BioVendor Group

ENG

Product Data Sheet:

HUMAN OBESTATIN ELISA

Catalogue number:
RA19025R

For research use only!

BioVendor R&D

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CONTENTS

1. HUMAN OBESTATIN ELISA

96 wells

Storage: -20°C

Expiry date: stated on the package

This kit contains:

Reagents	Colour code	Quantity	Form
Antibody Coated Microtiter Strips	Blister with zip	1	-
Conjugate Solution (Streptavidin AChE Tracer)	Green	1	lyophilized
Biotin-Labelled Antibody	Red	1	lyophilized
Human Obestatin Standard	Blue with red septum	2	lyophilized
Quality Control	Green with red septum	2	lyophilized
Dilution Buffer (EIA buffer)	Blue	1	lyophilized
Wash Solution	Silver	1	liquid
Substrate Solution (Ellman´s Reagent)	Black with red septum	2	lyophilized
Tween 20	Transparent	1	liquid
Cover Sheet	-		-

Each kit contains suffi cient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 35 samples in duplicate.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Quality Control and one of Substrate Solution.

2. PRECAUTION FOR USE

Users are recommended to carefully read all instructions for use before starting work.

Each time a new pipette tip is used, aspirate a sample or reagent and expel it back into the same vessel. Repeat this operation two or three times before distribution in order to equilibrate the pipette tip.

- For research laboratory use only.
- Not for human diagnostic use.
- Do not pipet liquids by mouth.
- Do not use kit components beyond the expiration date.
- Do not eat, drink or smoke in area in which kit reagents are handled.
- Avoid splashing.

The total amount of reagents contains less than 100 µg of sodium azide. Flush the drains thoroughly to prevent the production of explosive metal azides.

Wearing gloves, laboratory coat and glasses is recommended when assaying kit materials and samples.

Temperature:

Unless otherwise specified, all the experiments are done at room temperature (RT), that is around 20°C. Working at 25°C or more affects the assay and decreases its efficiency.

3. BACKGROUND

Acetylcholinesterase AChE® Technology

Acetylcholinesterase (AChE®), the enzymatic label for EIA, has the fastest turnover rate of any enzymatic label. This specific AChE is extracted from the electric organ of the electric eel, *Electrophorus electricus*, and it's capable of massive catalytic turnover during the generation of the electrochemical discharges. The use of AChE as enzymatic label for EIA has been patented by the French academic research Institute CEA [1, 2, 3].

AChE® assays are revealed with Substrate Solution (Ellman's reagent), which contains acetylthiocholine as a substrate. The final product of the enzymatic reaction (5-thio-2-nitrobenzoic acid) is bright yellow and can be read at 405-414 nm. AChE® offers several advantages compared to enzymes conventionally used in EIAs:

Kinetic superiority and high sensitivity:

AChE® shows true first-order kinetics with a turnover of 64,000 sec^{-1}. That is nearly 3 times faster than Horse Radish Peroxidase (HRP) or alkaline phosphate. AChE® allows a greater sensitivity than other labeling enzymes.

Low background:

non-enzymatic hydrolysis of acetylthiocholine in buffer is essentially absent. So, AChE® allows a very low background and an increased signal/noise ratio compared to other substrate of enzymes which is inherently unstable.

Wide dynamic range:

AChE® is a stable enzyme and its activity remains constant for many hours as, unlike other enzymes, its substrate is not suicidal. This permits simultaneous assays of high diluted and very concentrated samples.

Versatility:

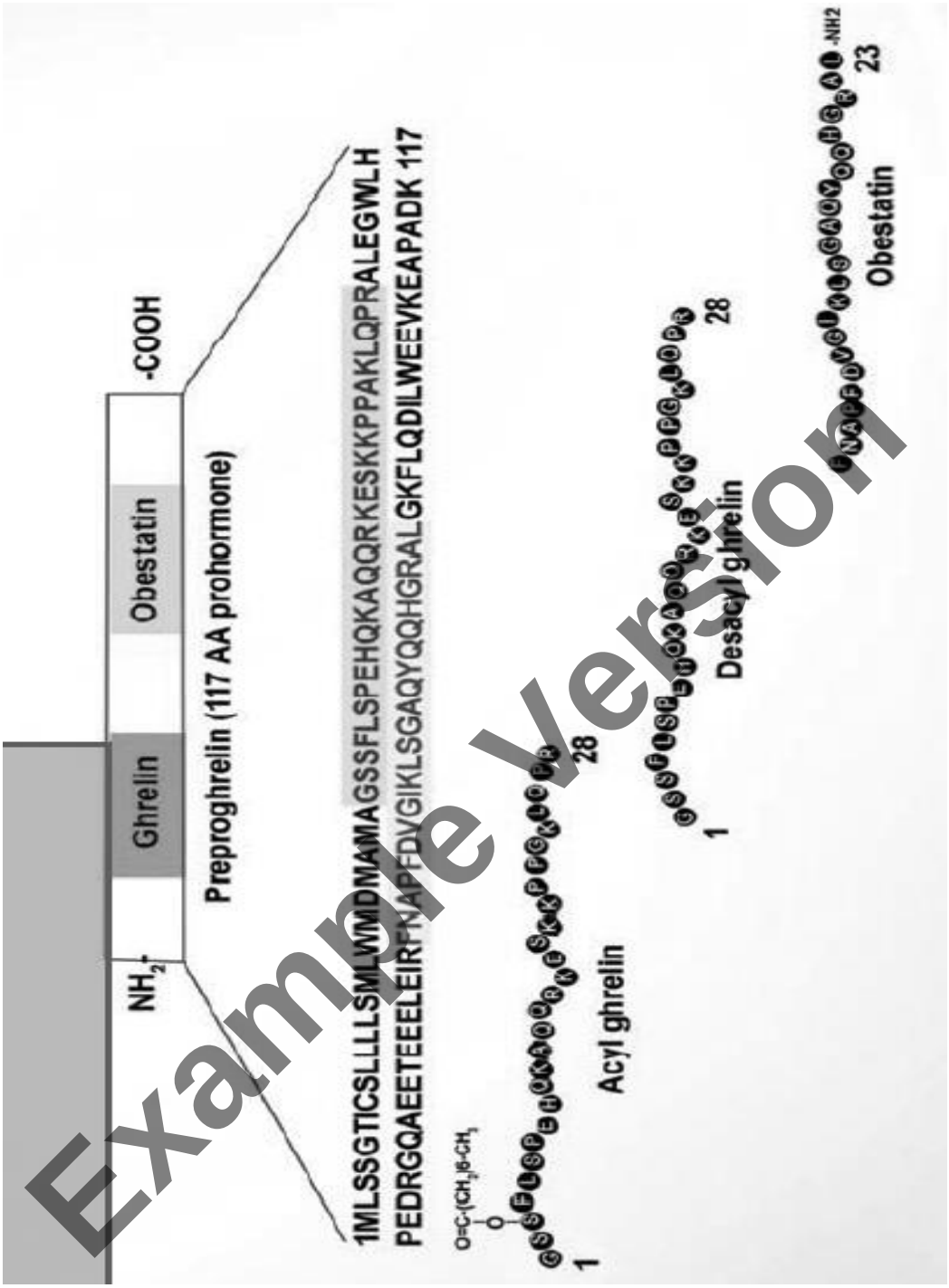
AChE® is a completely stable enzyme, unlike peroxidase which is suicidal. Thus, if a plate is accidentally dropped after dispatch of the AChE® Substrate Solution (Ellman's Reagent) or if it needs to be revealed again, one only needs to wash the plate, add fresh Substrate Solution and proceed with a new development. Otherwise, the plate can be stored at 4°C with Wash Buffer in wals while waiting for technical advice from the Bioreagent Department.

Obestatin

Obestatin is an amidated peptide made of 23 amino-acids with a secondary conformation in alpha-helix [4]. It was first isolated in 2005 from rat stomach [5]. Obestatin is a preproghrelin-derived peptide and is produced by many tissues or organs like stomach [6], pancreas [6], adipose tissue [6], skeletal muscle or heart.

Obestatin was identifi ed as an anorexigenic peptide with an action on the food intake [5]. The first studies have shown that the obestatin reduced food intake and body weight. It has

also been considered to be an antidiabetic peptide by positively influencing glucose and lipid metabolism [6].



Obestatin reduces the apoptosis and promote the proliferation of B-cells and human pancreatic islets [7].

