User Manual

cAMP Hunter™ Exenatide Bioassay Kit

For the Measurement of Control-Mediated cAMP Accumulation

For Bioassay Kits with control:

95-0062Y2-00101: 2-Plate Kit

95-0062Y2-00102: 10-Plate Kit

For Bioassay Kits without control

95-0062Y2-00192: 10-Plate Kit

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1. Overview

The cAMP Hunter Exenatide 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 control -mediated GPCR activation. The bioassay kit contains all the materials needed for a complete assay, including cells, detection reagents, cell plating reagent, assay plates, and for some kits, a positive control agonist. 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

Control-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. The cAMP Hunter Exenatide Bioassay monitors activation of the naturally $G\alpha_s$-coupled receptor, GLP1R, by measuring the cellular cAMP levels using a homogeneous, gain-of-signal competitive immunoassay based on Enzyme Fragment Complementation (EFC) technology, which is explained in the cAMP Detection Kit Principle section.

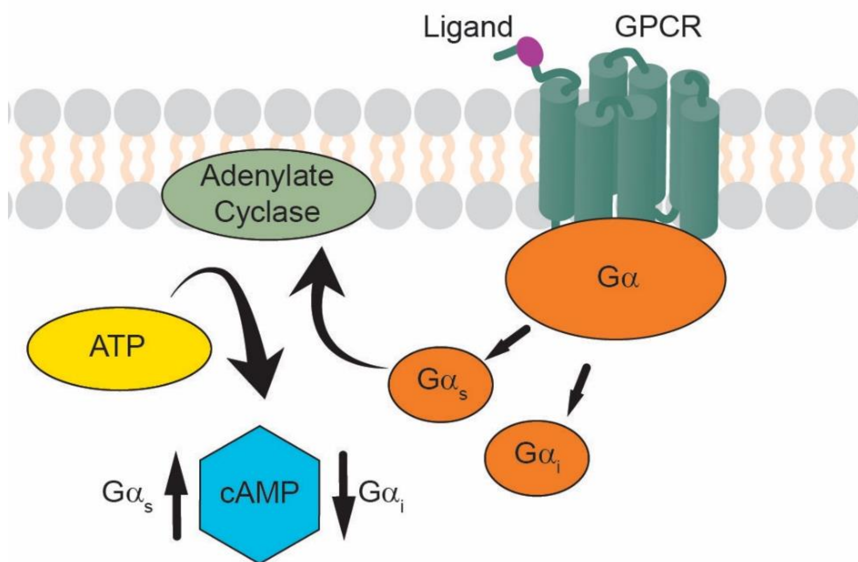


Figure 1. Assay Principle

The cAMP Hunter Bioassay has been developed to interrogate the GPCR cAMP Pathway. Control-mediated activation of GPCR either stimulates or inhibits adenylate cyclase to modulate cellular cAMP levels. In the case of GLP1R, its activation by Exenatide stimulates adenylate cyclase, which in turn leads to an increase in 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.

