User Manual

cAMP Hunter™ Tirzepatide (GIP RA) Bioassay Kit

For the Measurement of Ligand-Mediated cAMP Accumulation

For Bioassay Kits:

95-0146Y2-00207: 2-Plate Kit

95-0146Y2-00208: 10-Plate Kit

For Bioassay kits without control

95-0146Y2-00209: 10-Plate Kit

Document Number 70-450 Revision 0

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Important: Please read this entire user manual before proceeding with the assay

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1. Overview

The cAMP Hunter Tirzepatide (GIP RA) Bioassay Kit provides a robust and highly sensitive functional, cell-based assay to monitor 3'-5'-cyclic adenosine monophosphate (cAMP) production in cells as a result of ligand-mediated Gastric Inhibitory Polypeptide (GIP) Receptor Activation. The bioassay kit contains all the materials needed for a complete assay, including cells, detection reagents, cell plating reagent, dilution buffer, positive control agonist, and assay plates. A 10-Plate cAMP Hunter™ Tirzepatide (GIP RA) Bioassay Kit is also offered without the positive control agonist but contains all other components listed above to run the assay. The ready-to-use cryopreserved cells have been manufactured to ensure assay reproducibility and faster implementation from characterization to lot release. This bioassay has been optimized for a 96-well format.

2. Assay Principle

Ligand-mediated G protein coupled receptor (GPCR) stimulation leads to the activation of G-proteins, which trigger downstream signaling pathways by either recruiting, activating, or inhibiting cellular enzymes. One such enzyme is adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic AMP (cAMP). Adenylate cyclase is either stimulated or inhibited by the G-protein subunits, Gα*s* and Gα*i*, respectively. In the cAMP Hunter Tirzepatide (GIP RA) Bioassay, cells overexpressing Gastric Inhibitory Peptide Receptor (GIPR) utilize the natural coupling status of the GPCR to Gα*s* to monitor activation of the receptor. When GIPR is activated by Tirzepatide, it stimulates adenylate cyclase, which in turn enables the production of cAMP. The resulting increase in cellular cAMP levels is measured using a homogenous, gain-of-signal competitive immunoassay based on Enzyme Fragment Complementation (EFC) technology.

The EFC technology uses a β-galactosidase (β-gal) enzyme split into two fragments, the Enzyme Donor (ED) and Enzyme Acceptor (EA). Independently, these fragments have no β-gal enzymatic activity; however, in solution, they rapidly complement to form an active β-gal enzyme. In this assay, cAMP from cell lysates competes with ED-labeled cAMP (ED-cAMP) for the anti-cAMP antibody (Ab). Antibody-bound ED-cAMP cannot complement with EA, but unbound ED-cAMP is free to complement with EA and form active β-gal. Any β-gal formed subsequently acts upon the substrate in the detection reagent to produce a chemiluminescent signal. The signal from the assay is directly proportional to the amount of cellular cAMP in the well, i.e., the higher the GIPR activation, the greater the cellular cAMP levels, and the larger the signal produced in the assay.

