

**Human Cholinergic Receptor, Muscarinic 1
Inhibition Reporter Assay System
(CHRM1)**

96-well Format Assays
Product # IB44101

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Technical Manual
(version 7.2i)

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Human CHRM1 Inhibition Reporter Assay System 96-well Format Assays

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I. Description

▪ Background ▪

Muscarinic acetylcholine receptors are members of the G-protein-coupled receptor (GPCR) superfamily that respond to the endogenous neurotransmitter acetylcholine. They are involved in a wide range of processes in the central and peripheral nervous system, which makes them of interest as drug targets for conditions including Alzheimer's disease, schizophrenia, and drug addiction¹. The muscarinic acetylcholine receptor subfamily is composed of five subtypes, known as M1 – M5, and by their corresponding genes names (i.e., CHRM1 – CHRM5). However, targeting a specific subtype has proved challenging due to the high degree of homology of the orthosteric binding site for acetylcholine.

The CHRM1 subtype is expressed in the cerebral cortex and hippocampus regions of the brain, pointing to its key role in cognitive function. Targeting the less conserved allosteric binding sites of CHRM1 have resulted in the development of selective activators of this receptor enabling the specific targeting of the M1 subtype². These opportunities for novel drug development ensure that M1 remains a viable therapeutic target.

▪ The Assay System ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of the **Human Cholinergic receptor, muscarinic 1 (CHRM1)**.

CHRM1 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 NFAT³. CHRM1 activation of the Ca^{+2} -calcineurin > NFAT cascade is the signal transduction pathway exploited by the reporter cells provided in this kit.

INDIGO's CHRM1 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 luciferase gene expression. Quantifying relative changes in luciferase activity in the treated reporter cells relative to the untreated reporter cells provides a sensitive surrogate measure of drug-induced changes in CHRM1 activity. Accordingly, the principal application of this reporter assay is in the screening of test compounds to quantify inhibitory functional activities that they may exert against CHRM1, 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 CHRM1 Inhibition Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference inhibitor Atropine, Luciferase Detection Reagents, and a cell culture-ready assay plate.

Note that the recommended application for this assay kit is for the assessment of **inhibitors of CHRM1 only**. If the intended application is to test for activators of CHRM1, INDIGO offers a separate kit which has been optimized for the evaluation of activation activities of test compounds on CHRM1 (see "Related Products" on pg. 11 of this Technical Manual).

▪ 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). 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: 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.) As described in *Step 7* and depicted in Appendix 1 for the reference inhibitor Atropine, Compound Screening Medium (CSM) may be used directly as the diluent to prepare serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if small-molecule test compound solubility is expected to be problematic,

2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. DMSO-induced cytotoxicity can be expected above 0.4%.

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.

Protein samples (e.g., antibodies or polypeptides): For protein test samples it is recommended to solvate the test materials in aqueous buffered solutions supplemented with carrier protein (e.g., PBS + 0.1% BSA) at concentrations *no less* than 10x relative to the highest desired treatment concentration.

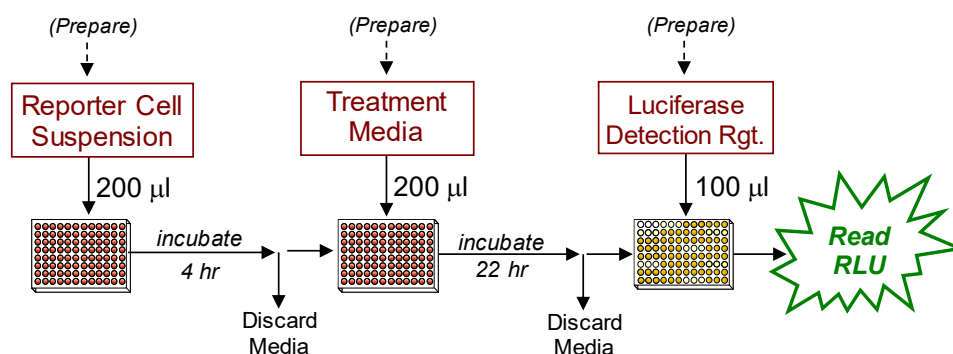
▪ **Considerations for Automated Dispensing** ▪

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 <i>(prepared from kit components)</i>	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml <i>(prepared from kit components)</i>	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ **Assay Scheme** ▪

Figure 1. Assay workflows for CHRM1 inhibition assays. Assay workflow. In brief, 200 µl/well of 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 200 µl/well of 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 terms of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪

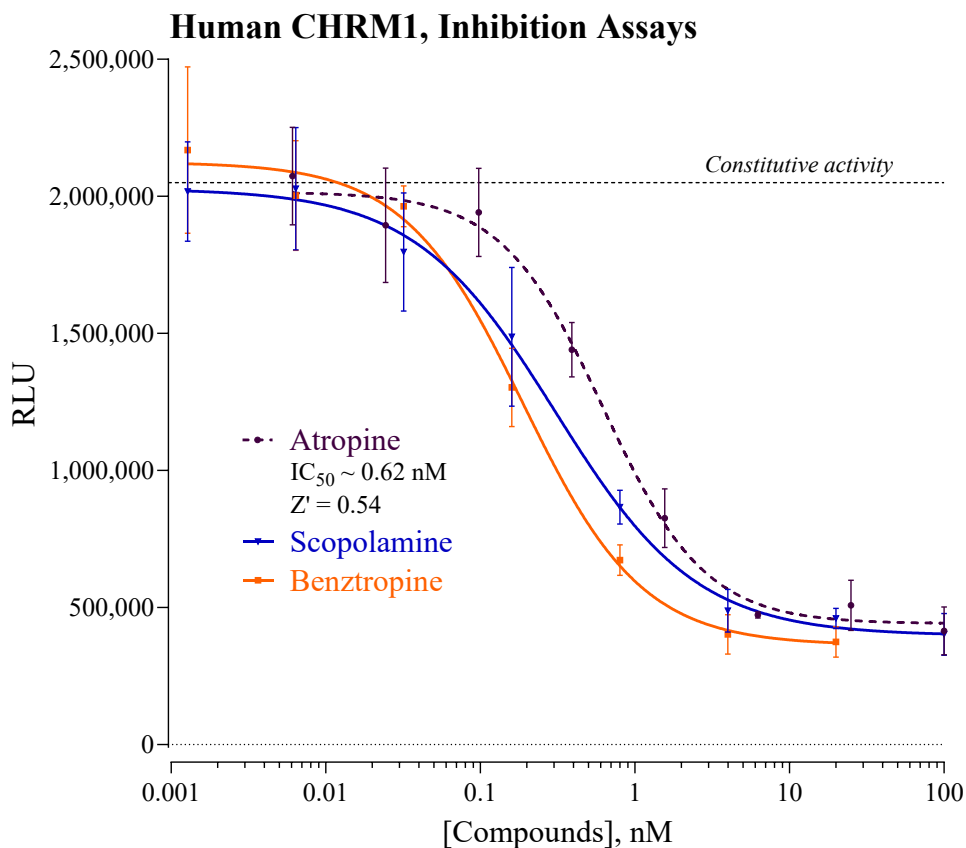


Figure 2. Inhibition of CHRM1. Reporter cells were treated with the reference inhibitors Atropine (provided), Scopolamine, and Benztropine (mesylate). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown).

Luminescence was quantified and values of average ($n = 4$) RLU, standard deviation (SD), and Z'^4 values were calculated. The least-squares method of non-linear regression was used to plot activity changes vs. Log_{10} [Compound, nM], and 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 CHRM1 Inhibition 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ CHRM1 Inhibition 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
▪ Atropine (100 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, <i>collagen-coated</i> 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:

- dry ice bucket (*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).
- plate-reading luminometer.
- *Optional:* clear 96-well assay plate, sterile, collagen-coated, for viewing cells on *Day 2*.

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 12-18* are performed on **Day 2** and require less than 1 hour to complete.

▪ A word about CHRMI Inhibition-mode assay setup ▪

The CHRMI Inhibition Reporter Cells have been optimized to test inhibitors of CHRMI activation. The Reporter Cells in this kit display high levels of CHRMI activity, *without* the need for an added activator (such as acetylcholine).

Due to the nature of an inhibition assay, in which the decrease in activity of a target resulting from exposure to a test substance is the assay readout, it is *highly recommended* that this assay be coupled with INDIGO's Live Cell Multiplex Assay (LCMA) (see "Related Products" on pg. 11 of this Technical Manual for product information for the LCMA). The LCMA is a fluorescence-based assay designed to quantify relative changes in live mammalian cell number following treatment with test compounds and can confirm that an observed decrease in CHRMI activity is due to receptor inhibition rather than cytotoxic effects.

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 **CHRMI Inhibition 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 - 10 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-channel 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% 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: 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. Inhibition-mode assays. This CHRM1 Inhibition Assay kit includes a concentrated stock (100 µM) of Atropine prepared in DMSO. The following 8-point treatment series, with concentrations generated using serial 4-fold dilutions, provides a complete dose-response: 100, 25.0, 6.25, 1.56, 0.391, 0.098, 0.024, and 0.006 nM. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') controls.

NOTE: Follow standard laboratory safety procedures and wear appropriate personal protective equipment while working with Atropine. Handle Atropine with **extreme care and caution** and dispose of any generated Atropine waste in the appropriate biohazardous waste disposal receptacles.

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-channel 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 μ l 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 CHRM1 Inhibition Assays	
IB44101	Human CHRM1 Inhibition Reporter Assay System 1x 96-well format assay
IB44101-32	Human CHRM1 Inhibition Reporter Assay System 3x 32-well format assay
Human CHRM1 Activation Assays	
IB44001	Human CHRM1 Activation Reporter Assay System 1x 96-well format assay
IB44001-32	Human CHRM1 Activation Reporter Assay System 3x 32-well format assay
Bulk volumes of CHRM1 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 available in 10 mL, 25 mL, 50 mL, 500 mL, or larger custom volumes.

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

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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.

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