Human Adrenoceptor Alpha 2A
(ADRA2A)
Reporter Assay System

384-well Format Assays
Product # IB34002

▪

Technical Manual
(version 8.0)

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Human ADRA2A Reporter Assay
384-well Format Assays

I. Description

▪ Background ▪

The adrenoceptors (*a.k.a*. adrenergic receptors) mediate the action of the sympathetic nervous system and are activated in response to "fight-or-flight" signals. They are divided into three types, adrenoceptor α1-, α2-, and β. Each type is further composed of three subtypes resulting in 9 different types (α1A, α1B, α1D, α2A, α2B, α2C, β1, β2, and β3)[1].

Adrenoceptors belong to the G-Protein-coupled receptor (GPCR) family. They all display the characteristic seven transmembrane helices, the extracellular loops which contribute to ligand binding, and the intracellular carboxy tail that associates with trimeric G proteins. All nine types of adrenoceptors are activated by the same endogenous catecholamines (epinephrine and norepinephrine); however, the specificity of their responses depends on the G-proteins and effector systems they associate with in a tissue and time specific manner[1].

Adrenoceptor Alpha 2A (ADRA2A) is expressed in the central nervous system and in peripheral organs such as spleen, kidney, heart, aorta, skeletal muscle, lung and liver[2,5]. ADRA2A agonists are used in general anesthesia and in the treatment of opiate withdrawal, attention deficit and hyperactive disorder (ADHD), post-traumatic stress disorder (PTSD), and Tourette's symdrome[6]. ADRA2A also plays a role in the regulation of blood pressure[2], in the regulation of glucose metabolism[7], and in some types of cancer[8].

▪ The Assay System ▪

This assay utilizes proprietary human cells engineered to provide constitutive, high-level expression of the **Human Adrenoceptor Alpha 2A (ADRA2A).**

ADRA2A signals mainly through the $G\alpha_i$ family of G proteins to negatively regulate the norepinephrine release[1,2]. However, ADRA2A can also signal through the Gαq > PLCβ > PKC signaling pathway[3,4]. This pathway leads to an increase in intracellular calcium and the concomitant activation of calcineurin, a calcium-dependent phosphatase. Ca^{+2}·calcineurin, in turn, acts to dephosphorylate and activate the transcription factor NFAT[9]. ADRA2A activation of the Ca^{+2}·calcineurin > NFAT cascade is the signal transduction pathway exploited by the reporter cells provided in this kit.

INDIGO's ADRA2A Reporter Cells contain an engineered luciferase reporter gene functionally linked to tandem consensus sequences of the NFAT genetic response element positioned upstream of a minimal promoter. When activated, NFAT binds to these response elements to seed the formation of a complete transcription complex that drives luciferase gene expression. Quantifying relative changes in luciferase activity in the treated reporter cells relative to the untreated cells provides a sensitive surrogate measure of drug-induced changes in ADRA2A activity. The principal application of this reporter assay is in the screening of test samples to quantify functional interactions, either activating or inhibitory, that they may exert against ADRA2A, or the coupled Ca^{+2}·calcineurin / NFAT signal transduction pathway.

The Reporter Cells in this kit are transiently transfected and prepared as frozen stocks using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method allows for the immediate dispensing of healthy, division-competent reporter cells into assay plates. There is no need for intermediate treatment steps such as spin-and-rinse of cells, viability determinations or cell titer adjustments prior to assay setup.

INDIGO's assay kits provide the convenience of an all-inclusive cell-based assay system. In addition to ADRA2A Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator Brimonidine (*a.k.a*. UK14,304), Luciferase Detection Reagents, and a cell culture-ready assay plate, and a detailed assay protocol.

▪ The Assay Chemistry ▪

INDIGO's 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-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.

▪ 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 setups in which a conventional **tip-based** instrument is used to dispense test compounds into assay wells (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**. The final concentration of DMSO carried over into assay reactions should not exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in 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.

or,

b.) Assay setups in which an **acoustic transfer** device is used to dispense test compounds into assay wells (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**.

