User Manual

cAMP Hunter™ Tirzepatide (GLP-1 RA) Bioassay Kit

For the Measurement of Ligand-Mediated cAMP Accumulation

For Bioassay Kits with control

95-0062Y2-00198: 2-Plate Kit

95-0062Y2-00199: 10-Plate Kit

For Bioassay Kits without control

95-0062Y2-00200: 10-Plate Kit

Document Number 70-447 Rev0

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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 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 G-protein coupled receptor (GPCR) 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 (GLP-1 RA) Bioassay Kit is also offered without 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 but can be adapted to a 384-well format if needed.

2. Assay Principle

Ligand-mediated GPCR stimulation leads to the activation of G-proteins, which in turn triggers downstream signaling pathways by recruiting, activating or inhibiting cellular enzymes. One such enzyme is adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) to cAMP. Adenylate cyclase is either stimulated or inhibited by the G-protein subunits, $G\alpha_s$ and $G\alpha_i$, respectively. In the cAMP Hunter Tirzepatide Bioassay Kit, cells overexpressing GLP-1R utilize the natural coupling status of the GPCR to monitor activation of the $G\alpha_s$-coupled receptor. Following Stimulation, the functional status of GLP1R is monitored by measuring cellular cAMP levels using a homogenous, gain-of-signal competitive immunoassay based on Enzyme Fragment Complementation (EFC) technology.

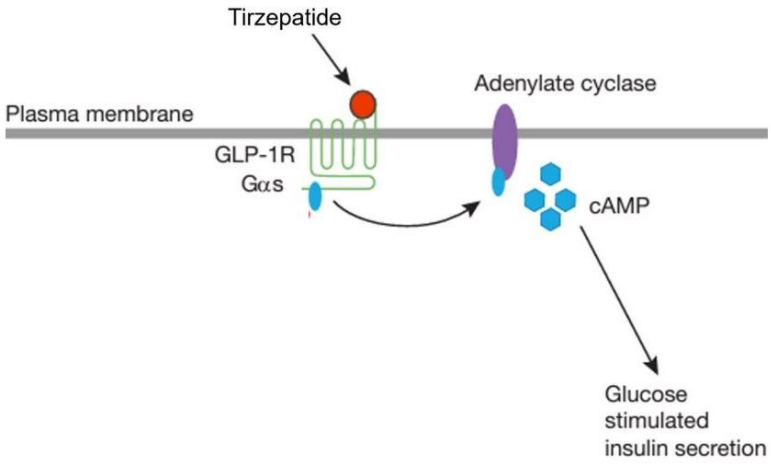


Figure 1. Assay Principle

The cAMP Hunter Bioassay has been developed to interrogate the GPCR cAMP Pathway. Ligand-mediated activation of a GPCR either stimulates or inhibits adenylate cyclase to modulate cellular cAMP levels. In the case of GLP1R, its activation by Tirzepatide stimulates adenylate cyclase, which in turn enables the production of cAMP.

cAMP Detection Kit Principle

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 and ED-labeled cAMP (ED-cAMP) compete for anti-cAMP antibody (Ab). Antibody-bound ED-cAMP does not complement with EA, but unbound ED-cAMP is free to complement with EA to form an active enzyme, which subsequently produces 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 GLP1R activation, the greater the cAMP levels inside the cells, and larger the signal in the assay.