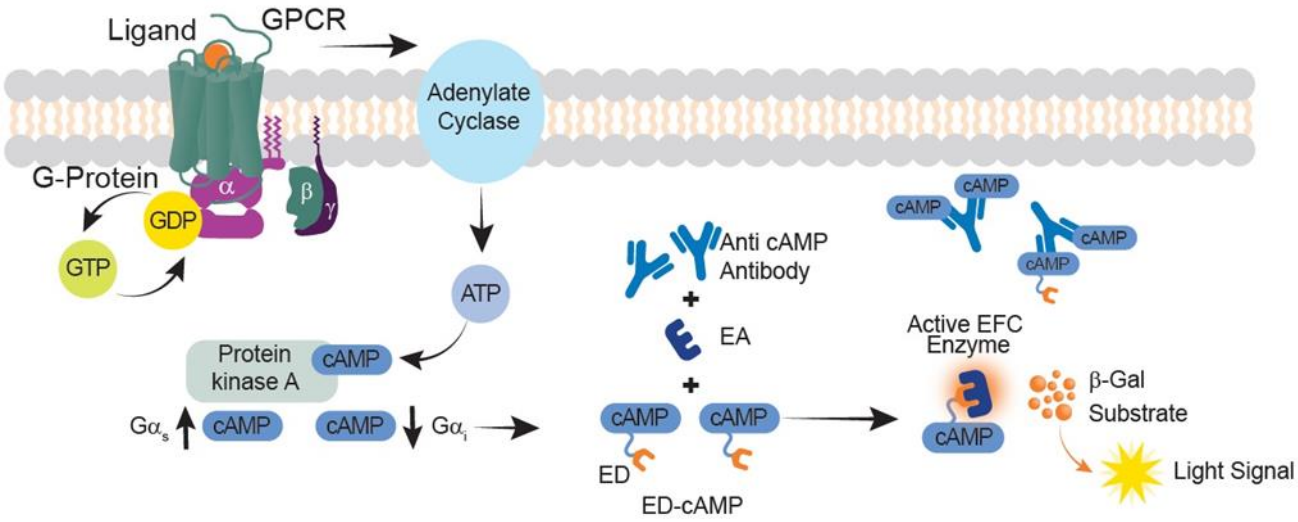


Figure 1. Assay Principle

When cellular cAMP levels are low, ED-labeled cAMP successfully binds with the anti-cAMP-antibody, as there are an insufficient number of cellular cAMP molecules to compete against it. In contrast, when cellular cAMP levels are high, the cellular cAMP molecules bind to the anti-cAMP antibody instead, leaving ED-labeled cAMP relatively free. Upon the addition of the detection reagent containing EA, the ED and EA fragments undergo complementation that successfully forms an active β-galactosidase enzyme that hydrolyzes the substrate and generates a chemiluminescent signal.

3. Materials Provided

List of Components	95-0146Y2-00207 (2 plate Kit)	95-0146Y2-00208 (10 plate kit)	95-0146Y2-00209 (10 plate kit without control)
cAMP Hunter CHO-K1 GIPR Bioassay Cells (2.2×10^6 cells in 0.2 mL per vial)	2 vials	10 vials	10 vials
AssayComplete™ Cell Plating 2 Reagent (CP2) (100 mL per bottle)	1 x 100 mL	2 x 100 mL	2 x 100 mL
AssayComplete Cell Assay Buffer (50 mL per bottle)	1 x 50 mL	2 x 50 mL	2 x 50 mL
Control Agonist (GIP), 500 µg	1 x 500	1 x 500 µg	N/A*
cAMP Detection Kit for Bioassays			
cAMP Standard (250 µM) (Bottle)	1 x 0.2 mL	1 x 1 mL	1 x 1 mL
cAMP Antibody Reagent (Bottle)	1 x 5 mL	1 x 25 mL	1 x 25 mL
cAMP Lysis Buffer (Bottle)	1 x 7.6 mL	1 x 38 mL	1 x 38 mL
Substrate Reagent 1 (Bottle)	1 x 2 mL	1 x 10 mL	1 x 10 mL
Substrate Reagent 2 (Bottle)	1 x 0.4 mL	1 x 2 mL	1 x 2 mL
cAMP Solution D (Bottle)	1 x 10 mL	1 x 50 mL	1 x 50 mL
cAMP Solution A (Bottle)	1 x 16 mL	1 x 80 mL	1 x 80 mL
96-well White, Flat-bottom Poly-D-Lysine-coated, Sterile Plates with Lid	2 Plates	10 Plates	10 Plates

***Note**: For 95-0146Y2-00209, control agonist is not provided in the kit and would need to be obtained separately if needed. Refer to additional materials required table for ordering information for the control agonist.

4. Storage Conditions

cAMP Hunter CHO-K1 GIPR Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, store the vials of bioassay cells in the vapor phase of liquid nitrogen as soon as possible upon receipt. Please contact technical support immediately, if the cells received were already thawed.

- Short-term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for longer than 24 hours).
- Long-term (greater than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen.

Safety Warning: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. Use tongs to remove cryovials from liquid nitrogen storage and place the vials immediately on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen that may be present inside the vial to evaporate. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

AssayComplete™ Cell Plating 2 Reagent (CP2)

Upon receipt, store at -20°C. Once thawed, the Cell Plating 2 Reagent can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C. Once aliquoted, do not freeze-thaw more than two times. Make aliquots suitable for testing one assay plate; for example, 20 mL of reagent per aliquot can be dispensed and stored at -20°C. Ensure that the reagent is equilibrated to room temperature before use in the assay for best performance.

AssayComplete Cell Assay Buffer

Upon receipt, store at -20°C. Once thawed, the Cell Assay Buffer can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C. Once aliquoted do not freeze-thaw more than twice. Make aliquots suitable for testing one assay plate; for example, 10 mL of reagent per aliquot can be dispensed and frozen down. This amount may vary depending on your stock sample concentrations and should be adjusted accordingly. Ensure that the reagent is equilibrated to room temperature before use in the assay for best performance.

