

**Human 5-Hydroxytryptamine Receptor 2A
Reporter Assay System
(Serotonin Receptor 5-HT_{2A}; HTR_{2A})**

96-well Format Assays
Product # IB54001

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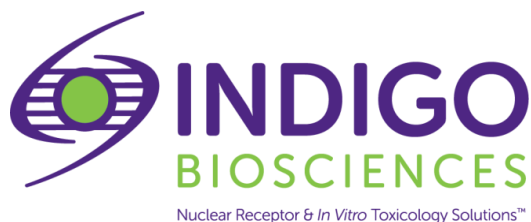
Technical Manual
(version 7.2i)

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Human HTR2A Reporter Assay System 96-well Format Assays

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I. Description

▪ Background ▪

Serotonin (5-hydroxytryptamine, 5-HT) receptors comprise seven receptor families (5-HT1 to 5-HT7) and 14 receptor subtypes in humans. With the exception of the 5-HT3 receptor, which is a ligand-gated ion channel, serotonin receptors are G-protein-coupled receptors (GPCRs), with the majority belonging to the class A family. Serotonin receptors are widely expressed throughout the central and peripheral nervous systems and are involved in diverse physiological processes, including cognition, mood, sensory perception, nociception, gastrointestinal function, cardiovascular regulation and sleep. Their broad tissue distribution and functional diversity make them important targets in drug discovery and pharmacological research^{1,2}.

Beyond primary drug discovery, serotonin receptors are also important in secondary pharmacology and safety assessment, where unintended interactions may identify potential off-target liabilities associated with central nervous system, cardiovascular, gastrointestinal, and other adverse pharmacological effects^{3,4}.

Among the serotonin receptors, 5-hydroxytryptamine receptor 2A (HTR2A, a.k.a. 5-HT2A) is an important target in CNS pharmacology and drug discovery. HTR2A is primarily coupled to Gq/11 signaling pathway, leading to phospholipase C activation, intracellular calcium mobilization, and downstream transcriptional responses². The receptor is highly expressed in the cerebral cortex and contributes to the regulation of cognition, perception, mood, and other CNS functions. HTR2A is targeted by diverse pharmacological agents, including psychedelic compounds that act as receptor agonists and atypical antipsychotics that exhibit antagonist or inverse agonist activity. Accordingly, HTR2A functional assays are useful for characterizing both on-target and off-target serotonergic activity and for evaluating compound potency, efficacy, and functional pharmacology²⁻⁵.

▪ The Assay System ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of the **Human 5-Hydroxytryptamine 2A (HTR2A)**.

INDIGO's HTR2A Reporter Cells contains an engineered luciferase reporter gene functionally linked to tandem consensus sequences of NFAT genetic response elements positioned upstream of a minimal promoter. 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 enzyme activity in the treated reporter cells relative to the untreated reporter cells provides a sensitive surrogate measure of drug-induced changes in HTR2A activity. The principal application of this reporter assay is in the screening of test compounds to quantify any functional activities or off target activities, either activating or inhibitory, that they may exert against HTR2A.

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 HTR2A Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator Serotonin, Luciferase Detection Reagents, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's 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).

INDIGO'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. The **Serotonin stock** included with this kit is prepared in DMSO at a 1,000x-concentration relative to the highest recommended treatment (as depicted in APPENDIX 1). 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. 10).

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.

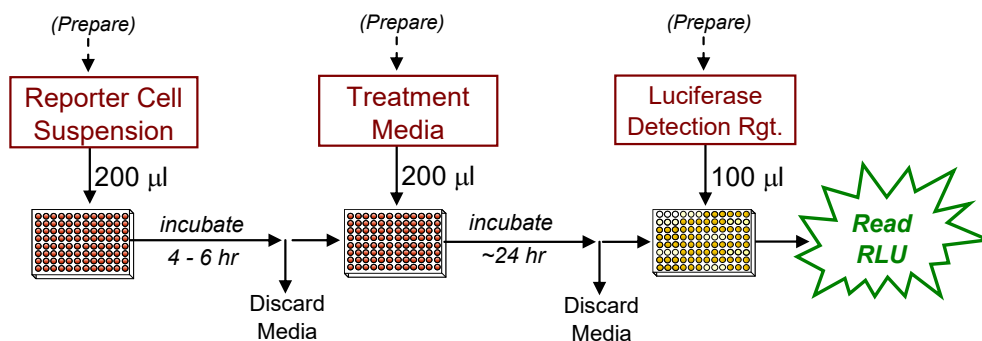
▪ Considerations for Automated Dispensing ▪

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. *In brief*, 200 µl of Reporter Cells is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded, and 200 µl/well of the prepared 1x-concentration treatment media are added. Following a 22–24-hours incubation, treatment 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.