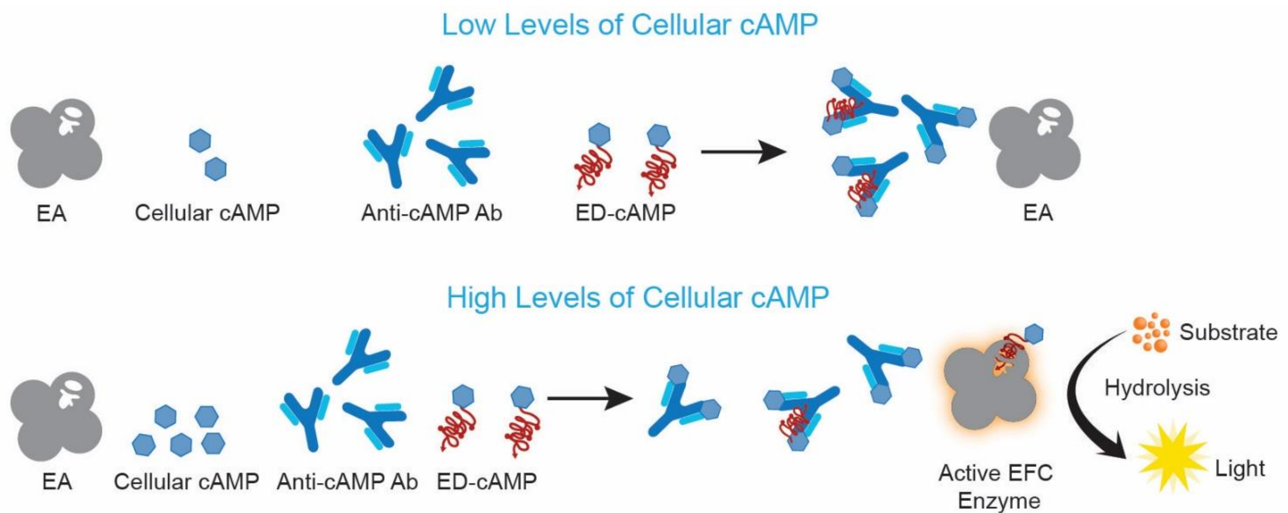


Figure 2. cAMP Detection Kit Principle

3. Materials Provided in cAMP Hunter™ Exenatide Bioassay Kit

List of Components	95-0062Y2-00101 (2-Plate Kit)	95-0062Y2-00102 (10-Plate Kit)	95-0062Y2-00192 (10-Plate Kit w/o control)
cAMP Hunter CHO-K1 GLP1R Bioassay Cells (3.75 x 10^6 cells in 0.2 mL per vial)	2	10	10
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	3 x 50 mL	3 x 50 mL
*Control Agonist (Human Exendin-4) (500 µg per vial)	1	1	No Control
cAMP Detection Kit for Bioassays cAMP Standard (250 µM) (Bottle) cAMP Antibody Reagent (Bottle) cAMP Lysis Buffer (Bottle) Substrate Reagent 1 (Bottle) Substrate Reagent 2 (Bottle) cAMP Solution D (Bottle) cAMP Solution A (Bottle)	 1 x 0.2 mL 1 x 5 mL 1 x 7.6 mL 1 x 2 mL 1 x 0.4 mL 1 x 10 mL 1 x 16 mL	 1 x 1 mL 1 x 25 mL 1 x 38 mL 1 x 10 mL 1 x 2 mL 1 x 50 mL 1 x 80 mL	 1 x 1 mL 1 x 25 mL 1 x 38 mL 1 x 10 mL 1 x 2 mL 1 x 50 mL 1 x 80 mL
96-Well White, Clear-Bottom, TC-Treated, Sterile Plates with Lid	2 Plates	10 Plates	10 Plates

***Note:** For 95-0062Y2-00192 control not provided in the kit, would need to be obtained separately if needed.

4. Storage Conditions

cAMP Hunter CHO-K1 GLP1R 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 always worn 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.

Assay Complete™ Cell Plating 2 Reagent (CP2)

Upon receipt, store at -20°C. Once thawed, the Cell Plating 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 until needed. Avoid multiple freeze-thaw cycles. To make aliquots suitable for testing one assay plate each, 20 mL of reagent per aliquot can be dispensed and frozen down.

AssayComplete Cell Assay Buffer

Upon receipt, store reagents at -20°C. It is important to thaw cell assay buffer from -20°C to room temperature at least 24 hours prior to using in the assay. After thawing to room temperature, leave the bottle at 2-8°C overnight before use. Ensure that the reagents are 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 until needed. Avoid multiple freeze-thaw cycles. To make aliquots suitable for testing one assay plate each, 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.

Human Exendin-4 Control Agonist

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 control datasheet. The reconstituted control 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 reagents at -20°C. It is important to thaw the kit from -20°C to room temperature at least 24 hours prior to using the kit. After thawing the kit to room temperature, leave it at 2-8°C overnight

before use. Ensure that the reagents are at room temperature for best performance.

After thawing, store reagents for up to 4 weeks at 2-8°C. 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 in opaque containers until needed. Avoid multiple freeze-thaw cycles.