Control Agonist (GIP)

Upon receipt, store at -20°C until ready to use (up to the expiration date listed on the kit’s Certificate of Analysis). Centrifuge the vial prior to opening, to maximize recovery, and reconstitute as recommended in the ligand datasheet with 0.200 mL of tissue culture grade water (as recommended by vendor) to make a stock concentration of 500 µM. Store in small, single-use aliquots at -20°C. The reconstituted ligand is stable for 12 months at -20 to -80°C, or 1 week at 2-8°C.

cAMP Detection Kit for Bioassays

Upon receipt, store the kit at -20°C. Once thawed, the kit components can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on the kit's Certificate of Analysis), the reagents should be aliquoted and stored at -20°C in opaque containers until needed. Once aliquoted do not freeze-thaw more than twice. Make aliquots suitable for testing one assay plate; for example,10 mL of reagent per aliquot can be dispensed and frozen down. This amount may vary depending on your stock sample concentrations and should be adjusted accordingly. Ensure that the reagents are equilibrated to room temperature before use in the assay for best performance.

96-well White, Flat-bottom Poly-D-Lysine-coated, Sterile Plates with Lid

Upon receipt, store at room temperature.

5. Additional Equipment and Materials Required

The below equipment and additional materials are required to perform these assays. Equivalent reagents may be used. All plastic materials should be stored at temperatures specified by suppliers.

Material	Ordering Information
Tirzepatide (research grade)	Selleckchem, Cat No. P1206, or similar
*Control Agonist (GIP)	DiscoverX, Cat No. 92-1078, or similar
DMSO, Anhydrous	Thermo Fisher Scientific, Cat No. 1003306536, or similar
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	DiscoverX, Cat No. 92-0011, or similar
15 mL LightSafe polypropylene tubes, sterile	Millipore Sigma (Cat No. Z688320), or similar
Multimode or luminescence plate reader	Refer to the Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Sterile disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094, or similar
50 mL and 15 mL Polypropylene tubes, sterile	
1.5 mL polypropylene microcentrifuge tubes, sterile	
Tissue culture disposable pipettes (1 mL - 25 mL), sterile	
Disposable pipette tips for P20, P100, P1000 pipettes (10 µL-1000 µL)	
Sterile biosafety cabinet	
Humidified tissue culture incubator	
Single and multichannel pipettes (10 µL-1000 µL)	

Note: *A positive control is only required for kits purchased with the bioassay kit configuration that does not include it. The positive control is used for QC testing of the bioassay cells in this kit, as reflected in the Certificate of Analysis.

6. Unpacking Cell Cryovials

Cryovials are shipped on dry ice and contain cells in 0.2 mL of AssayComplete™ Freezing Reagent. Upon receipt, the vials should be transferred to the vapor phase of liquid nitrogen for storage beyond 24 hours. The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage.

Appropriate personal protective equipment (PPE), including a face shield, gloves, and a lab coat should be worn during these procedures.

1. cAMP Hunter CHO-K1 GIPR Bioassay Cells must arrive in a frozen state on dry ice.

Contact technical support immediately, if cells received were already thawed.

2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For long-term storage, place vials in the vapor phase of liquid nitrogen storage.

Cryovials should be stored in the vapor phase of liquid nitrogen ONLY. These vials are not rated for storage in the liquid phase.

