BioVendor
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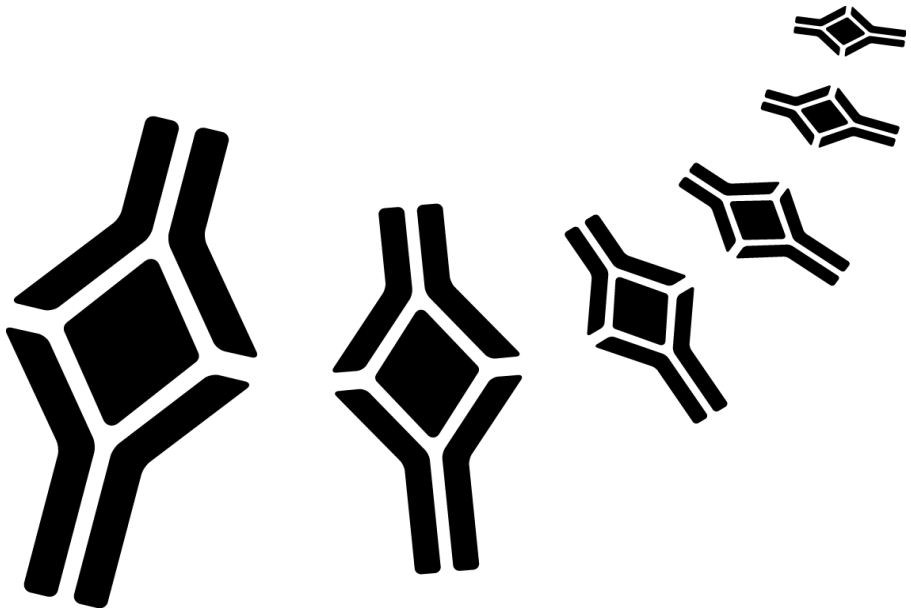


HUMAN ACYLATED GHRELIN EASY SAMPLING ELISA

Product Data Sheet

Cat. No.: RA194062500R

For Research Use Only

CONTENTS

» **This kit is manufactured by:**
BioVendor – Laboratorní medicína a.s.

» **Use only the current version of Product Data Sheet enclosed with the kit!**

1. HUMAN GHRELIN ACYLATED EASY SAMPLING 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 (Human, rat tracer easy sampling)	Green	1vial	lyophilized
Human Acylated Ghrelin Standard	Blue with red septum	2	lyophilized
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	

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 36 samples in duplicate.

If you want to use the kit in two times, we provide one addional vial of Standard, one of Quality Control and one of Substrate Solution (Ellman´s reagent).

2. PRECAUTION FOR USE

Users are recommended to 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 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.

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

Temperature:
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 effi ciency.

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 Bioreagent Department.

Ghrelin

Ghrelin discovered in 1999, is fast becoming an endokrinology target of the millennium. Ghrelin, identifi ed in rat stomach as an endogenous ligand for the GH secretagogue receptor, is mainly produced in stomach, but has been demonstrated in many other organs ***[4, 5]***. In addition to GH-releasing properties and its orexant action, Ghrelin could act as an hormone having effects on gastric motility (similarity with the peptide hormone motilin), acidic secretion, cardiovascular action, antiproliferative effects, pancreatic and glucose metabolism function, sleep ***[6, 7, 8]***...

Ghrelin gene raises to mRNA prepro-ghrelin of 117 amino acids. This precursor is processed into Ghrelin, 28 amino acids (human). Before being secreted, this peptide is octanoylated at Ser 3 by GOAT (Ghrelin Octanoyl Acyl Transferase). This step is Essentials for biological activity making GOAT a perfect target for drugs in feeding behaviour. Interestingly, the potential therapeutic importance of this hormone is not restricted to regulation of food intake ***[9]*** but also in cachexia (related to cancer treatment, anorexia nervosa or ischemia) ***[10]*** gastrin motility and may be involved in osteoporosis, somatopause, infertility and ovulation induction, neurological disorders (Alcoholism, Post Traumatic Stress disorders...) ***[11]*** and cardiovascular diseases.

4. PRINCIPLE OF THE ASSAY

This Enzyme Immunometric Assay (EIA) is based on a doubleantibody sandwich technique. The wells of the plate supplied are coated with a monoclonal antibody specifi c to the C-terminal part of Ghrelin.

