bertin bioreagent

ACYLATED GHRELIN (human)
EASY SAMPLING

For laboratory research only. Not for human or veterinary diagnostic use.

Bertin Bioreagent also markets pre-analytical products, ELISA kits, antibodies & biochemicals for:

- **Inflammation**
- **Oxidative injury**
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- **Pain**
- **Prion diseases**

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Acylated Ghrelin (human)
Easy Sampling ELISA kit
#A05306.96 wells

For research laboratory use only
Not for human diagnostic use

This assay was developed
& validated by Bertin Bioreagent

Fabriqué en France
Made in France

#A11306
Version: 0124

Table of contents

96 wells
Storage: -20°C
Expiry date: stated on the package

This kit contains:

Designation	Colour of cap	Item #	Quantity per kit	Form
Strip 96 well Microtiter plate, pre-coated with anti-Ghrelin mouse monoclonal antibody Easy Sampling	Blister with zip	A08306.1 ea	1	-
Acylated Ghrelin (human, rat) Tracer Easy Sampling	Green	A04306.100 dtn	1	Lyophilised
Acylated Ghrelin (human) Standard	Blue with red septum	A06106.1 ea	2	Lyophilised
Acylated Ghrelin (human) Quality Control	Green with red septum	A10106.1ea	2	Lyophilised
Acylated Ghrelin ELISA Buffer	Blue	A07106.1 ea	1	Lyophilised
Wash Buffer concentrated 400x	Silver	A17000.1 ea	1	Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Ellman's reagent 49+1	Black with red septum	A09000_49+1 .100 dtn	2	Lyophilised
Technical Booklet	-	A11306	1	-
Well 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.

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 where kit reagents are handled
- Avoid splashing

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

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

Temperature

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

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 is capable of providing a rapid catalytic turnover during the generation of the electrochemical discharges. The use of AChE as enzymatic label for EIA is patented by the French academic research Institute CEA ***[1, 2, 3]***, and Bertin Bioreagent has expertise to develop and produce EIA/ELISA kits using this technology.

AChE assays are revealed with Ellman's reagent, which contains acetylthiocholine as a substrate. The final product of the enzymatic reaction (5-thio-2-nitrobenzoic acid), is bright yellow in color and can be read at 405-414 nm using a spectrophotometer. AChE offers several advantages over other commonly used enzymes 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 phosphatase. AChE provides greater sensitivity than other labeling enzymes.
- **Low background**: Non-enzymatic hydrolysis of acetylthiocholine in buffer is essentially absent. Thus, AChE ensures a very low background and an

increased signal/noise ratio compared to other substrate of enzymes that are inherently unstable.

- **Wide dynamic range:** AChE is a stable enzyme and its activity remains constant for many hours. Unlike other enzymes, AChE has substrate that is not suicidal which permits simultaneous assays of high and low concentration samples.
- **Versatility:** AChE is a completely stable enzyme, unlike peroxidase which is suicidal. The accidentally dropped plate containing AChE substrate (Ellman's reagent) does not need to be discarded and experiment can be continued by adding washing buffer and fresh Ellman's reagent into the plate wells. As an option Otherwise, plate can be stored at +4°C containing washing buffer while waiting for technical advice from the Bioreagent Department.

Ghrelin

Ghrelin discovered in 1999, is fast becoming an endocrinology target of the millennium. Ghrelin, identified 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 essential 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]*** gastric motility and may be involved in osteoporosis, somatopause, infertility and ovulation induction, neurological disorders (Alcoholism, Post Traumatic Stress disorders...) ***[11]*** and cardiovascular diseases.

Principle of the assay

The Enzymatic Immunoassay (ELISA) is based on a sandwich technique. Wells of supplied plate are coated with a monoclonal antibody specific 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 then added to the wells.

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

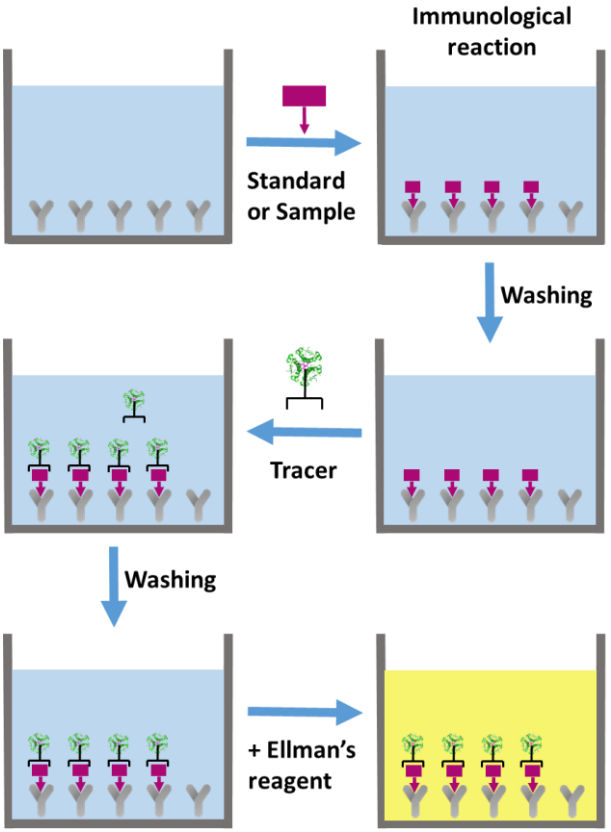
The sandwich is immobilised on the plate where excess reagents are washed away.