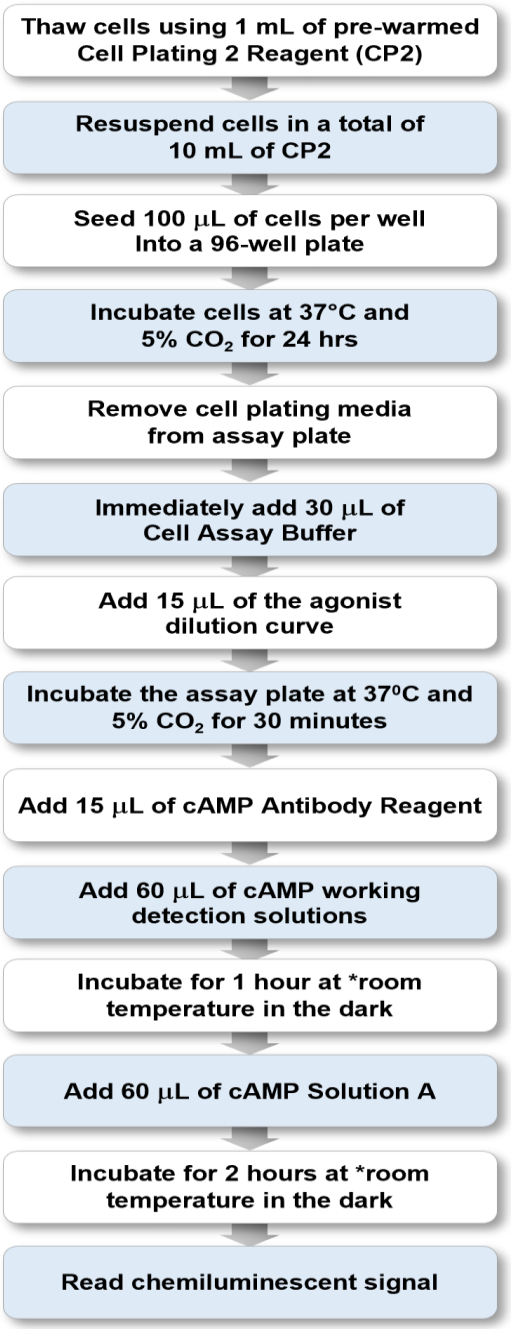
3. When ready to perform the assay, using tongs, remove the cryovials from the liquid nitrogen storage, and place them immediately in dry ice, in a covered container. Wait for at least one minute for any liquid nitrogen inside the vial to evaporate.

4. Proceed with the thawing protocol in the detailed assay protocol section 8.1.

7. Protocol Schematic

Quick-start Procedure: In a 96-well plate, perform the following steps.

Tip: *Use this sheet to note your assay specific conditions. Post it on your bench to use as a quick reference guide.*	Assay Name: _____Date: _____ Product Details: _____



*Room temperature refers to a range of 23-25°C

8. Detailed Protocol

This assay is performed under aseptic conditions. Cells and reference standards/test samples are prepared in a Biological Safety Cabinet following good aseptic technique. All appropriate materials are either certified sterile or prepared aseptically.

Prepared volumes may be scaled up or down if required. If purchasing the bioassay kit without control, it can be sourced per the details in the Additional Materials and Equipment Recommended for Assay.

8.1 Bioassay Cell Preparation

(Day 1)

The following protocol is for thawing and plating cryopreserved CHO-K1 GIPR bioassay cells from cryovials.

1. Before thawing the cells, ensure that all the materials required are set up in the tissue culture hood. These include:
   a. One sterile 25 mL reagent reservoir
   b. One sterile 15 mL conical tube
   c. A pipette (P1000) set to dispense 1 mL
   d. A multichannel pipette and tips set to dispense 100 µL
   e. A bottle of AssayComplete™ Cell Plating 2 Reagent (CP2), **pre-warmed in a 37°C water bath for 15 minutes and equilibrated to room temperature.**
   f. A 96-Well White, Flat-bottom Poly-D-Lysine-coated, Sterile Assay Plate (provided with the kit)
2. Dispense 10 mL of CP2 into the 15 mL conical tube.
3. Remove the cryovial from the liquid nitrogen vapor and immediately place it in dry ice.
4. Remove the cryovial from dry ice and ensure cap is tightened. Immediately thaw vial in 37°C water bath for 30 (+/- 5) seconds, gently shaking to thaw cells. **DO NOT LEAVE THE VIAL IN WATER BATH**.
5. Visually inspect bottom of vial after 20 seconds. If pellet is thawed, remove vial from water bath, wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood. If ice is still visible, return vial to water bath for additional 10-15 seconds.
6. Add 1 mL of pre-warmed CP2 from the 15 mL conical tube into the cryovial. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipette the cell suspension up and down, several times, to uniformly resuspend the cells.
7. Transfer the cell suspension to the conical tube containing the remaining 9 mL of CP2. Remove any remaining liquid from the cryovial to ensure maximum recovery of all the cells.
8. Replace the cap on the conical tube and mix by gentle inversion 2-3 times to ensure that the cells are properly resuspended in the reagent, without creating any froth in the suspension. Immediately transfer the suspension into the sterile 25 mL reagent reservoir.
9. Using a manual 12-channel multichannel pipette, transfer 100 µL of the cell suspension to each well

of the 96-well assay plate one row at a time, using reverse pipetting. Mix cells in reagent reservoir by pipetting up and down 2-3 times before aspirating and pipetting cells into each subsequent row in the assay plate.

10. Replace the lid and leave the plate at room temperature for 15 minutes, to allow the cells to settle uniformly in the well and minimize potential edge effects.
11. Incubate the assay plate in humidified tissue culture incubator at 37°C and 5% CO_2 for 24 ± 2 hours before proceeding with the assay.