Due to heterogeneity of these sources, obestatin has many different functions. Indeed obestatin could have a function in the regulation of blood pressure [8] and its plasmatic

concentration increases in case of hypertensive patients [9].

Finally obestatin could have a role in regulation of anxiety and improvement of the memory [10].

4. PRINCIPLE OF THE ASSAY

This Enzyme Immunometric Assay (EIA) is based on a sandwich technique. The plate supplied is coated with a monoclonal antibody (mAb) specifi c to the obestatin.

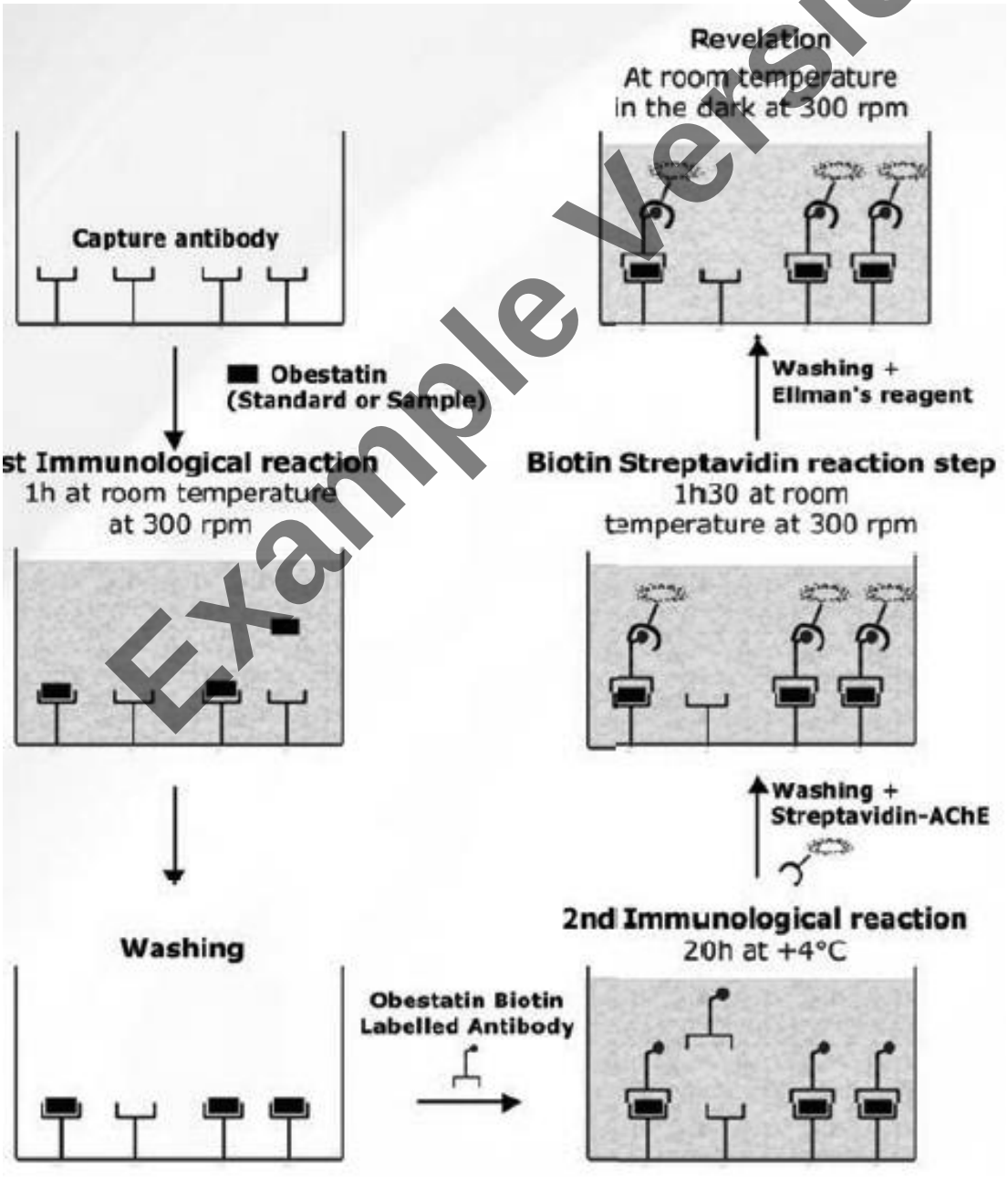
Obestatin from the standard or the samples is going to bind to the mAb coated on the plate and then is detected by a second mAb labelled with biotin also specifi c for the obestatin.