96-Well Clear Bottom Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

5. Additional Materials and Equipment Recommended for Assay

The following equipment and additional materials are required to perform these assays:

Material	Ordering Information
Human Exendin-4	DiscoverX (92-1115), or similar
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	92-0011
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
Single and multichannel micropipettes and pipette tips (10 µL - 1000 µL)	

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.

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.

1. 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.

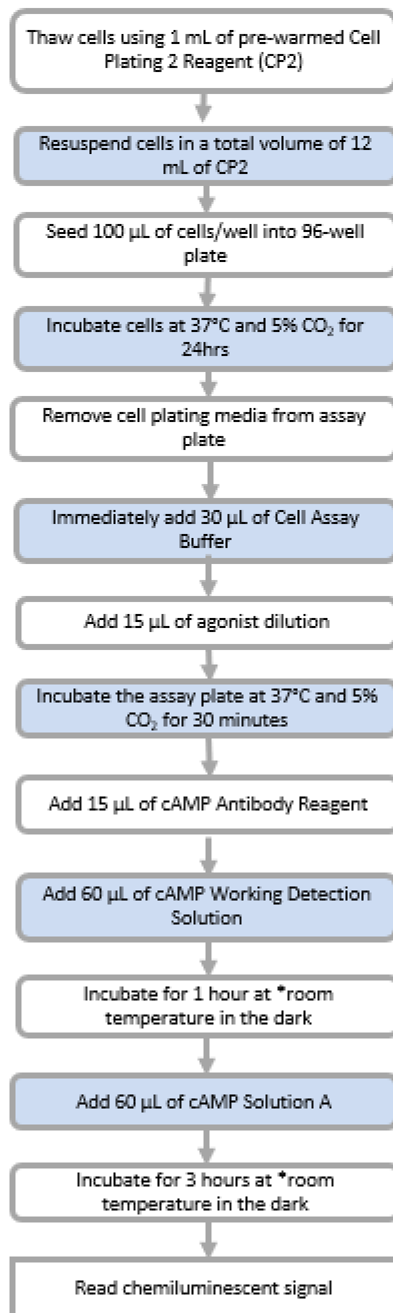
Cryovials should be stored in the vapor phase of liquid nitrogen ONLY. These vials are not rated for storage in the liquid phase.

2. 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.
3. 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

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: ____

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



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

8. Detailed Protocol

This user manual provides a protocol for determining potency of Exenatide in a 96-well format.

This user manual provides a protocol for the measurement of control-mediated cAMP accumulation. 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.

If purchasing the bioassay kit without control, Human Exendin-4 control can be sourced per the details in the table under Additional Materials Required.

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 tissue culture hood. These include:
    a. One sterile 25 mL reagent reservoir
    b. One sterile 15 mL conical tube
    c. A micropipette (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, Solid bottom, Tissue Culture-Treated Sterile Assay Plate (provided with the kit)
    g. A 96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plate, to be used as the master dilution plate.
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.

DO NOT use heated water bath to thaw the vial. Wipe down the cryovial quickly with 70% ethanol and bring it into the tissue culture hood right away. DO NOT touch the sides or bottom of the vial to avoid thawing of the cell pellet through body heat.

4. Thaw the pellet by immediately adding 1 mL (using P1000) of pre-warmed CP2 from the 15 mL conical tube to the cryovial, thawing the cell pellet. The medium should be added slowly along the side of the wall of cryovial tube. Mix the cells gently by pipetting up and down several times to break up any clumps. Transfer the cell suspension to the conical tube containing the remaining 11 mL of CP2. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
5. Gently invert the conical tube several times to ensure that the cells are properly resuspended in CP2, without creating any froth in the suspension. Immediately transfer the suspension into the sterile 25 mL reagent reservoir.

6. Transfer 100 µL of the cell suspension to each well of the 96-well assay plate using a multichannel pipette. 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.