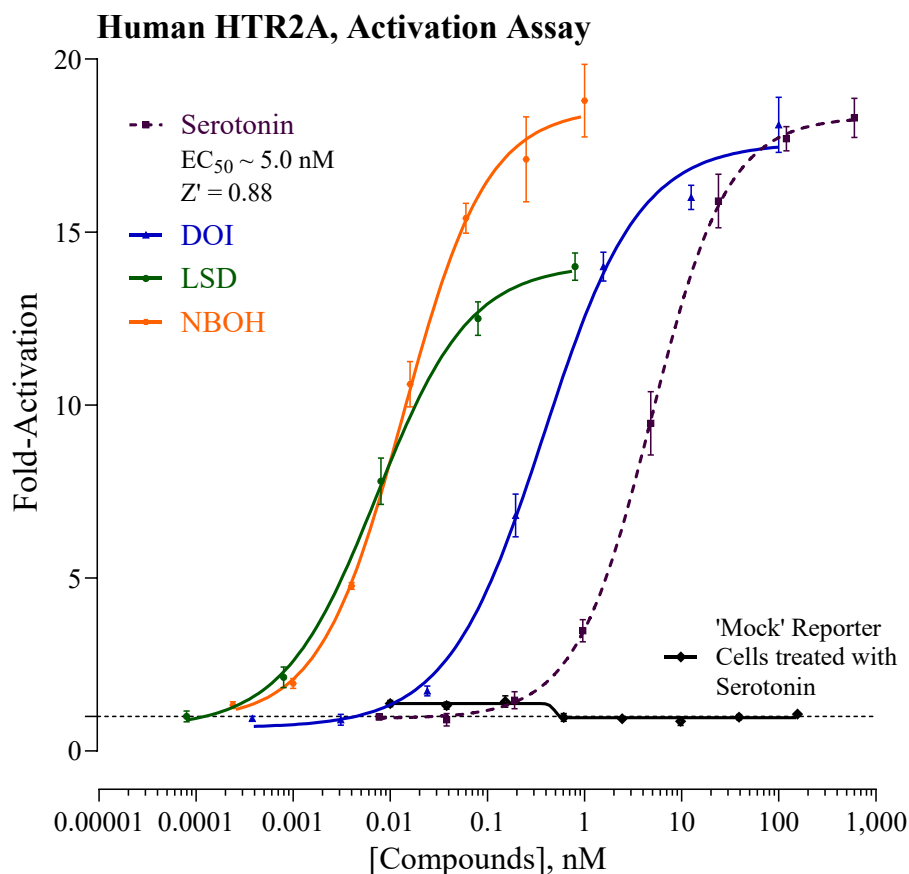


Figure 2. *Activation of HTR2A.* Activation assays were performed using the reference compounds Serotonin (provided), 2,5-Dimethoxy-4-iodoamphetamine (DOI), Lysergic Acid Diethylamide (LSD), and 25C-NBOH. The absence of signal in Serotonin treated 'Mock' cells (which contains the NFAT-Luc reporter vector, but do not express HTR2A) confirms that the observed ligand-dependent response is specific to HTR2A activation. Luminescence was quantified and values of average ($n = 3$) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, and Z' ⁶ values were calculated. The least-squares method of non-linear regression was used to plot Fold-Activation vs. Log₁₀ [Compound, nM] and EC₅₀ values were determined using GraphPad Prism software. All chemicals were procured from Cayman Chemical, Ann Arbor MI, USA.