This antibody will bind to any Ghrelin introduced into the wells (standard or sample). After a washing step, the acetylcholinesterase (AChE) - Fab' conjugate (Tracer) which recognises the N-terminal part of Acylated Ghrelin is also added to the wells.

The two antibodies then form a sandwich by binding on different parts of the Acylated Ghrelin.

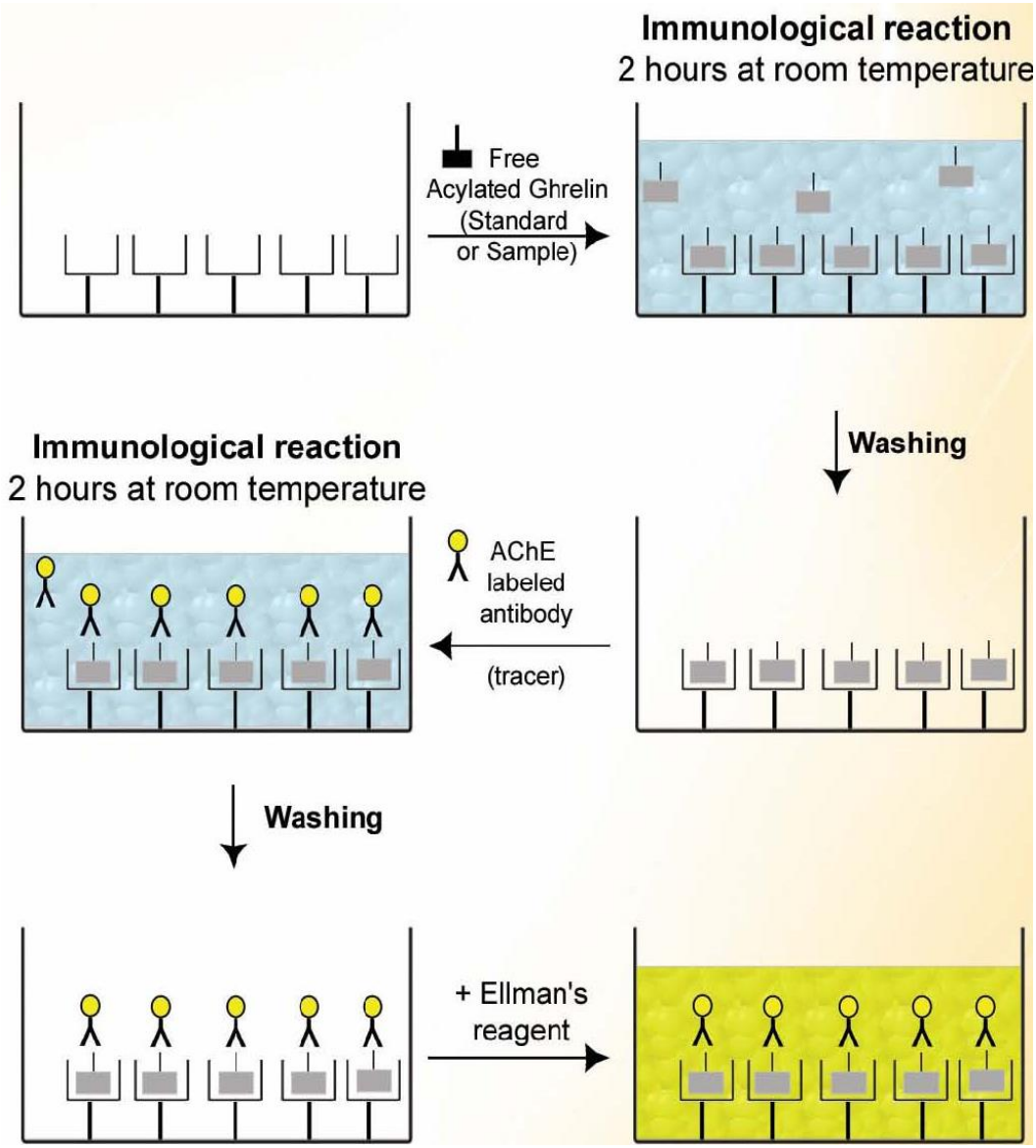
The sandwich is immobilised on the plate so reagents in excess may be washed away.

The concentration of Acylated Ghrelin (human) 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 414 nm.

The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of Acylated Ghrelin (human) present in the well during the immunological incubation.