7. Incubate the assay plate at 37°C and 5% CO_2 for 24 hours before proceeding with the assay.

8.2 Exenatide Reference Standard (RS) and Test Sample Preparation

Day 2

The following protocol describes the recommended steps for preparing the Exenatide reference standard and test sample working stocks and serial dilutions. Exenatide is supplied as a 250 µg/mL stock.

- Note that volumes may be scaled up or down as appropriate, based on number of plates to be run.
- Volumes recommended for Exenatide RS Working Stock are sufficient for two assay plates.

1. On day of assay, prepare working stocks of Exenatide as detailed in Table 1 below:

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

Sample	[Exenatide], ng/mL	Volume Exenatide, µL	Volume Dilution Buffer (PDB-B2), µL
Exenatide Reference Standard - Dilution 1	2500	10 µL of 250 µg/mL stock	990
Exenatide Reference Standard Dilution 2	25	10 µL of Dilution 1	990
Exenatide Reference Standard Working Stock	3	60 µL of Dilution 2	440

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

2. On the day of assay, prepare serial dilutions of the Exenatide Reference Standard (RS) in row A of the 96-well master dilution plate (MDP), at 3X the final concentration of each dilution, in PDB-B2 (i.e. the Control Diluent) as per Table 2 below. Sufficient volumes to run triplicate wells per dose in the assay plate are provided.
    a. Add volume of control diluent (PDB-B2) to row A of the MDP, (as indicated in column 6) of Table 2.
    b. Transfer 200 µL of Exenatide RS Working stock to the MDP in Row A, Well 1.
    c. Prepare the dilution series by transferring the volume of Exenatide RS sample (indicated in column 5 of Table 2) from the source well (indicated in column 4 of Table 2) to the destination well (indicated in column 1 of Table 2). Pipet up and down several times to mix in each destination we. Replace pipet tips between each serial dilution. No sample is added to well 12 of the MDP, as this serves as the negative control (vehicle only).

Table 2. Example of Preparation of Exenatide 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 RS on MDP Row A, ng/mL**	**Dilution Factor**	**Dilution (3X) RS Source Well**	**Volume (3X) of RS added, µL**	**Volume of Control Diluent, µL**	**Final Concentration (1X) of RS in Assay Plate, ng/mL**
Row A, Well 1	3	--	RS Working stock (3 ng/mL; see Table 1)	200	--	1
Row A, Well 2	1	3	Row A, Well 1	100	200	0.333
Row A, Well 3	0.333	3	Row A, Well 2	100	200	0.111
Row A, Well 4	0.1665	2	Row A, Well 3	150	150	0.0555
Row A, Well 5	0.083	2	Row A, Well 4	150	150	0.0278
Row A Well 6	0.042	2	Row A, Well 5	150	150	0.0139
Row A, Well 7	0.021	2	Row A, Well 6	150	150	0.0069
Row A, Well 8	0.010	2	Row A, Well 7	150	150	0.0035
Row A, Well 9	0.005	2	Row A, Well 8	150	150	0.0017
Row A, Well 10	0.0026	2	Row A, Well 9	150	150	0.0009
Row A, Well 11	0.0004	6	Row A, Well 10	30	150	0.00014
Row A, Well 12	0	--	--	--	150	--

3. On the day of assay, prepare appropriate intermediate dilutions of Test Sample (TS), as shown in Table 3, to generate a working stock of 3 ng/mL.
    - Note: if concentration of stock solution of test sample is lower than that of reference standard, adjust dilution volumes accordingly to create Dilution 1 stock.

Table 3. Example Preparation of Test Sample (TS) Intermediate Dilutions & Working Stock

Sample	[Test Sample], ng/mL	Volume Test Sample, µL	Volume Dilution Buffer (PDB-B2), µL
Test Sample -Dilution 1	2500	10 µL of 250 µg/mL stock	990
Test Sample- Dilution 2	25	10 µL of Dilution 1	990
Test Sample- Working Stock	3	60 µL of Dilution 2	440

