Human Adrenoceptor Alpha 1D Reporter Assay System (ADRA1D)

96-well Format Assays
Product # IB31201

▪

Technical Manual
(version 7.2i)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human ADRA1D Reporter Assay System
96-well Format Assays

I. Description

▪ Background ▪

The adrenoreceptors (*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, adrenoreceptor 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].

Adrenoreceptors 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 adrenoreceptors are activated by the same endogenous catecholamines (epinephrine and norepinephrine); however, the specificity of their responses depends on the G-proteins and effectors systems they associate with in a tissue and time specific manner[1].

Adrenoreceptor alpha 1D (**ADRA1D**) signals through the $G\alpha_{q/11}$ family of G proteins[2]. Upon binding to a catecholamine, ADRA1D undergoes a conformational change that triggers the activation of $G\alpha_{q/11}$ proteins *via* an exchange of GDP with GTP, followed by the activation of phospholipase C, the release of inositol triphosphate (IP3) which binds to its receptors on the endoplasmic reticulum and triggers the release of calcium and activation of the protein kinase C.

ADRA1D is abundantly expressed in the brain[3]. ADRA1D is highly distributed in dentate nucleus neurons of the cerebellum[3]. Major roles of ADRA1D include involvement in cardiovascular, urinary and central nervous system functions[4]. Although ADRA1D is closely related to ADRA1A and ADRA1B the precise physiological roles of ADRA1D have yet to be firmly established[5].

▪ The Assay System ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of the **Human Adrenoreceptor Alpha 1D (ADRA1D)**.

ADRA1D activation of the PLC pathways leads to an increase in intracellular calcium and the concomitant activation of calcineurin, a calcium-dependent phosphatase. Ca^{+2}-calcineurin acts to dephosphorylate and activate the transcription factor NFAT[6]. ADRA1D activation of the Ca^{+2}·calcineurin > NFAT cascade is the signal transduction pathway exploited by the reporter cells provided in this kit.

INDIGO's ADRA1D Reporter Cells contain an engineered luciferase reporter gene functionally linked to tandem consensus sequences of NFAT genetic response elements upstream of a minimal promoter. Activated NFAT binds to these response elements to seed the formation of a complete transcription complex that drives Luc 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 ADRA1D 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 ADRA1D, or the coupled Ca^{+2}·calcineurin / NFAT signal transduction pathway.

INDIGO's Reporter Cells are transiently transfected and prepared as frozen stocks using a 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 ADRA1D Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator L-Phenylephrine, Luciferase Detection Reagents, 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 O_2 and ATP as co-substrates to yield 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).

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.) **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 ▪

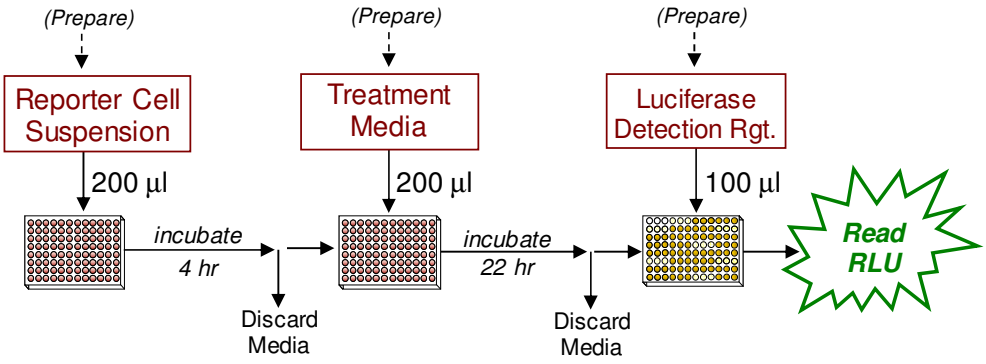


Figure 1. Assay workflow. Reporter Cells are dispensed into the assay plate and incubated for 4-6 hours. Following the pre-incubation period, the culture media are discarded, and the prepared treatment media are added. Following a 22–24-hour treatment period the media are discarded, and Luciferase Detection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

