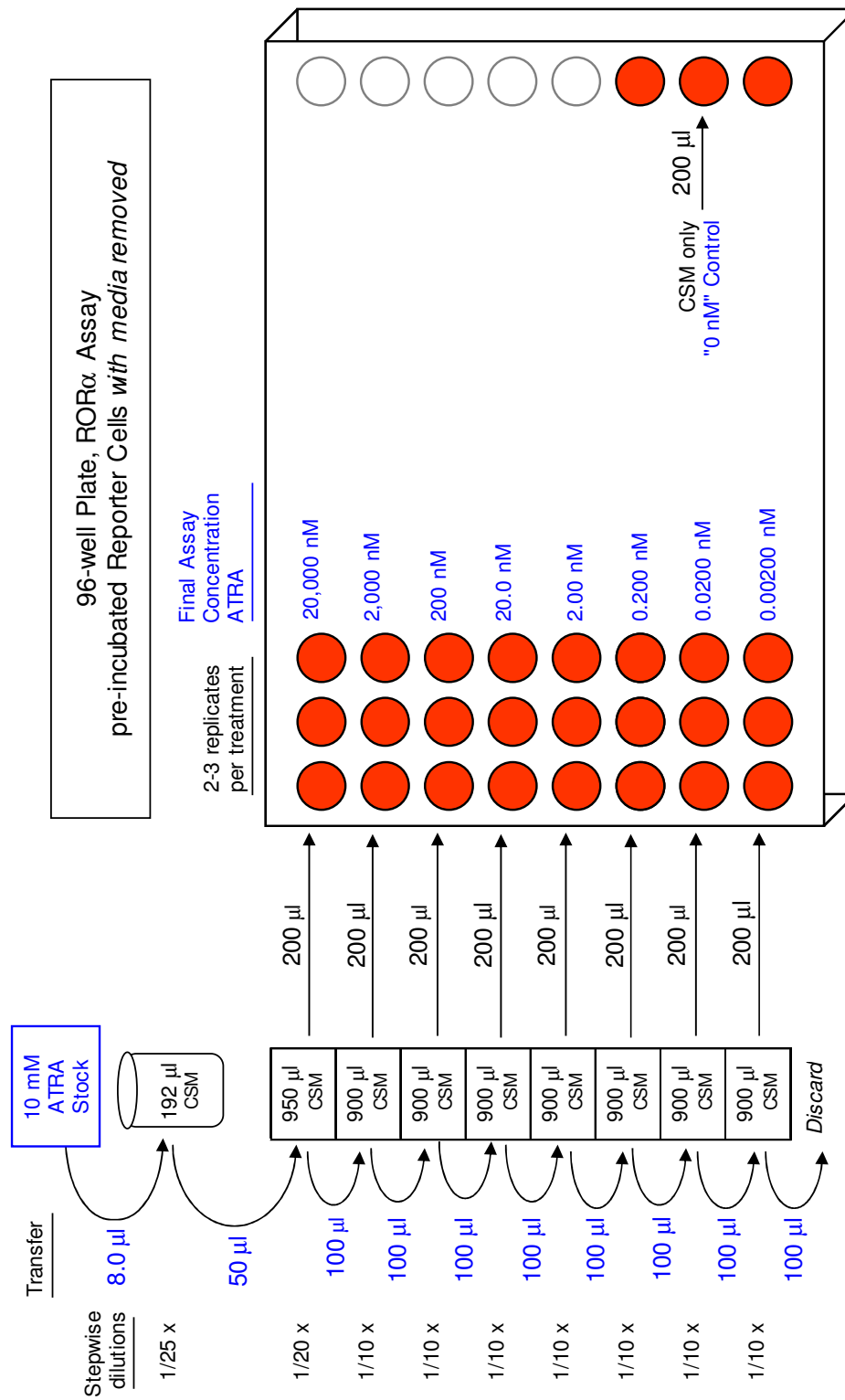
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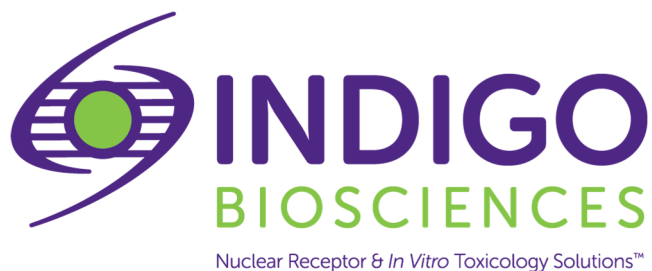
Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

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APPENDIX 1

Example scheme for the serial dilution of all *trans*-Retinoic Acid and the setup of an ROR α activation dose-response assay.