4. On the day of assay, prepare serial dilutions of the TS in row B of the 96-well master dilution plate (MDP), at 3X the final concentrations of each dilution, in PDB-B2 (i.e. the Control Diluent) as per Table 4 below. Sufficient volumes to run triplicate wells per dose in the assay plate are provided.
    a. Add volume of control diluent (PDB-B2) to row B of the MDP, as indicated in column 6 of Table 4.
    b. Transfer 200 µL of working stock of TS to the MDP Row B Well 1.
    c. Prepare dilution series by transferring indicated volume of TS (shown in column 5 of Table 4) from the source well (indicated in column 4 of Table 4) to the destination well (indicated in column 1 of Table 4). Pipet up and down several times to mix in each destination well. Replace pipet tips between each serial dilution. No sample is added to well 12 of the MDP as this serves as the negative control (vehicle only).

Table 4. Example of Preparation of Test Sample (TS) 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 TS on MDP Row B, ng/mL**	**Dilution Factor**	**Dilution (3X) TS Source Well**	**Volume (3X) of TS added, µL**	**Volume of Control Diluent, µL**	**Final Concentration (1X) of TS in Assay Plate, ng/mL**
Row B, Well 1	3	--	3 ng/mL working stock of TS	200	--	1
Row B, Well 2	1	3	Row B, Well 1	100	200	0.333
Row B, Well 3	0.333	3	Row B, Well 2	100	200	0.111
Row B, Well 4	0.1665	2	Row B, Well 3	150	150	0.0555
Row B, Well 5	0.083	2	Row B, Well 4	150	150	0.0278
Row B Well 6	0.042	2	Row B, Well 5	150	150	0.0139
Row B, Well 7	0.021	2	Row B, Well 6	150	150	0.0069
Row B, Well 8	0.010	2	Row B, Well 7	150	150	0.0035
Row B, Well 9	0.005	2	Row B, Well 8	150	150	0.0017
Row B, Well 10	0.0026	2	Row B, Well 9	150	150	0.0009
Row B, Well 11	0.0004	6	Row B, Well 10	30	150	0.00014
Row B, Well 12	0	--	--	--	150	--

5. Assay plate preparation:
   a. Remove assay plates with cells from incubator.
   b. Completely remove the Cell Plating Reagent from each assay plate by carefully aspirating the wells or covering the plate wells with an absorbant material (e.g. Kimwipes) then gently and briefly spinning plate upside down.
   c. Immediately add 30 µL of Cell Assay Buffer to all empty wells of the plate.
6. Transfer 15 µL of the 3X RS and Test sample serial dilutions from the MDP to the appropriate wells containing cells in the assay plate as indicated below, and as diagrammed into 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)
7. Incubate the assay plate in a humidified incubator at 37°C and 5% CO_2 for 30 minutes.

