Human G Protein-Coupled Bile Acid Receptor 1 Reporter Assay System (TGR5; GPBAR1)

96-well Format Assays
Product # IB26001

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Technical Manual
(version 7.2i)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human TGR5 / GPBAR1 Reporter Assay System
96-well Format Assays

I. Description

▪ Background ▪

This assay utilizes proprietary human cells that provide constitutive expression of the **Human G Protein Coupled Bile Acid Receptor 1 (GPBAR1)**, also commonly referred to as the **Takeda G-protein-coupled receptor 5 (TGR5)**. TGR5 is the designation used in this technical manual.

TGR5 was characterized in 2002 as the first cell surface receptor for bile acids[1]. It is encoded by the GPBAR1 gene, which is conserved among vertebrates, indicating its physiological importance[2]. TGR5 is expressed in various organs (such as gallbladder, intestine, spleen, brown adipose tissue, and skeletal muscles) and cell types (such as macrophages, liver endothelial, and Kupffer cells)[2,3].

As a member of the G Protein-coupled receptor family, the topology of TGR5 displays the characteristic seven transmembrane helices, three extracellular loops contributing to ligand binding, and an intracellular carboxy tail that associates with trimeric G proteins. Upon binding to bile acid ligands, TGR5 undergoes a conformational change that triggers the activation of $G\alpha_s$ proteins *via* an exchange of GDP with GTP, followed by the activation of adenylate cyclase and the production of cAMP[3].

TGR5 functions as a metabolic regulator involved in the homeostasis of bile acids, glucose metabolism, lipid metabolism and energy expenditure[3]. In addition, TGR5 regulates pathways related to inflammation, cancer, and liver regeneration[4]. Interestingly, The TGR5 promoter harbors the response element for the Farnesoid X Receptor (FXR), the predominant bile acid nuclear receptor, resulting in ligand-dependent cross-signaling between FXR and TGR5[5].

▪ 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 TGR5 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 TGR5.

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 TGR5 Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator Lithocholic Acid (LCA), Luciferase Detection Reagents, and a cell culture-ready assay plate.

[1] Maruyama, T et al (2002) Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun. **298**(5): 714-719.

[2]Duboc H et al (2014) The bile acid TGR5 membrane receptor: From basic research to clinical application. Dig. Liver Dis. **46**: 302-312.

[3] Pols, TWH et al (2011) The bile acid membrane receptor TGR5: A valuable metabolic target. Dig Dis. **29**(1): 37-44.

[4] Guo, C et al (2016) TGR5, not only a metabolic regulator. Front. Physiol. **7**: 646.

[5]Pathak P et al (2017) Farnesoid X receptor induces Takeda G-protein receptor 5 cross talk to regulate bile acid synthesis and hepatic metabolism. J. Biol. Chem. **292**(26): 11055-11069.

▪ 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. 8).

> *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.

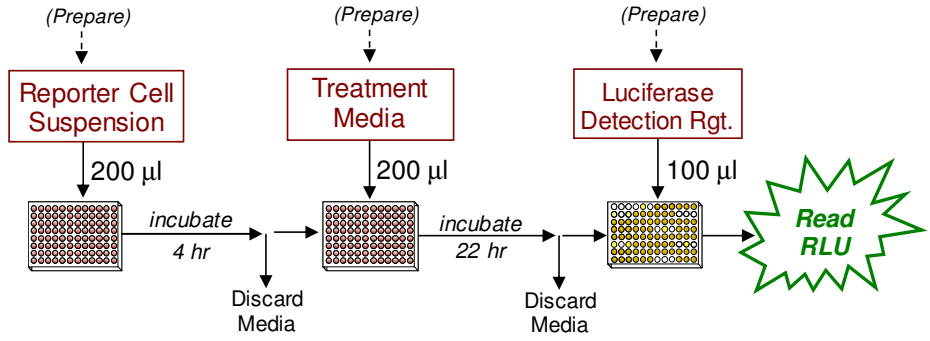
▪ Considerations for Automated Dispensing ▪

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*, 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 the prepared treatment media are added. Following a 22-24 hr treatment period the 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.



