User Manual

cAMP Hunter™ Semaglutide Bioassay Kit

For the Measurement of Ligand-Mediated cAMP Accumulation

For Bioassay Kits:

95-0062Y2-00175: 2-Plate Kit

95-0062Y2-00176: 10-Plate Kit

Document Number 70-441 Revision 0

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

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Overview

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

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. The cAMP Hunter Semaglutide 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.

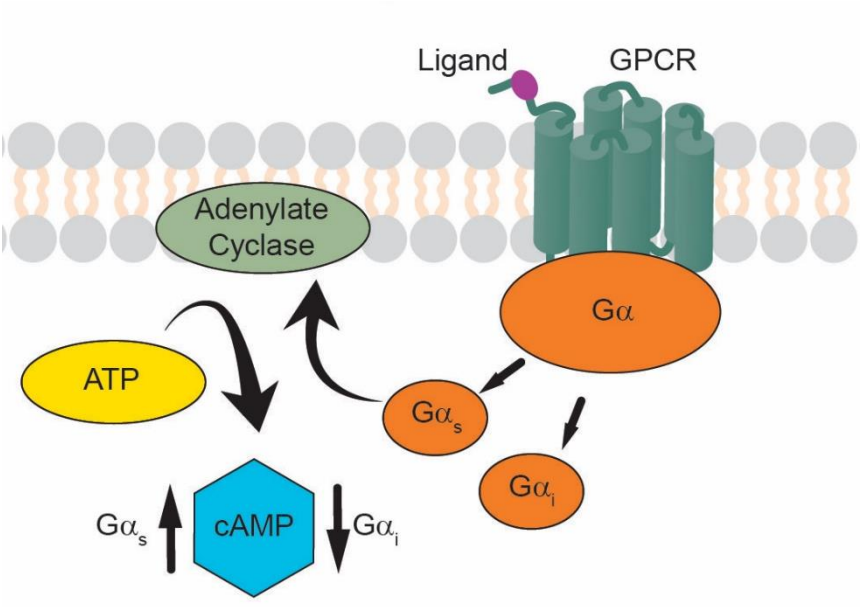


Figure 1. Assay Principle

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

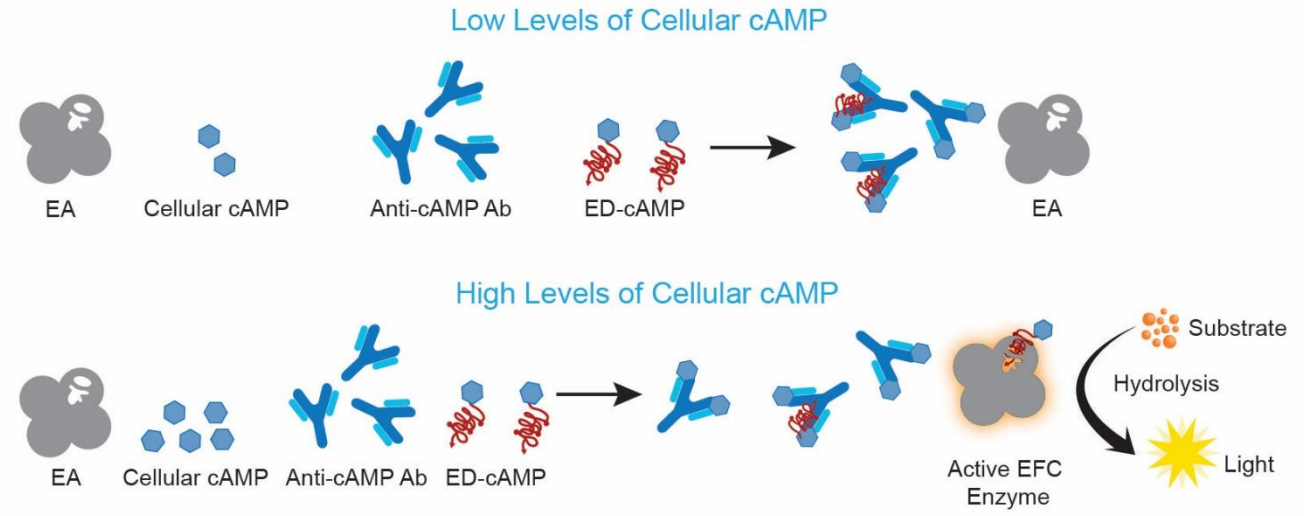


Figure 2. cAMP Detection Kit Principle

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. In contrast, when cellular cAMP levels are high, the 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.