The immunological complex (mAb-obestatin-mAb_biotin) is revealed by the interaction between biotin and streptavidin labelled with AChE (Tracer).

The concentration of obestatin is determined by measuring the enzymatic activity of immobilized tracer using Ellman's Reagent. AChE tracer acts on Ellman's Reagent to form a yellow compound that strongly absorbs at 405 or at 414 nm.

The intensity of colour, which is determined by spectrophotometry, is proportional to the amount of obestatin present in the well during the immunological reaction.

The principle of the assay is summarised below:



5. MATERIALS AND EQUIPMENT REQUIRED

In addition to standard laboratory equipment, the following material is required:

- Precision micropipettes (20 to 1000 µL)
- Multichannel pipette 100 µL or 200 µL and disposable tips 30- 300 µL
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Orbital microplate shaker
- UltraPure water
- Polypropylene tubes

Water used to prepare all EIA reagents and buffers must be UltraPure (deionized & free from organic contaminants traces).

Otherwise, organic contamination can significantly affect the enzymatic activity of the tracer AcetylCholinesterase (AChE). Do not use distilled water, HPLC-grade water or sterile water.

UltraPure water may be purchased from BioVendor #S0001.

6. SAMPLE COLLECTION AND PREPARATION

This assay has been validated to measure obestatin in plasma (K3-EDTA) or in Obestatin Dilution Buffer.

General precautions

All samples must be free from organic solvents prior to assay.

Samples should be assayed immediately after collection or should be stored at -20°C.

Sample collection

Blood samples are collected in tubes containing K3-EDTA. then, they are centrifuged at 3,500 rpm for 10 minutes at +4°C and supernatants are transfered in separate tubes.

Samples should be quickly assayed or stored at -20°C for later use.

Sample preparation

Plasma samples may be assayed directly without any extraction procedure after being diluted at least to **1:8 in Obestatin Dilution Buffer** in order to avoid matrix effect.

7. REAGENT PREPARATION

Each kit contains suffi cient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 35 samples in duplicate.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Quality Control and one of Ellman's Reagent.

All reagents need to be brought to room temperature, around +20°C, prior to the assay.

Dilution Buffer

Reconstitute the vial of Dilution Buffer with 50 mL of UltraPure water.

Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at 4°C: 1 month

Human Obestatin Standard

Reconstitute one vial of Obestatin Standard with 1 mL of UltraPure water.

Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

The concentration of the fi rst standard (S1) is 4000 pg/mL.

Prepare seven propylene tubes for the other standards and add 500 µL of Obestatin Dilution Buffer into each tube. Then prepare the standards by serial dilutions as follows:

Standard	Volume of Standard	Volume of Dilution Buffer	Standard concentration
S1	-	-	4000 pg/mL
S2	500 µL of S1	500 µL	2000 pg/mL
S3	500 µL of S2	500 µL	1000 pg/mL
S4	500 µL of S3	500 µL	500 pg/mL
S5	500 µL of S4	500 µL	250 pg/mL
S6	500 µL of S5	500 µL	125 pg/mL
S7	500 µL of S6	500 µL	62.5 pg/mL
S8	500 µL of S7	500 µL	31.3 pg/mL

Stability at 4°C: 48 hours

Obestatin Quality Control

The Quality Control provided in this kit has been prepared by spiking Obestatin (human) peptide in Obestatin Dilution Buffer.

Reconstitute one Quality Control vial with 1 mL of Dilution Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at +4°C: 48 hours

Obestatin Biotin-Labelled Antibody

Reconstitute one vial with 10 mL of Dilution Buffer Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at +4°C: 1 month

Conjugate Solution

Reconstitute the vial with 10 mL of Dilution Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at 4°C: 1 week

Wash Buffer

Dilute 3.5 mL of concentrated Wash Buffer with 1400 mL of UltraPure water. Add 700 µL of Tween20.

Use a magnetic stirring bar to mix the content.

