Human RAR-related Orphan Receptor, Alpha
(NR1F1, RORA, RORα)
Reporter Assay System

384-well Format Assays
Product # IB04012

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Technical Manual
(version 8.0)

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Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human RORα Reporter Assay
384-well Format Assays

I. Description

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

II. Product Components & Storage Conditions ……6

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

IV. Assay Protocol

- *DAY 1 Assay Protocol*……...……..…..7
- *DAY 2 Assay Protocol*……...……....……9

V. Related Products……..……..…....10

VI. Limited Use Disclosure……..……..10

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

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

I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary non-human 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 RORα 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 RORα in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of RORα 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 RORα 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 RORα.

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 RORα 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 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 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 RORβ and RORγt. 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 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**
when using tip dispensing of test cmpds **Reporter Cell Suspension** 7.5 ml	15 µl / well 5.8 ml / plate	~ 1.7 ml
when using acoustic dispensing of test cmpds **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.

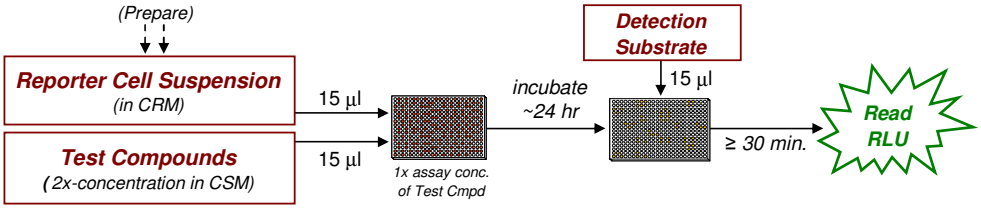
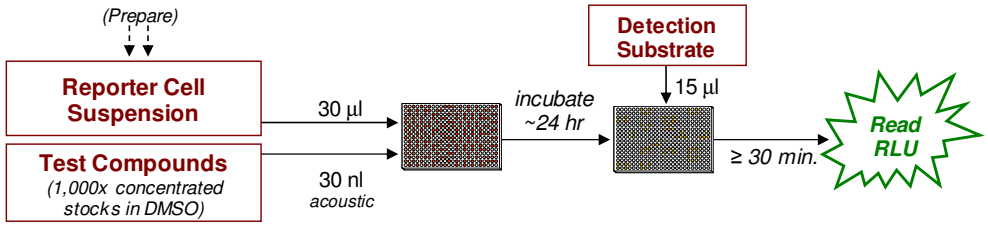


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



▪ Assay Performance ▪

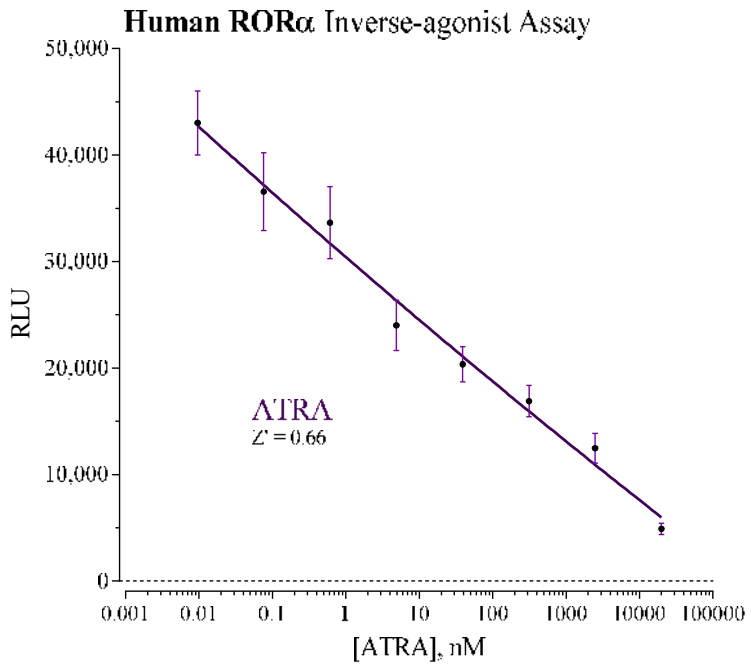


Figure 2. Inverse-agonist dose-response performance of the Human RORα assay.

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.

