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

384-well Format Assays
Product # IB26002

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Technical Manual
(version 8.0)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human TGR5 / GPBAR1 Reporter Assay
384-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 ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of the **Human TGR5 (*a.k.a.* GPBAR1)**.

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.

▪ 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 Receptor Assays feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction. Incorporating a 30-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.

▪ Considerations for the Preparation and Automated Dispensing of Test compounds ▪

Small molecule compounds are typically solvated at high concentration (ideally 1,000x-concentrated) in DMSO and stored frozen as master stocks. For **384-well format assays** these master stocks will be diluted by one of two alternative methods, the selection of which will be dictated by the type of dispensing instrument that is to be used. This Technical Manual provides detailed protocols for each of these two alternative methods:

a.) Assay setups in which a conventional **tip-based** instrument is used to dispense test compounds into assay wells (in black text). Use **Compound Screening Medium (CSM)** to generate a series of **2x-concentration** test compound treatment media, as described in *Step 2a* of the **Assay Protocol**. The final concentration of DMSO carried over into assay reactions should not exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in any organic solvent.

NOTE: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup and are considered to be 'single-use' reagents.

or,

b.) Assay setups in which an **acoustic transfer** device is used to dispense test compounds into assay wells (in blue text). Use DMSO to make a series of **1,000x-concentrated** test compound stocks that correspond to each desired final assay concentrations, as described in *Step 2b* of the **Assay Protocol**.

▪ Considerations for Automated Dispensing of Other Assay Reagents ▪

When dispensing into a small number of assay plates, first carefully consider the dead volume requirement of your tip-based dispensing 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 on a *per kit* basis. Always pool the individual reporter cell suspensions and all other respective assay kit reagents before processing multiple 384-well assay plates.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(384-well plate)*	**Excess rgt. volume available for instrument dead volume**
when using tip dispensing of test cmpds **Reporter Cell Suspension** 7.5 ml	15 µl / well 5.8 ml / plate	~ 1.7 ml
when using acoustic dispensing of test cmpds **Reporter Cell Suspension** 15 ml	30 µl / well 11.5 ml / plate	~ 3.4 ml
Detection Substrate 7.8 ml	15 µl / well 5.8 ml / plate	~ 2 ml

▪ Assay Scheme ▪

The *Day 1* preparation, volumes, and chronology of dispensed cells and test compounds are different between assay setups using a *tip-based dispenser* (**1a**) and those using an *acoustic transfer device* (**1b**). Following 22 -24 hours incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

Figure 1a. Assay workflow if using conventional **tip-based** dispensing of test compounds.

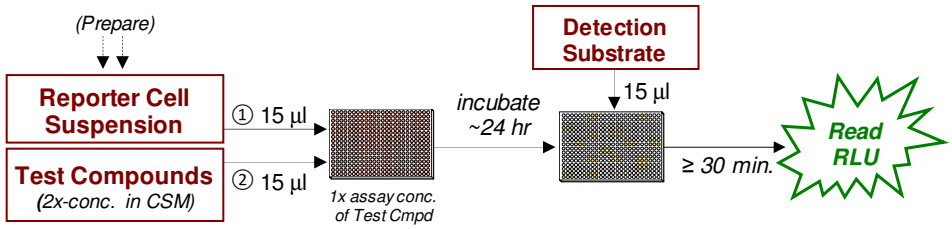
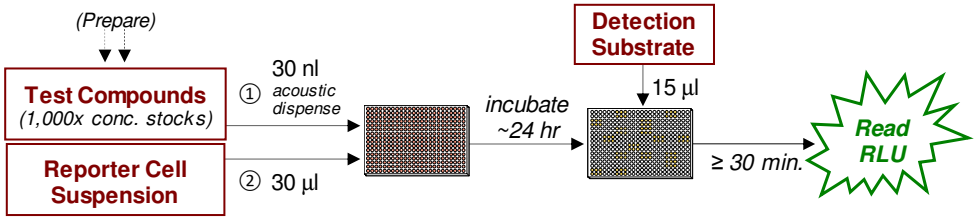