▪ Assay Performance ▪

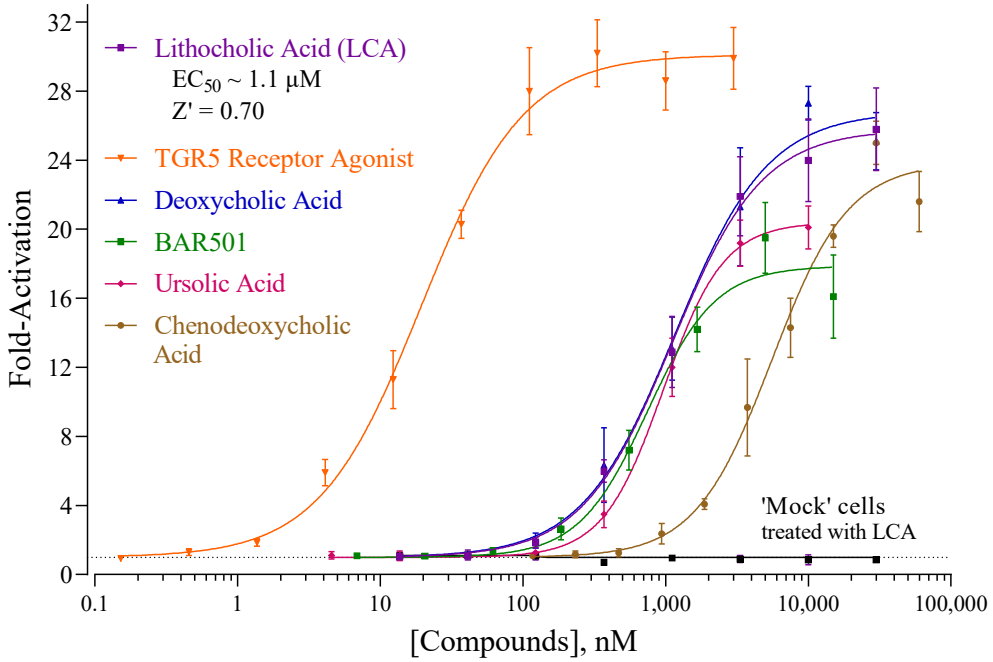


Figure 2. Human TGR5 Agonist Dose Response. Agonist assays were performed using the reference compounds Lithocholic Acid (LCA, provided), TGR5 Receptor Agonist (Adooq Biosciences, Irvine, CA, USA), BAR501, Deoxycholic Acid, Chenodeoxycholic Acid, and Ursolic Acid (all from Cayman Chemical, Ann Arbor MI, USA). 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. Non-linear regression analyses of Fold-Activation *vs.* Log_{10} [Agonist, nM] and EC_{50} values were determined using GraphPad Prism software. The absence of signal in LCA treated 'Mock' cells (which contains the CRE-Luc reporter vector, but do *not* express TGR5) confirms that the agonist-dependent response observed in the TGR5 Reporter Cells is specific to TGR5.

[6] 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.

II. Product Components & Storage Conditions

This Human TGR5 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.*
▪ TGR5 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
▪ Lithocholic Acid (LCA; 30 mM in DMSO) (Physiological bile acid activator of TGR5)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well assay plate (white, sterile, cell-culture ready)	1	ambient

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:* clear 96-well assay plate, cell culture treated, for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

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-hour 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 ▪

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 TGR5 Assay kit includes a 30 mM stock solution of **Lithocholic Acid** (**LCA**), a bile acid that may be used to setup inhibition-mode assays. 3.0 µM LCA approximates EC_{80} in this assay. Hence, it is a suitable concentration of challenge agonist to use when screening test materials for inhibitory activities.

Add LCA 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 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 **TGR5 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, then transfer the cell suspension into a reservoir. Using an electronic, repeat-dispensing 8-chanel 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 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 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. Agonist*-mode assays**. This TGR5 Assay kit includes a concentrated stock (30 mM) of LCA prepared in DMSO. The following 7-point treatment series, with concentrations generated using serial 3-fold dilutions, provides a complete dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123, and 0.0412 µM. **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 **LCA** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). 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-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-chanel 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.

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.

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

Product No.	*Product Descriptions*
Human TGR5 Assays	
IB26001	Human TGR5 Reporter Assay System 1x 96-well format assay
IB26002	Human TGR5 Reporter Assay System 1x 384-well format assays
Bulk volumes of TGR5 Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

FXR Assay (recommended for counter-screening)	
IB00601	Human Farnesoid X Receptor 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 in 10 mL, 25 mL, 50 mL, and 500 mL 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.

APPENDIX 1

Example scheme for the serial dilution of the reference agonist Lithocholic Acid (LCA) and the setup of a TGR5 dose-response assay.