



SemaBridge ELISA Kit

1 Plate Kit Catalog # AB000226

Complete kit for the conformational comparability analysis of Semaglutide.

Please read this insert completely prior to performing the assay.

This kit is intended for research use only. Not for use in diagnostic procedures.

Background information

Semaglutide is an anti-diabetic medication used for the treatment of type 2 diabetes, and an anti-obesity medication used for long-term weight management and to reduce the risk of major adverse cardiovascular events. It is a peptide similar to the hormone glucagon-like peptide-1 (GLP-1), modified with a side chain. It can be administered by subcutaneous injection or taken orally. It is sold by Novo Nordisk under the brand names **Ozempic** and **Rybelsus** for diabetes, and under the brand name **Wegovy** for weight management, weight loss, and the treatment of metabolic-associated steatohepatitis (nonalcoholic steatohepatitis). Semaglutide is an incretin mimetic.

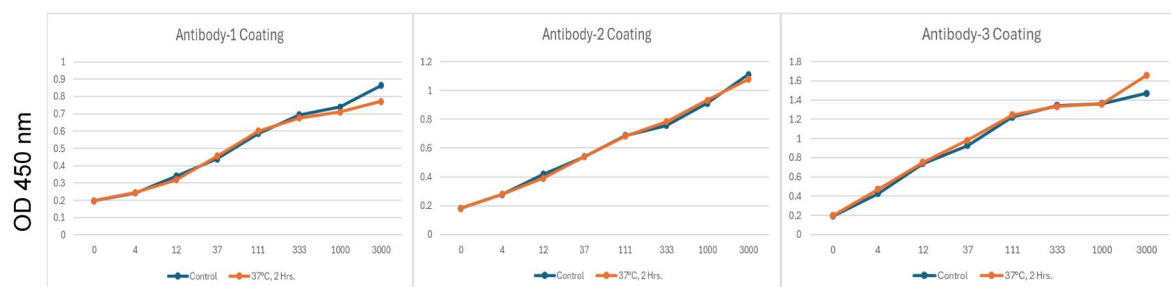


Assay Principle

The assay is in a sandwich ELISA format where the plate is coated with three (3) antibodies raised against peptides derived from the full length protein sequence of Semaglutide. Taken individually, each of these antibodies is strongly antigenic to the peptide sequence that was used in its production. However, when these peptides are incorporated into a full length correctly folded Semaglutide, the antigenicity of many of them may be masked by the three dimensional structure of the peptide and a partial level of epitopes are detected by this assay. The result is three separate standard curves which can be likened to a 'fingerprint' for correctly folded Semaglutide. For a Semaglutide Biosimilar, if the peptide is correctly folded, the 'fingerprint' will match that of Semaglutide. If it is not correctly folded, previously masked peptide sequences will be exposed or previously exposed will be missing and will be recognized by the reporting antibody made to the three peptide sequences. In this way, changes in the 'fingerprint' generated by the ELISA will point out differences between the Biosimilar and authentic Semaglutide.

The assay is performed by making a 3 µg/mL solution of Semaglutide Biosimilar and reference material respectively, perform a 1:3 serial dilution to dilute down to 4 ng/mL and adding to the 96-well plates. Following a 1-hour incubation to allow capture of the Biosimilar and Semaglutide reference proteins by the panel of antibodies on the plate, three reporting polyclonal anti-semaglutide peptide antibody, conjugated with biotin, is added and incubated for 45 min. to allow it to bind to any captured proteins. After this incubation, the plate is washed and a Streptavidin-HRP (Horse Radish Peroxidase) conjugate is added and incubated for 30 minutes. The Streptavidin-HRP conjugate will be captured by any biotin labeled antibody bound to the plate. Following a wash step to remove unbound conjugate, TMB substrate is added and is converted by the captured HRP to a colored product in proportion to the amount of HRP bound to the plate. After a short incubation to allow color development, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450nm wavelength. The color development will be proportional to the captured Biosimilar or Semaglutide reference protein. A typical ELISA with the Semaglutide reference protein without any treatment and mild stress is shown in the figure on next page.

Semaglutide PCAELISA Graph



In the 96-well plate, Column 1-4 is coated with 10 µg/mL Antibody1; Column 5-8 is coated with 10 µg/mL Antibody2; Column 9-12 is coated with 10 µg/mL Antibody-3.