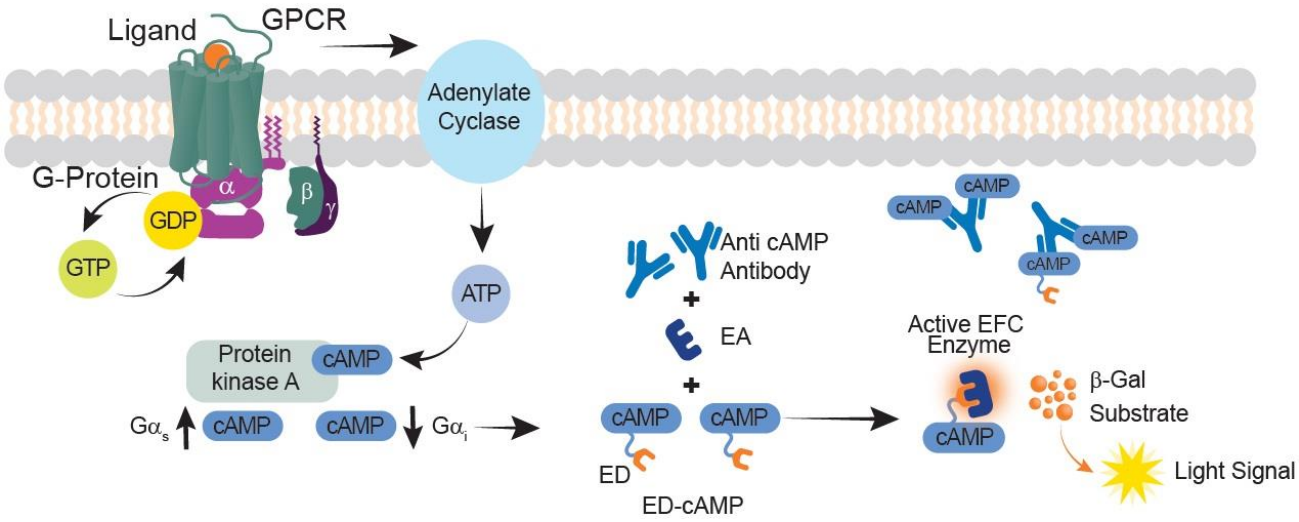


Figure 2. cAMP Detection Kit Principle

When cellular cAMP levels are low, ED-labeled cAMP successfully binds with the anti-cAMP-antibody, as there aren't enough cellular cAMP molecules to compete against it. The bound ED-labeled cAMP will not be available to undergo complementation. 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, which successfully forms an active β-galactosidase enzyme that hydrolyzes the substrate and generates a chemiluminescent signal proportional to the level of cAMP in the cell.

3. Materials Provided

List of Components	95-0062Y2-00198 (2-Plate Kit)	95-0062Y2-00199 (10-Plate Kit)	95-0062Y2-00200 (10 plate Kit without control)
cAMP Hunter CHO-K1 GLP1R Bioassay Cells (3.75 x 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
AssayComplete Protein Dilution Buffer B2 (PDB-B2) (50 mL per bottle)	1 x 50 mL	2 x 50 mL	2 x 50 mL
Control Agonist (Recombinant Human Exendin-4)	1 x 500 µg	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, Clear-Bottom, TC-Treated, Sterile Plates with Lid	2 Plates	10 Plates	10 Plates

***Note**: For 95-0062Y2-00200 control agonist is not provided in the kit and would need to be obtained separately if required. For ordering information for the control agonist, refer to the Additional Materials Required table in section 5.

4. Storage Conditions

cAMP Hunter CHO-K1 GLP1R Bioassay Cells

Bioassay 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 should be dispensed and frozen down. Ensure that the reagent is equilibrated to room temperature before use in the assay for best performance.

AssayComplete Protein Dilution Buffer B2 (PDB-B2)

Upon receipt, store at -20°C. Once thawed, the Protein Dilution Buffer B2 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 should 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 (Recombinant Human Exendin-4)

Upon receipt, store at -20°C until ready to use (up to the expiration date listed on the kit’s Certificate of Analysis). To avoid condensation, equilibrate the vial to ambient temperature before opening. Centrifuge the vial prior to opening, to maximize recovery, and reconstitute as recommended in the ligand datasheet with 0.478 mL of reconstitution buffer to make a stock concentration of 250 µM. The reconstituted ligand is stable for 12 months at -20 to -80°C (store in small aliquots; do not refreeze), 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 . Ensure that the reagents are equilibrated to room temperature before use in the assay for best performance.

96-Well Clear Bottom Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

5. Additional Materials Required

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

Material	Ordering Information
Tirzepatide (research grade)	Sellekchem, Cat No. P1206, or similar
***Control Agonist (Recombinant Human Exendin-4)	DiscoverX, Cat No. 92-1115, 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
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 pipet tips for P20, P100, P1000 micropipettors	
Sterile biosafety cabinet	
Automated cell counter or hemocytometer	
Humidified tissue culture incubator	
Single and multichannel micropipettors (e.g. P20, P100, P1000)	

Note: ***Positive control used for QC testing of the bioassay cells in this kit as reflected in Certificate of Analysis; only needed if the kit purchased was the variant without control included

6. Unpacking Cell Cryovials

Cryovials are shipped on dry ice and contain cells in 0.1 mL of AssayComplete™ Freezing Reagent (refer to the CoA for cell number provided in the vial). 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 GLP1R 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.