This ELISA so called Easy Sampling ELISA kit works with any sample collected on any kind of protease inhibitors, without extraction but a simple dilution.

The principle of the assay is summarised below:



5. MATERIAL REQUIRED BUT NOT PROVIDED

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

FOR SAMPLE PREPARATION

- EDTA tubes for blood collection
- Protease inhibitor (AEBSF, PMSF, Aprotinin, Pefabloc®P800, PHMB)
- UltraPure water (cat. number S0001)

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 able to perform at 600 rpm
- 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 (cat. number S0001)

6. SAMPLE COLLECTION AND PREPARATION

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.

Blood Collection

- Blood samples are collected in tubes containing EDTA and a protease inhibitor to prevent the degradation of Acylated Ghrelin.
- Choice of protease inhibitor
- We suggest adding AEBSF at 0.2 mg/mL blood during blood collection. We suggest preparing a 100 times concentrated solution of protease inhibitor and then adding 10 µL of this solution per mL of blood. For example, for the AEBSF, prepare a mother solution at 20 mg/mL in UltraPure water and add 10 µL of this solution per mL of blood. The mother solution may be stored one month at -20°C. We suggest using aliquots for AEBSF solution in order to avoid freezing/thawing cycles.

Other protease inhibitors could be used with the assay like Aprotinin (up to 0,6 TIU/mL blood), PMSF (around 0.1 mg/mL blood according to literature), PHMB, Pefabloc® or Pefabloc SC® (up to 0.2 mg/mL blood) as indicated in the section “Protease inhibitor compatibility table” at the end of this booklet. For the use of these different products, please refer to the vendor’s instructions.

- Collection tubes are mixed by inversion 5 folds.

Samples should be kept on ice between collection and centrifugation (15 minutes max).

• Blood samples are centrifuged at 3,500 rpm for 10 minutes at +4°C and then, supernatants are transferred in separate tubes. Samples should be quickly assayed or stored at -20°C or at -80°C for later use within 6 months.
• The best way is to assay the samples within 3 weeks after the collection date. Moreover, we suggest using aliquots for plasma samples (we suggest 250 µl per aliquot) in order to avoid freezing/thawing cycles.

Plasma samples prepared as above-mentioned can be assayed for Acylated Ghrelin with Acylated Ghrelin EIA kit or for Unacylated Ghrelin with Unacylated Ghrelin EIA kit.

Sample preparation
Plasma samples may be assayed directly without any extraction procedure after being diluted at **least to 1:2 in Dilution Buffer** in order to avoid matrix effect.

7. REAGENT PREPARATION

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 36 samples in duplicate.

If you want to use the kit in two times, we provide one addional vial of Standard, one of Quality Control and one of Substrate Solution (Ellman´s reagent).

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

• **Acylated Ghrelin (human) Standard**
Reconstitute the Standard 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 250 pg/mL.
Prepare seven propylene tubes for the other standards and add 500 µL of 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	-	-	250
S2	500 µL of S1	500 µL	125
S3	500 µL of S2	500 µL	62.5
S4	500 µL of S3	500 µL	31.3
S5	500 µL of S4	500 µL	15.6
S6	500 µL of S5	500 µL	7.8
S7	500 µL of S6	500 µL	3.9
S8	500 µL of S7	500 µL	2.0

- **Acylated Ghrelin (human) Quality Control**

The Quality Control provided in this kit has been prepared by spiking Acylated Ghrelin (human) peptide in Dilution Buffer.
Reconstitute the Quality Control 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: 1 week

- **Acylated Ghrelin Conjugate Solution**

Reconstitute the vial Conjugate Solution 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 2 mL of concentrated Wash Buffer with 800 mL of UltraPure water. Add 400 µL of Tween20. Use a magnetic stirring bar to mix the content.
Stability at +4°C: 1 week

- **Substrate Solution (Ellman's Reagent)**

5 minutes before use (development of the plate), reconstitute one vial of Substrate Solution (Ellman's Reagent_49+1) with 49 mL of UltraPure water and 1 mL of concentrated Wash Buffer. The tube kontent 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.

Plate preparation

Prepare the Wash Buffer as indicated in the reagent preparation section. Open the plate packet and select the suffi cient strips for your assay and place the unused strips back in the packet

Stability at +4°C: 1 month

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

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

Plate set-up

A plate set-up is suggested on the following page.