Materials Provided

List of Components	95-0062Y2-00175	95-0062Y2-00176
cAMP Hunter CHO-K1 GLP1R Bioassay Cells (3.75 x 10^6 cells in 0.2 mL per vial)	2	10
AssayComplete™ Cell Plating 2 Reagent (CP2) (100 mL per bottle)	1	2
AssayComplete Cell Assay Buffer (50 mL per bottle)	1	2
AssayComplete Protein Dilution Buffer B2 (PDB-B2) (50 mL per bottle)	1	2
Control Agonist (Human Exendin-4)	1 x 500 µg	1 x 500 µg
cAMP Detection Kit for Bioassays		
cAMP Standard (250 µM) (Bottle)	1 x 0.2 mL	1 x 1 mL
cAMP Antibody Reagent (Bottle)	1 x 5 mL	1 x 25 mL
cAMP Lysis Buffer (Bottle)	1 x 7.6 mL	1 x 38 mL
Substrate Reagent 1 (Bottle)	1 x 2 mL	1 x 10 mL
Substrate Reagent 2 (Bottle)	1 x 0.4 mL	1 x 2 mL
cAMP Solution D (Bottle)	1 x 10 mL	1 x 50 mL
cAMP Solution A (Bottle)	1 x 16 mL	1 x 80 mL
96-Well White, Clear-Bottom, TC-Treated, Sterile Plates with Lid	2	10

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 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 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 ligand datasheet. 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 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.

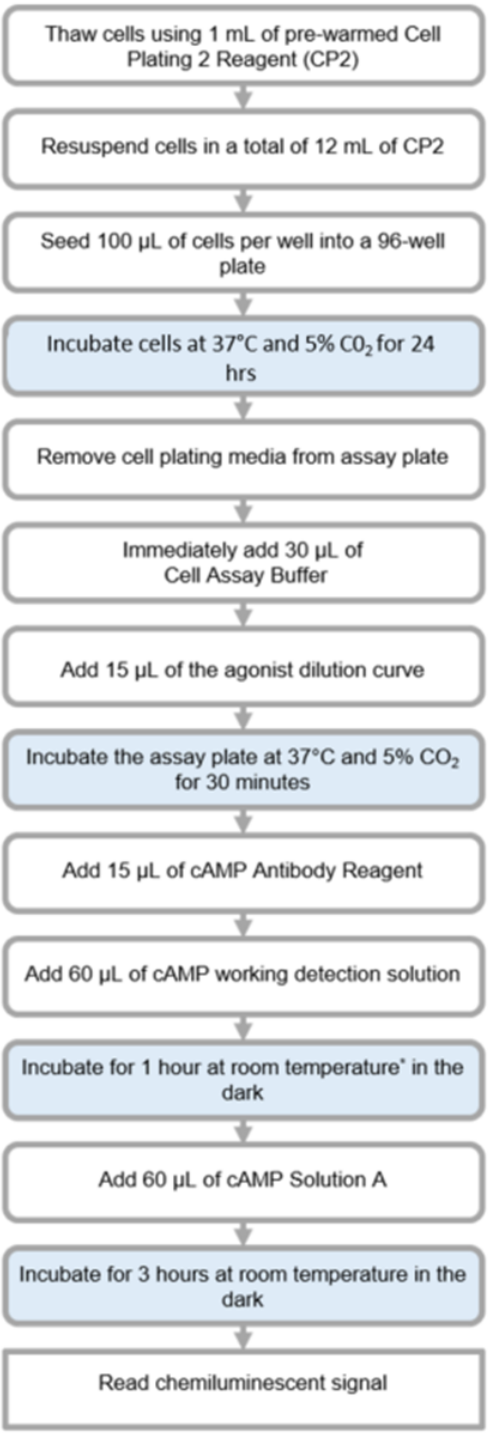
Additional Materials Required

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

Material	Ordering Information
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)	

Protocol Schematic

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



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

Detailed Protocol

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

Day 1: Bioassay Cell Preparation

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.
4. Remove the cryovial from dry ice and ensure cap is tightened. Immediately thaw vial in 37°C water bath for 30 seconds, gently shaking to thaw cells. DO NOT LEAVE IN WATER BATH.
5. 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
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 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.
8. 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.
9. 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.
10. Incubate the assay plate at 37°C and 5% CO_2 for 24 hours before proceeding with the assay.