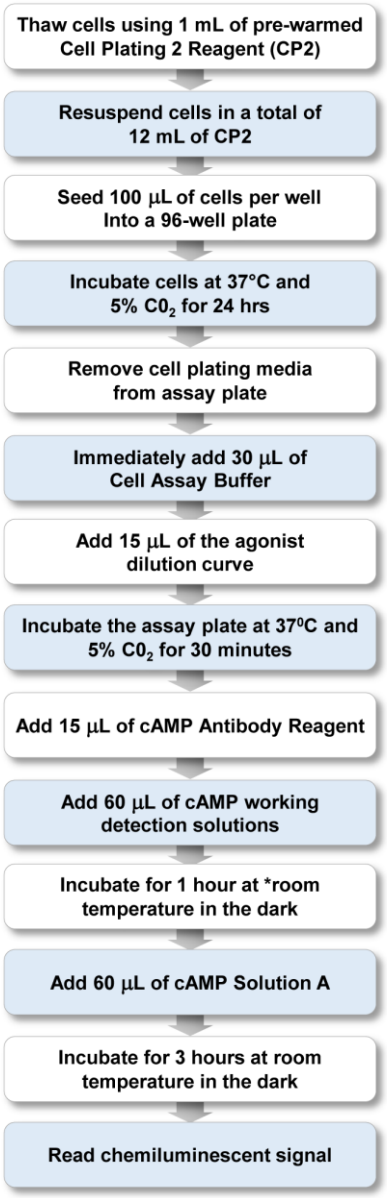
3. Using tongs, remove the cryovials from the liquid nitrogen storage, and place them immediately in dry ice, in a covered container. Wait for at least one minute for any liquid nitrogen inside the vial to evaporate.

4. Proceed with the thawing protocol in the detailed assay protocol section. Refer to the CoA for the appropriate AssayComplete products mentioned in the protocol below.

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. Preparation of cells, Reference Standards/Test Samples are performed 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 GLP1R bioassay cells from cryovials.

1. Before thawing the cells, ensure that all the materials required are set up in the biosafety cabinet. These include:
   a. One sterile 25 mL reagent reservoir
   b. One sterile 15 mL conical tube
   c. A micropipettor (P1000) set to dispense 1 mL
   d. A multichannel pipette and tips set to dispense 100 µL
   e. An aliquot 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, Clear bottom, Tissue Culture-Treated Sterile Assay Plate (provided with the kit)
2. Dispense 12 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.

   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 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 1.0 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 pipet 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 11 mL of CP2. Remove any remaining liquid from the cryovial to ensure maximum recovery of all the cells.

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.

8. Using a manual 12-channel multichannel pipet, 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 dispensing cells into each subsequent row in the assay plate.
9. 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.
10. 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 Sample Preparation

(Day 2)

Tirzepatide Reference Standard (RS) and Test Sample (TS) Preparation

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. Micropipettors (P20, P100, P1000)
   b. A multichannel pipette and tips set to dispense 20 µL.
   c. An aliquot of AssayComplete™ Protein Dilution Buffer-B2, **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 reference or test samples.
2. On the day of assay, prepare Tirzepatide RS or test sample intermediate and working stock solutions in PDB-B2 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 and is recommended by vendor 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 reccomedations to prepare 4.813 mg/mL stock concentration.

Reconstituted Tirzepatide stock from Step 2a may be stored in small aliquots at -20°C.

   b. It is recommended to use a minimum aliquot size of 10 µL for preparation of all sample / stock dilutions.