Stability at +4°C: 1 month

Substrate Solution (Ellman's Reagent)

5 minutes before use (development of the plate), reconstitute one vial of **Substrate Solution** with 49 mL of UltraPure water and 1 mL of concentrated Wash Buffer. The tube content should be thoroughly mixed.

Stability at +4°C and in the dark: 24 hours

8. ASSAY PROCEDURE

It is recommended to perform the assays in duplicate and to follow the instructions hereafter.

8.1 Plate preparation

Prepare the Wash Buffer as indicated in the reagent preparation section.

Open the plate packet and select the sufficient strips for your assay.

Put unused strips back in the zip lock bag with the absorbant pocket and properly close zip lock bag.

Store at -20°C for 1 month.

Rinse each well 5 times with the Wash Buffer 300 µL/well.

Just before distributing samples, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

8.2 Plate set-up

A plate set-up is suggested hereafter.

We suggest to assay each Blank and each Non-Specifi c Binding in four different wells.

The contents of each well may be recorded on the template sheet provided at the end of this technical booklet.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bk	S1	S5	*	*	*	*	*	*	*	*	*
B	Bk	S1	S5	*	*	*	*	*	*	*	*	*
C	Bk	S2	S6	*	*	*	*	*	*	*	*	*
D	Bk	S2	S6	*	*	*	*	*	*	*	*	*
E	NSB	S3	S7	*	*	*	*	*	*	*	*	*
F	NSB	S3	S7	*	*	*	*	*	*	*	*	*
G	NSB	S4	QC	*	*	*	*	*	*	*	*	*
H	NSB	S4	QC	*	*	*	*	*	*	*	*	*

Bk: Blank

NSB: Non Specific Binding

QC: Quality Controls

S1-S8: Standards 1-8

*: Samples

8.3 Pipetting the reagents

All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipette the buffer, standard, sample, tracer, biotin-labelled antibody and other reagents.

Before pipetting, equilibrate the pipette tips in each reagent.

Do not touch the liquid already in the well when expeling with the pipette tip.

- **Dilution Buffer**

  Dispense 100 µL to Non Specific Binding (NSB) wells.

- **Human Obestatin Standards**

  Dispense 100 µL of each of the eight standards S8 to S1 in duplicate to appropriate wells.

  Start with the lowest concentration standard S8 and equilibrate the tip in the next higher standard before pipetting.

- **Quality Control and Samples**

  Dispense 50 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in Dilution Buffer.

8.4 Incubating the plate

Incubate the plate 1 hour at room temperature under agitation on an orbital plate shaker at 300 rpm.

8.5 Washing the plate

Rinse each well 5 times with the Wash Buffer (300 µL/well).

Just before distributing reagents, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

8.6 Pippeting the reagents

- **Biotion Labelled Antibody**

  Dispense 100 µL into each well, except Blank (Bk) wells.

8.7 Incubating the plate

Incubate the plate overnight (20 hours) at +4°C.

Bring the plate back to room temperature 1 hour before the end of the incubation time

8.8 Washing the plate

Rinse each well 5 times with the Wash Buffer (300 µL/well).

Just before distributing reagents, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

8.9 Pippeting the reagents

- **Conjugate Solution**

Dispense 100 µL into each well, except Blank (Bk) wells.

8.10 Incubating the plate

Incubate the plate 1 hour 30 minutes at room temperature under agitation on an orbital plate shaker at 300 rpm.

8.11 Developing and reading the plate

- Reconstitute Substrate Solution as mentioned in the Reagent preparation section.
- Empty the plate by turning over. Rinse each well five times with 300 µL of Wash Buffer. At the end of the last washing step, empty the plate and blot the plate on a paper towel to discard any trace of liquid.
- Add 200 µL of Substrate Solution to each well. Cover the plate with an aluminium sheet and incubate in the dark at room temperature. Optimal development is obtained using an orbital shaker.
- Wipe the bottom of the plate with a paper towel, and make sure that no liquid has splashed outside the wells.
- Read the plate at a wavelength between 405 and 414nm (yellow colour).

  After addition of Substrate Solution the absorbance has to be checked periodically (every 30 minutes) until the maximum absorbance of the STD1 is

  between 2.500-3.000 A.U (blank subtracted).

