BioVendor Group®

ENG

Product Data Sheet:

MOUSE/RAT OBESTATIN ELISA

Catalogue number:

RA19014R

For research use only!

Example version

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Example Version

1. MOUSE/RAT OBESTATIN ELISA

96 wells

Storage: -20°C

Expiry date: stated on the package

This kit contains:

REAGENTS (Store at 2-8°C)	COLOUR CODE	Quantity	Form
Antibody Coated Microtiter Strips (pre-coated with Obestatin MAb	Blister with zip	1	
Streptavidin AChE tracer	Green	1	lyophilized
Mouse/Rat Obestatin Biotin-Labelled Antibody	Red	1	lyophilized
Mouse/Rat Obestatin Standard	Blue with red septum	2	lyophilized
Mouse/Rat Obestin Quality Control	Green with red septum	2	lyophilized
Dilution Buffer (EIA buffer)	Blue	1	lyophilized
Wash Solution Conc. (400x)	Silver	1	liquid
Substrate Solution (Ellman´s reagent)	Black with red septum	2	lyophilized
Tween 20	Transparent	1	liquid
Cover Sheet		1	

Example Version

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

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.

<u>Temperature:</u>

Unless otherwise specifi ed, 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.

Example Version

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 fi rst-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®

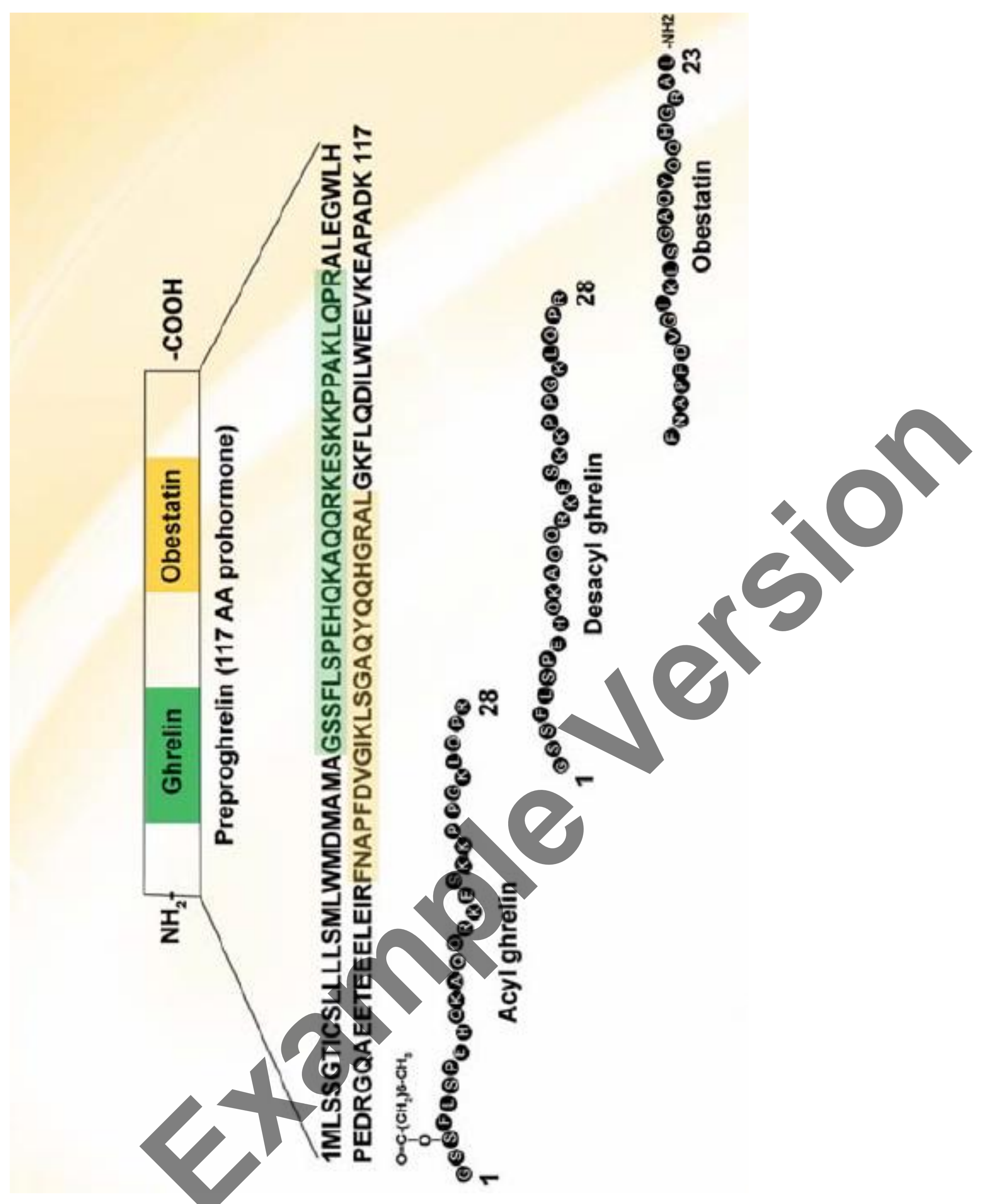
is a completely stable enzyme, unlike peroxidase which is suicidal. Thus, if a plate is accidentally dropped after dispatch of the AChE® substrate solution (Ellman's reagent) or if it needs to be revealed again, one only needs to wash the plate, add fresh Substrate Solution (Ellman's reagent) and proceed 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 Technical Support.