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



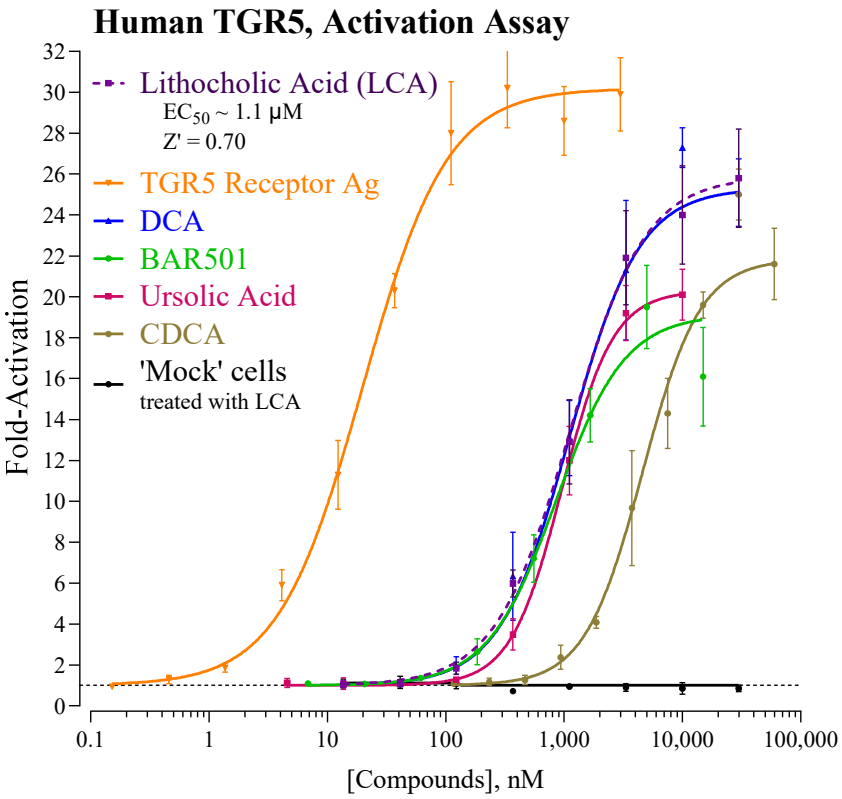


Figure 2. Activation of Human TGR5. Activation assays were performed using the reference compounds Lithocholic Acid (LCA, provided), TGR5 Receptor Agonist (Adooq Biosciences, Irvine, CA, USA), Deoxycholic Acid (DCA), BAR501, Chenodeoxycholic Acid (CDCA), and ursolic Acid (all from Cayman Chemical, Ann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

II. Product Components & Storage Conditions

This assay kit contains materials to perform assays in a single 384-well assay plate.

Cryopreserved mammalian cells are temperature sensitive! To ensure maximal viability the tube of Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in 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 inventory the individual kit components be sure to first transfer and submerge the tube of cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, the 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*	*minimum Storage Temp.*
▪ TGR5 Reporter Cells	1 x 1.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 7 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 80 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	1 x 7.8 mL	**-80°C**
▪ 384-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

▪ dry ice container

▪ 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 & tips suitable for dispensing 15 µl.

▪ disposable media basins, sterile.

▪ sterile multi-channel media basins *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).

▪ *Optional:* clear 384-well assay plate 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 1-8* are performed on ***Day 1***, requiring less than 2 hours to complete. *Steps 9-13* 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 5b* of this protocol, and *Step 6b* of the protocol when using an acoustic transfer device to dispense test compounds.

Note that when using a *tip-based instrument* for the dispensing of 2x-concentrated test compounds the cell suspension must also be supplemented with a **2x-**concentration (~6.0 µM) of the challenge agonist LCA.

When using an *acoustic transfer* device for the dispensing of 1,000x-concentrated test compounds the cell suspension should be supplemented with a **1x-**concentration (~3.0 µM) of the challenge agonist LCA.

DAY 1 Assay Protocol:

All steps should be performed using proper aseptic technique.

1.) Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

2.) Prepare dilutions of treatment compounds: Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens. NOTE that test and reference compounds will be prepared differently when using tip-dispensing *vs.* acoustic dispensing. Regardless of the method, residual DMSO carried over into assay reactions should not exceed 0.4%.

a. *Tip dispensing method*: In *Step 6*, 15 µl / well of the prepared treatment media is added to the assay that has been pre-dispensed with 15 µl /well of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a **2x-**concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Plan dilution volumes carefully; this assay kit provides 45 ml of CSM.

b. *Acoustic dispensing method*: In *Step 6*, 30 nl / well of **1,000x**-concentrated test compound solutions are added to the assay plate using an acoustic transfer device.

