Human Adrenoceptor Alpha 2A (ADRA2A) Reporter Assay System

96-well Format Assays
Product # IB34001

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Technical Manual
(version 7.2j)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human ADRA2A Reporter Assay System
96-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 that have been engineered to provide constitutive 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 nuclear receptor assays capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

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

INDIGO's Receptor Assays 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.) **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired assay concentration series, as described in *Step 7* (pg. 9).

Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions to produce 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the concentration of total DMSO (or any organic solvent) carried over into assay wells should not exceed 0.4%. Emerging cytotoxicity can be expected above 0.4% DMSO exposure over the 24-hour treatment period.

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

▪ Considerations for Automated Dispensing ▪

When using an automated dispensing instrument to process a small number of assay plates, first carefully consider the dead volume requirement of your 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 ▪

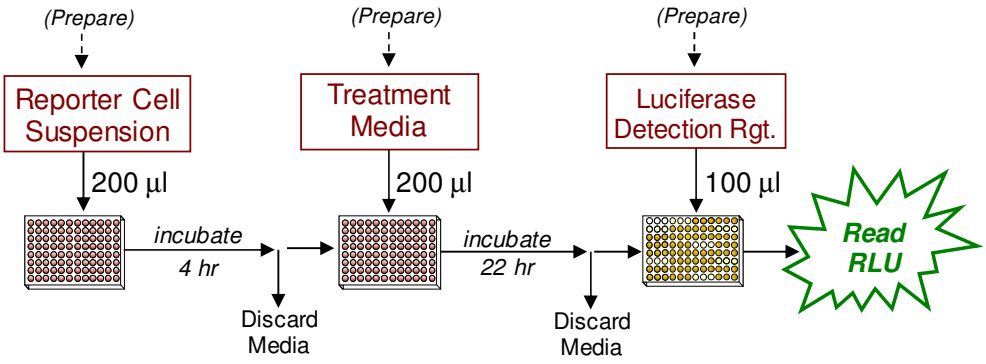

Figure 1. Assay workflow. Reporter Cells are dispensed into the assay plate and incubated for 4-6 hours. Following the pre-incubation period, the culture media are discarded, and the prepared treatment media are added. Following a 22-24 hour treatment period the media are discarded, and Luciferase Detection 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.

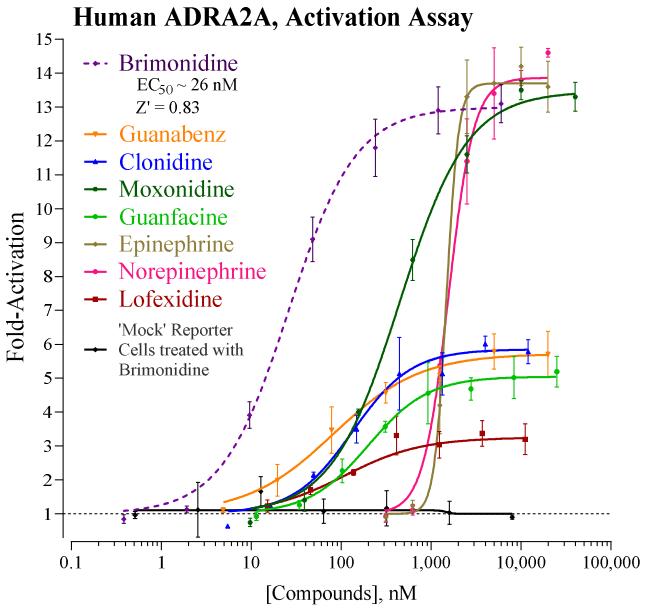

Figure 2. Activation of ADRA2A. Reporter cells were treated with the reference activators Brimonidine (*a.k.a.* UK14,304; provided), Guanabenz, Clonidine, Moxonidine, Guanfacine, Epinephrine, Norepinephrine, and Lofexidine. The absence of signal in Brimonidine treated 'Mock' cells (which contains the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