Obestin

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 identified 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].

Example Version



Example Version

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) specific 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 specific 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 Substrate Solution (Ellman´s reagent). AChE tracer acts on Substrate Solution (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 bellow:

Example version

5. MATERIAL REQUIRED BUT NOT PROVIDED

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

FOR THE ASSAY

- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or wash-bottles)
- Orbital Microplate shaker
- Multichanel pipette and disposable tips 30-300 µl
- Ultra pure water
- Polypropylene tubes

Water used to prepare all EIA reagents and buffers must be Ultra Pure, deionized & free from organic contaminants traces.

Otherwise, organic contamination can signifi cantly affect the enzymatic activity of the tracer AcetylCholinesterase. Do not use distilled water, HPLC-grade water or sterile water.

- Ulta pure water may be purchased from BioVendor

6. SAMPLE COLLECTION AND PREPARATION

This assay has been validated to measure obestatin in plasma (K_3 -EDTA) or in Obestatin Dilution buffer.

General precautions

All samples must be free of 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 K -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 **1:4 in Obestatin Dilution buffer** in order to avoid matrix effect.

Example Version

7. REAGENT PREPARATION

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

Dilution Buffer

Reconstitute the vial 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

Mouse/Rat Obestatin Standard

Reconstitute the vial 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 first standard (S1) is 20 000 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 pg/mL
S1	-	-	20 000
S2	500 µL of S1	500 µL	10 000
S3	500 µL of S2	500 µL	5 000
S4	500 µL of S3	500 µL	2 500
S5	500 µL of S4	500 µL	1 250
S6	500 µL of S5	500 µL	625
S7	500 µL of S6	500 µL	312.5
S8	500 µL of S7	500 µL	156.3

Stability at 4°C: 8 days

Mouse/Rat Obestatin Quality Control

The Quality Control provided in this kit has been prepared by spiking Obestatin (mouse, rat) peptide in Obestatin EIA Buffer.

Reconstitute the vial with 1 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at +4°C: 8 days

Example Version

Mouse/Rat Obestatin Biotin-Labelled Antibody

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

Stability at +4°C: 1 month

Streptavidin AChE Tracer

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

Stability at +4°C: 1 month

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 Elman's Reagent 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

Example Version

8. ASSAY PROCEDURE

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

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.

Distribution of reagents and samples

A plate set-up is suggested on the following page. We suggest to assay each Blank and each Non-Specific 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.

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 Specifi c Binding NSB wells.

Mouse/Rat Obestatin Standards

Dispense 100 µL of each of the eight standards S1 to S8 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 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in Dilution Buffer.

Incubating the plate

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

Example Version

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.