▪ 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, and chronology of dispensed cells and test compounds are different between assay setups using a *tip-based dispenser* (**1a**) and those using an *acoustic transfer device* (**1b**). Following 22 -24 hours incubation Detection Substrate is added. 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.

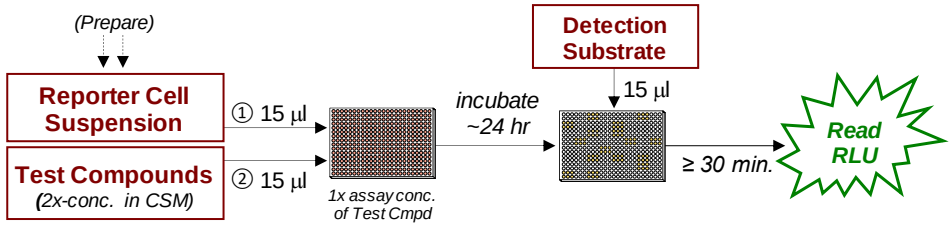
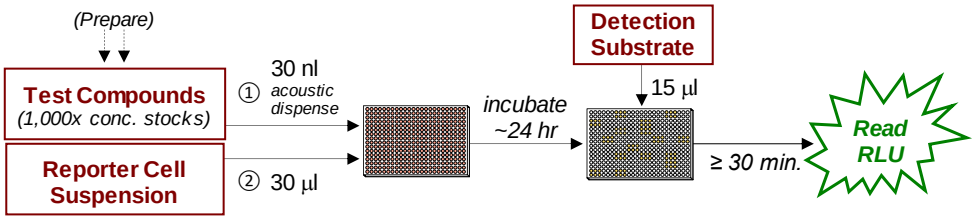


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



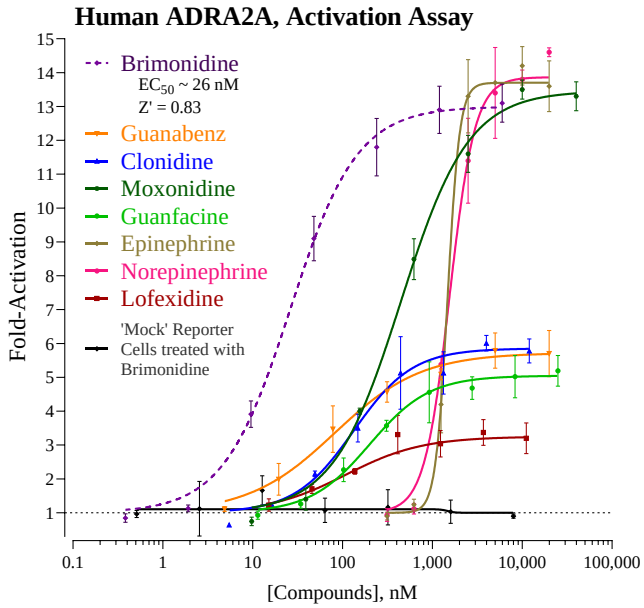


Figure 2. Activation of ADRA2A. Reporter cells were treated with the reference activators Brimonidine (*a.k.a.* UK14,304, provided), Guanabenz, Clonidine, Moxonidine, Guanfacine, Epinephrine, Norepinephrine, and Lofexidine. The absence of signal in Brimonidine treated 'Mock' cells (which contains the NFAT-Luc reporter vector, but do *not* express ADRA2A) confirms that the observed ligand-dependent response is specific to ADRA2A activation.