8.2: Tirzepatide Reference Standard and Test Sample Preparation

(Day 2)

The following protocol gives the recommended dilutions for preparing the Tirzepatide reference standard and test sample working stocks and serial dilutions.

1. Before beginning, ensure all required reagents are assembled in biosafety cabinet:
    a. Pipettes (P20, P100, P1000)
    b. A multichannel pipette and tips set to dispense 20 µL.
    c. An aliquot of AssayComplete™ Cell Assay Buffer (CAB), **pre-warmed in a 37˚C water bath for 15 minutes, then equilibrated to room temperature.**
    d. A 96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plate, labeled ‘Master dilution plate’ (MDP).
    e. Sterile microfuge tubes, for preparation of intermediate, working, or nominal testing concentrations of Tirzepatide.
2. On day of assay, prepare Tirzepatide intermediate and working stock solutions in Cell Assay Buffer as shown in examples in Table 1 below.
    a. Tirzepatide used for qualification of this assay (see Additional Materials Required table) is supplied as a lyophilized powder to be dissolved in 207.8 µL of DMSO to prepare a 4.813 mg/mL stock.

Note: For Tirzepatide sourced from other vendors, stock concentration and reconstitution buffer volumes may differ. Follow vendor’s recommendations to prepare 4.813 mg/mL stock concentration.

    b. Reconstituted Tirzepatide stock form 2a may be stored in small aliquots at -20°C. Avoid multiple freeze-thaws.
    c. It is recommended to use a minimum aliquot size of 10 µL for preparation of all sample/stock dilutions.

Table 1. Example Preparation of Tirzepatide Intermediate Dilutions & Working Stocks

Intermediate and Working Stock Solutions	Final Concentration, µg/mL	Volume of Tirzepatide (Stock concentration)	Volume Dilution Buffer (CAB), µL
Reference Standard (Tirzepatide), Intermediate Stock 1	120.329	10 µL of 4.813 mg/mL	390
Reference Standard (Tirzepatide), Intermediate Stock 2	6.016	10 µL of 120 µg/mL	190
Reference Standard (Tirzepatide), Intermediate Stock 3	0.602	20 µL of 6.016 µg/mL	180
Working Stock of Tirzepatide	0.150	88 µL of 0.602 µg/mL	265

Note: Use a vortex to mix (10-20 sec) for each dilution before preparing next serial dilution.

3. On the day of assay, prepare serial dilutions of the Tirzepatide Reference Standard (RS) in row A of the 96-well master dilution plate (MDP), at 3X the final concentration of each dilution, in CAB (i.e. the Ligand Diluent) as per Table 2 below.

   Table 2 below indicates an example sample dilution scheme. Following this dilution scheme should result in sufficient volumes to run triplicate wells per dose in the assay plate.

   a. Starting at Row A, Well 1 of the MDP, add appropriate volume of ligand diluent (CAB) as indicated in column 6 of Table 2.

   b. Transfer appropriate volume (indicated in column 5 of Table 2) of Tirzepatide Working Stock to the MDP Row A, Well 2.

   c. Prepare the dilution series by transferring the indicated volume of Tirzepatide RS sample (column 5, Table 2) from the source well (indicated in column 4) to the destination well (indicated in column 1). Pipette up and down several times to mix in destination wells. Replace pipette tips between each serial dilution.

   d. No Tirzepatide working stock is added to well 1 (vehicle only), as this serves as the negative control.

Table 2. Sample concentration in Master Dilution prepared as a 3X stock: Example of Preparation of Tirzepatide RS Serial Dilutions

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Well location on Master Dilution Plate (MDP)	Concentration (3X) of Tirzepatide on MDP Row A, ng/mL	Dilution Factor	Dilution (3X) Tirzepatide Source Well	Volume (3X) of Tirzepatide added, µL	Volume of Ligand Diluent, µL	Final Concentration (1X) of Tirzepatide in Assay Plate, ng/mL
Row A, Well 1	0	--	--	--	180	--
Row A, Well 2	150	--	Working Stock (150 ng/mL; see Table 1)	300.0	-	50.000
Row A, Well 3	50.000	3	Row A, Well 2	100	200	16.667
Row A, Well 4	16.667	3	Row A, Well 3	100	200	5.556
Row A, Well 5	5.556	3	Row A, Well 4	100	200	1.852
Row A Well 6	2.778	2	Row A, Well 5	150	150	0.926
Row A, Well 7	1.389	2	Row A, Well 6	150	150	0.463
Row A, Well 8	0.694	2	Row A, Well 7	150	150	0.231
Row A, Well 9	0.347	2	Row A, Well 8	150	150	0.116
Row A, Well 10	0.174	2	Row A, Well 9	150	150	0.058
Row A, Well 11	0.043	4	Row A, Well 10	75	225	0.014
Row A, Well 12	0.011	4	Row A, Well 11	75	225	0.004

4. On the day of assay, prepare appropriate dilutions of Test Sample (TS), as needed, to generate 300 µL of a working stock of 150 ng/mL (as prepared for Tirzepatide Reference Standard Working Stock; in Table 1).