9. DATA ANALYSIS

Make sure that your plate reader has subtracted the absorbance readings of the blank well (absorbance of Ellman's reagent alone) from the absorbance readings of the rest of the plate. If it is not the case, please do it.

- Calculate the average absorbance for each NSB, standard and sample.
- For each standard, plot the absorbance on y axis versus the concentration on x axis. Draw a best-fi t line through the points.
- To determine the concentration of your samples, fi nd the absorbance value of each sample on the y axis.
- Read the corresponding value on the x axis which is the concentration of your unknown sample.
- Samples with a concentration greater than 4000 pg/mL should be re-assayed after dilution in Dilution Buffer.
- Most plate readers are supplied with curve-fitting software capable of graphing these data (4-parameter logistic fit 4PL). If you have this type of software, we recommend using it. Refer to it for further information.

Two vials of Quality Control are provided with this kit.

Your standard curve is validated only:

- **If the Quality Control obtained with the assay is +/- 25% of the expected concentration (see the label of QC vial).**
- **If the NSB is lower than 0.200 A.U.**

10. ACCEPTABLE RANGE

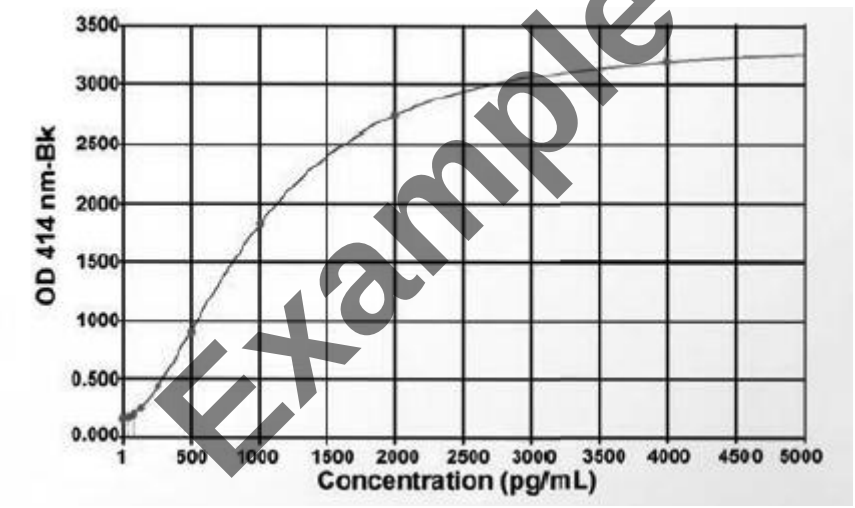
- NSB absorbance < 0.200 A.U.
- Limit of detection < 500.0 pg/mL
- QC sample: ±25% of the expected concentration (see the label of QC vial)

11. TYPICAL RESULTS

The following data are for demonstration purpose only. Your data may be different and still correct.

These data were obtained using all reagents as supplied in this kit under the following conditions: 60 minutes developing at RT, reading at 414 nm. A 5-parameter logistic fi tting with a 1/Y ponderation was used to determine the concentrations.

Standard	Obestatin (human) pg/mL	Absorbance A.U.
S1	4,000.0	3.192
S2	2,000.0	2.737
S3	1,000.0	1.833
S4	500.0	0.906
S5	250.0	0.437
S6	125.0	0.242
S7	62.5	0.180
S8	31.3	0.164
NSB	0	0.146



Typical Obestatin standard curve

12. ASSAY VALIDATION AND CHARACTERISTICS

The Enzyme Immunometric assay of Obestatin (human) has been validated in plasma collected on K3-EDTA.

For additional information regarding the validation of imunoassay for protein biomarkers in biological samples, please refer to bibliography [11, 12].

The Limit of Detection (LOD)

calculated as the concentration of Obestatin (human) corresponding to the NSB average plus three standard deviations is 62.5 pg/mL.

Due to the minimal plasma dilution (1:8), the limit of detection in plasma is less than 500.0 pg/mL.

Intra-assay variation

	QC n°1	QC n°2	QC n°3
Means of measured concentrations (pg/mL)	172.1	265.7	731.5
Means of measured concentrations (pg/mL) X dil	1377.1	2125.8	5851.9
Cv %	6.9%	2.3%	1.8%

Unspiked plasma (K3-EDTA) has been diluted at 1:8 in Obestatin Dilution Buffer. The number of replicates (n) is equal to 20, for the three quality controls. The three validation levels were analysed along with the calibration curve for a unique experiment.

