INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human
Cannabinoid Type 1 Receptor
Reporter Assay System
(CB1R; CNR1)

3x 32 Assays in 96-well Format
Product # IB19001-32

▪

Technical Manual
(version 7.2)

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I. Description

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▪ The Assay System ▪

This assay utilizes proprietary human cells that provide constitutive expression of the **Human Cannabinoid Type 1 Receptor,** referred to herein as **CB1R**.

CB1R is one of the two main cannabinoid receptors identified as part of the endocannabinoid system. CB1R is highly expressed in the central nervous system, but also in skeletal muscles, gastrointestinal tract, liver, pancreas, skin, reproductive and cardiovascular systems[1]. The CB1R is involved in a wide range of physiological and pathophysiological processes related to neurological disorders, energy metabolism, obesity, diabetes, cardiovascular and reproductive disorders, inflammation, and cancer[1].

CB1R is a member of the super-family of G-protein coupled receptors (GPCR). Its structure includes seven transmembrane helices, with an extracellular amino end and an intracellular carboxy tail associated with trimeric G proteins. Upon ligand binding, the receptor undergoes conformational change that triggers the activation of G proteins *via* an exchange of GDP to a GTP.

Modes of CB1R signal transduction are diverse and may involve a variety of signaling molecules and kinases that vary based on the type of ligand, and the type of cells in which the receptor is expressed[1]. The first signaling pathway described for CB1R involved the activation of a pertussis toxin sensitive G protein ($G\alpha_{i/o}$), leading to the inhibition of forskolin-stimulated cyclic AMP, activation of G-protein coupled inwardly rectifying potassium channels (GIRKs) and an inhibition of several calcium channels. This is followed by recruitment of β-arrestin, a cytosolic protein involved in receptor desensitization and internalization. However, β-arrestin activation is also involved in the activation of downstream kinases such as MAPK[1,2].

CB1R can also couple with stimulatory G proteins such as $G\alpha_s$ and $G\alpha_{q/11}$, resulting in the activation of ERK and PLC pathways and increased levels of intracellular calcium[1,2]. An outcome of CB1R activation is that calcineurin, a calcium-dependent phosphatase, dephosphorylates and activates the transcription factor NFAT[3]. Signal transduction *via* the Ca^{+2}•calcineurin / NFAT cascade is exploited by the reporter cells included in this kit.

INDIGO's Reporter Cells contains an engineered luciferase reporter gene functionally linked to tandem NFAT genetic response elements (GRE) and a minimal promoter. Activated NFAT binds to its corresponding GRE's to initiate 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, dose-dependent surrogate measure of drug-induced changes in CB1R activity. Accordingly, the principal application of this reporter assay is in the screening of test compounds to quantify any functional activities, either activating or inhibitory, that they may exert against CB1R or its associated 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 CB1R Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator synthetic cannabinoid CP-55,940, Luciferase Detection Reagents, and a cell culture-ready assay plate.

[1] Haspula D. and Clark, M.A. (2020) Cannabinoid Receptors: An update on cell signaling, pathophysiological roles and therapeutic opportunities in neurological, cardiovascular, and inflammatory diseases. Int. J. Mol. Sci. **21**: 7693-7753.

[2] Turu, G. and Hunyady L. (2010) Signal transduction of the CB1 cannabinoid receptor, J. of Mol. Endocrinol. **44**: 75-85.

[3]Gwack Y et al (2007) Signalling to transcription: store-operated Ca2+ entry and NFAT activation in lymphocytes. Cell Calcium. 42:145-56.

▪ 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 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. Note that the final concentration of DMSO carried over into assay wells should *not* exceed 0.4%.

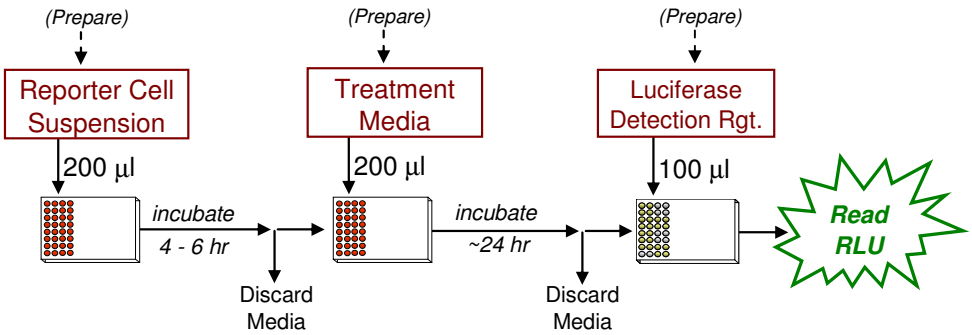
Immediately prior to setting up an assay the prepared stocks are serially diluted using **Compound Screening Medium (CSM)** to achieve the desired assay concentrations, as described in *Step 7* (pg. 9).

> *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.

▪ Assay Scheme ▪

Figure 1. Assay workflow.

In brief, 200 µl of Reporter Cells is dispensed into wells of the assay plate and for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared treatment media are added. Following 22-24 hr incubation, discard the treatment media and add Luciferase Detection Reagent. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



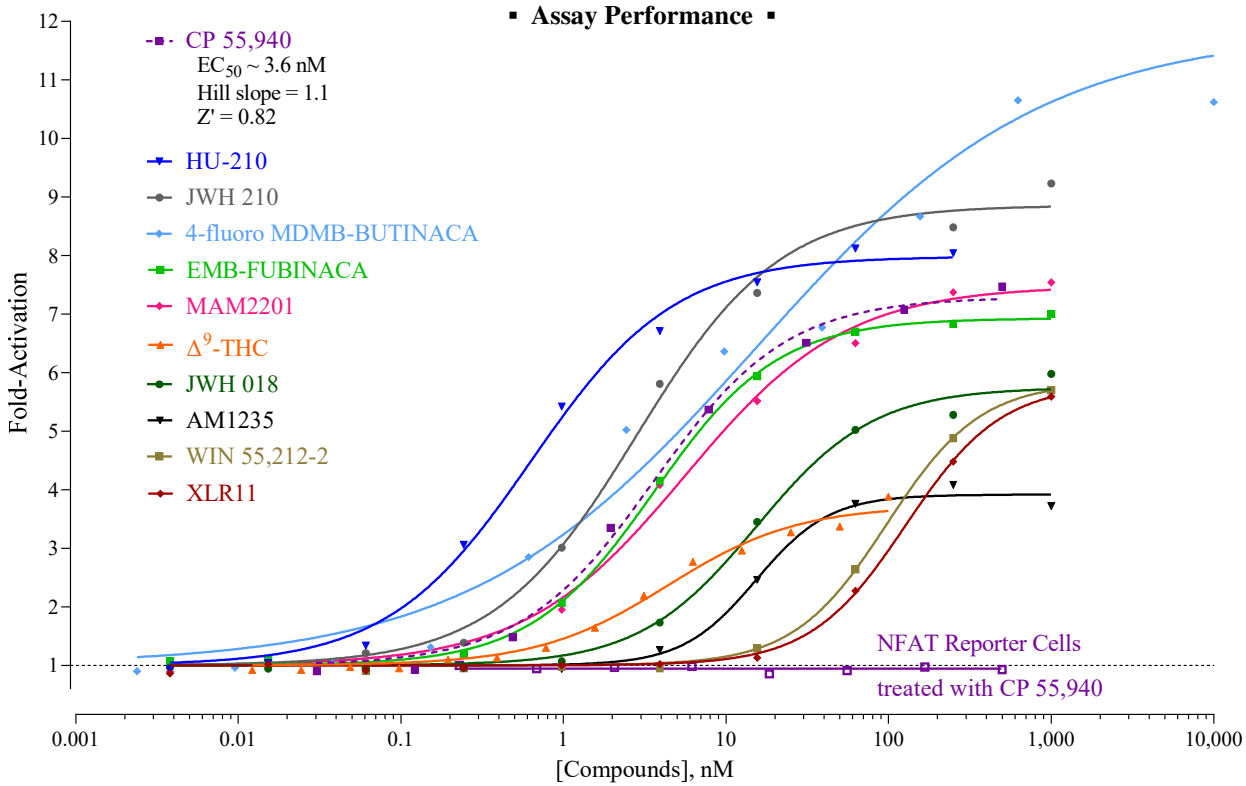


Figure 2. Human CB1R Agonist dose-response analyses. Agonist dose-response assays were performed using the reference compounds CP 55,940 (provided), HU-210, JWH 210, 4-fluoro MDMB-BUTINACA, EMB-FUBINACA, MAM2201, Δ^9-THC, JWH 018, AM1235, WIN 55,212-2 and XLR11 (all compounds from Cayman Chemical, Ann Arbor MI, USA). Luminescence was quantified and values of average relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, and Z'[4] values were calculated. Non-linear regression analyses of Fold-Activation *vs.* Log_{10} [Agonist, nM] were plotted and EC_{50} values determined using GraphPad Prism software. Coefficients of variation ranged between 3 – 12% for the various treatment concentrations; n = 3. Error bars are omitted for visual clarity. The absences of signal in CP 55,940 treated NFAT Reporter cells (which do not express CB1R) confirms that the response observed in the CB1R Reporter Cells is specific to cannabinoid receptor function.