Kit Components	*Amount*	*Storage Temp.*
▪ 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% CO_2 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 (≥ 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

Product No.	*Product Descriptions*
Human RORα Assay Products	
IB04011-32	Human RORα Assay System; 3x 32 assays in 96-well format
IB04011	Human RORα Assay System; 1x 96-well format assay
IB04012	Human RORα 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.

www.indigobiosciences.com

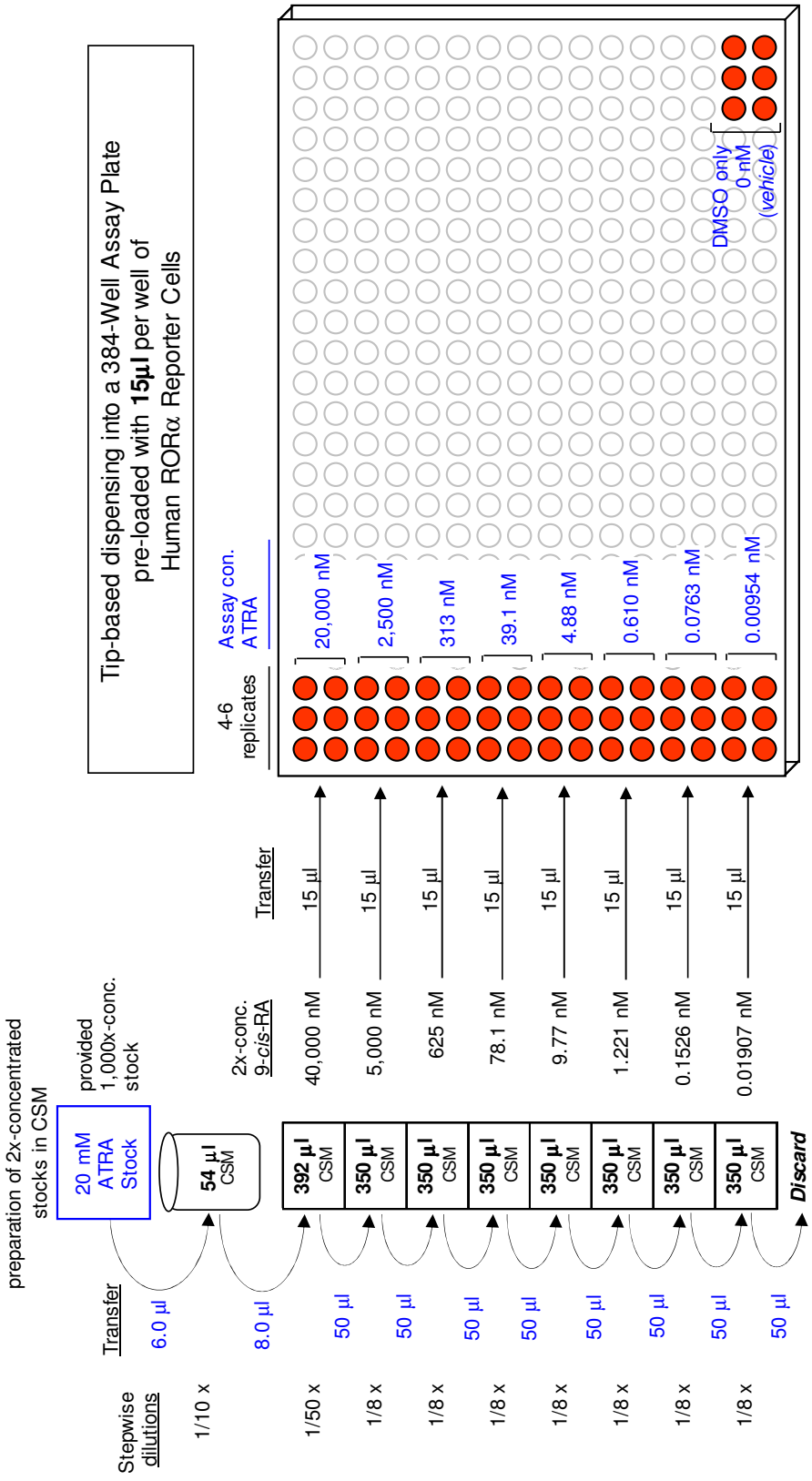
VI. Limited Use Disclosures

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

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

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

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 µl / well into an assay plate that has been *pre-dispensed* with 15 µl / well of RORα 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 µl / well of RORα reporter cells.