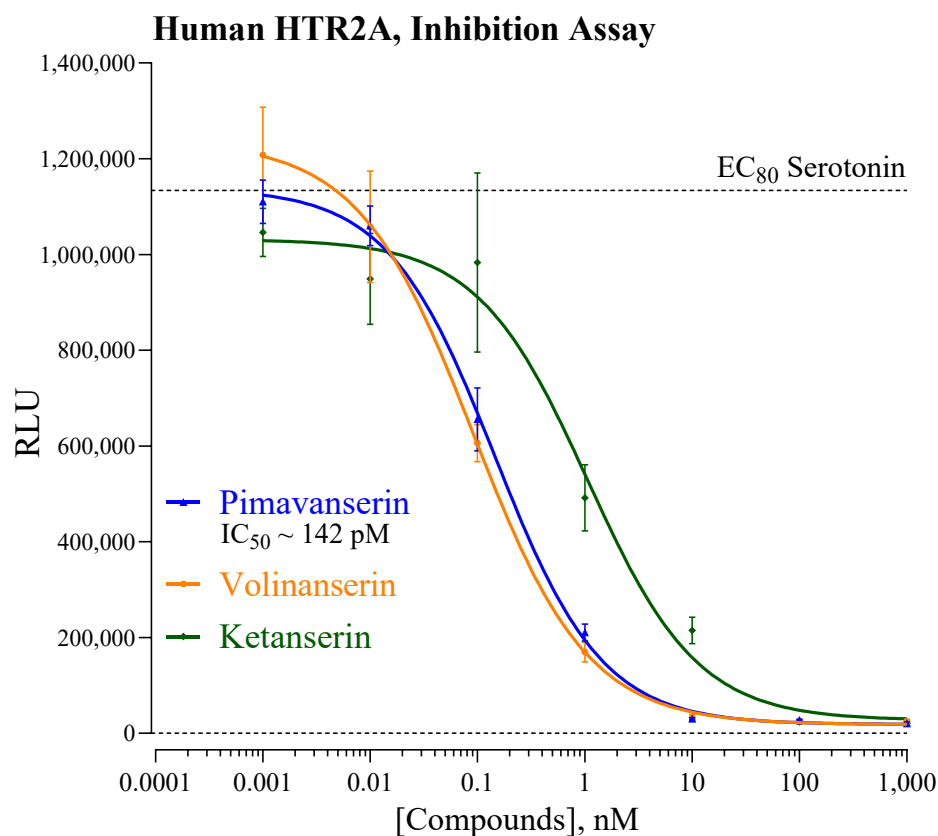


Figure 3. *Inhibition of HTR2A.* HTR2A reporter cells were co-treated with an EC₈₀ concentration of the reference activator Serotonin and varying concentrations of the HTR2A specific inhibitors Pimavanserin and Volinanserin, as well as a broad antagonist Ketanserin. INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown).

Luminescence was quantified and values of average ($n = 3$) relative light units (RLU), and corresponding standard deviation (SD) values were calculated. The least-squares method of non-linear regression was used to plot RLU vs. Log_{10} [Compound, nM] IC₅₀ 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 HTR2A 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 CANNOT 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ HTR2A 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
▪ Serotonin (600 µM 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% CO₂ 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* sterilized 96 deep-well blocks (*e.g.*, Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference and test compound(s).
- *Optional:* reference antagonist (refer to Fig. 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

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 inhibition-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 HTR2A Assay kit includes a 600 μ M stock solution of **Serotonin** that may be used to setup inhibition-mode assays. ~15-20 nM of Serotonin approximates EC_{80} in this assay. Hence, it is a suitable concentration of challenge activator to use when screening test materials for inhibitory activities.

Add the challenge agonist (Serotonin) to a bulk volume of CSM at an EC_{50} – EC_{85} Concentration. This medium is then used to prepare serial dilutions of test compounds 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 **HTR2A 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. Transfer the cell suspension into a reservoir. Using an electronic, repeat-dispensing 8-channel pipette, dispense **200 μ l / well** of cell suspension into the 96-well 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. 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, collagen coated 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, $\geq 70\%$ humidity, 5% CO₂) 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 at the desired assay concentrations: Use **CSM** to prepare an appropriate dilution series of reference and test compound stocks. Prepare treatment media at the desired assay concentrations. In *Step 9*, **200 μ l / well** of the prepared treatment media are dispensed into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should not exceed 0.4%.

a. Activator-mode assays. This HTR2A Assay kit includes a concentrated stock (600 μ M) of Serotonin prepared in DMSO. The following 8-point treatment series, with concentrations generated using serial 5-fold dilutions, provides a complete dose-response: 600, 120, 24.0, 4.80, 0.960, 0.192, 0.038 and 0.0077 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 **Serotonin** to achieve an EC₅₀ – EC₈₀ concentration (refer to "*A word about inhibition-mode assay setup*", pg. 9). 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-incubation 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 μ l / 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-channel dispenser and dispense 100 μ l 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 ($\geq 70\%$) 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.

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

<i>Product No.</i>	<i>Product Descriptions</i>
Human HTR2A Assays	
IB54001	Human HTR2A Reporter Assay System 1x 96-well format assay
IB54001-32	Human HTR2A Reporter Assay System 3x 32-well format assay
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 10 mL, 25 mL, 50 mL, and 500 mL; custom volumes available
Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Citations

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- ³ Whitebread S., et. al. (2016) Secondary pharmacology: screening and interpretation of off-target activities - focus on translation. *Drug Discov Today.* Aug;21(8):1232-42. doi: 10.1016/j.drudis.2016.04.021. Epub 2016 Apr 29. PMID: 27140035.
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- ⁵ Gumpfer RH, Nichols DE. (2026) Chemistry/structural biology of psychedelic drugs and their receptor(s). *Br J Pharmacol.* Jul;183(14):3920-3934. doi: 10.1111/bph.17361. Epub 2024 Oct 2. PMID: 39354889.
- ⁶ 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

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Example scheme for the serial dilution of the reference agonist Serotonin and the setup of an HTR2A dose-response assay.