The concentration of Acylated Ghrelin (human) 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 414 nm.

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

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:



Assay characteristics

Validated for use:

- in buffer
- in plasma (without extraction but diluted at least at 1/2).
  A 5-parameter logistic fitting with ponderation $1/Y^2$ 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]***.

> ***Limit of detection (LOD):*** 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 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 concentra-tions (pg/mL)**	**CV (%)**	**Recovery (%)**	**Mean of observed concentra-tions (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 LLOQ: Lower Limit of Quantification
HQC : High QC MQC: Mid QC LQC : Low QC

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 ELISA Buffer before assay. For within-run precision and accuracy, the number of replicates (***n***) is equal to 6 for each level of QC, the five QC samples were analyzed 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 level of QC, the five QC samples were analyzed 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 sample 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 five times concentrated in each matrix (free of Ghrelin) and then diluted to 1/5 in ELISA buffer in order to obtain a final concentration of 5 pg/mL and analyzed against a calibration curve.

> *Specificity*

Specificity 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 final 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 ELISA 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 ELISA 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	1	1/5	13.30	66.3	29.3
		1/10	6.60	66.0	
		1/20	5.32	106.0	
	2	1/5	13.00	65.0	12.7
		1/10	6.01	60.1	
		1/20	2.52	50.4	
	3	1/5	10.80	54.0	10.0
		1/10	4.70	47.0	
		1/20	2.87	57.3	
2	1	1/2	13.80	27.6	18.8
		1/5	4.06	20.3	
		1/10	2.02	20.2	
	2	1/2	13.50	27.0	11.8
		1/5	4.32	21.6	
		1/10	2.29	22.9	
	3	1/2	15.20	30.4	16.7
		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 (%)
3	1	1/2	14.80	29.7	15.5
		1/5	4.72	23.6	
		1/10	2.24	22.4	
	2	1/2	14.20	28.4	12.0
		1/5	4.72	34.4	
		1/10	2.29	22.9	
	3	1/2	13.90	27.7	9.2
		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 AEBSF 0.4 mg/mL	10	9.5	3.48	-4.53
	150	165	1.93	10.30
Freeze/thaw 3 cycles AEBSF 0.4 mg/mL	10	11.0	10.90	10.10
	150	157	8.31	4.89
Freeze/thaw 1 cycle Aprotinin 1.2 TIU/mL	10	10.3	9.12	3.18
	150	156	8.81	4.18
Freeze/thaw 3 cycle Aprotinin 1.2 TIU/mL	10	9.5	12.90	-4.49
	150	150	3.79	-0.15
24h at +5°C AEBSF 0.4 mg/Ml	10	8.0	3.52	-20.20
	150	138	3.91	8.27
24h at 20/25°C AEBSF 0.4 mg/mL	10	3.3	7.82	-66.80
	150	65.4	3.90	-56.40

> ***Long term stability (3 months 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

> *Cross-reactivity*

Molecule/Species	Cross-reactivity
Acylated Ghrelin (rat)	100%
Acylated Ghrelin (dog)	85%
UnAcylated Ghrelin (human)	<1 %
UnAcylated Ghrelin (rat)	<1 %
Uncylated 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
A05306.96 wells	YES	YES	YES	YES	YES	YES
A05106.96 wells	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 define according to its needs which inhibitor to be used.

Acidification 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 ELISA Buffer or neutralisation is performed.