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, conjugate, antiserum 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.

- **Acylated Ghrelin (human) 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
Cover the plate with the cover sheet and incubate for 2 hours at room temperature on a orbital shaker (at 600rpm).

Washing the plate
Empty the plate by turning over. Rinse each well fi ve times with 300 µL Wash Buffer. The 5th time, slightly shake the plate for 5 minutes on an orbital shaker. Then rewash fi ve times with 300 µL 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.

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

B : Blank
S1-S8: Standards 1-8
* : Samples

NSB : Non Specific Binding
QC: Quality Controls

Pipetting the reagents
- **Acylated Ghrelin Conjugate Solution**

Dispense 100 µL to each well, except blank (Bk) wells.

Incubating the plate
Cover the plate with the cover sheet and incubate for 2 hours at room temperature on a orbital shaker (at 600rpm).

Developing and reading the plate
- Reconstitute Substrate Solution (Ellman's reagent) as mentioned in the Reagent preparation section.
- Empty the plate by turning over. Rinse each well five times with 300 µL Wash Buffer. The 5th time, slightly shake the plate for 5 minutes on an orbital shaker. Then rewash five times with 300 µL 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 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 has reached a minimum of 0.5 A.U. blank subtracted.

Easy Sampling Enzyme Immunoassay Protocole (volumes are in µL)				
	Blank	NSB	Standard	Sample or QC
Dilution Buffer	-	100	-	-
Standard	-	-	100	-
Sample or QC	-	-	-	100
Cover plate, incubate 2 hours at 600 rpm				
Wash plate 5 times, shake 5 min, wash 5 times & discard liquid from the wells				
Tracer	-	100	100	100
Cover plate, incubate 2 hours at 600 rpm				
Wash plate 5 times, shake 5 min, wash 5 times & discard liquid from the wells				
Ellman's reagent	200			
Incubate with an orbital shaker in the dark at RT				
Read the plate between 405 and 414 nm				

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 not, do it now.
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, 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. Do not forget to integrate the dilution factor of your own samples (due notably to the minimal dilution for the assay 1:2).
Samples with a concentration greater than 250 pg/mL should be re-assayed after dilution in Dilution Buffer.
Most plate readers are supplied with curve-fi tting software capable of graphing these data (logit/log or 4-parameter logistic fi t 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 (written on the Quality Control Sheet)