Note: depending on stock concentration of TS, intermediate dilutions of TS may need to be prepared (as demonstrated in Table 1 for the Tirzepatide Reference Standard) to generate the 150 ng/mL TS working stock, which is used as the starting material for the top dose in the dose response curve.

   a. All dilutions should be prepared in CAB diluent. Volumes may be scaled up or down as needed. For preparing all sample/stock dilutions, it is recommended to use a minimum aliquot size of 10 µL.

5. On the day of assay, prepare serial dilutions of TS in row B of the 96-well master dilution plate (MDP), at 3X the final concentration of each dilution, in CAB (i.e., the Ligand Diluent) using the same dilution

series as for the Tirzepatide RS, as per the example in Table 2 above. Sufficient volumes to run triplicate wells per dose in the assay plate will be the result.

6. If using the supplied GIP (500 µM stock= 2500 µg/mL) as positive control, prepare GIP intermediate and working stock solutions in CAB on day of assay, as shown in examples in Table 3 below.
   a. Volumes may be scaled up or down as needed. For preparing all stock dilutions, it is recommended to use a minimum aliquot size of 10 µL.

Table 3. Example Preparation of GIP Intermediate Dilutions & Working Stocks

Intermediate and Working Stock Solutions	Final Concentration, µg/mL	Volume of GIP (Stock concentration)	Volume Dilution Buffer (CAB), µL
GIP ligand, Intermediate Stock 1	125.0	10 µL of 2500 µg/mL	190
GIP ligand, Intermediate Stock 2	12.5	10 µL of 125.0 µg/mL	90
GIP ligand, Intermediate Stock 3	1.25	10 µL of 12.5 µg/mL	90
Working Stock of GIP	0.600	48 µL of 1.25 µg/mL	52

Note: Use a vortex to mix (10-20 sec) for each dilution before preparing next serial dilution.

7. On the day of assay, prepare serial dilutions of the GIP ligand in row C of the 96-well master dilution plate (MDP), at 3X the final concentrations of each dilution, in CAB (i.e. the Ligand Diluent) as per Table 4 below. Sufficient volumes to run duplicate wells per dose in the assay plate will be the result.
   a. Add appropriate volume of ligand diluent (CAB) to row C of the MDP, (as indicated in column 6) of Table 4.
   b. Transfer indicated volume of Working Stock of GIP to the MDP Row C Well 2; the volume that should be transferred is indicated in column 5 of Table 4.
   c. Prepare the dilution series by transferring the volume of GIP ligand (indicated in column 5) from the source well (indicated in column 4) to the destination well (indicated in column 1). Pipette up and down several times to mix in destination wells. Replace pipette tips between each serial dilution.
   d. No sample is added to well 1 (vehicle only), as this serves as the negative control.

Table 4. Sample concentration in Master Dilution Plate prepared as a 3X stock: Example Preparation of GIP Sample Serial Dilutions

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Well location on Master Dilution Plate (MDP)	Concentration (3X) of GIP on MDP Row C, ng/mL	Dilution Factor	Dilution (3X) GIP Source Well	Volume (3X) of GIP added, µL	Volume of Ligand Diluent, µL	Final Concentration (1X) of GIP in Assay Plate, ng/mL
Row C, Well 1	0	--	--	--	180	--
Row C, Well 2	600		Working Stock of GIP (600 ng/mL; see Table 3)	100	-	200
Row C, Well 3	200	3	Row C, Well 2	40	80	66.667
Row C, Well 4	66.667	3	Row C, Well 3	40	80	22.222
Row C, Well 5	22.222	3	Row C, Well 4	40	80	7.407
Row C Well 6	11.111	2	Row C, Well 5	60	60	3.704
Row C, Well 7	5.556	2	Row C, Well 6	60	60	1.852
Row C, Well 8	1.852	3	Row C, Well 7	40	80	0.617
Row C, Well 9	0.617	3	Row C, Well 8	40	80	0.206
Row C, Well 10	0.206	3	Row C, Well 9	40	80	0.069
Row C, Well 11	0.069	3	Row C, Well 10	40	80	0.023
Row C, Well 12	0.023	3	Row C, Well 11	40	80	0.008