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

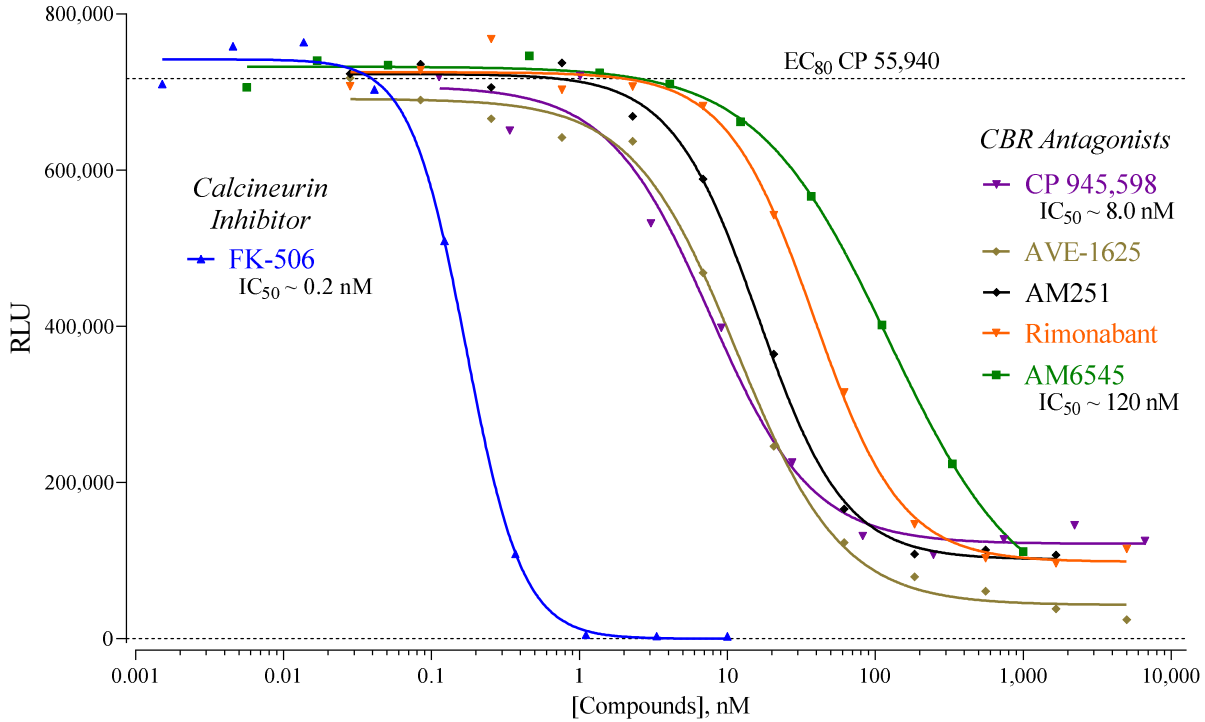


Figure 3. Human CB1R Antagonist dose-response analyses. CB1R reporter cells were co-treated with an EC_{80} concentration of the reference agonist CP 55,940 and varying concentrations of the CB1R reference antagonists CP 945,598, AVE-1625, AM251, Rimonabant, and AM6545. The range of determined IC_{50} values is shown. Treating the CB1R reporter cells with the calcineurin inhibitor FK-506 reaffirms that agonist-activated CB1R signals *via* the Ca^{+2}•Calcineurin / NFAT signal transduction cascade. INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown). Non-linear regression analyses of RLU *vs.* Log_{10}[Antagonist, nM] were plotted and IC_{50} determinations made using GraphPad Prism software. Coefficients of variation ranged between 3 – 11% for the various treatment concentrations; n = 3. Error bars are omitted for visual clarity. All compounds were obtained from Cayman Chemical (Ann Arbor MI, USA).

II. Product Components & Storage Conditions

This Human CB1R Assay kit contains materials to perform three distinct groups of assays in the format of a 96-well plate. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays.

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

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first 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.*
▪ CB1R Reporter Cells	3 x 0.6 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ CP 55,940 (1.0 mM in DMSO) (Synthetic cannabinoid, activator of CB1R)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate Frame	1	ambient
▪ Snap-in 8-well strips (white, sterile, collagen-coated wells)	12	**-20°C**

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

III. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

DAY 1

▪ 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.,* Fig. 3)
▪ *Optional:* clear 96-well assay plate, cell culture treated, for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

The assay protocol begins on the next page. 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 hr 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 ▪

Receptor inhibition assays expose the Reporter Cells to a fixed, 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 chemical inhibition. This CB1R Assay kit includes a 1 mM stock solution of **CP 55,940**, a potent synthetic cannabinoid activator of CB1R, that may be used to setup inhibition-mode assays. 16 nM CP 55,940 approximates EC_{80} in this assay. Hence, it presents a suitable concentration of challenge agonist to use when screening test materials for inhibitory activities.

Add CP-55,940 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 final assay concentrations. This is an efficient and precise method of setting up CB1R 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 **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **7.0 ml** per tube.

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

4.) *Gently* invert the tube(s) of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an electronic, repeat-dispensing 8-chanel pipette, dispense **200 µl / well** of cell suspension into the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 '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 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(s) and Reference Compound treatment media: Use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 9*, the prepared treatment media will be dispensed at 200 µl/well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO, or any other organic solvent, carried over into assay reactions should not exceed 0.4%.

***a. Agonist*-mode assays**. This CB1R Assay kit includes a concentrated stock of CP 55,940, 1 mM prepared in DMSO. The following 7-point treatment series, with concentrations generated using serial 6-fold dilutions, provides a complete dose-response: 1000, 167, 27.8, 4.63, 0.772, 0.129, and 0.0214 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 **CP 55,940** 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 cell pre-culture period:*** **Discard the culture media**. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (*e.g.,* Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

9.) Dispense **200 µl** of each treatment media into appropriate wells of the assay plate.

10.) 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.

11.) For greater convenience on *Day 2*, retrieve the appropriate number of vials of **Detection Substrate** *and* **Detection Buffer** from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

DAY 2 Assay Protocol: Subsequent manipulations do *not* require special regard for aseptic technique and may be performed on 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. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

14.) *Immediately before proceeding to Step 15*: To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

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

16.) Add 100 µl of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
Human CB1R Assays	
IB19001-32	Human CB1R Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB19001	Human CB1R Reporter Assay System 1x 96-well format assay
IB19002	Human CB1R Reporter Assay System 1x 384-well format assays
Bulk volumes of CB1R Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

NFAT Assays (recommended for receptor specificity screening)	
IB18001-32	NFAT Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
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 in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in **5x bulk volume** to perform **480** Live Cell Assays contained in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays contained in 10 x 96-well assay plates

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

Products 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 current version available.

APPENDIX 1

Example scheme for the serial dilution of the reference agonist CP 55,940 and the setup of a CB1R dose-response assay.