Preparing the positive control: This assay kit includes a 30 mM stock solution of LCA, an agonist of TGR5. The following 7-point treatment series, with concentrations presented in **3-fold** decrements, provides a complete dose-response: 30,000, 10,000, 3,333, 1,111, 370, 123, and 41.2 nM. Always include 'no treatment' (or 'vehicle') control wells.

APPENDIX 1a provides an example for generating such a dilution series to be used when *tip-dispensing* compound solutions prepared in CSM (15 µl / well).

APPENDIX 1b provides an example for generating such a series of 1,000x-concentrated solutions of compounds prepared in DMSO to be used when performing *acoustic dispensing* (transfer 30 nl / well).

(continued)

When using *tip-based* instrumentation for dispensing test compounds …

3.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a *rapid thaw* of the frozen cells by transferring a 6.5 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab, then transfer it into the cell culture hood.

5.) *Gently* invert the tube of cells several times to gain a homogenous suspension.

a. **for *Agonist*-mode assays**: Dispense **15 µl / well** of cell suspension into the assay plate.

~ or ~

b. **for *Antagonist*-mode assays**: First supplement the bulk volume of Reporter Cell suspension with a 2x-concentration of the challenge agonist LCA (refer to "*A word about antagonist-mode assay setup*", pg. 8). Dispense **15 µl / well** of cell suspension into the assay plate.

6.) Dispense **15 µl / well** of 2x-concentrated treatment media (from *Step 2a*) into the assay plate.

When using an *acoustic transfer* device for dispensing test compounds …

3.) Dispense **30 nl / well** of the 1,000x-concentrated compounds (in DMSO solutions, from *Step 2b*) into the assay plate.

4.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a *rapid thaw* of the frozen cells by transferring a **6.5 ml** volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 ml.

5.) Retrieve the tube of cell suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab. Add an additional **7.5 ml** of **CSM** to the tube. The resulting volume of cell suspension will be 15 ml.

6.) *Gently* invert the tube of cells several times to gain a homogenous cell suspension.

a. **for *Agonist*-mode assays**: Dispense **30 µl / well** of cell suspension into the assay plate that has been pre-dispensed with test compounds.

~ or ~

b. **for *Antagonist*-mode assays**: First supplement the bulk volume of Reporter Cell suspension with the challenge agonist LCA to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 8). Then dispense **30 µl / well** of the supplemented cell suspension into the assay plate that has been pre-dispensed with test compounds.

NOTE: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate *will* cause well-to-well variation (= increased Standard Deviation) in the assay.

(continued)

NOTE: Following the dispensing of Reporter Cells and test compounds INDIGO recommends performing a *low-speed* spin of the assay plate (with lid) for ≤ 1 minute using a room temperature centrifuge fitted with counter-balanced plate carriers.

7.) Transfer the assay plate into a 37°C, humidified, 5% CO_2 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.

8.) For greater convenience on *Day 2*, retrieve **Detection Substrate** from freezer storage and place 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**.

9.) Approximately 30 minutes before intending to quantify receptor activity remove **Detection Substrate** from the refrigerator and place it in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure a homogenous solution.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If this solution was not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

10.) 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*.

11.) Following 22 - 24 hours of incubation dispense **15 µl / well** of **Detection Substrate** into all wells of the assay plate.

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 dispensing Detection Substrate into the assay plate and throughout the 'plate rest' period.

NOTE: Perform this reagent transfer carefully to avoid bubble formation! Scattered micro-bubbles will not pose a problem. However, bubbles covering the surface of the reaction mix, or large bubbles clinging to the side walls of the well, will cause lens-effects that will degrade the accuracy and precision of the assay data. INDIGO recommends performing a final *low-speed* spin of the assay plate (with lid) for ≤ 1 minute using a room temperature centrifuge fitted with counter-balanced plate carriers.

12.) Allow the plate(s) to rest at room temperature for 30 minutes. Do not shake the assay plate(s) during this period.

NOTE: the luminescent signal is unstable during the first 30 minutes of the luciferase reaction, however, after the initial 30-minute reaction period the luminescence signal achieves a stable emission output.

13.) Quantify luminescence.

14.) 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
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 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.

www.indigobiosciences.com

VI. Literature Citations

[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.

[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.