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

Intermediate and Working Stock Solutions	Final Concentration, µg/mL	Volume of Tirzepatide (Stock concentration)	Volume Dilution Buffer (PDB-B2), µL
Reference Standard (Tirzepatide), Intermediate Stock 1	120.329	10 µL (of 4.813 mg/mL stock)	390
Reference Standard (Tirzepatide), Intermediate Stock 2	6.016	10 µL (of 120.329 µg/stock)	190
Reference Standard (Tirzepatide), Intermediate Stock 3	0.602	20 µL (of 6.016 µg/mL stock)	180
Working Stock of Tirzepatide	0.024	12 µL (of 0.602 µg/mL stock)	288

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 the master dilution plate (MDP), **at 3X the final concentrations of each dilution**, in PDB-B2 (i.e. the Ligand Diluent).
   An example sample dilution scheme is indicated in Table 2 below. 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 PDB-B2; as indicated in column 6 of Table 2.
   b. Transfer appropriate volume (indicated in column 5, Table 2) of Tirzepatide Working Stock to the MDP Row A Well 2.
   c. Transfer indicated volume (column 5, Table 2) of Tirzepatide sample from the source well (indicated in column 4) to the destination well (indicated in column 1). Pipet up and down several times after each transfer to ensure dilutions are mixed well. Replace pipet tips between each serial dilution.
   d. No Tirzepatide working stock is added to Row A Well 1 (PDB-B2 only), to serve 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 sample on MDP Row A, ng/mL	Dilution Factor	Dilution (3X) sample Source Well	Volume (3X) of sample added, µL	Volume of Ligand Diluent, µL	Final Concentration (1X) of sample in Assay Plate, ng/mL
Row A, Well 1	0	--	--	0	180	0 (Negative Control)
Row A, Well 2	24.0	--	Working Stock (0.024 µg/mL; from Table 1)	300	0	8.00
Row A, Well 3	9.60	2.5	Row A, Well 2	120	180	3.20
Row A, Well 4	3.84	2.5	Row A, Well 3	120	180	1.28
Row A, Well 5	1.54	2.5	Row A, Well 4	120	180	0.512
Row A Well 6	1.02	1.5	Row A, Well 5	200	100	0.341
Row A, Well 7	0.683	1.5	Row A, Well 6	200	100	0.228
Row A, Well 8	0.455	1.5	Row A, Well 7	200	100	0.152
Row A, Well 9	0.228	2.0	Row A, Well 8	150	150	0.076
Row A, Well 10	0.114	2.0	Row A, Well 9	150	150	0.038
Row A, Well 11	0.0455	2.5	Row A, Well 10	120	180	0.0152
Row A, Well 12	0.0182	2.5	Row A, Well 11	120	180	0.0061

4. On the day of assay, prepare appropriate dilutions of Test Sample (TS), as needed, to generate 300 µL of a working stock of 24 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 24 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 PDB-B2 diluent. It is recommended to use a minimum aliquot size of 10 µL for preparation of all sample / stock dilutions.

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 PDB-B2 (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 supplied Exendin-4 as positive control, prepare Exendin-4 intermediate and working stock solutions in PDB-B2 as shown in examples in Table 3 below:
   a. Exendin-4 (see Additional Materials Required table if using kit# 95-0062Y2-00200, which does not include the control agonist) is supplied as a lyophilized powder and is dissolved in 0.478 mL of supplied reconstitution buffer (PDB) (as recommended in datasheet) to prepare a 1.046 mg/mL stock concentration.
      i. Reconstituted Exendin-4 stock from 6a may be stored in small aliquots at -20°C. Avoid mutiple freeze thaws.
   b. It is recommended to use a minimum aliquot size of 10 µL for preparation of all sample / stock dilutions.

Table 3. Example Preparation of Exendin-4 Intermediate Dilutions & Working Stocks

Intermediate and Working Stock Solutions	Final Concentration, µg/mL	Volume of Exendin-4 (Stock concentration)	Volume Dilution Buffer (PDB-B2), µL
Exendin-4, Intermediate Stock 1	20.8	10 µL of 1.046 mg/mL	493
Exendin-4, Intermediate Stock 2	1.04	15 µL of 20.8 µg/mL	285
Exendin-4, Intermediate Stock 3	0.052	15 µL of 1.04 µg/mL	285
Working Stock of Exendin-4	0.003	23 µL of 0.052 µg/mL	377