Day 2: Semaglutide Reference Standard and Test Sample Preparation

The following protocol is the recommended dilutions for preparing the Semaglutide reference standard and test sample working stocks and serial dilutions. Semaglutide is supplied as a 1.34 mg/mL stock

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

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

Sample	[Semaglutide], µg/mL	Volume Semaglutide, µL	Volume Dilution Buffer, µL
Reference Standard (Semaglutide)- Dilution 1	26.8	10 µL of 1.34 mg/mL	490
Reference Standard (Semaglutide) Dilution 2	1.0	10 µL of Dilution 1	258
Reference Standard (Semaglutide) Dilution 3	0.1	20 µL of Dilution 2	180

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 Semaglutide Reference Standard (RS) in row A 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 2 below. Sufficient volumes to run triplicate wells per dose in the assay plate will be the result.
    a. Add volume of ligand diluent (PDB-B2) to row A of the MDP, (as indicated in column 6) of Table 2.
    b. Transfer Semaglutide RS Dilution3 to the MDP Row A Well 1; The volume that should be transferred is (indicated in column 5) of Table 2.
    c. Prepare the dilution series by transferring the volume of Semaglutide RS sample (indicated in column 5) from the source well (indicated in column 4) to the destination well (indicated in column 1). Pipet up and down several times to mix in destination wells. Replace pipet tips between each serial dilution. No sample is added to well 12 (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 Semaglutide 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	9		RS Dilution 3 (100 ng/mL; see Table 1)	18	182	3
Row A, Well 2	3	3	Row A, Well 1	50	100	1
Row A, Well 3	1	3	Row A, Well 2	50	100	0.333

Row A, Well 4	0.333	3	Row A, Well 3	80	160	0.111
Row A, Well 5	0.167	2	Row A, Well 4	150	150	0.055
Row A Well 6	0.111	1.5	Row A, Well 5	200	100	0.037
Row A, Well 7	0.0741	1.5	Row A, Well 6	200	100	0.0247
Row A, Well 8	0.0370	2	Row A, Well 7	100	100	0.0123
Row A, Well 9	0.0185	2	Row A, Well 8	100	100	0.00617
Row A, Well 10	0.00617	3	Row A, Well 9	50	100	0.00206
Row A, Well 11	0.00154	4	Row A, Well 10	40	120	0.000514
Row A, Well 12	0	--	--	--	100	--

3. On the day of assay, prepare working stock of Test Sample, as needed, to generate a working stock of 100 ng/mL (as for Semaglutide Reference Standard Dilution 3; in Table 1)
4. On the day of assay, prepare serial dilutions of the Test Sample 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 Ligand Diluent) as per Table 3 below. Sufficient volumes to run triplicate wells per dose in the assay plate will be the result.
    a. Add volume of ligand diluent (PDB-B2) to row B of the MDP, as indicated in column 6 of Table 3.
    b. Transfer Test Sample to the MDP Row B Well 1; The volume that should be transferred is indicated in column 5 of Table 3.
    c. Prepare the dilution series by transferring the volume of Test Sample (indicated in column 5) from the source well (indicated in column 4) to the destination well (indicated in column 1). Pipet up and down several times to mix in destination wells. Replace pipet tips between each serial dilution. No sample is added to well 12 (vehicle only), as this serves as the negative control.