Pipetting the reagents

Dilution Buffer

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

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

Washing the plate

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

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

Pipetting the reagents

Streptavidin-AChE Tracer

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

Example Version

Incubating the plate

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	S1	S5	QC	*	*	*	*	*	*	*	*
B	B	S1	S5	QC	*	*	*	*	*	*	*	*
C	B	S2	S6	*	*	*	*	*	*	*	*	*
D	B	S2	S6	*	*	*	*	*	*	*	*	*
E	NSB	S3	S7	*	*	*	*	*	*	*	*	*
F	NSB	S3	S7	*	*	*	*	*	*	*	*	*
G	NSB	S4	S8	*	*	*	*	*	*	*	*	*
H	NSB	S4	S8	*	*	*	*	*	*	*	*	*

B : Blank

S1-S8: Standards 1-8

* : Samples

NSB : Non Specific Binding

QC: Quality Controls

Example Version

Developing and reading the plate

- Reconstitute Substrate Solution (Ellman's reagent) as mentioned in the Reagent preparation section.
- Rinse each well 5 times with the Wash Buffer (300 µL/ well). Just before distributing the Substrate Solution (Ellman´s reagent), remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.
- Add 200µL of Substrate Solution (Ellman´s reagent) to each 96 well. Cover the plate with 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 (Ellman´s reagent), the absorbance has to be checked periodically (every 30 minutes) until the maximum absorbance of the STD1 has reached 1.800 A.U (blank subtracted).

Enzyme Immunoassay Protocole (volumes are in µL)					
	Blank	NSB	Standard	QC	Sample
Obestatin Dilution buffer	-	100	-	-	-
Standard	-	-	100	-	-
QC	-	-	-	100	-
Sample	-	-	-	-	100
Cover plate, incubate 1 hour at RT under agitation at 300 rpm					
Wash strips 5 times, with 300 µL and discard the liquid from the wells					
Biotin- Labelled Ab	-	100			
Cover plate, incubate overnight (20 hours) at +4°C					
Wash strips 5 times, with 300 µL and discard the liquid from the well					
Streptavidin AChE Tracer	-	100			
Cover plate, incubate 1 hour 30 under agitation at 300 rpm					
Wash strips 5 times, with 300 µL and discard the liquid from the well					
Substrate Solution (Ellman´s reagent)	200				
Incubate 1 hour at RT under agitation at 300 rpm					
Read the plate between 405 and 414 nm					

Example version

9. DATA ANALYSIS

Make sure that your plate reader has subtracted the absorbance readings of the blank well (absorbance of Substrate Solution (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-fit line through the points.
- To determine the concentration of your samples, find 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 than20 000 pg/mL should be re-assayed after dilution in Obestatin Dilution buffer.
- Most plate readers are supplied with curve-fitting software capable of graphing these data (logit/log or 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 calculated concentration of the Quality Control obtained with the assay is +/- 25% of the expected concentration (see the Quality Control Sheet).

- If the NSB is lower than 0.200 A.U.

10. ACCEPTABLE RANGE

NSB absorbance: < 0.200 A.U.

Limit of detection: < 625.0 pg/mL in undiluted plasma sample

< 156.3 pg/mL in Dilution buffer

QC sample: ±25% of the expected concentration (see the Quality Control Sheet)

