Human Gastric Inhibitory Polypeptide Receptor Reporter Assay System (GIPR)

96-well Format Assays
Product # IB38001

▪

Technical Manual
(version 7.2)

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

INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human GIPR Reporter Assay System
96-well Format Assays

I. Description

▪ Background ▪

This assay utilizes proprietary human cells that provide constitutive expression of the **Human Gastric Inhibitory Polypeptide Receptor (GIPR)**.

Gastric Inhibitory Polypeptide (GIP), the primary activator of GIPR, is a gut-derived hormone that is secreted from the enteroendocrine K cells of the small intestine in response to nutrient intake[1]. GIP, along with a related hormone, glucagon-like peptide-1 (GLP-1), constitute the incretin hormones that regulate glucose tolerance / levels by stimulating insulin release from pancreatic β-cells[2].

GIPR belongs to the class B1 G protein-coupled receptor (GPCR) superfamily and signals through $G\alpha_s$/adenylyl cyclase activation, leading to an increase in concentration of the second messenger molecule cyclic adenosine monophosphate (cAMP)[1].

Historically, GLP-1R agonists have shown clinical success in treating obesity and type 2 diabetes (T2D). GIP, and its receptor GIPR, are also associated with the pathophysiology of obesity and T2D[3]. As such, GIPR is an important therapeutic target. As an example, the anti-obesity injectable drug Tirzepatide is a single-molecule co-activator of GLP-1R and GIPR[4]. Clinical studies for this compound showed efficacy for glucose lowering and weight loss in T2D patients as compared to control groups. Interestingly, Tirzepatide displayed higher affinity for GIPR compared to GLP-1R, with signaling studies demonstrating similar action as the native GIP peptide[4]. GIPR and GLP-1R continue to command considerable interest in therapeutics development and drug safety screening.

▪ 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 GIPR 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 GIPR. 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 GIPR Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator GIP, Luciferase Detection Reagents, and a cell culture-ready assay plate.

▪ 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 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.

Stocks of test materials that are **Protein** or **Poly-peptide** ligands, or **Antibodies**, should be solvated in aqueous buffered solutions with carrier protein (*e.g.,* PBS + 0.1% BSA) at concentrations *no less* than 10x-concentrated relative to the highest desired treatment concentration.

Immediately prior to setting up an assay, the above master stocks are serially diluted using one of two alternative strategies:

1.) For both **small-molecule** and **proteinaceous** test samples, **Compound Screening Medium (CSM)** may be used as the diluent to achieve the desired assay concentration series, as described in *Step 7* (pg. 9).

Alternatively, if **small-molecule** test compound solubility is expected to be problematic, *2.)* DMSO may be used to make serial dilutions to produce 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make 1,000-fold dilutions of the prepared DMSO dilution series. Note: Do not use DMSO as the diluent for proteinaceous test compounds.

Regardless of the dilution method used, the concentration of total DMSO (or any organic solvent) carried over into assay wells should not exceed 0.4%. Emerging cytotoxicity can be expected above 0.4% DMSO exposure over the 24-hour assay period.

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 ▪

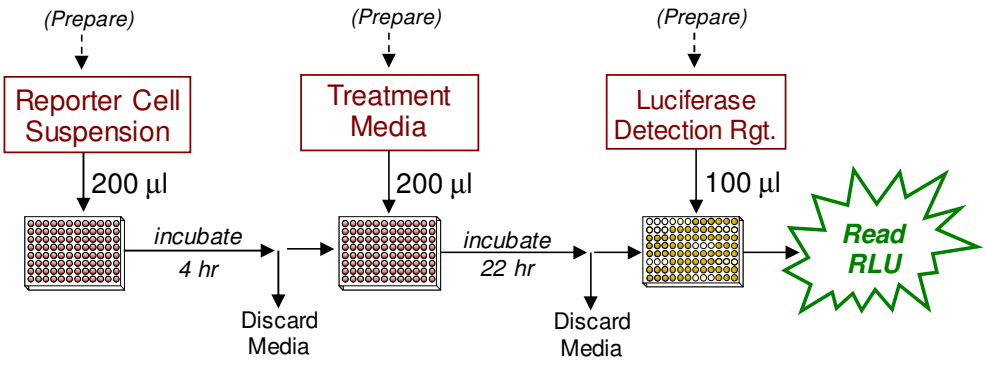

Figure 1. Assay workflow. Reporter Cells are dispensed into the assay plate and incubated for 4-6 hours. Following the pre-incubation period, the culture media are discarded, and the prepared treatment media are added. Following a 22–24-hour treatment period the media are discarded, and Luciferase Detection 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.

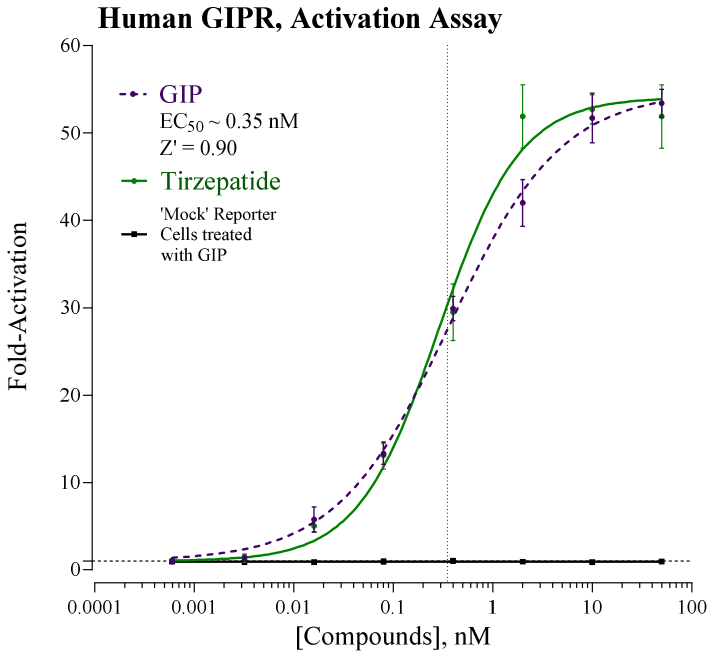

Figure 2. Activation of GIPR. Activation assays were performed using the reference polypeptides GIP (provided) and Tirzepatide.

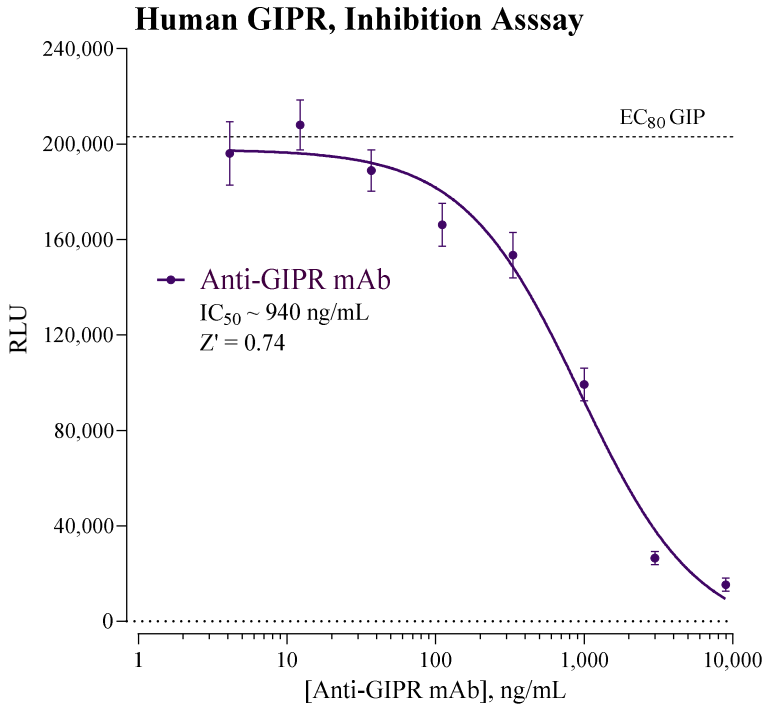

Figure 3. Inhibition of GIPR. GIPR reporter cells were co-treated with an EC_{80} concentration of the reference activator GIP and varying concentrations of anti-GIPR specific monoclonal antibody (R&D System, Minneapolis, MN). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown).

For both Activation and Inhibition assays, luminescence was quantified and values of average (n=3) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, and Z'[5] values were calculated. GraphPad Prism software was used to plot data using the least-squares method of non-linear regression for Fold-Activation or RLU *vs.* Log_{10} [Cmpd, nM], and to determine EC_{50} / IC_{50} values. Polypeptide activators were procured from Cayman Chemical (Ann Arbor MI, USA.)

II. Product Components & Storage Conditions

This Human GIPR 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.*
▪ GIPR 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
▪ GIP (5.0 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 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 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 Inhibition-mode assay setups ▪

Receptor inhibition assays expose the Reporter Cells to a fixed, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known activator AND varying concentrations of the test compound(s) to be evaluated for chemical inhibition. This GIPR Assay kit includes a 5.0 µM stock solution of GIP, the physiological activator of GIPR, that may be used to set up inhibition-mode assays. ~1.9 nM GIP approximates EC_{80} in this assay and, therefore, is a suitable *final assay concentration* of activator to be used when screening test compounds for inhibitory activity.

Add GIP to a bulk volume of **CSM**, as described above. This activator-supplemented medium is then used to prepare serial dilutions of test material stocks to achieve the desired respective final assay concentrations. This is an efficient and precise method of setting up GIPR inhibition assays, and it is the method presented in *Step 7b* of this protocol.

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

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 the clear 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% 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(s) 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 carried over into assay reactions should not exceed 0.4%.

***a. Activation*-mode assays**. This GIPR Assay kit includes a concentrated stock of GIP, 5.0 µM prepared in PBS/0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 3-fold dilutions, provides a complete dose-response: 50.0, 10.0, 2.00, 0.400, 0.080, 0.016, and 0.0032 nM. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' control wells.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition assays, first supplement a bulk volume of CSM with the challenge activator GIP to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setup*", pg. 8). The GIP-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 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. Set the read time 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 µl of the prepared **LDR** into all wells of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes. Do not shake the assay plate during this period.

17.) Quantify luminescence.

18.) Data analyses.

V. Related Products

Product No.	*Product Descriptions*
Human GIPR Assays	
IB38001	Human GIPR Reporter Assay System 1x 96-well format assay
IB38002	Human GIPR Reporter Assay System 1x 384-well format assays
Bulk volumes of GIPR Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

Product No.	*Product Descriptions*
Human GLP-1R Assays	
IB33001	Human GLP-1R Reporter Assay System 1x 96-well format assay
IB33002	Human GLP-1R Reporter Assay System 1x 384-well format assays
Bulk volumes of GLP-1R 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 in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

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.

"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 current version available.

VII. Citations

[1] Kizilkaya HS *et al*., (2021) Loss of Function Glucose-Dependent Insulinotrophic Polypeptide Receptor Variants Are Associated With Alterations in BMI, Bone Strength and cardiovascular Outcomes. Frontiers in Cell and Developmental biology **9**- 2021. http://doi.org/10.3389/fcell.2021.749607.

[2] Campbell JE (2020) Targeting the GIPR for obesity: To agonize or antagonize? Potential mechanisms. Molecular Metabolism. http://doi.org/10.1016/j.molmet.2020.101139.

[3] Liskiewicz A *et al*., (2023) Glucose-dependent insulinotropic polypeptide regulates body weight and food intake via GABAergic neurons in mice. Nature Metabolism **5**: 2075-2085.

[4] Willard FS *et al*., (2020) Tirzepatide is an imbalanced and biased dual GIP and GLP-1 receptor agonist. JCI insight. http://doi.org/10.1172/jci.insight.140532.

[5] 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 GIP and the setup of a GIPR dose-response assay.