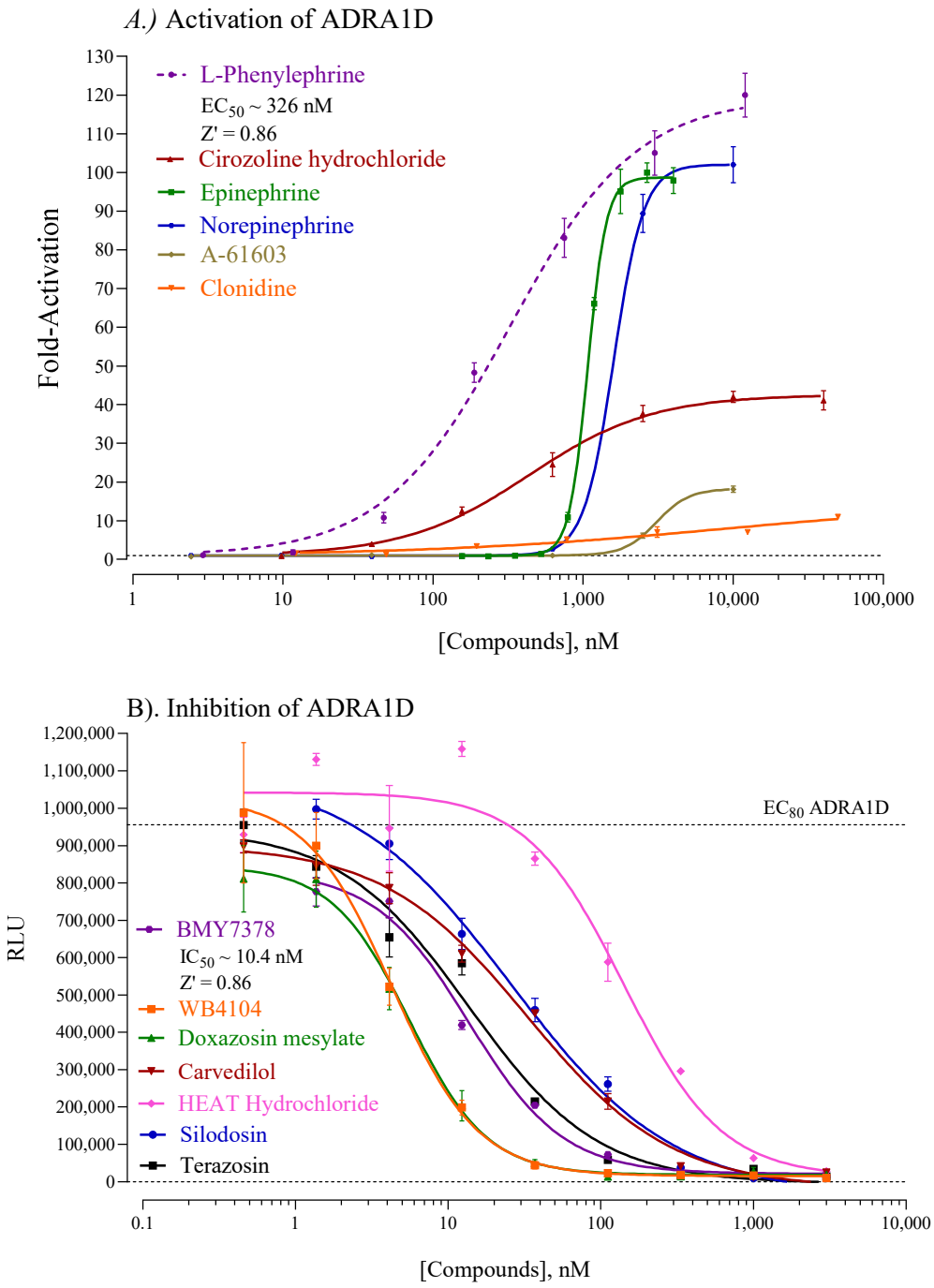


Figure 2. *A.) Activation of ADRA1D.* Activation assays were performed using the reference compounds L-phenylephrine (provided), Cirozoline•HCl, Epinephrine, Norepinephrine, A-61603, and Clonidine.

B.) Inhibition of ADRA1D. ADRA1D reporter cells were co-treated with an EC_{80} concentration of the reference activator L-phenylephrine and varying concentrations of the general alpha adrenergic receptor inhibitors, BMY7378, WB4104, Doxazosin mesylate, Carvedilol, HEAT Hydrochloride, Silodosin and Terazosin. INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown).