Inter-assay variation

	QC n°1	QC n°2	QC n°3
Means of measured concentrations (pg/mL)	142.0	225.7	744.1
Means of measured concentrations (pg/mL) X dil	1135.6	1805.4	5952.8
Cv %	16.2%	13.9%	5.6%

Unspiked plasma (K3-EDTA) have been diluted at 1:8 in Obestatin Dilution Buffer. The number of replicates (n) is equal to 5, for the three quality controls. The three validation levels were analysed along with the calibration curve for a total of 5 independent runs.

Cross-reactivity [6]

Obestatin (mouse, rat)	19.1 %
Obestatin (dog)	10.1 %
Obestatin (pig)	0.0 %

Linearity

Single donor plasma are spiked with 6,000 pg/mL of Obestatin (human) and then diluted by serial dilutions in Obestatin Dilution Buffer. Each dilution is run in duplicate.

Matrix	Dilution (1:x)	Endogenous obestatin (human) measured conc (pg/mL)	Spiked obestatin (human) (pg/mL)	Endogenous obestatin (human) + Spiked measured conc (pg/mL)	Endogenous obestatin (human) + Spiked measured conc X Dilution (pg/mL)	Accuracy (%)	CV %
1		1297.3	6000.0	-	-	-	-
	8	-	-	740.3	5922.6	77.1%	13.2%
	16	-	-	420.4	6725.6	90.5%	
	32	-	-	229.1	7331.5	100.6%	
	64	-	-	106.6	6823.9	92.1%	
	128	-	-	66.0	8454.3	119.3%	
2		1334.7	6000.0	-	-	-	-
	8	-	-	886.8	7094.4	96.0%	6.1%
	16	-	-	421.3	6740.8	90.1%	
	32	-	-	211.7	6775.7	90.7%	
	64	-	-	121.8	7796.2	107.7%	
	128	-	-	57.4	7343.0	100.2%	
3		5122.5	6000.0	-	-	-	-
	8	-	-	1388.2	11105.6	99.7%	16.9%
	16	-	-	738.1	11810.1	111.5%	
	32	-	-	364.1	11651.0	108.8%	
	64	-	-	245.9	15736.4	176.9%	
	128	-	-	ND	ND	N/A	

ND: range outside of the standard curve

Stability tests

			1	2	3
Number of freezing / thawing cycles	0	pg/mL	596.3	4590.2	9051.0
		Accuracy	N/A	N/A	N/A
	1	pg/mL	444.6	4719.1	8875.8
		Accuracy	74.7%	102.8%	98.1%
	2	pg/mL	445.7	4384.3	8822.1
		Accuracy	74.7%	95.5%	97.5%
	3	pg/mL	631.0	4369.7	9255.1
		Accuracy	105.8%	95.2%	101.8%
	4	pg/mL	525.5	4455.9	9217.3
		Accuracy	88.1%	97.1%	101.8%
24 hous at RT		pg/mL	647.7	4420.2	8779.7
		Accuracy	108.6%	96.3%	97.0%
Means		pg/mL	548.5	4489.9	9000.2
CV %			15.0%	2.8%	2.1%

Each sample is diluted at 1:8 in Obestatin Dilution Buffer.

The concentration indicated in the table above is the measured concentration multiplied by the dilution factor 1:8.

13. TROUBLESHOOTING

Absorbance values too low:

- organic contamination of water,
- one reagent has not been dispensed,
- incorrect preparation/dilution,
- assay performed before reagents reached room temperature,
- reading time not long enough.

High signal and background in all wells:

- inefficient washing,
- overdeveloping (incubation time should be reduced),
- high ambient temperature.

High dispersion of duplicates:

- poor pipetting technique,
- irregular plate washing.

If a plate is accidentally dropped after dispatch of the AChE® substrate (Substrate Solution) or if it needs to be revealed again:

- one only needs to wash the plate, add fresh Substrate Solution and proceed with a new development.
- otherwise, the plate can be stored at 4°C with Wash Buffer in wells while waiting for technical advice from the Bioreagent Department.

These are a few examples of troubleshooting that may occur.

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