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 Exendin-4 in Row C of the 96-well master dilution plate (MDP), at 3X the final concentrations of each dilution, in PDB-B2 (i.e. the Ligand Diluent) as per Table 4 below. Following this dilution scheme should result in sufficient volumes to run triplicate wells per dose in the assay plate.

   a. Starting at Row C, Well 1 of the MDP, add appropriate volume of PDB-B2; as indicated in column 6 of Table 4.
   b. Transfer appropriate volume (indicated in column 5, Table 4) of Exendin-4 Working Stock (3X stock= 0.003 µg/mL or 3 ng/mL) to the MDP Row C, Well 2.
   c. Transfer indicated volume (column 5, Table 4) of Exendin-4 sample from the source well (indicated in column 4) to the destination well (indicated in column 1). Pipet up and down several times after each transfer to ensure dilutions are mixed well. Replace pipet tips between each serial dilution.
   d. No sample is added to Well 1 (vehicle only), as this serves as the negative control.

Table 4. Exendin-4 concentration in Master Dilution prepared as a 3X stock: Example of Preparation of Exendin-4 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 Exendin-4 on MDP Row A, ng/mL	Dilution Factor	Dilution (3X) Exendin-4 Source Well	Volume (3X) of sample added, µL	Volume of Ligand Diluent, µL	Final Concentration (1X) of sample in Assay Plate, ng/mL
Row C, Well 1	0	--	--	--	180	--
Row C, Well 2	3.000		Working Stock (3 ng/mL) from Table 3	300.0	-	1.0000
Row C, Well 3	1.000	3	Row C, Well 2	100	0.0	0.3333
Row C, Well 4	0.333	3	Row C, Well 3	100	200	0.1111
Row C, Well 5	0.111	2	Row C, Well 4	150	200	0.0556
Row C Well 6	0.074	2	Row C, Well 5	150	150	0.0278
Row C, Well 7	0.049	1.5	Row C, Well 6	200	150	0.0185
Row C, Well 8	0.033	1.5	Row C, Well 7	200	100	0.0123
Row C, Well 9	0.022	2	Row C, Well 8	150	100	0.0062
Row C, Well 10	0.011	2	Row C, Well 9	150	150	0.0031
Row C, Well 11	0.004	2.5	Row C, Well 10	120	150	0.0012
Row C, Well 12	0.001	2.5	Row C, Well 11	120	180	0.0005

9. 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 1000rpm. 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 wells of the plate.

10. 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 (Exendin-4 Ctrl)