Luminescence was quantified and values of average (n = 3) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, and Z'[7] values were calculated. The least-squares method of non-linear regression was used to plot Fold-Activation or RLU *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.

II. Product Components & Storage Conditions

This Human ADRA1D 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*	*Storage Temp.*
▪ ADRA1D 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
▪ L-Phenylephrine (12 mM in DMSO)	1 x 30 µL	-20°C
▪ Detection Substrate	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:* 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 ADRA1D Assay kit includes a 12 mM stock solution of **L-Phenylephrine** that may be used to set up inhibition-mode assays. 1.0 µM of L-phenylephrine approximates $EC_{70\text{-}80}$ in this assay. Hence, it is a suitable concentration of challenge agonist to use when screening test materials for inhibitory activities.

Add L-phenylephrine 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 **ADRA1D 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 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 ADRA1D Assay kit includes a concentrated stock (12 mM) of L-phenylephrine prepared in DMSO. The following 7-point treatment series, with concentrations generated using serial 4-fold dilutions, provides a complete dose-response: 12,000, 3,000, 750, 187.5, 46.8, 11.7, and 2.93 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 **L-phenylephrine** 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.

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.

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 ADRA1B Assays	
IB31101	Human ADRA1B Reporter Assay System 1x 96-well format assay
IB31102	Human ADRA1B Reporter Assay System 1x 384-well format assays
Human ADRA1A Assays	
IB31001	Human ADRA1A Reporter Assay System 1x 96-well format assay
IB31002	Human ADRA1A Reporter Assay System 1x 384-well format assays
Human ADRA1D Assays	
IB31201	Human ADRA1D Reporter Assay System 1x 96-well format assay
IB31202	Human ADRA1D Reporter Assay System 1x 384-well format assays
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 assays
Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

NFAT Assays (recommended for receptor specificity screening)	
IB18001	NFAT Reporter Assay System 1x 96-well format assay

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 in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Citations

[1] Perez DM (2020) α1-Adrenergic Receptors in Neurotransmission, Synaptic Plasticity, and Cognition. Front. Pharmacol. 11:581098. doi: 10.3389/fphar.2020.581098.

[2] Wu, D., *et. al*. (1992) Activation of Phospholipase C by α1-Adrenergic Receptors is mediated by the α subunits of Gq family. JBC 267(36):25798-25802.

[3] Schambra UB, *et. al.* (2005) Nueron specifi alpha-adrenergic receptor expression in human cerebellum: implications for emerging cerebellar roles in neurologic disease. Neuroscience: 135 (2) 507-523.

[4] Camp ND, *et. al.* (2015) Individual protomers of a G protein-coupled receptor dimer integrate distinct functional modules. Cell Discov: 1: 15011

[5] Janezic EM ., *et. al*. (2020) N-glycosylation of α_{1D} -adrenergic receptor N-terminal domain is required for correct trafficking, function and biogenesis. Sci Rep: 10: 7209.

[6] Park JY, *et. al.* (2020) The Role of Calcium-Calcineurin-NFAT Signaling Pathway in Health and Autoimmune Disease, Frontiers in Immunology.:doi:10.3389/fimmu.2020.00195