Typical Semaglutide Conformational Array ELISA Graph

Supplied Components:

Coated Clear 96 Well Plates

One clear plastic microtiter plates coated with the three antibodies (Antibody-1, Antibody-2 and Antibody-3) against Semaglutide peptides.

Kit AB-000226 (1 plate)

5x Dilution Buffer

Buffer used for dilution of testing samples and Streptavidin-HRP conjugate. The 30 mL of concentrate should be diluted to 150 mL with 120 mL deionized or distilled water.

Kit AB-000226 (30 mL)

10x PBS-T

After dilution, it is used as wash solution. The 50 mL of concentrate should be diluted to 500 mL with 450 mL deionized or distilled water.

Kit AB-000226 (50 mL)

Reporting antibody buffer

A special reporting antibody buffer is supplied to reduce nonspecific binding and background signal (15 mL).

Reporting antibody

A biotin labeled rabbit polyclonal antibody against Semaglutide peptides. Immediately prior to the assay, dilute the whole 150 μ l into 15 mL of reporting buffer supplied to give a 6 μ g/mL working stock.

Kit AB-000226 (0.6 mg/mL, 150 μ l / tube)

Streptavidin-HRP Conjugate

A Streptavidin – Horse Radish Peroxidase conjugate in a special stabilizing solution. Immediately prior to the assay, dilute the whole 375 μ l into 15 mL of 1x Dilution buffer to give a 0.1 μ g/mL working stock.

Kit AB-000226 (4 μ g/mL, 375 μ l / tube)

TMB Substrate

Use directly without dilution.

Kit AB-000226 (15 mL)

Stop Solution

A 1M solution of sulfuric acid. CAUSTIC. Use directly without dilution.

Kit AB-000226 (15 mL)

Plate Sealer

Kit AB-000226 (1)

Other Materials Required

Distilled or deionized water.

Single- and multi-channel micro-pipettes with disposable tips to accurately dispense volumes 5-250 μ L.

Plastic tubes (i.e. 1.5 mL – 15 mL) for sample dilution

Reagent reservoirs for sample addition

Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.

Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturer's Wash Buffers, may contain sodium azide that will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on Page 4

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

Procedural Notes

Allow diluted reagents and buffers to reach room temperature (18-25°C) prior to starting the assay. Once the assay has been started, all steps should be completed in sequence and without interruption. You do not want the plate to dry out in between steps as this can cause high backgrounds or erroneous results. Make sure that required reagents and buffers are ready when needed. Prior to adding to the plate, reagents should be mixed gently (not vortexed) by swirling.

Avoid contamination of reagents, pipette tips and wells. Use new disposable tips and reservoirs, do not return unused reagent to the stock bottles / vials and do not mix caps of stock solutions.

Incubation time can affect results. All wells should be handled in the same order for each step.

Microplate washing is important and can affect results by giving erroneous results or high backgrounds. We recommend a multichannel pipette to add 250 μ l of buffer to each well across the plate, followed by a dumping out of contents (to a sink or other receptacle) with a rapid wrist motion. The plate should then be tapped firmly on a paper towel to shake out any remaining liquid. Avoid prolonged incubation with wash buffer when performing wash steps.

When making additions to the plate, be careful to avoid damaging the antibody coating, for example by scratching the bottoms or the sides of the wells. One technique to avoid this is to make additions (for a right-handed person) from left to right across the plate, supporting the pipette tips on the right edge of the well with each addition and thus avoiding contact with the bottom or sides of the wells.

During the incubation times, the plate should be covered to minimize evaporation from the wells. This can be done with the adhesive covers provided or by stacking an empty plate on top.

After the last wash step and prior to adding the TMB substrate, wipe the bottom of the plate with a clean paper towel to ensure that moisture or fingerprints do not interfere with the OD reading.

Once the TMB substrate is added it will be converted by the captured HRP to a blue colored product. Generally we find that a 12 minute incubation is sufficient for enough color development to discern differences between the standards and the reaction should be stopped at this point. Bear in mind that, given sufficient time, even a small amount HRP is capable of converting all the TMB to product. Keeping OD₄₅₀ values well below 2.0 will result in greatest accuracy as at high absorbance values very little light is reaching the detector and measurements are error prone. (Remember that at an OD of 1.0 only 10% of the light is being detected and at an OD of 2.0 only 1% of the light is reaching the detector).