**Human RAR-related Orphan Receptor, Alpha
(NR1F1, RORA, ROR α)
Reporter Assay System**

384-well Format Assays
Product # IB04012

■

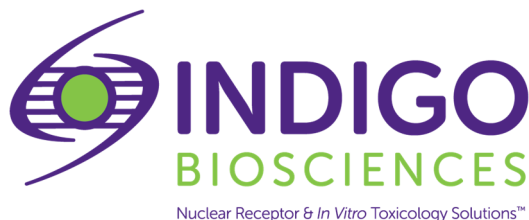
Technical Manual
(version 8.0)

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Human ROR α Reporter Assay 384-well Format Assays

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I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary non-human mammalian cells engineered to provide high-level expression of a hybrid form of the **Human RAR-related Orphan Receptor Alpha (NR1F1)**. The N-terminal DNA binding domains (DBD) of the native ROR α has been substituted with that of the yeast GAL4-DBD. The reporter gene is beetle luciferase functionally linked to the GAL4 upstream activation sequence (UAS).

As is true *in vivo*, these reporter cells express ROR α in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of ROR α in the absence of added ligand, and the dose-dependent *loss* of constitutive activity upon treatment with compounds exerting inverse-agonist activity. Conversely, **Figure 3** demonstrates that ligand interactions can also result in agonist responses, producing moderate increases in ROR α activity above its already high level of constitutive activity. Therefore, the principal applications of this assay are in the screening of test samples to quantify *inverse-agonist* and/or *agonist* activities that they may exert against human ROR α .

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's Nuclear Receptor Assays are all-inclusive cell-based assay systems. In addition to ROR α Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, the reference inverse-agonist all-*trans*-retinoic acid (ATRA)¹, 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 O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO₂, 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 minutes 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.

¹ Gege C., Schlüter T., Hoffmann T. (2014) Identification of the first inverse agonist of retinoid-related orphan receptor (ROR) with dual selectivity for ROR β and ROR γ t. Bioorganic & Med. Chem. Letters: **24**, 5265-5267.

▪ **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 steps in which a conventional **tip-based** instrument is used to dispense test compounds into assay wells are denoted 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 (refer to APPENDIX 1a as an example). The final concentration of DMSO carried over into assay reactions should never exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO, or any organic solvent.

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 steps in which an **acoustic transfer** device is used to dispense test compounds into assay wells are denoted in blue text. 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. Also refer to APPENDIX 1b as an example.

▪ **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.

Stock Reagent & Volume provided	Volume to be Dispensed (384-well plate)	Excess rgt. volume available for instrument dead volume
<i>when using tip dispensing of <u>test cmpds</u></i> Reporter Cell Suspension 7.5 ml	15 µl / well 5.8 ml / plate	~ 1.7 ml
<i>when using acoustic dispensing of <u>test cmpds</u></i> Reporter Cell Suspension 15 ml	30 µl / well 11.5 ml / plate	~ 3.4 ml
Detection Substrate 7.8 ml	15 µl / well 5.8 ml / plate	~ 2 ml

▪ Assay Scheme ▪

The *Day 1* preparation, volumes used, and chronology of dispensing cells and test compounds are different between assay setups using a *tip-based dispenser* (**1a**) and those using an *acoustic transfer device* (**1b**). On *Day 2*, following a 22 -24 hr treatment period, Detection Substrate is added and 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.

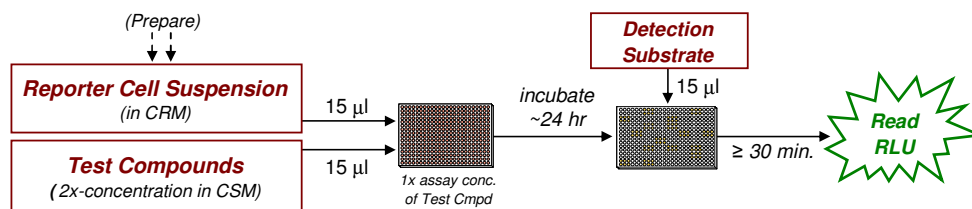
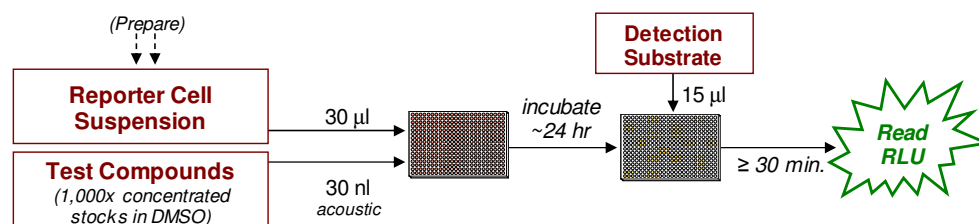