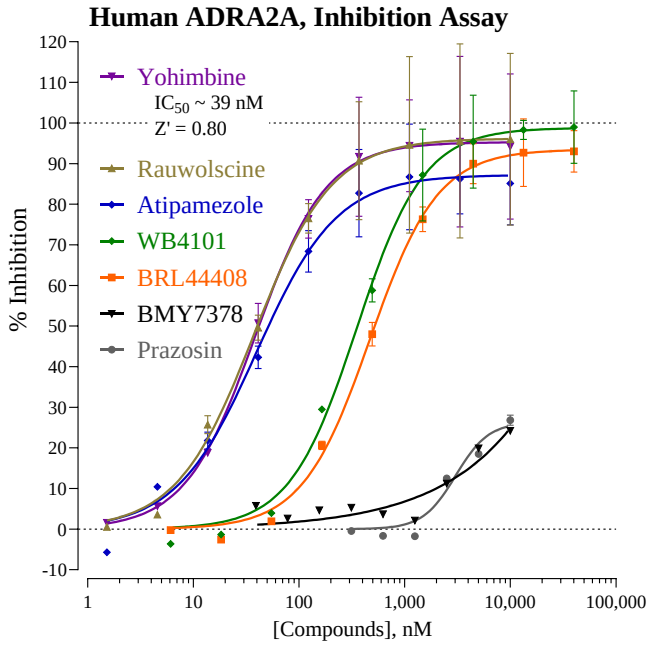


Figure 3. Inhibition of ADRA2A. Reporter cells were co-treated with an EC_{80} concentration of the reference activator Brimonidine and varying concentrations of the α_2 adrenoceptors selective inhibitors Rauwolscine, Yohimbine, Atipamezole, BRL44408, and the general alpha adrenoceptor inhibitors WB4101, BMY7378, and Prazosin. INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown).

For both the activation assay (Figure 2) and inhibition assay (Figure 3), luminescence was quantified and values of average (n=4) RLU, standard deviation (SD), Fold-Activation or % Inhibition, and Z'[10] values were calculated. The least-square method of non-linear-regression was used to plot activity changes *vs.* Log_{10}[Compound, nM], and EC_{50}/IC_{50} values were determined, using GraphPad Prism software. All chemicals were procured from Cayman chemical (Ann Arbor, MI, USA) except for Epinephrine, which was procured from Sigma Aldrich (Allentown, PA, USA).

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 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 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***	***minimum Storage Temp.***
▪ ADRA2A 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
▪ Brimonidine, 6.0 mM (in DMSO)	1 x 80 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	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

▪ 8-channel electronic, repeat-dispensing pipettes & tips suitable for dispensing 15 µl.

▪ disposable media basins, 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).

▪ *Optional:* antagonist reference compound (*e.g.*, Fig. 3.)

▪ *Optional:* clear 384-well assay plate for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-8* are performed on ***Day 1***, requiring less than 2 hours to complete. *Steps 9-13* are performed on ***Day 2*** and require less than 1 hour to complete.

▪ A word about antagonist-mode assay setups ▪

When setting up receptor inhibition assays the Reporter Cells are co-treated with a fixed sub-maximal concentration (typically between EC_{50} – EC_{85}) of the reference agonist AND varying concentrations of the test compound(s). This ADRA2A Assay kit includes a 6.0 mM stock solution of **Brimonidine** that may be used to setup inhibition-mode assays. ~110 nM of Brimonidine approximates EC_{80} in this assay. Hence, it is a suitable concentration of challenge agonist to use when screening test materials for inhibitory activities.

Add Brimonidine to a bulk volume of **CSM**, as described above. This agonist-supplemented medium is then used to prepare serial dilutions of test material stocks to achieve the desired respective assay concentrations. This is an efficient and precise method of setting up inhibition assays, and it is the method presented in *Step 5b* of this protocol, and *Step 6b* of the protocol when using an acoustic transfer device to dispense test compounds.

Note that when using a *tip-based instrument* for the dispensing of 2x-concentrated test compounds the cell suspension must also be supplemented with a **2x-**concentration (~220 nM) of the challenge agonist Brimonidine.

When using an *acoustic transfer* device for the dispensing of 1,000x-concentrated test compounds the cell suspension should be supplemented with a **1x-**concentration (~110 nM) of the challenge agonist Brimonidine.

DAY 1 Assay Protocol:

All steps should 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, residual DMSO carried over into assay reactions should not 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 45 ml of CSM.

b. *Acoustic dispensing method*: In *Step 6*, 30 nl / well of **1,000x**-concentrated test compound solutions are added to the assay plate using an acoustic transfer device.