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

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

VII. Limited Use Disclosures

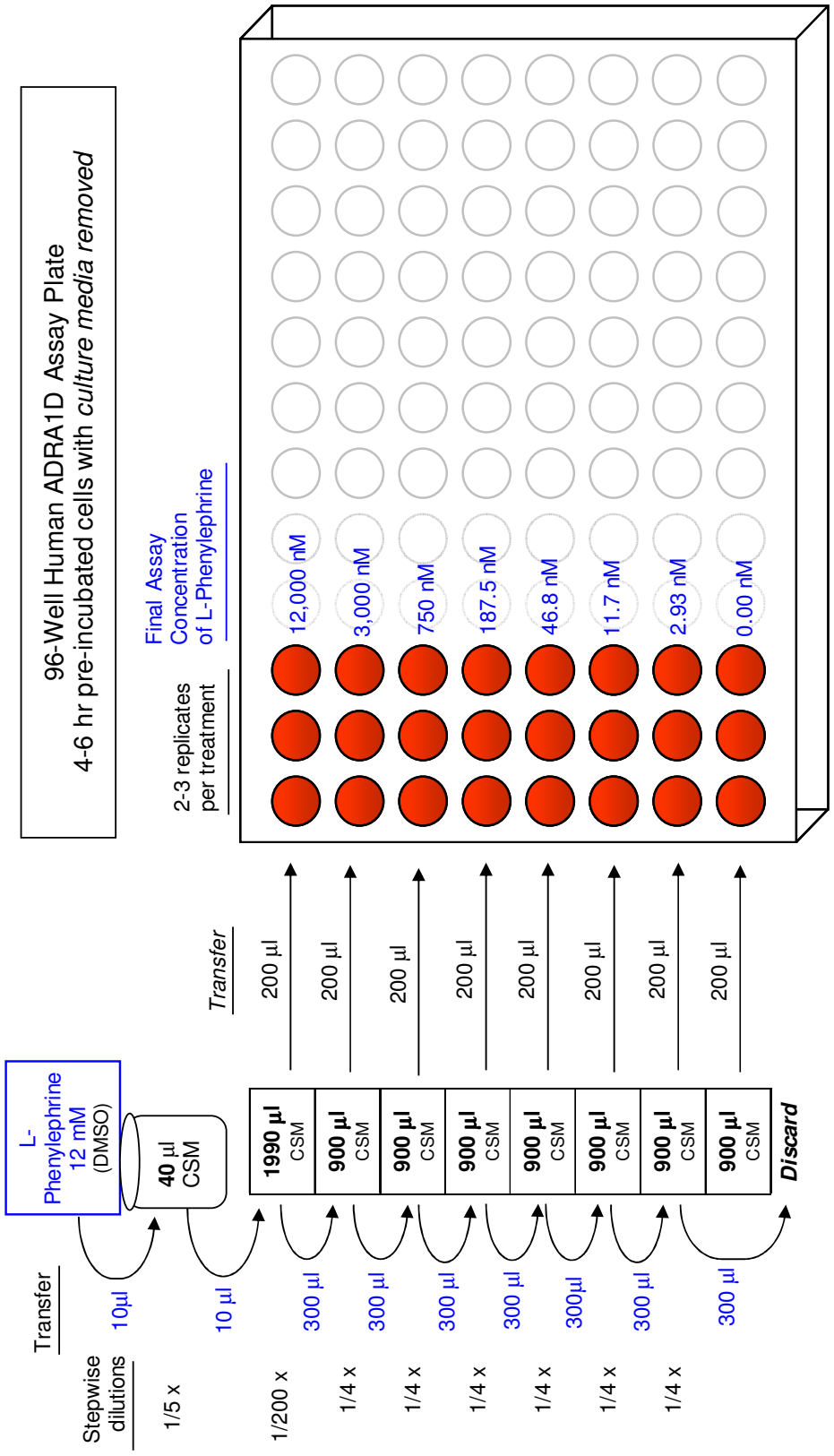
Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

“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 1

Example scheme for the serial dilution of the reference agonist L-Phenylephrine and the setup of an ADRA1D dose-response assay.



Human Adrenoceptor Alpha 1D Reporter Assay System (ADRA1D)

384-well Format Assays
Product # IB31202

▪

Technical Manual
(version 8.0)

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▪ 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** the user will choose to dilute master stocks using one of two alternative methods. The selection of dispensing method to be used will be dictated by the type of instrument that will 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 **µL volumes** of test compounds into assay wells (protocol is presented 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 never exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO.

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.) **Acoustic transfer** or **Pin-based dispensing of nL volumes** of test compounds into assay wells (protocol is presented 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.

(continued …)

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.