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



▪ Assay Performance ▪

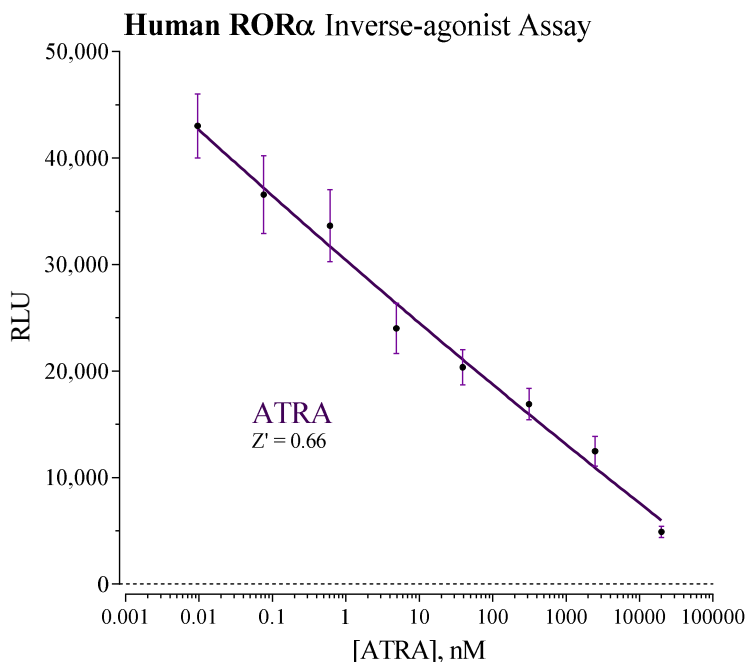


Figure 2. Inverse-agonist dose-response performance of the Human ROR α assay.

Treatment media containing the reference inverse-agonist all *trans*-Retinoic Acid (ATRA; provided) was prepared as described in **Appendix 1a**. Luminescence intensity was quantified as relative light units. Average RLU values and corresponding standard deviation (SD) values were determined for each treatment concentration (n = 4). Z' value was calculated as described by Zhang, *et al.* (1999)².

² 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.

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 [Step 3](#) or [Step 4](#).

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 cells in dry ice.

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

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ RORα Reporter Cells	1 x 1.0 mL	-80°C
▪ Cell Recovery Medium (CRM)	1 x 7 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ ATRA, 20 mM (in DMSO)	1 x 60 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

- 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
- auto-dispenser suitable for 15 µl dispense volume, or [acoustic transfer device](#) for 30 nl dispense volume.
- disposable media reservoirs, sterile.
- sterile multi-channel media basins *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- agonist 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.

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 never 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 assay kit includes a 20 µM stock of ATRA, an inverse-agonist of RORα. The following 8-point treatment series, with concentrations presented in 8-fold decrements, provides a complete dose-response: 20000, 2500, 313, 39.1, 4.88, 0.610, 0.0763, and 0.00954 nM. Always include ‘untreated’ (or ‘Vehicle only’) control wells.

APPENDIX 1a provides an example for generating such a dilution series to be used when *tip-dispensing* compound solutions prepared as 2x concentrates in CSM (dispense 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* (transfer 30 nl / well).

When using *tip-based* instrumentation for dispensing test compounds ...

3.) 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 **6.5 ml** volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter 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 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.) Gently invert the tube of cell suspension several times to gain a homogenous suspension. Transfer onto an appropriate media reservoir, then 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.) *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 **6.5 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.

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. Transfer into an appropriate media reservoir, 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 it is recommended to perform 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 for 22 - 24 hours.

NOTE: Ensure a high-humidity ($\geq 70\%$) 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. It is recommended to perform 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 minutes reaction period the luminescence signal achieves a stable emission output.