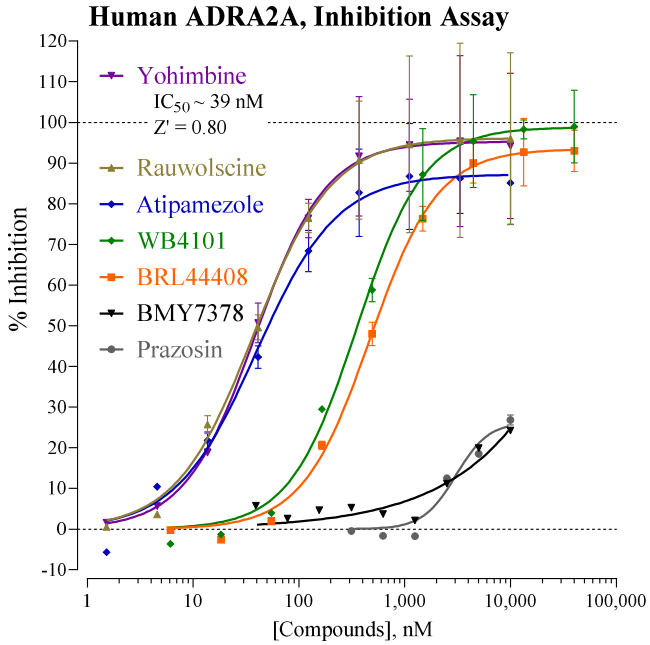

Figure 3. Inhibition of ADRA2A. Reporter cells were co-treated with an EC_{80} concentration of the reference activator Brimonidine and varying concentrations of the α_2 adrenoceptors selective inhibitors Rauwolscine, Yohimbine, Atipamezole, BRL44408, and the general alpha adrenoceptor inhibitors WB4101, 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 = 3) RLU, standard deviation (SD), Fold-Activation or % Inhibition, and Z'[10] values were calculated. The least-squares 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 Human ADRA2A Assay kit contains materials to perform assays in a single 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.

Kit Components	***Amount***	***minimum Storage Temp.***
▪ ADRA2A 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
▪ Brimonidine, 6 mM (in DMSO)	1 x 30 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well, *collagen-coated* assay plate (white, sterile, cell-culture ready)	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

▪ container of dry ice (see *Step 2*)

▪ 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 & sterile tips

▪ disposable media basins, sterile.

▪ sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).

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

▪ *Optional:* clear 96-well assay plate, collagen-coated, and cell culture treated, for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Please 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-hour incubation step to complete. *Steps 12-17* 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 6mM 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 7b* of this protocol.

DAY 1 Assay Protocol: All steps should 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 **ADRA2A Reporter Cells** from -80°C storage, place it directly into dry ice for transport to the laminar flow hood. When ready to begin, 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 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 electronic, repeat-dispensing 8-chanel pipette, dispense **200 µl / well** of cell suspension into wells of the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'cell blank' wells (meaning cell-free but containing CSM) must be included in the assay plate to allow quantification of plate-specific 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 this plate in an 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% CO_2) for 4 - 6 hours.

6.) Near the end of the pre-culture 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: Use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. 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, or any other organic solvent, carried over into assay reactions should not exceed 0.4%.

***a. Agonist*-mode assays**. This ADRA2A Assay kit includes a concentrated stock (6 mM) of Brimonidine prepared in DMSO. The following 7-point treatment series, with concentrations generated using serial 5-fold dilutions, provides a complete dose-response: 6,000, 1,200, 240, 48.0, 9.60, 1.92, and 0.384 nM. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') controls.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition assays, first supplement a bulk volume of CSM with the challenge activator **Brimonidine** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 8). The supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired series of treatment concentrations.

8.) At the end of the 4 - 6 hours 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-chanel 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 a bench top or in a **fume hood.**

12.) Approximately 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. 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*.

14.) Immediately before proceeding to *Step 15*, prepare **Luciferase Detection Reagent** (**LDR**). Combine 'Detection Buffer' and 'Detection Substrate' by pouring-over their entire volumes into a media basin; rock the basin gently to mix the reagent. The resulting volume of LDR is 12 ml.

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 preparing LDR, and subsequently when dispensing it into the assay plate followed by a 'plate rest' period (*Step 16*).

15.) Following 22 - 24 hours incubation in treatment media, discard the media contents by manually 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 the prepared **LDR** into all wells of the assay plate. Allow the assay plate to rest at room temperature for 5 – 10 minutes following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

18.) Data analyses.

V. Related Products

Product No.	*Product Descriptions*
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
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 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 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
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.

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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 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, and claims are subject to change without prior notice.

APPENDIX 1

Example scheme for the serial dilution of the reference agonist Brimonidine and the setup of a ADRA2A dose-response assay.