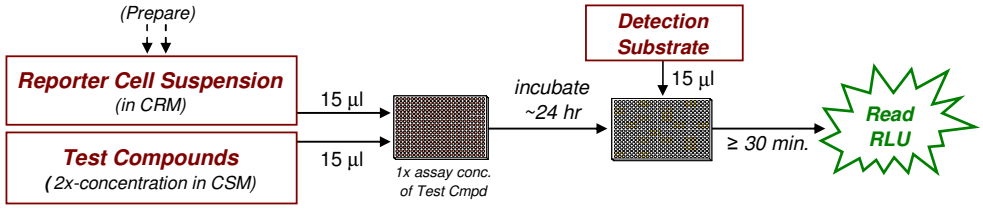
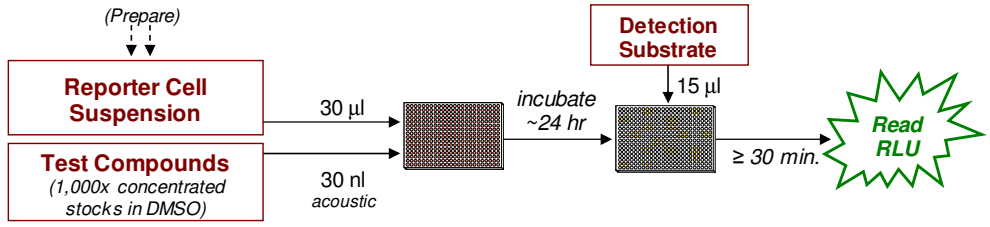