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

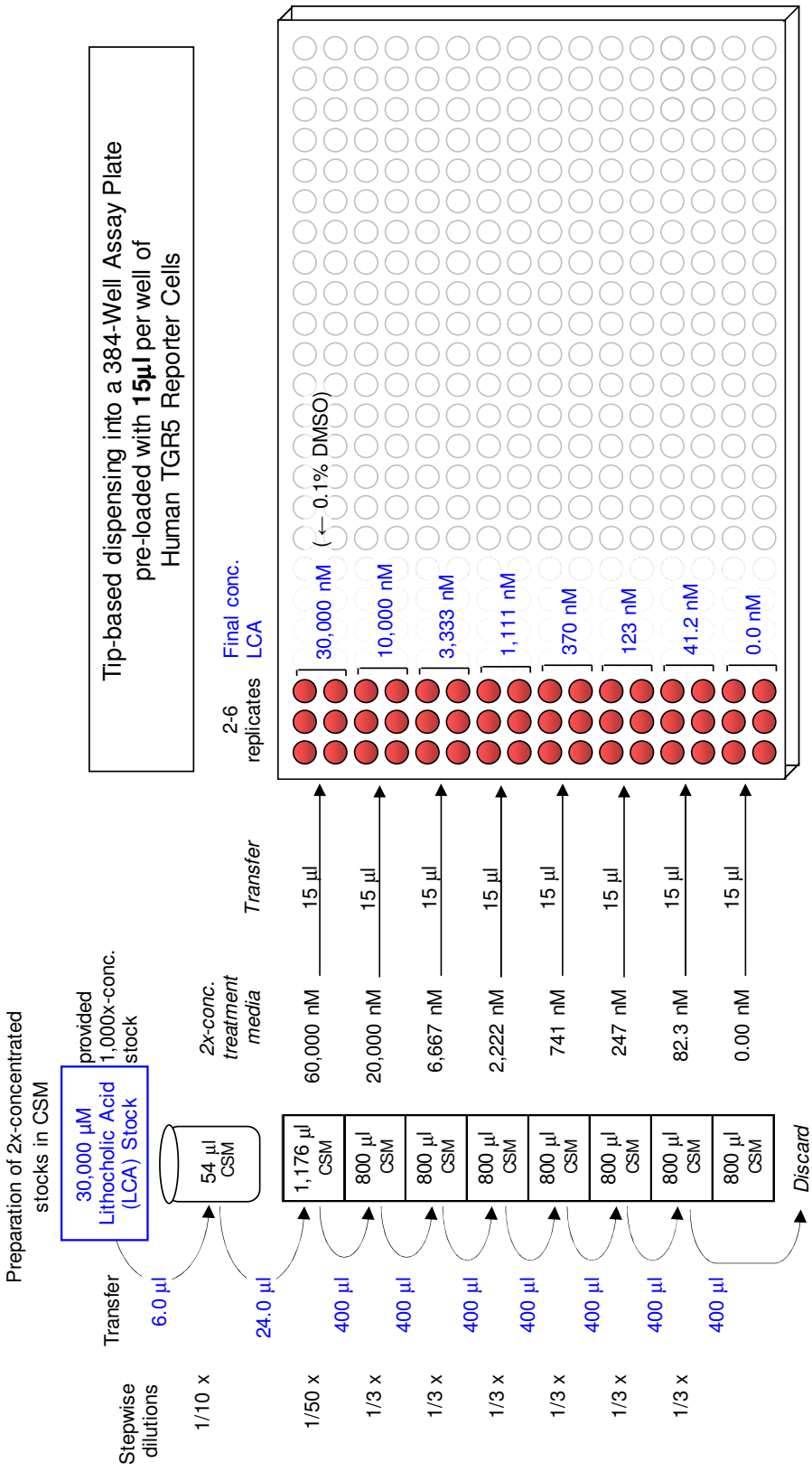
VII. Limited Use Disclosures

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

"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA).

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 recently updated version available.

APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of Lithocholic Acid using **CSM** to generate **2x-concentrated** treatment media. A *tip-based* instrument is used to dispense 15 µl / well into an assay plate that has been *pre-dispensed* with 15 µl / well of TGR5 Reporter Cells suspension.



APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the Lithocholic Acid using **DMSO** to generate **1,000x-concentrated** stocks. 30 nl / well of these prepared stocks are first dispensed into *empty* wells of the assay plate using an acoustic transfer device, followed by the dispensing of 30 µl / well of TGR5 reporter cells.