Human Calcitonin Receptor Reporter Assay System (CTR)

96-well Format Assays
Product # IB55001

▪

Technical Manual
(version 7.2i)

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Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human CTR Reporter Assay System
96-well Format Assays

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I. Description

▪ Background ▪

This assay utilizes proprietary human cells that provide constitutive expression of the **human Calcitonin Receptor (CTR).**

The Calcitonin Receptor (CTR) is a Class B G-protein coupled receptor (GPCR) activated by the peptide hormone calcitonin. CTR also serves as the core component of the Amylin receptors (AMY1-3R), which are formed by the heterodimerization of CTR with one of the three Receptor Activity Modifying Proteins (RAMP1-3)[1]. The binding of Calcitonin to CTR stimulates G-Protein Gαs, which activates adenylate cyclase (AC), leading to an increase in cellular cAMP[2].

CTR is an important drug target due to its roles in regulating bone and energy homeostasis. The receptor is highly expressed on the surface of osteoclasts, where its activation inhibits bone resorption[3]. CTR is also expressed in regions of the brain, such as the hypothalamus, where it contributes to the regulation of food intake and pain perception[4].

Given these roles, CTR has been exploited in multiple therapeutic approaches. Calcitonin analogs are used to treat bone disorders such as osteoporosis and Paget's disease[5]. Importantly, the development of Dual Amylin and Calcitonin Receptor Agonists (DACRAs) is currently at the forefront of pharmaceutical strategies aimed at providing more effective and sustainable metabolic control for patients with obesity and Type 2 Diabetes[6]. DACRAs have been shown to suppress appetite, delay gastric emptying, and reduce fat accumulation in organs such as the liver[7].

▪ The Assay System ▪

INDIGO's Reporter Cells contains an engineered luciferase reporter gene functionally linked to tandem Cyclic AMP Response Elements (CRE) and a minimal promoter. Activated adenylate cyclase results in the production of cAMP, which binds the transcription factor CREB (cAMP Response Element-Binding Protein). Activated CREB binds to CRE sequences, seeding 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 CTR 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 CTR.

INDIGO's Reporter Cells are transiently transfected and prepared as cryopreserved 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 CTR Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator Calcitonin, 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).

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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 Calcitonin, 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. The **Calcitonin stock** included with this kit is prepared in PBS + 0.1% BSA at a 100x-concentration relative to the highest recommended treatment (as depicted in APPENDIX 1).

▪ 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** ▪

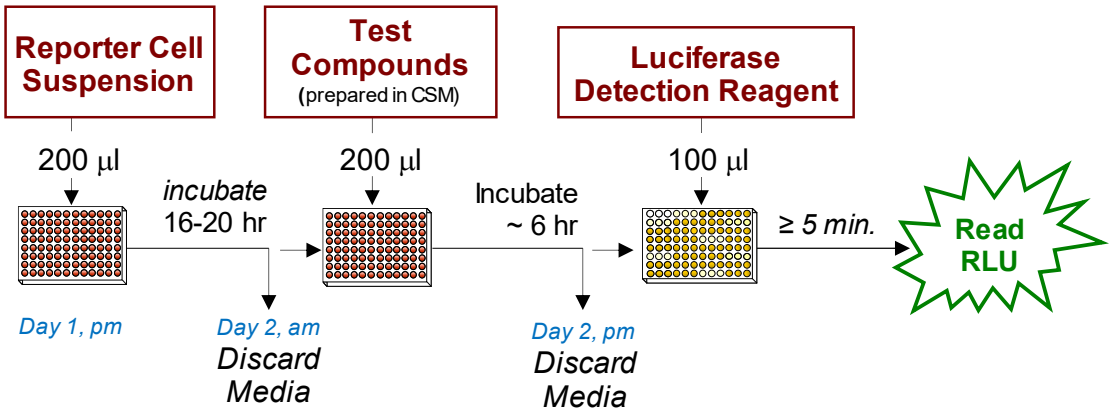


Figure 1. Assay workflows for CTR activation and inhibition assays. It is recommended to begin assay setups in the late afternoon (pm) of *Day 1*. In brief, 200 µl/well of Reporter Cells are dispensed into the assay plate, which is then incubated overnight (16-20 hours). In the morning (am) of *Day 2*, the culture media are discarded and 200 µl/well of the prepared treatment media are added. Following an incubation period of ~ 6 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.