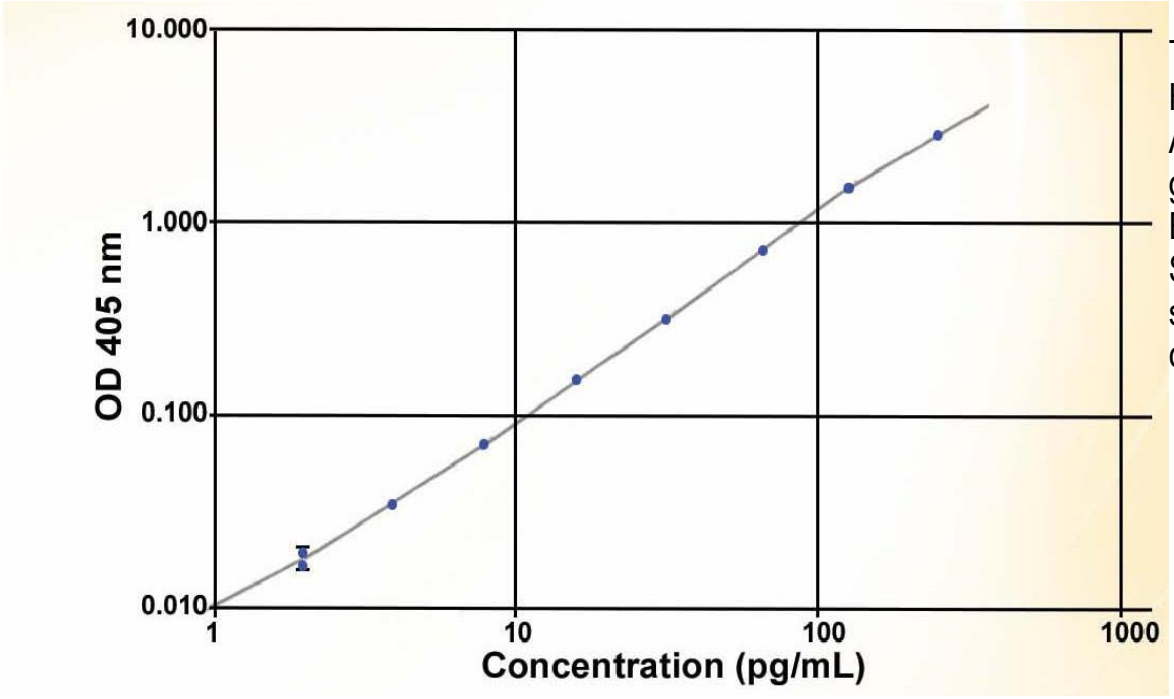
10. ACCEPTABLE RANGE

Non Specifi c Binding < 50 mA.U.
Limit of detection in the sample before dilution <2 pg/mL
QC sample: ±25% of the expected concentration (see on the Quality Control Sheet)

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 developping, fading at 414 nm. A 5-parameter logistic fitting ponderation 1/Y2 was used to determine the concentrations.

	Acylated Ghrelin (human) pg/mL	Absorbance (mAU)
Standard S1	250	2875
Standard S2	125	1518
Standard S3	62.5	741
Standard S4	31.3	324
Standard S5	15.6	163
Standard S6	7.8	81
Standard S7	3.9	43
Standard S8	2.0	28
Blank	0	10



Typical Human Acylated ghrelin Easy Sampling standard curve

12. ASSAY VALIDATION AND CHARACTERISTICS

Immunometric assay of Acylated Ghrelin (human) has been validated for its use in buffer and in plasma (without extraction but diluted at least 1:5). A sigmoidal logistic fitting ponderation 1/Y2 was used to determine the concentrations .
For additional information regarding the validation of imunoassay for protein biomarkers in biological samples, please refer to bibliography ***[12, 13]***.

The **limit of detection**, calculated as the concentration of Acylated Ghrelin corresponding to the NSB average (n = 8) plus three standard deviations is 2 pg/mL. Due to the minimal plasma dilution (1:2), the limit of detection in the samples is less than 4 pg/mL.

Intra-assay & inter-assay variations and recovery:

	Inter-assay			Intra-assay		
QC levels after 1:5 dilution (pg/mL)	**Mean of observed concentrations (pg/mL)**	**CV (%)**	**Recovery (%)**	**Mean of observed concentrations (pg/mL)**	**CV (%)**	**Recovery (%)**
200 (ULOQ)	217.0	10.0	8.6	228.0	2.6	13.8
150 (HQC)	163.0	7.0	8.5	167.0	2.8	11.6
50 (MQC)	45.9	13.5	-8.3	45.8	4.8	-8.3
10 (LQC)	9.6	20.1	-4.2	9.0	15.1	-10.1
5 (LLOQ)	5.1	27.6	-2.2	4.2	21.6	-16.6

ULOQ: Upper Limit of Quantification HQC: High QC MQC: Mid QC
LQC: Low QC LLOQ: Lower Limit of Quantification

The intra-assay and inter-assay variations were studied on a pool of human plasma containing AEBSF 0.4 mg/mL (free of Ghrelin) spiked samples for each level of QC. QC were prepared five times concentrated from a pool of human plasma and then diluted to 1:5 in Dilution Buffer before assay. For within-run precision and accuracy, the number of replicates (***n***) is equal to 6 for each levels of QC, the fi ve QC samples were analysed along with the calibration curve for a unique experiment. For between-run precision and accuracy, the number of replicates (n) is equal to 6 for each levels of QC, the fi ve QC samples were analysed along with the calibration curve for a total of 9 independent experiments.

Selectivity

Matrix	Mean of measured concentration (pg/mL)	CV (%)	Recovery (%)
1	6.49	3.18	29.7
2	6.05	3.53	20.9
3	6.14	4.95	22.7
4	6.35	2.18	26.9
5 (haemolysed)	3.96	10.70	-20.7
6	5.44	3.83	8.8
7	5.54	6.69	8.8
8	5.71	9.73	14.2
9	5.13	10.70	2.5
10 (haemolysed)	3.66	4.26	-26.7

Selectivity was tested by spiking 10 sources of samples matrix containing AEBSF at 0.4 mg/mL at the LLOQ (n=3).
These sources included 2 haemolysed samples (matrix 5 and 10).
QC samples (n=3) were prepared fi ve times concentrated in each matrix (free of Ghrelin) and then diluted to 1:5 in Dilution buffer in order to obtain a fi nal concentration of 5 pg/mL and analysed against a calibration curve.