Example Version

11. TYPICAL RESULTS

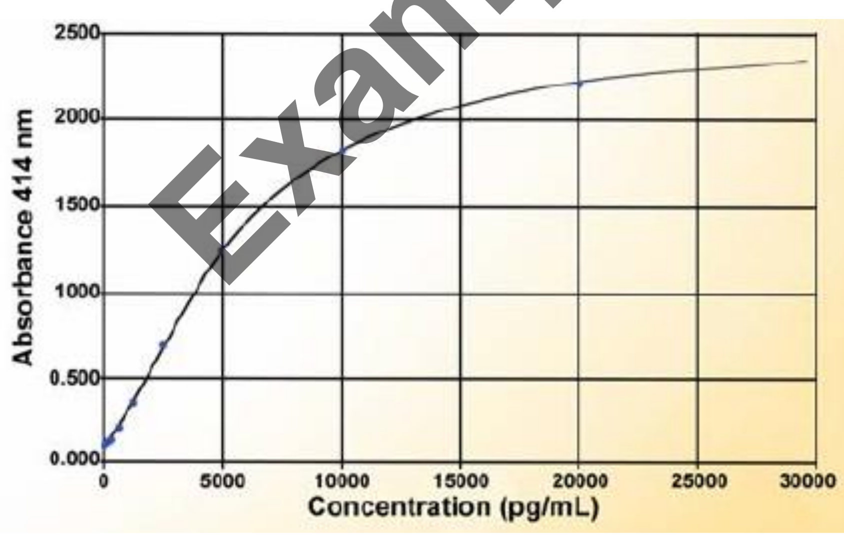
The following data are for demonstration purpose only. Your data may be different and still correct.

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 fitting with a 1/Y ponderation was used to determine the concentrations.

	Mouse/Rat Obestatin pg/mL	Absorbance (A.U.)
Standard S1	20 000.0	2.221
Standard S2	10 000.0	1.811
Standard S3	5 000.0	1.233
Standard S4	2 500.0	0.695
Standard S5	1 250.0	0.364
Standard S6	625.0	0.206
Standard S7	312.5	0.144
Standard S8	156.3	0.126
NSB	0	0.106

Example Version

Typical Mouse/Rat Obestatin standard curve



12. ASSAY VALIDATION AND CHARACTERISTICS

The Enzyme Immunometric assay of Obestatin (mouse, rat) has been validated in mouse plasma collected on K -EDTA.

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

The **limit of detection**, calculated as the concentration of Obestatin (mouse, rat) corresponding to the NSB average plus three standard deviations is 156.3 pg/mL. Due to the minimal plasma dilution (1:4), the limit of detection in plasma is less than 625.0 pg/mL.

Inter-assay variation

	QC n°1	QC n°2	QC n°3	QC n°4
Means of measured concentrations (pg/mL)	229,5	1 308,0	3 173,9	5 626,8
Means of measured concentrations (pg/mL) X dil	918,2	5 232,1	1 2695,7	2 2507,4
Cv %	12,7	8,1	9,0	10,2

QC are a mix of mouse plasma (K -EDTA) spiked with Obestatin (mouse, rat).

Each QC is tested in duplicate, in five different experiments, diluted at 1:4 in Obestatin Dilution buffer.

Intra-assay variation

	QC n°1	QC n°2	QC n°3	QC n°4
Means of measured concentrations (pg/mL)	244,2	1 163,7	2 848,6	4 913,1
Means of measured concentrations (pg/mL) X dil	976,9	4654,7	1 1394,4	19 652,6
Cv %	19,3	6,8	3,8	3,4

QC are a mix of mouse plasma (K -EDTA) spiked with Obestatin (mouse, rat).

Each QC is tested in 10 duplicate, in five different experiments, diluted at 1:4 in Obestatin Dilution buffer.

Example Version

Cross-reactivity

Obestatin (human)	> 100.0 %
Obestatin (dog)	42.6 %
Obestatin (pig)	0.0 %

Linearity

Matrix	Dilution (1:x)	Endogenous obestatin (mouse, rat) measured conc (pg/mL)	Spiked obestatin (mouse, rat) (pg/mL)	Endogenous obestatin (mouse, rat) + Spiked measured conc (pg/mL)	Endogenous obestatin (mouse, rat) + Spiked measured conc X Dilution (pg/mL)	Accuracy (%)	CV %
1		-	12 000,0	-	-	-	-
	4	489,3	-	2 970,0	11 880,1	94,9	5,3
	8	-	-	1 602,6	12 821,1	102,8	
	16	-	-	822,5	13 159,9	105,6	
	32	-	-	419,9	13 436,2	107,9	
2		-	12 000,0	-	-	-	-
	4	1613,8	-	2 965,3	11 861,4	85,40	2,6
	8	-	-	1 577,1	12 616,5	91,69	
	16	-	-	754,2	12 067,3	87,11	
	32	-	-	381,2	12 197,0	88,19	
3		-	12 000,0	-	-	-	-
	4	516,4	-	356,5	11 406,8	90,8	4,5
	8	-	-	699,7	11 195,7	89,0	
	16	-	-	1 475,8	11 806,1	94,1	
	32	-	-	2 645,0	10 580,1	83,9	

Example Version

13. ASSAY TROUBLE SHOOTING

Absorbance values too low: organic contamination of water, incubation in wrong conditions (time or temperature), reading time not long enough. Standard or Conjugate Solution or Substrate Solution´s reagent have not been dispensed.