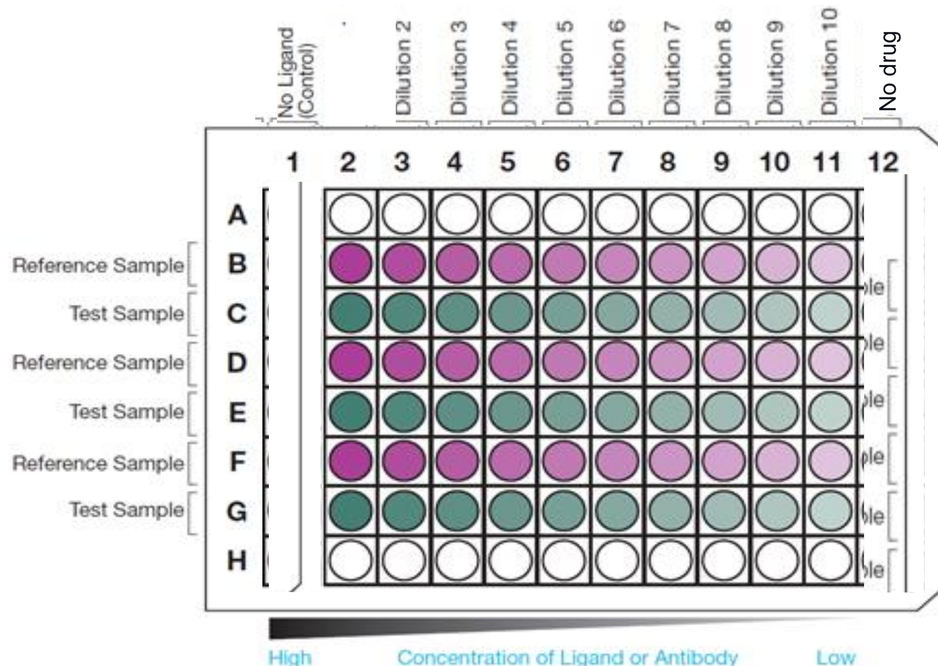


Figure 3. Representative Assay Plate Map

This plate map shows two interdigitated 11-point dose curves, with three replicates per dose point, for one test sample and a reference sample prepared using the same dilution scheme. Column A contains the highest dose of each sample, while column 11 contains the lowest dose. Column 12 contains no drug (vehicle only).

8.3 cAMP Detection

Day 2

1. Following agonist incubation, add 15 µL of the cAMP Antibody Reagent to all wells of the assay plate.
2. Prepare a stock of the 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 the table below for the recommended volumes of each component.

    - Note: cAMP Working Detection Solution is light sensitive. Store in the dark before use.
    - Note: indicated volumes below are sufficient for 1 assay plate.

Working cAMP 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 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.

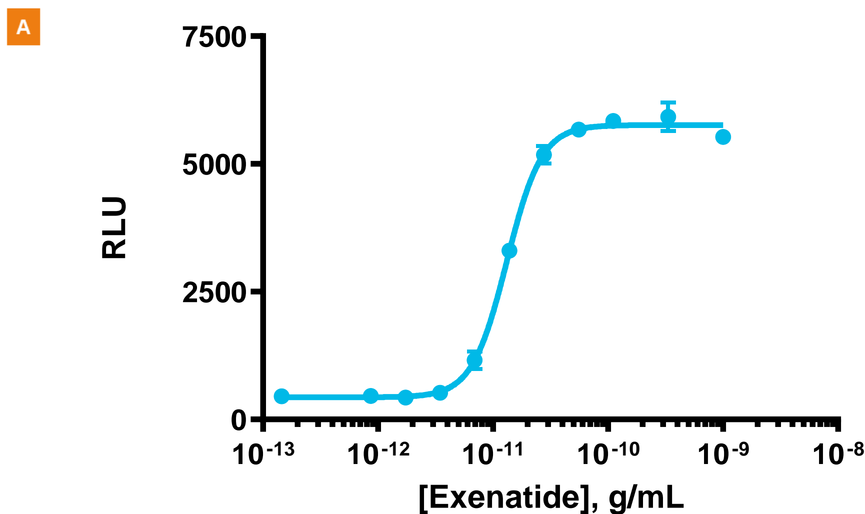
Room temperature refers to a range of 23-25°C.

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 assay plate on a Perkin Elmer Envision, or similar instrument, at 0.2 sec/well integration time.
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 Exenatide Bioassay generated using the protocol outlined in this manual. The data shows a potent, dose-dependent increase in cAMP production when treated with Exenatide. The plate was read on the EnVision® Multimode Plate Reader and data analysis was conducted using GraphPad Prism.

A



B

S/B	EC_{50}, pg/mL
13.2	13.2

Figure 4: Typical Results

Representative A, dose-response curve and B, EC_{50} and assay window (S/B) for Exenatide-mediated GLP1R 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 control used or improper control incubation time	Refer to the Certificate of Analysis for recommended control and assay conditions.
	Incorrect preparation of the control (agonist or antagonist)	Refer to the vendor-specific datasheet to ensure proper handling, dilution, and storage of the control.
	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 control (agonist or antagonist)	Some controls are difficult to handle. Confirm the final concentration of controls.
EC_{50} is right-shifted	Improper control handling or storage	Ensure that the controls are stored and incubated at the proper temperature.
	Difference in agonist binding affinity	Confirm that the control used is comparable to the control 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 control 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 controls.
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/

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