▪ **Assay Performance** ▪

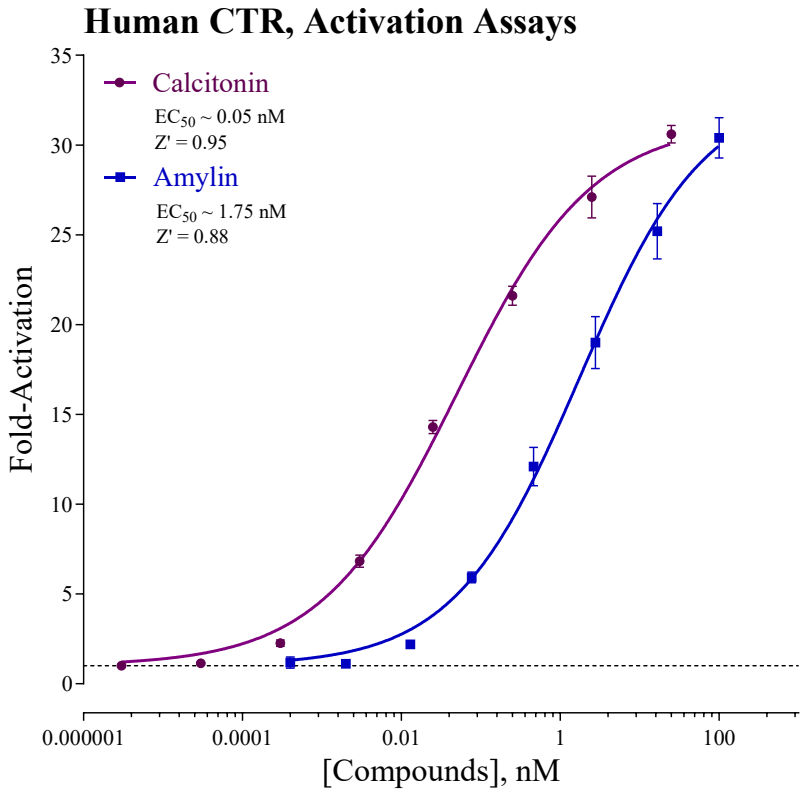


Figure 2. Activation of CTR. Assay was performed using the reference compounds Calcitonin (provided) and Amylin (Amidated). All chemicals were procured from Cayman Chemical (Ann Arbor, MI, USA. Luminescence was quantified and values of average (n=4) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, and Z'[8] values were calculated. GraphPad Prism software was used to plot data using the least-squares method of non-linear regression for Fold-Activation *vs.* Log_{10} [Compound, nM], and to determine the EC_{50} value.

II. Product Components & Storage Conditions

This Human CTR 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.***
▪ CTR 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
▪ Human Calcitonin (2.5 µM in PBS+0.1%BSA)	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:

- dry ice bucket (*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* 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*.
- Plate-reading luminometer

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 CTR *Activation* and *Inhibition*-assays are performed using a 6-hour treatment period, with the quantification of luciferase activity in the afternoon of *Day 2*.

▪ A word about Inhibition-mode assay setup ▪

Inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a CTR activator AND varying concentrations of the test compound(s) to be evaluated for inhibitory activities. This assay kit includes a 2.5 µM stock solution of **Calcitonin,** a potent activator of CTR that may be used to setup inhibition-mode assays. ~ 0.60 nM Calcitonin typically approximates EC_{80} in this cell-based assay. Hence, it presents a suitable co-treatment concentration to be used to screen test compounds for inhibitory activity.

Add the challenge activator, Calcitonin, 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 final assay concentrations. We find that this is an efficient and precise method of setting up CTR inhibition assays, and it is the method presented in *Step 8b* of this protocol.

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 the tube of **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, 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. Transfer the cell suspension into a media basin and, using an 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 'blank' wells (meaning cell-free but containing 'CSM') must be included in the assay plate to allow quantification of 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 basin during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

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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 the clear plate in an identical manner to the white assay plate.

NOTE 4.4: 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.

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 CTR Assay kit includes a 2.5 µM stock solution of (Calcitonin) a potent activator of CTR. The following 8-point treatment series, prepared in serial 10-fold decrements, provides a complete dose-response: 25.0, 2.50, 0.250, 0.025, 0.0025, 0.00025, 0.000025, and 0.0000025 nM. Always include a 'no treatment' (or 'Vehicle only') control. **APPENDIX 1** provides an example for generating such a dilution series.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition assays, first supplement a bulk volume of CSM with the challenge activator, Calcitonin, to achieve the desired final assay-concentration (refer to "*A word about inhibition-mode assay setup*", pg. 7). The Calcitonin-supplemented CSM is then used to generate dilutions of test compound stocks to achieve their final assay concentrations.

9.) At the end of the 16 – 20 hours cell incubation period, discard the culture media. The preferred method is to use a 'wrist flick' to manually eject media into an appropriate waste collection container. *Gently* tamp the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

10.) 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.

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11.) Transfer the assay plate into a cell culture incubator (37°C, ≥70% humidity, 5% CO_2). Incubate the assay plate 6 hours for **activation** or **inhibition** 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 6-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*, 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 and throughout the 'plate rest' period (*Step 15*).

14.) Following the 6-hour treatment period, 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.

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 CTR Assays	
IB55001-32	Human CTR Reporter Assay System 3x 32-well format assay
IB55001	Human CTR Reporter Assay System 1x 96-well format assay
Human AMY1R Assays	
IB48101-32	Human AMY1R Reporter Assay System 3x 32-well format assay
IB48101	Human AMY1R Reporter Assay System 1x 96-well format assay
Human AMY3R Assays	
IB48001-32	Human AMY3R Reporter Assay System 3x 32-well format assay
IB48001	Human AMY3R Reporter Assay System 1x 96-well format assay
Bulk volumes of AMYR 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.

www.indigobiosciences.com

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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VII. Citations

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

APPENDIX 1

Example scheme for the serial dilution of the reference agonist Calcitonin and the setup of a CTR dose-response assay.

