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

3x 32 Assays in 96-well Format
Product # IB44001-32

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Technical Manual
(version 7.2i)

www.indigobiosciences.com

3006 Research Drive, Suite A1, State College, PA 16801, USA

Customer Service:
814-234-1919; FAX 814-272-0152
customerserv@indigobiosciences.com

Technical Service:
814-234-1919
techserv@indigobiosciences.com

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Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human CHRM1 Activation Reporter Assay System
3x 32 Assays in 96-well Format

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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 addiction[1]. 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 subtype[2]. 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 NFAT[3]. 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 activating 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 Activation Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator Acetylcholine (chloride), Luciferase Detection Reagents, and a cell culture-ready assay plate.

Note that the recommended application for this assay kit is for the assessment of **activators of CHRM1 only**. If the intended application is to test for inhibitors of CHRM1, INDIGO offers a separate kit which has been optimized for the evaluation of inhibitory activities of test compounds on CHRM1 (see "Related Products" on pg. 10 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 8* and depicted in Appendix 1 for the reference activator Acetylcholine (chloride), 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.

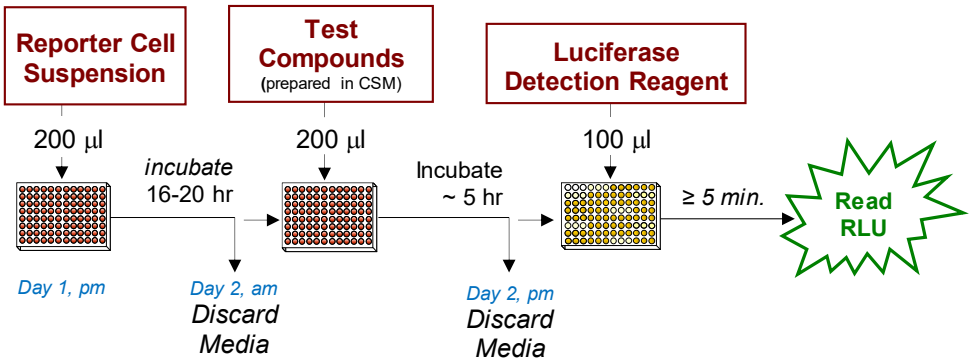
Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. DMSO-induced cytotoxicity can be expected above 0.4%.

> *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.

P**rotein samples** (*e.g.,* antibodies or activator 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.

▪ Assay Scheme ▪

Figure 1. Assay workflows for CHRM1 activation assays. It is recommended to begin assay setups in the late afternoon (pm) of *Day 1*. In brief, 200 µl/well of Reporter Cells are dispensed into the assay plate, which is then incubated overnight (16-20 hours). In the morning (am) of *Day 2*, the culture media are discarded and 200 µl/well of the prepared treatment media are added. Following an incubation period of ~ 5 hours, treatment 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.



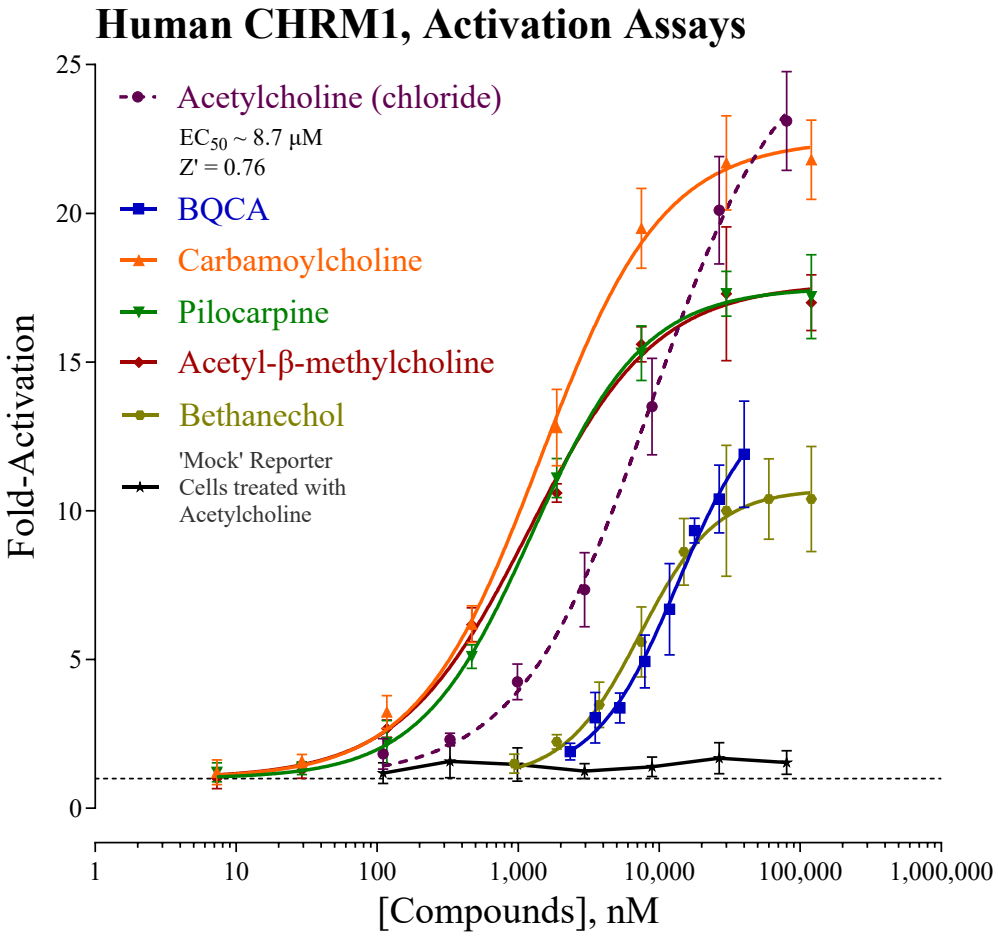


Figure 2. Activation of CHRM1. Reporter cells were treated with the reference activators Acetylcholine (chloride) (provided), BQCA (a selective positive allosteric modulator of CHRM1), Carbamoylcholine (chloride), Pilocarpine (chloride), Acetyl-β-methylcholine (chloride), and Bethanechol (chloride) for 5 h after an overnight pre-incubation (as depicted in Figure 1). All chemicals were procured from Cayman Chemical (Ann Arbor, MI, USA). Luminescence was quantified and values of average (n = 4) RLU, standard deviation (SD), Fold-Activation, 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 EC_{50} values were determined, using GraphPad Prism software. The absence of signal in Acetylcholine (chloride) treated 'Mock' cells (which express the NFAT-Luc reporter vector, but do *not* express CHRM1) confirms that the observed ligand-dependent response is specific to CHRM1 activation.

II. Product Components & Storage Conditions

This Human CHRM1 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.*
▪ CHRM1 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
▪ Acetylcholine (chloride) (20 mM in DMSO)	1 x 30 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	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:

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

IV. Assay Protocol

Review the entire Assay Protocol before starting. As noted in **Figure 1** (page 5), it is recommended that *Steps 1-6* are performed in the late afternoon on ***Day 1***; these will require less than one hour of bench work to complete. An overnight incubation (16-20 hours) is required. *Steps 7-17* are performed in the morning of ***Day 2***; approximately 2 hours of preliminary benchwork is required.

It is recommended that CHRM1 *Activation* assays are performed using a 5-hour treatment period, with the quantification of luciferase activity in the afternoon of *Day 2*.

DAY 1 Assay Protocol: It is recommended to begin mid- to late afternoon. 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.

Third, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips per tube of cells being used into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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-channel 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 16 - 20 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.

6.) For greater convenience on *Day 2*, transfer **Compound Screening Medium (CSM)** from freezer storage into a refrigerator (+4°C) to thaw overnight.

DAY 2 Assay Protocol: It is recommended to begin first thing in the morning.

7.) Near the end of the preliminary overnight incubation period remove **Compound Screening Medium (CSM)** from the refrigerator and allow it to warm to room temperature.

8.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations. 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 10*, 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 organic solvent) carried over into assay reactions should not exceed 0.4%.

***a. Activation*-mode assays**. This CHRM1 Assay kit includes a 20 mM stock solution of Acetylcholine (chloride), a potent activator of CHRM1. The following 7-point treatment series, prepared in serial 3-fold decrements, provides a complete dose-response: 80000, 26667, 8889, 2963, 988, 329 and 110 nM. Always include a 'no treatment' (or 'Vehicle only') control. **APPENDIX 1** provides an example for generating such a dilution series.

9.) At the end of the 16 – 20 hours cell incubation 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.

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

11.) Transfer the assay plate into a cell culture incubator (37°C, ≥70% humidity, 5% CO_2). Incubate the assay plate 5 hours for **activation** assays.

NOTE: At this time, retrieve **Luciferase Detection Buffer** and **Luciferase Detection Reagent** from freezer storage and place them in a low-light area so that they may thaw and equilibrate to room temperature for ready use in *Step 13*. Do NOT actively warm Detection Substrate above room temperature; if needed, a room temperature water bath may be used to expedite thawing.

12.) Near the end of the 5-hour treatment period, turn on the plate-reader and set the instrument to "luminescence" mode. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Set the read-time per assay well to 0.5 second (500 mSec), *or less*.

13.) Immediately before proceeding to *Step 14*: 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.

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 and throughout the 'plate rest' period (*Step 15*).

14.) Following 6 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 9*)

15.) Use an 8-channel pipette to dispense 100 µl of **LDR** to each well of the assay plate. Allow the plate to rest at room temperature for 5-10 minutes following the addition of LDR. Do not shake the plate during this period.

16.) Quantify luminescence.

17.) Data analyses.

V. Related Products

Product No.	*Product Descriptions*
Human CHRM1 Assays	
IB44001	Human CHRM1 Activation Reporter Assay System 1x 96-well format assay
IB44001-32	Human CHRM1 Activation Reporter Assay System 3x32-well format assay
IB44101	Human CHRM1 Inhibition Reporter Assay System 1x 96-well format assay
IB44101-32	Human CHRM1 Inhibition Reporter Assay System 3x32-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

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

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Product prices, availability, specifications, 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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VII. Citations

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[4] 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.

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

APPENDIX 1

Example scheme for the serial dilution of the reference agonist Acetylcholine (chloride) and the setup of a CHRM1 Activation dose-response assay.