Assay Protocol

1. Take out the precoated microplate.
2. Use the plate layout sheet on the back page to plan sample layout on plate and also aid in proper sample and reference identification after the assay. The plate is laid out as shown on the plate map on the following page, with each unique antibody appearing in 4 columns on the plate. Column 1 to 4 is coated with antibody-1; column 5-8 is coated with antibody-2, and column 9 to 12 is coated with antibody-3. There are a total of 32 wells coated for each antibody. If take two wells as blank, you can analyze 15 samples in duplicate, **the recommended testing sample concentration is 100 ng/mL prepared in the supplied diluent solution**. The same set of samples will also be analyzed in the antibody-2 and antibody-3 regions to provide an overall picture for the testing samples.

Alternatively, in our recommended analysis, two samples will be compared in 7 different concentrations, i.e. 3000, 1000, 333, 111, 37, 12 and 4 ng/mL testing sample prepared in 1% BSA in 1xPBS-T, these will be analyzed in duplicates. For example, column 1 and 2 will be for reference and column 3 and 4 will be for testing sample, the same set will be analyzed in the antibody-2 and antibody-3 regions respectively, this approach will provide a better picture of the testing sample.

3. Dilute the 10x PBST and 5x Dilution buffer with water to 1x-strength. Check both concentrate bottles for precipitates before proceeding and if found warm slightly in a water bath to dissolve before proceeding. The 30 mL of 10xPBST should be diluted to 300 mL with 270 mL water and the 10 mL of 5x Dilution Buffer should be diluted to 50 mL with 40 mL water.
4. Dilute your sample and Semaglutide standard to a concentration of 3 $\mu\text{g/mL}$ in 1.2 mL volume in the dilution buffer, label 6 additional tubes as 1, 0.33, 0.11, 0.037, 0.012 and 0.004 $\mu\text{g/mL}$ respectively and add 0.8 mL to each vial. Take 0.4 mL solution from the 3 $\mu\text{g/mL}$ vial and transfer to the vial labeled 1 $\mu\text{g/mL}$, mix well with pipette, repeat the process for the serial 1:3 dilution all the way down to 4 ng/mL. The reference set samples will be added to column 1,2, 5,6 and 9,10; the testing samples will be added to column 3,4, 7,8 and 11,12 respectively, starting with dilution buffer at row H and backward from 4 to 3000 ng/mL from G to the A rows stepwise.

Alternatively, if the samples are analyzed in one concentration, prepare the reference and testing samples at 100 ng/mL in diluent solution and added to the plate. Incubate the plate at room temperature for 60 min.

5. During the above incubation, dilute the 0.6 mg/mL reporting antibody to 6 µg/mL by adding 150 µL to 15 mL of Reporting Antibody Buffer.
6. Wash plate by emptying contents and adding 250 µL of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat.
7. Pipette 100 µL of 6 µg/mL Reporting Antibody into each well. Cover plate and incubate plate 45 min. at room temperature.
8. During the above incubation, dilute the 4 µg/mL Streptavidin-HRP conjugate to 0.1 µg/mL by adding 375 µL to 15 mL of Dilution Buffer.
9. Wash plate by emptying contents and adding 250 µL of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat.
10. Pipette 100 µL of 0.1 µg/mL Streptavidin-HRP conjugate into wells. Cover plate and incubate plate 30 min hour at room temperature.
11. Wash plate by emptying contents and adding 250 µL of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat 2 more times
12. Add 100 µL of TMB substrate to each well. Allow color development to proceed for exactly 15 minutes and then stop reaction by adding 100 µL of Stop Solution to each well. Upon addition of stop solution, developed color will change from blue to yellow.
13. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
14. Export the plate reader data into Excel and calculate an average and variance for each set of replicates. If the variance is large inspect the raw data to determine the problem. With data in triplicate, one outlier may be evident, but if data is in duplicate, the higher value is generally suspect (it's easier to get a high value in error than a low value). Graph the data as a bar graph so that for each array antibody the response can be compared between your sample and Semaglutide standard. Any differences between your sample and the Semaglutide standard should be apparent.

Plate Layout

Reference compound suggested use in wells marked

	1	2	3	4	5	6	7	8	9	10	11	12
A	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000	3000
B	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
C	333	333	333	333	333	333	333	333	333	333	333	333
D	111	111	111	111	111	111	111	111	111	111	111	111
E	37	37	37	37	37	37	37	37	37	37	37	37
F	12	12	12	12	12	12	12	12	12	12	12	12
G	4	4	4	4	4	4	4	4	4	4	4	4
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank