Semaglutide (Ozempic™) ELISA

For the quantitative determination of Semaglutide in human serum and plasma.

Store the Streptavidin HRP conjugate at -20°C.

For Research Use Only. Not for Use in Diagnostic Procedures.

Catalog Number: 07-SEMHU-E01
Size: 96 determinations
Version: 6.6 **ALPCO**: 1.0

26-G Keewaydin Drive, Salem, NH 03079|P: (800) 592-5726|F: (603) 898-6854|ts@alpco.com|www.alpco.com

INTENDED USE

The Semaglutide (Ozempic™) ELISA kit is used for the quantitative determination of Semaglutide in human serum and plasma. For Research Use Only. Not for use in diagnostic procedures.

INTRODUCTION

Semaglutide (trade name Ozempic) is a pharmaceutical drug in development by a Danish company, Novo Nordisk, for the treatment of type 2 diabetes. It is marketed by the name Ozempic. As a glucagon-like peptide-1 receptor agonist, it lowers the blood sugar level by increasing the production of insulin. It was discovered in 2012, by a team of researchers at Novo Nordisk as a longer-acting alternative to liraglutide. Clinical trials started in 2015, and phase 3 was completed in 2016. FDA approval was applied for in December 2016, and in October 2017 an FDA Advisory Committee voted 16-0 in favor. Semaglutide can be used as both an injection-type or oral-type drug. Trade names include Ozempic, Rybelsus, and Wegovy.

PRINCIPLE OF THE ASSAY

The Semaglutide ELISA is a competitive immunoassay for the determination of Semaglutide. The anti-GLP-1 antibodies are coated on a 96-well plate. A constant concentration of Biotinylated GLP-1 and varying concentration of unlabeled Semaglutide or sample compete for binding to anti-GLP-1 antibodies. Biotinylated GLP-1–antibody complexes are subsequently bound by Streptavidin-HRP which produces a soluble colored product after addition of TMB substrate. The enzyme reaction is stopped by dispensing of stop solution into the wells. The optical density (OD) of the solution at 450 nm is inversely proportional to the amount of bound Semaglutide molecule present in standards or samples.

MATERIALS SUPPLIED

07-SEMHU-E01			
Component	**Description**	**Quantity**	**Preparation**
Anti-GLP-1 Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-GLP-1 monoclonal antibody	1 x 96 wells	Ready-to-Use
Semaglutide Standard	Recombinant Semaglutide standard and preservative sodium azide < 0.1% (lyophilized; 4 ug/ml)	2 vials	Lyophilized, see Reagent Preparation
GLP-1 Biotin	Biotinylated GLP-1 prepared in buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	6 mL	Ready-to-Use
Standard Diluent	Buffered protein base with protein stabilizer and 1:100 human serum and preservative sodium azide < 0.1%	10 mL	Ready-to-Use
Sample Diluent	Buffered protein base with BSA and preservative sodium azide < 0.1%	50 mL	Ready-to-Use
Streptavidin HRP Conjugate	Concentrated Streptavidin HRP, (10 ul). Note: The dilution factor provided is lot-specific. *Store at -20 C.*	1 vial	See lot-specific Reagent Preparation Sheet included in kit.

26-G Keewaydin Drive, Salem, NH 03079 | P: (800) 592-5726 | F: (603) 898-6854 | ts@alpco.com | www.alpco.com

Streptavidin HRP Diluent	Buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 mL	Ready-to-Use
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersol < 0.01%. May turn yellow over time.	25 mL	See Reagent Preparation
TMB Substrate	Stabilized Chromogen	12 mL	Ready-to-Use
Stop Solution	0.73M Phosphoric Acid	12 mL	Ready-to-Use
Instructions	Instructions for Use	1	
Reagent Preparation Sheet	Reagent Preparation Sheet	1	

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

- Microtiter Plate Reader able to measure absorbance at 450 nm.
- Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul
- Deionized (DI) water
- Wash bottle or automated microplate washer
- Graph paper or software for data analysis
- Tubes for standard/sample dilutions
- Absorbent paper
- 37°C incubator
- Timer

PRECAUTIONS

- This kit is for Research Use Only. Follow the work instructions carefully.
- The expiration dates stated on the kit must be observed. The same relates to the stability stated for reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shifts during pipetting of reagents.
- All reagents should be kept in the original shipping containers.
- Some of the reagents contain a small amount of sodium azide (< 0.1 % w/w) as preservative. They must not be swallowed or allowed to touch skin or mucosa.

- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all samples as if potentially hazardous.

- Since the kit contains potentially hazardous materials, the following precautions should be observed
    - Do not smoke, eat, or drink while handling kit material
    - Always use protective gloves
    - Never pipette material by mouth
    - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case Good Laboratory Practice should be applied with all general and individual regulations to the use of this kit.
- Reagents that contain preservatives may be harmful if ingested, inhaled, or absorbed through the skin. Refer to SDS for more details.

REAGENT HANDLING and STORAGE CONDITIONS

26-G Keewaydin Drive, Salem, NH 03079 | P: (800) 592-5726 | F: (603) 898-6854 | ts@alpco.com | www.alpco.com

1. Store the Streptavidin HRP conjugate at -20°C.
2. Store other kit components at 2-8°C.
3. Use all reagents and wash solutions within 12 months of manufacturing date.
4. Before use, bring all components to room temperature (18-25ºC).
5. Upon assay completion, ensure all kit components are returned to appropriate storage conditions.

SAMPLE COLLECTION, PREPARATION, AND STORAGE

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma may also be used. Avoid lipemic, hemolytic, or contaminated samples. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, aliquot samples and keep at -20°C.

Serum and Plasma
Samples must be **diluted 1:100 (v/v)**, e.g. **1 ul sample + 99 ul (1X) Sample Diluent** prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires -20°C.

REAGENT PREPARATION

All reagents should be diluted immediately prior to use.

Bring all kit components and samples to room temperature (18-25°C) before use. If the kit will not be used all at once, take out only the number of strips and reagents that are needed and store the remaining strips and reagents as indicated.

1. **To make 1X working Wash Buffer**:

   Dilute 25 ml of (20X) Wash Buffer in 475 ml of DI water.

2. **Standards Preparation**

   Reconstitute the lyophilized standard in 1000 ul of Standard Diluent to get a concentration of 4000 ng/ml. Let sit for 15 minutes. 4000 ng/ml is the top standard. Prepare the remaining standards as per the below table. 1X working Standard Diluent serves as the Zero Standard (0 ng/ml).

Standard Concentration	Standard Vial	Preparation Instructions
4000 ng/ml	Reconstituted standard	Lyophilized Standard provided in the Kit + 1000 ul of 1X working Standard Diluent .
3000 ng/ml	Standard No. 6	750ul of Reconstituted standard + 250 ul of Standard Diluent (1X)
2000 ng/ml	Standard No.5	666.6 ul of Standard No. 6 + 333.4 ul Standard Diluent (1X)
1000 ng/ml	Standard No.4	500 ul of Standard No. 5 + 500 ul Standard Diluent (1X)
500 ng/ml	Standard No.3	500 ul of Standard No. 4 + 500 ul Standard Diluent (1X)
100 ng/ml	Standard No.2	200 ul of Standard No. 3 + 800 ul Standard Diluent (1X)
50 ng/ml	Standard No.1	500 ul of Standard No. 2 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No. 0	Only Standard Diluent (1X)

26-G Keewaydin Drive, Salem, NH 03079|P: (800) 592-5726|F: (603) 898-6854|ts@alpco.com|www.alpco.com

3. Streptavidin HRP Conjugate Working Solution Preparation:
See **Reagent Preparation Sheet** for lot-specific dilution factor (enclosed in the kit).

General notes: To use in the assay, it is recommended to do a two-step (2-step) dilution, prior to running the assay to avoid dilution error. Always prepare the Streptavidin HRP Conjugate fresh. Discard unused reagents. Do not store 1x working Streptavidin HRP Conjugate.

NOTE THE DILUTION FACTOR PROVIDED IS LOT-SPECIFIC AND WILL VARY FROM LOT TO LOT.

Store the Streptavidin HRP conjugate at -20°C.

QUALITY CONTROL

It is recommended that each laboratory assay appropriate quality control samples with each run to ensure that all reagents and procedures are correct.

PROCEDURAL NOTES

1. To achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess reagents is essential.
2. Avoid assay of samples containing sodium azide (NaN3), as it could destroy the HRP activity resulting in underestimation of the amount of Semaglutide.
3. It is recommended that all Standards and Samples be assayed in duplicate or triplicate.
4. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are the same for each well.
5. If the Substrate has a distinct blue color prior to use, it may be contaminated and use of such substrate can lead to inaccurate results.
6. The plates should be read within 30 minutes after adding the Stop Solution.
7. Make a plate map to identify the location of standards and samples.

ASSAY PROCEDURE

It is strongly recommended that all Standards and Samples be run in duplicate or triplicate. A standard curve is required for each assay.

1. Add **100 µl prepared Standards** or **diluted Samples** to respective wells.
2. Cover the plate and incubate for 90 minutes at 37°C.
3. Pipette 50 ul of **Biotinylated GLP-1** into each well.
4. Cover the plate and incubate for 90 minutes at 37°C.
5. Aspirate and wash plate 4 times with 1x working **Wash Buffer** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.
6. Add 100 ul of 1x working **Streptavidin-HRP Conjugate Solution** (freshly prepared using the 2-step method. See Reagent Preparation sheet attached to CoA) in each well.
7. Incubate the microplate for 60 minutes at 37°C.
8. Aspirate and wash plate 4 times with 1x working **Wash Buffer** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.
9. Add **100 ul** of **TMB Substrate** in each well.
10. Incubate the plate at 37°C for 30 minutes in the dark. DO NOT SHAKE. Shaking may result in high background or imprecision. Positive wells should turn bluish in color.
11. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.
12. Read the absorbance at 450 nm with a microplate reader.

26-G Keewaydin Drive, Salem, NH 03079|P: (800) 592-5726|F: (603) 898-6854|ts@alpco.com|www.alpco.com

CALCULATION OF RESULTS

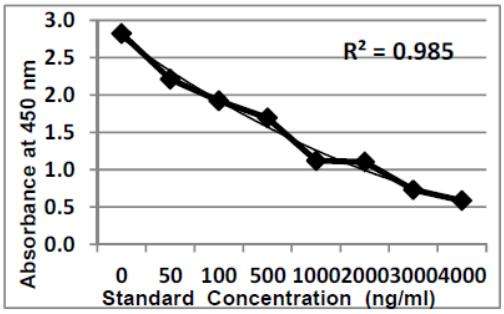
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Semaglutide concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Semaglutide Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which can generate a linear regression like cubic spline or 4PL (2nd order) is best recommended for automated results.

Note: It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 4000 ng/ml standard.

Typical Data

Standards (ng/mL)	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	2.82	-	-
50	2.21	36.7	73.3
100	1.92	147.8	147.8
500	1.69	334.3	66.9
1000	1.12	1489.6	149.0
2000	1.10	1548.0	77.4
3000	0.73	3185.0	106.2
4000	0.59	4061.9	101.5

Typical Graph



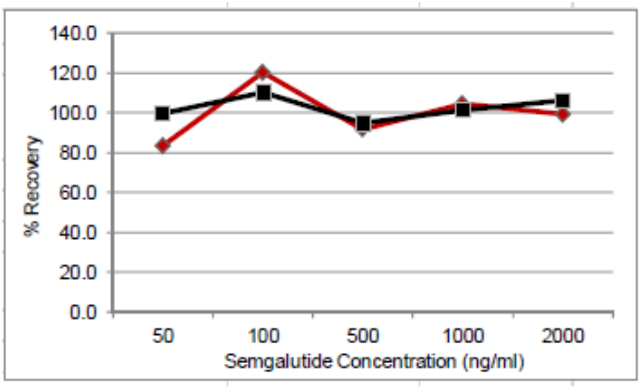
PERFORMANCE CHARACTERISTICS

This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

Sensitivity: Limit Of Detection: LoD is defined as the lowest detectable concentration corresponding to a signal of Mean of Zero Standard plus 2 x SD. Ten replicates of Zero Standards were evaluated and the LOD was found to be less than 50 ng/ml.

26-G Keewaydin Drive, Salem, NH 03079 | P: (800) 592-5726 | F: (603) 898-6854 | ts@alpco.com | www.alpco.com

Specificity: The capture monoclonal GLP-1 antibody used in the kit reacts with the mid-molecular epitope of GLP-1 (Glucagon-like peptide 1(7-36)). It has 100% cross reactivity to Semaglutide. The kit calibrators are certified against commercially-available Ozempic™ Injection.



Kit Standard Concentration (ng/ml)	Ozempic Calibrator % Recovery	Kit Calibrator % Recovery
50	83.5	99.9
100	120.4	110.1
500	91.8	94.8
1000	104.7	101.5
2000	99.4	106.3

Note: Semaglutide is chemically similar to human glucagon-like peptide-1 (GLP-1), but there are some differences in their peptide chains. Namely, Semaglutide has two amino acid substitutions compared with human GLP-1 which is at Position 8 where Alanine is replaced by 2-aminoisobutyric acid (Aib) and at Position 34 where Lysine is replaced by arginine. Also Lysine at position 26 is acylated with a spacer and C-18 fatty di-acid chain and the first six amino acids of GLP-1 are missing in Semaglutide.

The antibody used in the kit is not only specific to Semagalutide but has demonstrated cross reactivity of 100-120% against other forms of GLP-1 including exogenous GLP-1, Tirzapatide, and Semaglutide salts like Semaglutide Acetate. Hence the kit has limitations when detecting Semaglutide in the presence of other GLP-1 agonists.

Linearity: The standard graph range optimized is 0 ng/ml to 4000 ng/ml. Samples outside the assay range must be diluted using the diluent provided to bring the expected results within the given assay range.

Precision: Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (50 ng/ml), medium (1000 ng/ml) and high (4000 ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

Pool	Intra Assay % CV	Inter Assay % CV
Low	<10%	<12%
Medium	<8%	<10%
High	<8%	<10%

Cross Reactivity: The Semaglutide ELISA was validated for cross-reactivity with different markers and the results are below.

Marker	% Cross Reactivity
Liraglutide	100-120%
Endogenous GLP-1	<0.1%

26-G Keewaydin Drive, Salem, NH 03079 | P: (800) 592-5726 | F: (603) 898-6854 | ts@alpco.com | www.alpco.com