8. Assay plate preparation:
   a. Remove assay plates with cells from incubator.
   b. With a flicking motion, remove liquid from the assay wells in the plate. Then, place the assay plate upside down on a lab-grade low lint tissue/paper towel and spin in a plate centrifuge set at 1000 rpm. Stop once it reaches 1000 rpm. Alternately, completely remove the Cell Plating Reagent from each assay plate by carefully aspirating the wells.
   c. Immediately add 30 µL of Cell Assay Buffer to all empty wells of the plate.
9. Transfer 15 µL of the 3X sample dilutions from the MDP to the appropriate wells containing cells in the assay plate. Refer to Figure 3. Representative Assay Plate Map:
   a. Row A in MDP: transfer to Rows B, D and F in the assay plate (Reference Standard)
   b. Row B in MDP: transfer to Rows C, E and G in the assay plate (Test Sample)
   c. Row C in MDP: transfer to Rows A and H in the assay plate (GIP control)
10. Incubate the assay plate in a humidified incubator at 37°C and 5% CO_2 for 30 minutes.

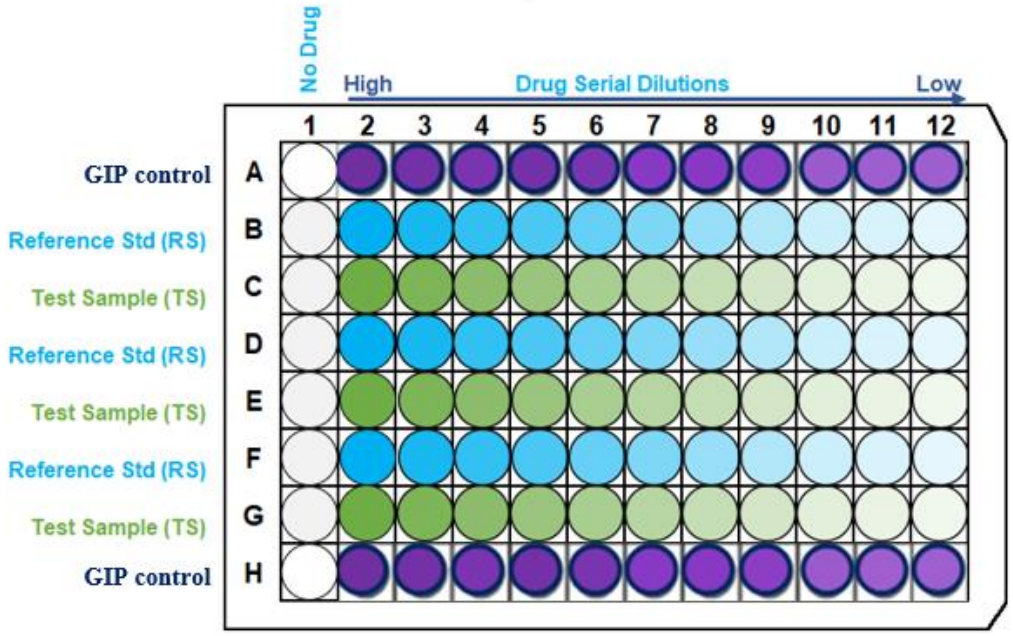


Figure 2. Representative Assay Plate Map

This plate map shows two interdigitated 11-point dose curves, with three replicates per dose point, for a test sample (TS) and reference standard (RS) prepared using the same dilution scheme. Rows A and H contain GIP control which uses an independent dilution scheme. Column 2 contains the highest dose of each sample, while column 12 contains the lowest dose. Column 1 contains no drug (CAB only).

8.3 Addition of Detection Reagent

Signal Detection

1. During the 30-minute agonist incubation period, prepare cAMP working detection solution in a separate 15 mL polypropylene tube by mixing 19-parts of cAMP Lysis Buffer, 5-parts of Substrate Reagent 1, 1-part Substrate reagent 2, and 25-parts of cAMP Solution D. Refer to Table 5 below for the volume of each component required for one 96-well plate. Adjust volumes according to your assay set-up, keeping the ratios consistent. Store in the dark before use.
2. Following agonist incubation, add 15 µL of the cAMP Antibody Reagent to all wells of the assay plate.