11. Incubate the assay plate in a humidified incubator at 37°C and 5% CO_2 for 30 minutes.

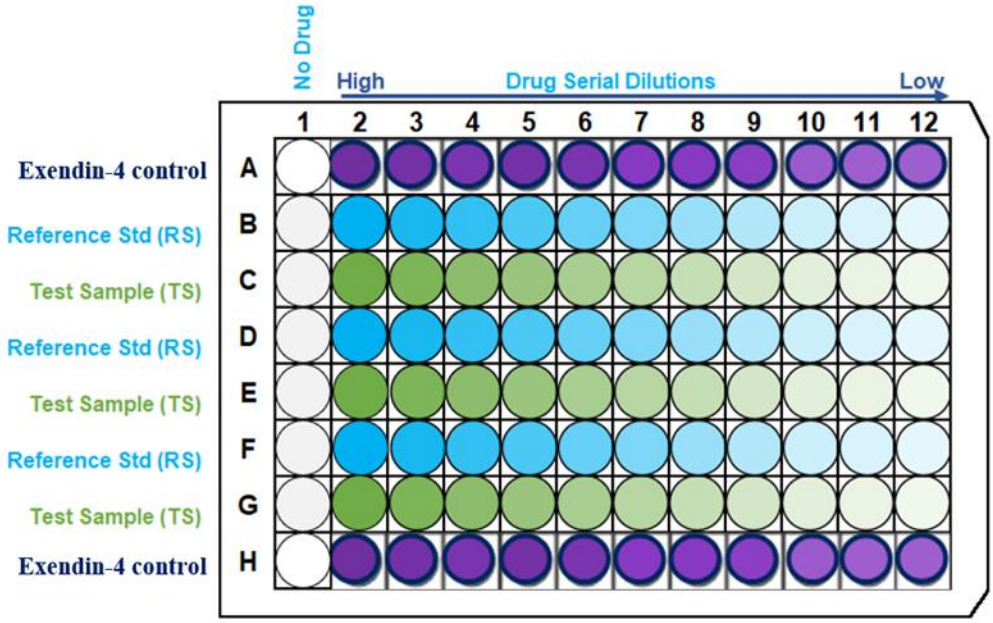


Figure 3. **Representative Assay Plate Map**

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 Exendin-4 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 (PDB-B2 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.

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

2. Following agonist incubation, add 15 µL of the cAMP Antibody Reagent to all wells of the assay plate.
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.

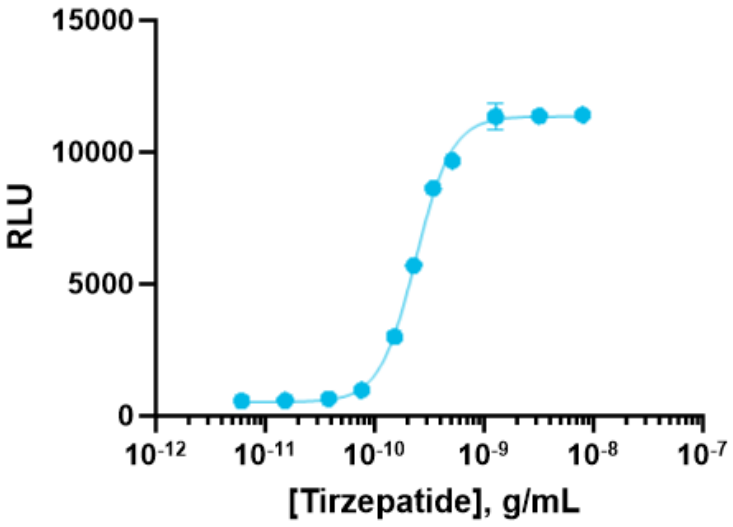
Room temperature refers to a range of 23-25°C.

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 3 hours at room temperature in the dark.
7. Read the sample on a Perkin Elmer Envision, with a 0.2 sec/well integration time, or on a similar bench top 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 Result

The following graph is an example of a typical dose-response curve for the Tirzepatide 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

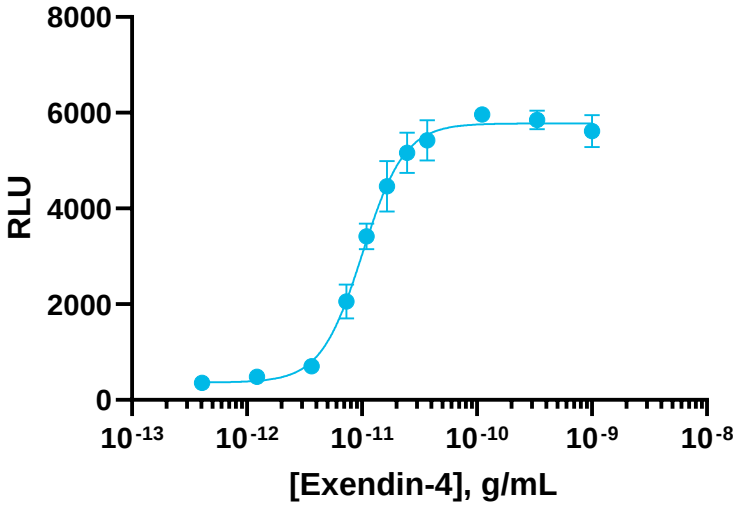


B

S/B	EC_{50}, pg/mL
20	237

Figure 4. Representative A, dose-response curve, and B, EC_{50} and assay window (Signal/Background, S/B) for Tirzepatide-mediated GLP1R activation, as measured in this bioassay.

A



B

S/B	EC_{50}, pg/mL
17	10.4

Figure 5. Representative A, dose-response curve, and B, EC_{50} and assay window (Signal/Background, S/B) for Exendin-4-mediated GLP1R activation, as measured in this bioassay.

10. 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	April 2024	New Document

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