Table 3. Sample concentration in Master Dilution Plate prepared as a 3X stock: Preparation of Test 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 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 B, Well 1	9	--	100 ng/mL working stock of Test Sample	18	182	3
Row B, Well 2	3	3	Row B, Well 1	50	100	1
Row B, Well 3	1	3	Row B, Well 2	50	100	0.333
Row B, Well 4	0.333	3	Row B, Well 3	80	160	0.111
Row B, Well 5	0.167	2	Row B, Well 4	150	150	0.055
Row B Well 6	0.111	1.5	Row B, Well 5	200	100	0.037
Row B, Well 7	0.0741	1.5	Row B, Well 6	200	100	0.0247
Row B, Well 8	0.0370	2	Row B, Well 7	100	100	0.0123
Row B, Well 9	0.0185	2	Row B, Well 8	100	100	0.00617
Row B, Well 10	0.00617	3	Row B, Well 9	50	100	0.00206
Row B, Well 11	0.00154	4	Row B, Well 10	40	120	0.000514
Row B, Well 12	0	--	--	--	100	--

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

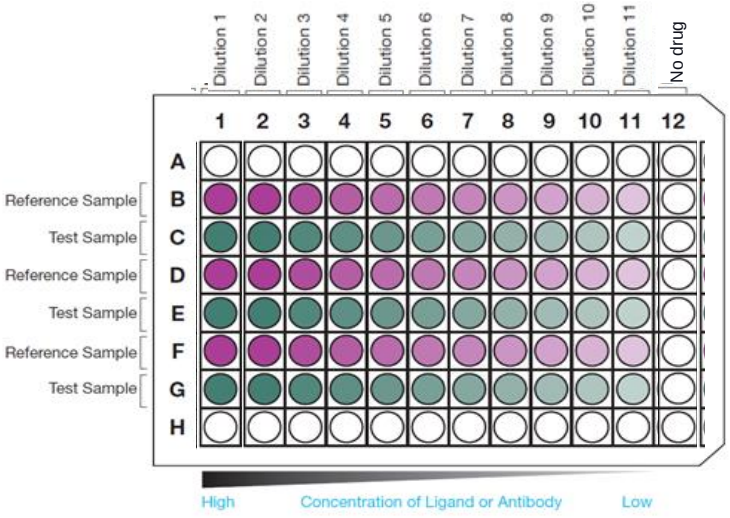


Figure 3. Representative Assay Plate Map

This plate map shows two interdigitated 11-point dose curves, with three replicates per dose point, for a test and 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).

Day 2: cAMP Detection

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. Store in the dark before use. Refer to the table below for the volume of each component.

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.

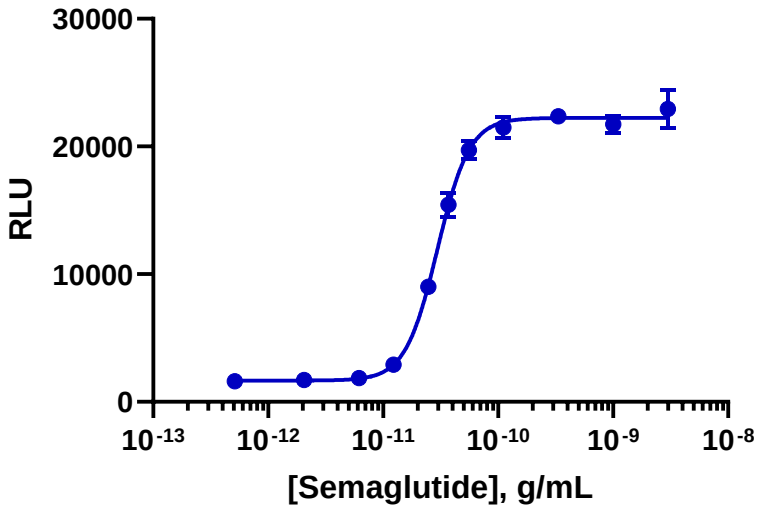
5. Add 60 µL of cAMP Solution A to all wells of the assay plate. Do not pipette up and down or vortex the plate to mix.
6. Incubate the assay plate for 3 hours at room temperature in the dark.

7. Read the sample on a Perkin Elmer Envision, 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.

Typical Results

The following graph is an example of a typical dose-response curve for the Semaglutide Bioassay generated using the protocol outlined in this manual. The data shows a potent, dose-dependent increase in cAMP production when treated with Semaglutide. 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
13.9	29.7

Typical Results. Representative A, dose-response curve and B, EC_{50} and assay window (S/B) for Semaglutide-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 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 1.866.448.4864 or DRX_SupportUS@eurofinsUS.com

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