13.) Quantify luminescence.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Human RORα Assay Products	
IB04011-32	Human ROR α Assay System; 3x 32 assays in 96-well format
IB04011	Human ROR α Assay System; 1x 96-well format assay
IB04012	Human ROR α Assay System; 1x 384-well format assays
LIVE Cell Multiplex (LCM) Assay	
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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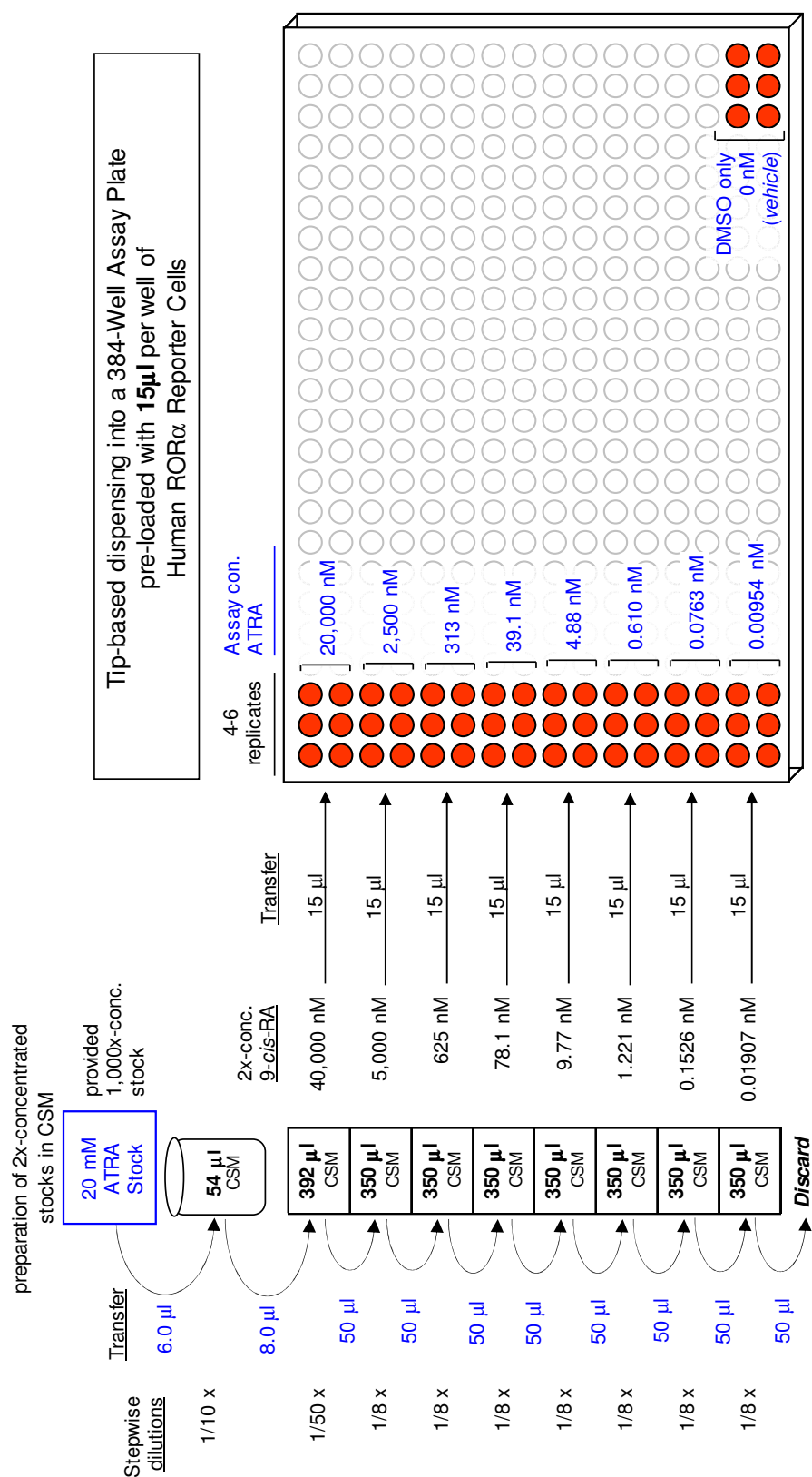
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APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference inverse-agonist ATRA into CSM to generate **2x-concentrated** treatment media. A *tip-based* instrument is used to dispense 15 μ l / well into an assay plate that has been *pre-dispensed* with 15 μ l / well of ROR α Reporter Cells suspension.



APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the reference inverse-agonist ATRA into DMSO to generate **1,000x-concentrated** stocks. 30 nl / well of these prepared stocks are first dispensed into *empty* wells of the assay plate using an acoustic transfer device, followed by the dispensing of 30 μ l / well of ROR α reporter cells.