Specificity

Specifi city was tested by adding AEBSF at 0.4 mg/mL (recommended use concentration = reference) and 2 mg/mL (high concentration) or aprotinin at 1.2 TIU/mL with or without HCl 0.1 N fi nal into sample matrix (a pool of human plasma samples) and measuring the accuracy of the Acylated Ghrelin (human) at both LLOQ and ULOQ (n=3).

Matrix	QC level after 1:5 dilution (pg/mL)	Mean of measured concentration (pg/mL)	CV (%)	Recovery (%)
AEBSF 0.4 mg/mL	5	5.25	9.23	4.91
	200	224	1.36	11.80
AEBSF 0.4 mg/mL + HCl 0.1N	5	5.17	7.62	3.32
	200	228	0.97	14.00
AEBSF 2 mg/mL	5	4.35	13.30	-13.00
	200	200	1.09	-0.13
AEBSF 2 mg/mL + HCl 0.1N	5	4.91	11.20	-1.72
	200	209	4.81	4.48
Aprotinin 1.2 TIU/mL	5	4.38	5.05	-12.50
	200	211	1.32	5.66
Aprotinin 1.2 TIU/mL + HCl 0.1N	5	4.88	3.99	-2.31
	200	215	3.54	7.31

Dilution tests

Dilution linearity was tested by spiking a pool of human plasma samples (free of Ghrelin) containing AEBSF at 0.4 mg/mL at 2000 pg/mL (n=3) and measuring precision and accuracy after serial dilution in Dilution buffer to bring the Acylated Ghrelin concentrations into the validated range for analysis (between ULOQ and LLOQ).

Dilution factor	Theoretical concentration (pg/mL)	Measured concentration (pg/mL)	Corrected concentration (pg/mL)	Recovery (%)	Mean recovery (%)	CV%
1:10	200	188.6	1886	-5.69	-1.33	5.27
		206.6	2066	3.32		
		196.8	1968	-1.62		
1:20	100	90.9	1 817	-9.14	-5.98	
		99.7	1 995	-0.25		
		91.5	1 829	-8.55		
1:40	50	46.3	1 852	-7.42	-8.64	
		46.7	1 868	-6.61		
		44.1	1 763	-11.90		
1:80	25	22.2	1 776	-11.20	-9.60	
		22.2	1 776	-11.20		
		23.4	1 872	-6.41		
1:160	12.5	11.1	1 768	-11.60	-4.32	
		12.7	2 028	1.39		
		12.2	1 945	-2.74		

Parallelism

Parallelism between the calibration standard curve and serial diluted samples was tested by diluting 3 samples containing AEBSF at 0.4 mg/mL in Dilution buffer (n=3) to bring the Acylated Ghrelin concentrations into the validated range for analysis (between ULOQ and LLOQ).

Sample	Dilution series	Dilution factor	Measured concentration (pg/mL)	Corrected concentrations (pg/mL)	CV (%)
		1:5	13.30	66.3	29.3
	1	1:10	6.60	66.0	
		1:20	5.32	106.0	
		1:5	13.00	65.0	12.7
1	2	1:10	6.01	60.1	
		1:20	2.52	50.4	
		1:5	10.80	54.0	10.0
	3	1:10	4.70	47.0	
		1:20	2.87	57.3	
		1:2	13.80	27.6	18.8
	1	1:5	4.06	20.3	
		1:10	2.02	20.2	
		1:2	13.50	27.0	11.8
2	2	1:5	4.32	21.6	
		1:10	2.29	22.9	
		1:2	15.20	30.4	16.7
	3	1:5	4.52	22.6	
		1:10	2.34	23.4	

Sample	Dilution series	Dilution factor	Measured concentration (pg/mL)	Corrected concentrations (pg/mL)	CV (%)
		1:2	14.80	29.7	15.5
	1	1:5	4.72	23.6	
		1:10	2.24	22.4	
		1:2	14.20	28.4	12.0
3	2	1:5	4.72	34.4	
		1:10	2.29	22.9	
		1:2	13.90	27.7	9.2
	3	1:5	4.82	24.1	
		1:10	2.34	23.4	

Stability test (freezing/thawing, 24h at +5°C and 24h at +20/+25°C)

Stability of Acylated Ghrelin was evaluated by using Low and High QC samples. These QC samples (n=3) were prepared from a pool of human plasma (free of Ghrelin) containing AEBSF at 0.4 mg/mL or Aprotinin at 1.2 TIU/mL and then frozen at -20°C for freeze/thaw stability or stored 24h at +5°C or at 20/25°C for short-term stability.