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



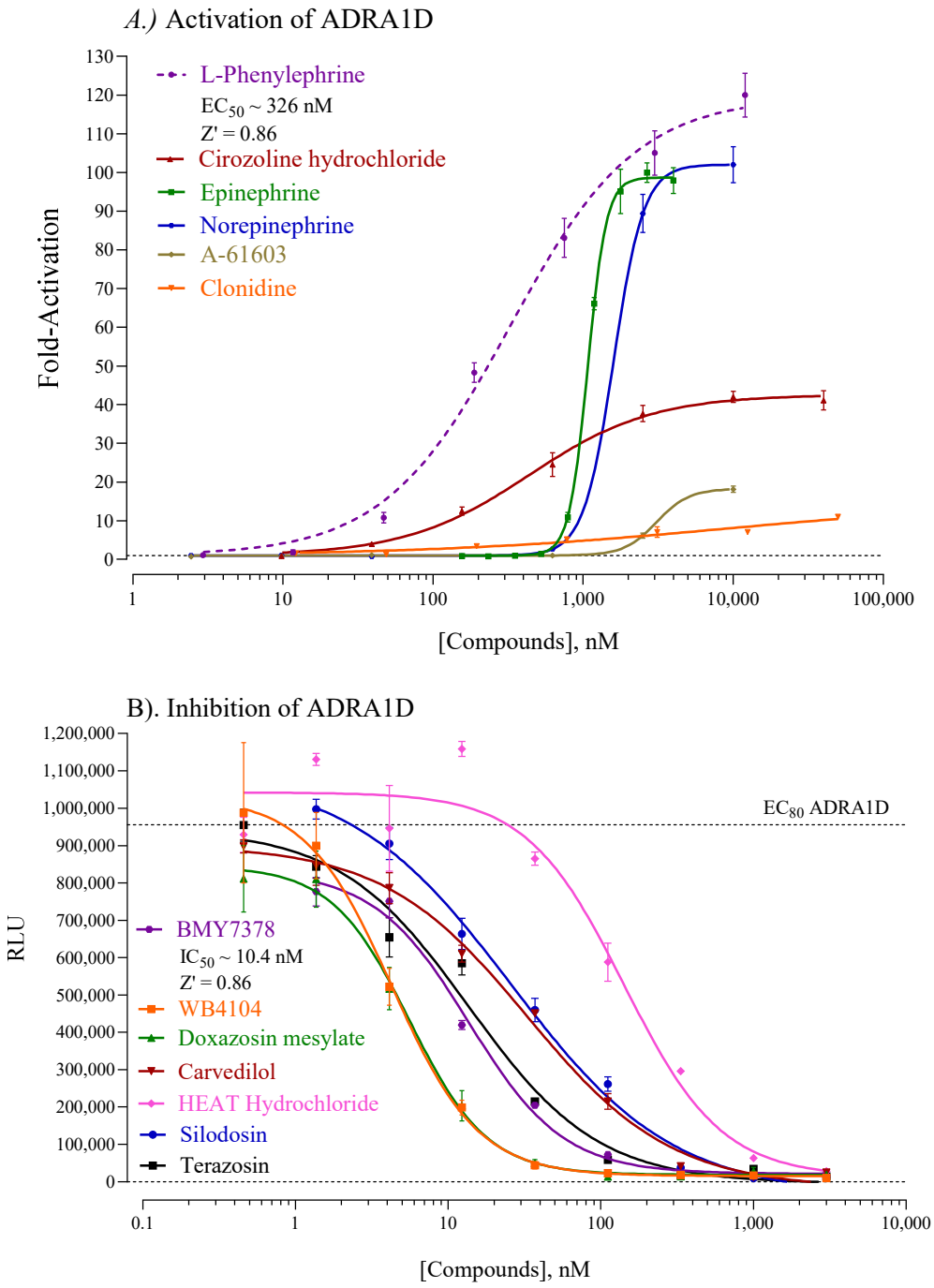


Figure 2. *A.) Activation of ADRA1D.* Activation assays were performed using the reference compounds L-phenylephrine (provided), Cirozoline•HCl, Epinephrine, Norepinephrine, A-61603, and Clonidine.

B.) Inhibition of ADRA1D. ADRA1D reporter cells were co-treated with an EC_{80} concentration of the reference activator L-phenylephrine and varying concentrations of the general alpha adrenergic receptor inhibitors, BMY7378, WB4104, Doxazosin mesylate, Carvedilol, HEAT Hydrochloride, Silodosin and Terazosin. INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown).

Luminescence was quantified and values of average (n = 3) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, and Z'[7] values were calculated. The least-squares method of non-linear regression was used to plot Fold-Activation or RLU *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.

II. Product Components & Storage Conditions

This Human ADRA1D Reporter 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*	*Storage Temp.*
▪ ADRA1D 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
▪ L- Phenylephrine, 12 mM (in DMSO) reference agonist for ADRA1D	1 x 80 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.

▪ mammalian cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2)

▪ 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. 2B)

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 setup ▪

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known agonist AND varying concentrations of the test compound(s) to be evaluated for antagonist activity. This ADRA1D Reporter Assay kit includes a 12 mM stock solution of L-Phenylephrine, a potent agonist of ADRA1D that may be used to set up antagonist-mode assays. 1.0 µM L-Phenylephrine typically approximates $EC_{70\text{-}80}$ in this assay. Hence, it presents a suitable *final assay concentration* of agonist to be used when screening test compounds for inhibitory activity.

Adding the challenge agonist L-Phenylephrine to the bulk suspension of Reporter Cells (*i.e.,* prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 5b* of the protocol when performing tip-based dispensing, 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 (2.0 µM) of the challenge agonist L-Phenylephrine.

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 1.0 µM of the challenge agonist L-Phenylephrine.

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 both the 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 wells should not exceed 0.4%.

a. *Tip dispensing method*: In *Step 6*, 15 µl / well of the prepared treatment media is added into assay wells that have 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 (prepared in DMSO) are added to the assay plate using an acoustic transfer device.

Preparing the positive control: This assay kit includes a 12 mM stock solution of **L-Phenylephrine**, a potent reference agonist of ADRA1D The following 7-point treatment series, with concentrations presented in 4-fold decrements, provides a complete dose-response: 12,000, 3,000, 750, 187.5, 46.8, 11.7, and 2.93 nM. Always include 'no treatment' (or 'vehicle') control wells. **APPENDIX 1** provides guidance for generating such a dilution series.

APPENDIX 1a provides an example for generating this dilution series to be used when *tip-dispensing* compound solutions prepared in CSM (15 µl / well).

APPENDIX 1b provides an example for generating a series of 1,000x-concentrated solutions of compounds prepared in DMSO to be used when performing *acoustic dispensing* (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 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**: Supplement the bulk volume of Reporter Cells suspension with a 2x-concentration of the challenge agonist (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 ADRA1D Reporter Cells suspension with the challenge agonist **L-Phenylephrine** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). 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.

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. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Set read-time 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 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 30-minute rest period allows the luminescence signal to achieve stable emission output.

13.) Quantify luminescence.

14.) Analyze data.

V. Related Products

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

NFAT Assays (recommended for receptor specificity screening)	
IB18001	NFAT Reporter Assay System 1x 96-well format assay

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 in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Citations

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[3] Schambra UB, *et. al.* (2005) Nueron specifi alpha-adrenergic receptor expression in human cerebellum: implications for emerging cerebellar roles in neurologic disease. Neuroscience: 135 (2) 507-523.