Table 5. Working Detection Solution Preparation

Working Detection Solution		
Components	**Volume Ratio**	**Volume per 96-well Plate (mL)**
cAMP Lysis Buffer	19	3.8
Substrate Reagent 1	5	1.0
Substrate Reagent 2	1	0.2
cAMP Solution D	25	5.0
Total Volume		**10**

3. Add 60 µL of the prepared cAMP working detection solution to all wells of the assay plate. **Do not pipette up and down or vortex the plate to mix.**
4. Incubate the assay plate for 1 hour at room temperature in the dark.

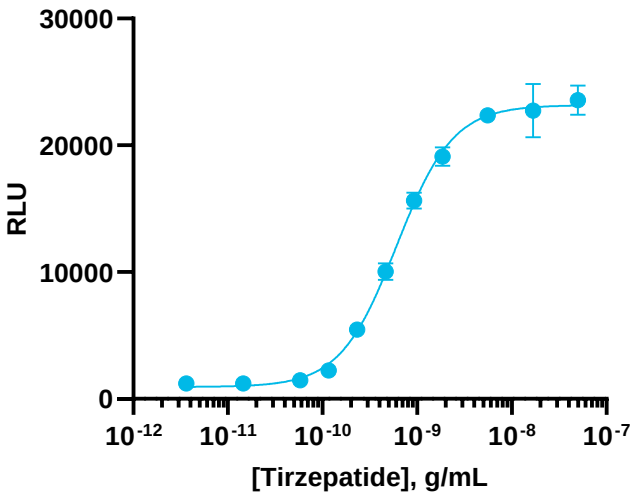
   Room temperature refers to a range of 23-25°C.

5. Add 60 µL of cAMP Solution A to all wells of the assay plate. **Do not pipette up and down or vortex the plate to mix.**
6. Incubate the assay plate for 2 hours at room temperature in the dark.
7. Read the sample on a Perkin Elmer Envision, SpectraMax ID3 or similar plate reader, 0.2 sec/well integration time or on a similar benchtop instrument. Refer to the Instrument Compatibility Chart at discoverx.com/instrument-compatibility/
8. Data analysis can be performed using any statistical analysis software such as GraphPad Prism, SoftMax Pro, Gen5, or Microsoft Excel etc.

9. Typical Results

The following graph is an example of a typical dose-response curve for the Tirzepatide (GIP RA) Bioassay generated using the protocol outlined in this manual. The data shows a potent, dose-dependent increase in cAMP production when treated with Tirzepatide. The plate was read on the EnVision® Multimode Plate Reader, with a 0.2 sec/well integration time, and data analysis was conducted using GraphPad Prism.

A



B

S/B	EC_{50}, ng/mL
19.3	0.6039

Figure 3: Representative A, dose-response curve and B, EC_{50} and assay window (S/B) for Tirzepatide-mediated GIPR activation, as measured in this bioassay.

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Incorrect thawing procedure	Refer to the thawing instructions in the Bioassay Cell Preparation section of this user manual.
	Incorrect ligand used or improper ligand incubation time	Refer to the Certificate of Analysis for recommended ligand and assay conditions.
	Incorrect preparation of the ligand (agonist or antagonist)	Refer to the vendor-specific datasheet to ensure proper handling, dilution, and storage of the ligand.
	Sub-optimal time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased or no response	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using a microscope.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 0.1-1 seconds/well.
Low or no signal	Incorrect preparation of detection reagents	Detection reagents are sensitive to light and should ideally be prepared just prior to use.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 0.1-1 second/well.
Experimental S/B does not match the value noted in the Certificate of Analysis provided	Incorrect incubation temperature	Confirm assay conditions.
		Check and repeat the assay at the correct incubation temperature, as indicated on the assay datasheet.
	Incorrect preparation of ligand (agonist or antagonist)	Some ligands are difficult to handle. Confirm the final concentration of ligands.
EC_{50} is right-shifted	Improper ligand handling or storage	Ensure that the ligands are stored and incubated at the proper temperature.
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Certificate of Analysis.
	Problems with dynamic range or dilutions	Changing tips during dilution can help in avoiding carryover. Include a cAMP standard curve in the assay to ensure that the ligand dose curve is in the dynamic range of the cAMP detection kit. Moving out of the dynamic range may shift the EC_{50} of the ligands.
High variability between replicates	Instrument calibration	Ensure that the dispensing equipment is correctly calibrated, and proper pipetting technique is used.

For questions on using this product, please contact Technical Support at discoverx.com/support/

Document Revision History

Revision Number	Date Released	Revision Details
0	May 2024	New Document

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