Preparing the positive control: This assay kit includes a 6 mM stock solution of Brimonidine, an agonist of ADRA2A. The following 7-point treatment series, with concentrations presented in **5-fold** decrements, provides a complete dose-response: 6,000, 1,200, 240, 48.0, 9.60, 1.92, and 0.384 nM. Always include 'no treatment' (or 'vehicle') control wells.

APPENDIX 1a provides an example for generating such a dilution series to be used when *tip-dispensing* compound solutions prepared in CSM (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).

(continued)

When using t*ip-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 cells several times to gain a homogenous suspension.

a. **for *Agonist*-mode assays**: Dispense **15 µl / well** of cell suspension into the assay plate.

~ or ~

b. **for *Antagonist*-mode assays**: First supplement the bulk volume of Reporter Cell suspension with a 2x-concentration of the challenge agonist Brimonidine (refer to "*A word about antagonist-mode assay setup*", pg. 8). 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.

a. **for *Agonist*-mode assays**: Dispense **30 µl / well** of cell suspension into the assay plate that has been pre-dispensed with test compounds.

~ or ~

b. **for *Antagonist*-mode assays**: First supplement the bulk volume of Reporter Cell suspension with the challenge agonist Brimonidine to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 8). Then dispense **30 µl / well** of the supplemented 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.

(continued)

NOTE: Following the dispensing of Reporter Cells and test compounds INDIGO recommends performing 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 37°C, humidified, 5% CO_2 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 or in a **fume hood**.

Approximately 30 minutes before intending to quantify receptor activity remove **Detection Substrate** from the **9.)** 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. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time is 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** into all wells of the assay plate.

NOTE: 'Detection Substrate' contains a high concentration of DTT, which produces a strong odor that some users may find objectionable. It is advised to work in a **fume hood** when dispensing Detection Substrate into the assay plate and the following 'plate rest' period.

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. INDIGO recommends performing 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-minute reaction period the luminescence signal achieves a stable emission output.

13.) Quantify luminescence.

14.) Data analyses.

V. Related Products

Product No.	*Product Descriptions*
Human ADRA2A Assays	
IB34001	Human ADRA2A Reporter Assay System 1x 96-well format assay
IB34002	Human ADRA2A Reporter Assay System 1x 384-well format assay
Human ADRA1A Assays	
IB31001	Human ADRA1A Reporter Assay System 1x 96-well format assay
IB31002	Human ADRA1A Reporter Assay System 1x 384-well format assay
Human ADRA1B Assays	
IB31101	Human ADRA1B Reporter Assay System 1x 96-well format assay
IB31102	Human ADRA1B Reporter Assay System 1x 384-well format assay
Human ADRA1D Assays	
IB31201	Human ADRA1D Reporter Assay System 1x 96-well format assay
IB31202	Human ADRA1D Reporter Assay System 1x 384-well format assay
Human ADRB1 Assays	
IB32001	Human ADRB1 Reporter Assay System 1x 96-well format assay
IB32002	Human ADRB1 Reporter Assay System 1x 384-well format assays
Human ADRB2 Assays	
IB32101	Human ADRB2 Reporter Assay System 1x 96-well format assay
IB32102	Human ADRB2 Reporter Assay System 1x 384-well format assay
Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

Human ADRB3 Assays	
IB32201	Human ADRB3 Reporter Assay System 1x 96-well format assay
IB32202	Human ADRB3 Reporter Assay System 1x 384-well format assay
NFAT Assays (recommended for receptor specificity screening)	
IB18001	NFAT Reporter Assay System 1x 96-well format assay
Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays
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

INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents 10 mL, 25 mL, 50 mL, and 500 mL; custom volumes available

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Literature Citations

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$$Z' = 1 - [3*(SD^{Ref\ EC100} + SD^{Untreated}) / (RLU^{Ref\ EC100} - RLU^{Untreated})]$$