High signal and background in all wells: Inefficient washing or overdeveloping (incubation time should be reduced) or high ambient temperature.

High dispersion of duplicates: Poor pipetting technique or irregular plate washing.

14. BIBLIOGRAPHY

1. Grassi J, Pradelles P,

Compounds labelled by the acetylcholinesterase of Electrophorus Electricus. Its preparation process and its use as a tracer or marquer in enzymo-immunological determinations.

United States patent, N° 1,047,330. September 10, 1991

2. Grassi J, Pradelles P,

The use of Acetylcholinesterase as a Universal marker inEnzyme-Immunoassays

Proceedings of the Third International Meeting on

Cholinesterases, American Chemical Society (1991)

3. Pradelles P, Grassi J, Maclouf J.

Enzyme Immunoassays of Eicosanoids Using Acetylcholinesterase

Methods in Enzymology (1990), vol. 187, 24-34

4. Alen B, Nieto L, Gurriaran-Rodriguez U, Mosteiro C.S, Alvarez- Perez J.C, Otero-Alen M, Camina J.P, Gallego R, Garcia-Caballero T, Martin-Pastor M, Casanueva F. F, Jimenez-Barbero J, Pazos Y

The NMR Structure of Human Obestatin in Membrane-

Like Environments: Insights into the Structure-Bioactivity

Relationship of Obestatin

PLOS ONE | www.plosone.org, vol. 7, Issue 10, e45434 2012

5. Zhang J.V, Ren P.G, Avsian-Kretchmer O, Luo C.W, Rauch R, Klein C, and Hsueh A.J.

Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake.

Science 310 996-999, 2005

6. Gronberg M, Tsolakis AV, Magnusson L, Jonson ET, Saras J.

Distribution of obestatin and ghrelin in human tissues: immunoreactive cells in the gastrointestinal tract, pancreas, and mammary glands.

J Histochem Cytochem ;56 :793-801, 2008

7. Granata R, Baragli A, Settanni F, Scarlatti F, Ghigo E

Unraveling the role of the ghrelin gene peptides in the endocrine pancreas.

J Mol Endocrinol ;45 :107-118, 2010

8. Ren AJ, He Q, SHI JS, Guo Zf, Zheng X, Lin L, Wang YK, XIA SY, Sun LL, Zhang, Yuan WJ

Association of obestatin with blood pressure in the third trimesters of pregnancy.

Peptides ;30 :1742-1745 ;2009

9. Li ZF, Guo ZF, Cao J, Hu JQ, Zhao XX, Xu RL, Huang XM, Qin YW, Zheng X

Plasma ghrelin and obestatin levels are increased in spontaneously hypertensive rats

Peptides ;31 :297-300 ;2010

10. Carlini VP, Schioth HB and Debarioglio SR

Obestatin improves memory performance and causes anxiolytic effects in rats.

Biochem Biophys Res Commun ;352 :907-12, 2007

Example Version

11. Valentin MA, Ma S, Zhao A, Legay F, Avrameas A

Validation of immunoassay for protein biomarkers: Bioanalytical study plan implementation to support pre-clinical and clinical studies.

J Pharm Biomed Anal. (2011) 55(5) : 869-877

15. EXPLANATION OF SYMBOLS

Symbol	Explanation
REF	Catalogue number
LOT	Batch code
	Caution
	Use by date
2 °C 8 °C	Temperature limit
	Manufacturer
www.biovendor.com	Read electronic instructions for use - eIFU
Σ 96	The content is sufficient for 96 tests
	Biological risks

Example Version

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Example Version

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