Conditions	QC level after 1:5 dilution (pg/mL)	Mean of measured concentration (pg/mL)	CV (pg/mL)	Recovery (%)
Freeze/thaw 1 cycle	10	9.5	3.48	-4.53
AEBSF 0.4 mg/mL	150	165	1.93	10.30
Freeze/thaw 3 cycles	10	11.0	10.90	10.10
AEBSF 0.4 mg/mL	150	157	8.31	4.89
Freeze/thaw 1 cycle	10	10.3	9.12	3.18
Aprotinin 1.2 TIU/mL	150	156	8.81	4.18
Freeze/thaw 3 cycles Aprotinin 1.2TIU/mL	10	9.5	12.90	-4.49
	150	150	3.79	-0.15
24h at +5°C	10	8.0	3.52	-20.20
AEBSF 0.4 mg/mL	150	138	3.91	8.27
24h at 20/25°C	10	3.3	7.82	-66.80
AEBSF 0.4 mg/mL	150	65.4	3.90	-56.40

Long term stability (3 and 6 monts at -20°C &-80°C)

Conditions	QC level after 1:10 dilution (pg/mL)	Mean of measured concentration (pg/mL)	CV (pg/mL)	Recovery (%)
3 months at -20°C	10	8.33	4.17	-16.70
	150	159	4.23	6.28
3 months at -80°C	10	9.64	5.66	-3.60
	150	171	2.19	14.20
6 months at -20°C	10	In progress		
	150	In progress		
6 months at -80°C	10	In progress		
	150	In progress		

Cross-reactivity

Acylated Ghrelin (rat)	100%
Acylated Ghrelin (dog)	85%
Unacylated Ghrelin (human)	<1%
Unacylated Ghrelin (rat)	<1 %
Unacylated Ghrelin (dog)	<1%
Ghrelin (1-14) (human)	<0.001 %
Ghrelin (1-11) (rat)	<0.001 %
Ghrelin (17-28) (human, rat)	<0.001 %
GHRF (human)	<0.001 %
Insulin (human)	<0.001 %
Motiline	<0.001 %
Leptin (human)	<0.001 %
Somatostatine	<0.001 %
CRF (human, rat)	<0.001 %
Glucagon (human, rat)	<0.001 %

Protease Inhibitor compatibility table

	AEBSF	PMSF	Pefabloc	P800	Aprotinin	PHMB
RA194062500R	YES	YES	YES	YES	YES	YES
RA194062400R	NO	YES	NO	NO	YES	YES

Plasma samples were collected on different protease inhibitors according to vendors instruction and measured with the appropriate kit. Recovery is different from one inhibitor to the other and it belongs to the end user to defi ne according to its Leeds which inhibitor to be used. Acidifi cation has also been tested with most inhibitors and may also change recovery, but will not affect the assay performances providing that 1:5 dilution with Dilution Buffer or neutralisation is performed.

13. ASSAY TROUBLE SHOOTING

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 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 wells while waiting for technical advice from the Bioreagent Department.

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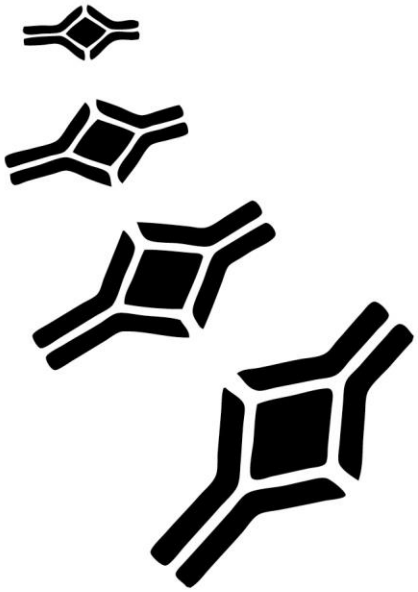

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