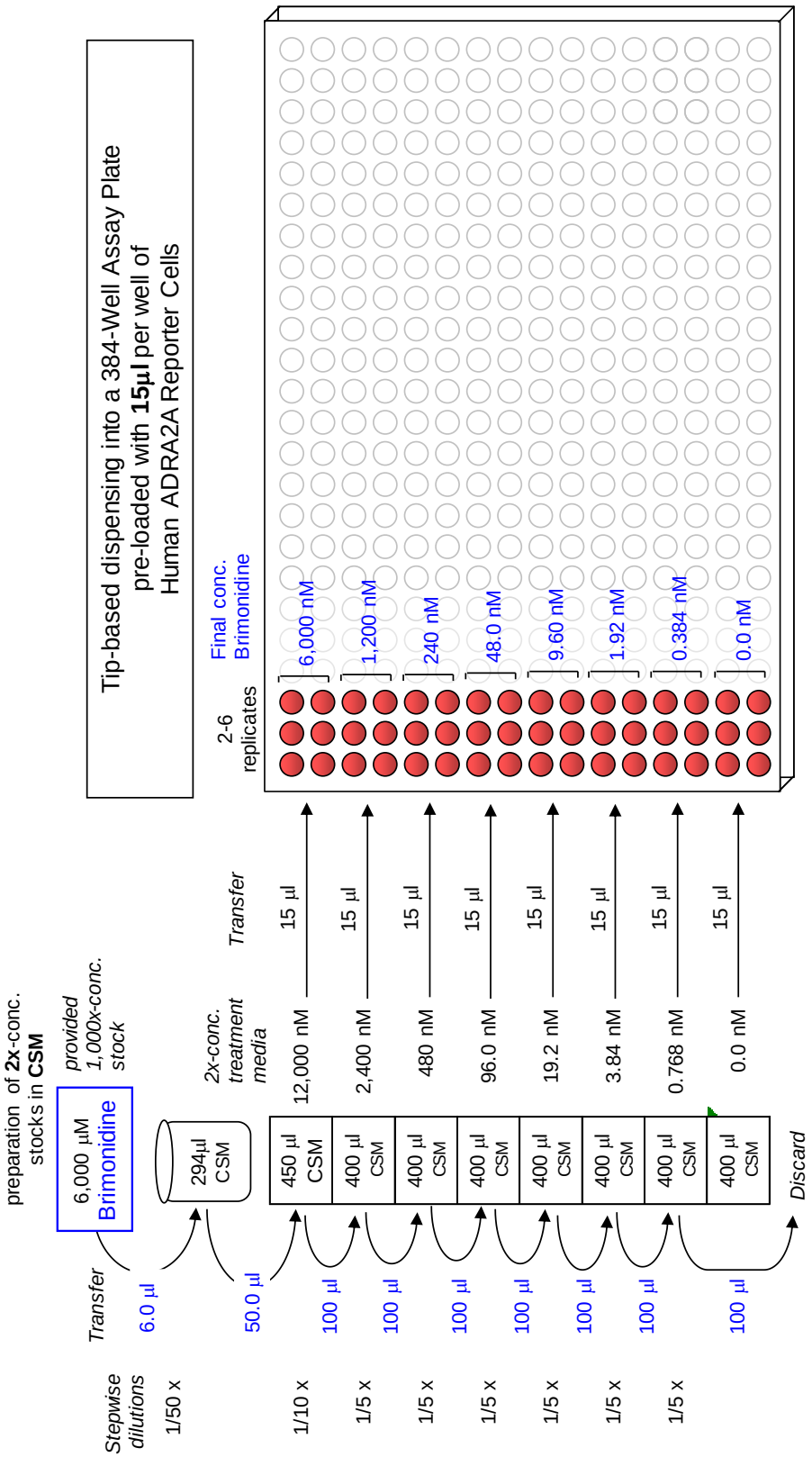
VII. Limited Use Disclosures

Products offered 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, 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 recently updated version available.

APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of Brimonidine using **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 ADRA2A Reporter Cells suspension.



APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the Brimonidine using **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 ADRA2A reporter cells.