[4] Camp ND, *et. al.* (2015) Individual protomers of a G protein-coupled receptor dimer integrate distinct functional modules. Cell Discov: 1: 15011

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

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

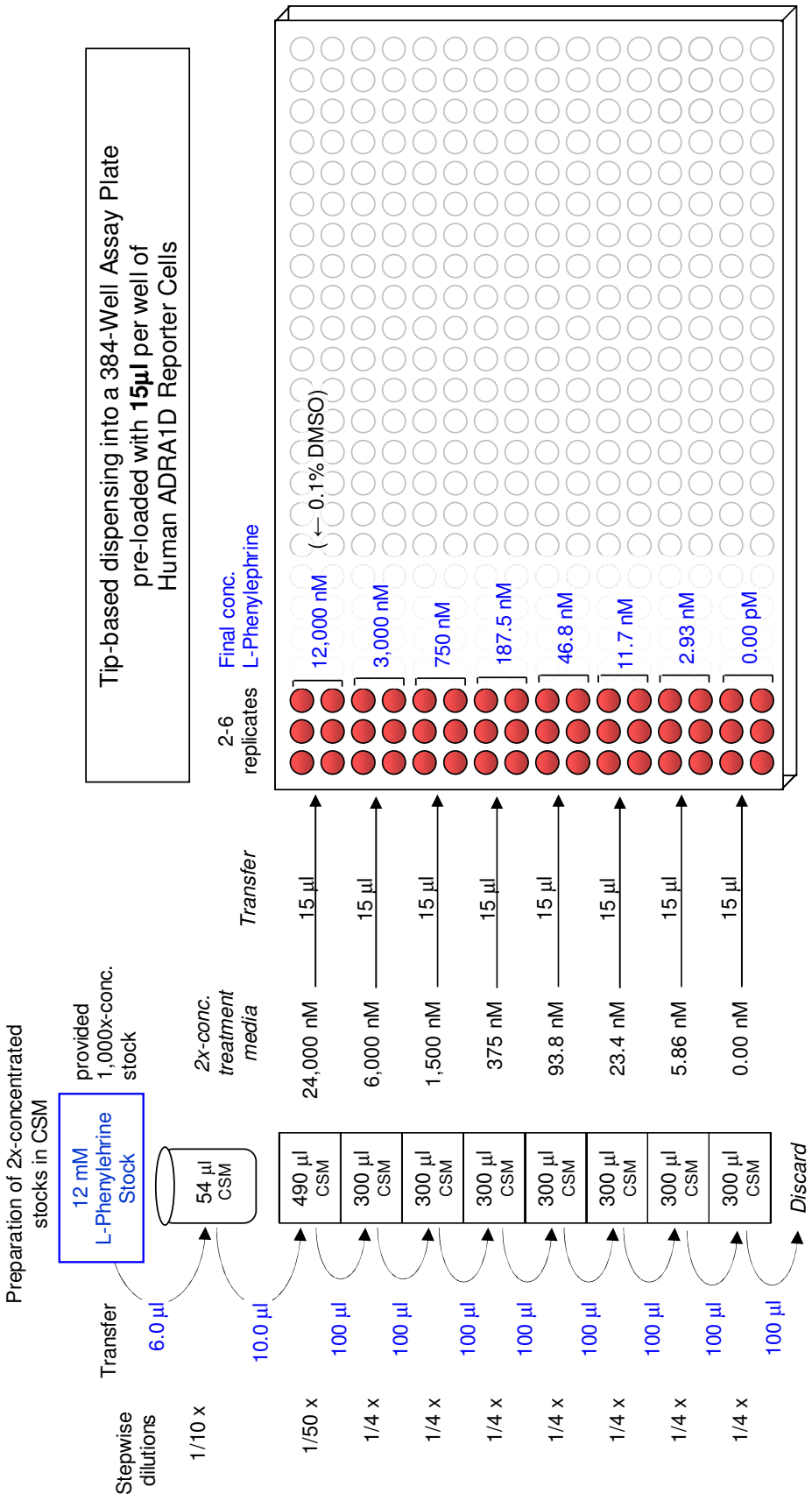
VII. Limited Use Disclosures

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 the reference agonist L-Phenylephrine 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 ADRA1D Reporter Cells suspension.



APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the reference agonist L-Phenylephrine into DMSO to generate **1,000x-concentrated** stocks. 30 nl / well are pre-dispensed into an empty assay plate using an acoustic transfer device.