> *Related products*

Item Reference	Designation	Application
A05106.96 wells	Acylated Ghrelin (human) Express ELISA kit	PHMB, PMSF, Aprotinin samples
A05106.384 wells	Acylated Ghrelin (human) 384w ELISA kit	GOAT inhibitor screening
A05117.96 wells	Acylated Ghrelin (mouse, rat) Express ELISA kit	PHMB, PMSF, Aprotinin samples
A05118.96 wells	UnAcylated Ghrelin (mouse, rat) Express ELISA kit	PHMB, PMSF, Aprotinin samples
A05119.96 wells	UnAcylated Ghrelin (human) Express ELISA kit	PHMB, PMSF, Aprotinin samples
A05306.96 wells	Acylated Ghrelin (human) Easy Sampling ELISA kit	Any kind of sample
A05317.96 wells	Acylated Ghrelin (mouse, rat) Easy Sampling ELISA kit	
A05318.96 wells	UnAcylated Ghrelin (mouse, rat) Easy sampling ELISA kit	
A05319.96 wells	UnAcylated Ghrelin (human) Easy Sampling ELISA kit	
A05320.96 wells	UnAcylated Ghrelin (dog) Easy Sampling ELISA kit	
A05321.96 wells	Acylated Ghrelin (dog) Easy Sampling ELISA Kit	
A05401.96 wells	Acylated Ghrelin (pig) ELISA kit	PHMB, PMSF, Aprotinin samples
A05402.96 wells	UnAcylated Ghrelin (pig) ELISA kit	PHMB, PMSF, Aprotinin samples
D31009	Sampling Tubes with PHMB	Sample preparation

Materials and equipment required

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

For the sample preparation:

- EDTA tubes for blood collection
- Protease inhibitor (AEBSF, PMSF, Aprotinin, Pefabloc®, P800, PHMB ...)
- UltraPure water #A07001

For the assay:

- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 nm or 414 nm filter)
- Microplate washer (or wash bottles)
- Orbital microplate shaker able to perform at 600 rpm
- Multichannel pipette and disposable tips 30-300µL
- UltraPure water #A07001.1L
- Polypropylene tubes

Water used to prepare all ELISA reagents and buffers must be UltraPure (deionized & free from organic contaminant traces).
Do not use distilled water, HPLC-grade water or sterile water.

- UltraPure water may be purchased from Bertin Bioreagent (item #A07001.1L).

Sample collection and preparation

This assay has been validated to measure Acylated Ghrelin in buffer and in plasma samples (see validation data paragraph).

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 or at -80°C prior the use with the assay.

Sample 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 10µL of this solution per mL of blood. For example, for 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". For the use of these different products, please refer to the vendor's instructions.

- Collection tubes are mixed by inversion 5 times.

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 Easy Sampling EIA kit or for UnAcylated Ghrelin with UnAcylated Ghrelin Easy Sampling EIA kit.

Sample preparation

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

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 according to suggested plate layout.

An additional vial of Standard, Quality Control and Ellman's Reagent are provided in case you need to perform 2 assays with the kit.

All reagents must be brought to room temperature (around +20°C) prior the use in assay.

ELISA Buffer

Reconstitute the ELISA Buffer #A07106 with 50 mL of UltraPure water. Allow buffer to stand for 5 minutes or until it is completely dissolved. Mix buffer thoroughly by gentle inversions.
Stability at 4°C: 1 month.

Acylated Ghrelin (human) Standard

Reconstitute the Standard vial #A06106 with 1 mL of UltraPure water. Allow standard to stand for 5 minutes or

until it is completely dissolved. Mix standard thoroughly by gentle inversions.

The concentration of the first standard (S1) is 250 pg/mL. Prepare seven polypropylene tubes (for the seven other standards) and add 500 µL of ELISA Buffer into each tube. Then prepare the standards by serial dilutions as indicated in following table. Mix each tube thoroughly before the next transfer.

Standard	Volume of Standard	Volume of ELISA 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

Stability at +4°C: 1 week

Acylated Ghrelin (human) Quality Control

The Quality Control provided in this kit has been prepared by spiking Acylated Ghrelin (human) peptide in ELISA Buffer.

Reconstitute the Acylated Ghrelin (human) QC vial #A10106 with 1 mL of UltraPure water. Allow quality control to stand

for 5 minutes or until it is completely dissolved. Mix quality control thoroughly by gentle inversions.
Stability at 4°C: 1 week.

Acylated Ghrelin Tracer Easy Sampling

Reconstitute the Ghrelin Tracer vial #A04306 with 10 mL of ELISA Buffer. Allow tracer to stand for 5 minutes or until it is completely dissolved. Mix tracer thoroughly by gentle inversions.
Stability at +4°C: 1 weeks.

Wash Buffer

Dilute 2 mL of concentrated Wash Buffer #A17000 with 800 mL of UltraPure water. Add 400 µL of Tween 20 #A12000. Use a magnetic stirring bar to mix the content. Note that concentrated Wash Buffer is also used for Ellman's reagent preparation.
Stability at +4°C: 1 week.

Ellman's Reagent

5 minutes before use (development of the plate), reconstitute one vial of Elman's Reagent #A09000_49+1 with 49 mL of UltraPure water and 1 mL of **concentrated** Wash Buffer#A17000. The tube content should be thoroughly mixed.
Stability a +4°C and in the dark: 24 hours.

Assay procedure

It is recommended to measure the samples in duplicate following the instruction below.

Plate preparation

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

Open the plate pouch and select enough strips for your assay. Place unused strips back in the pouch.

Stability at +4°C: 1 month.

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

Just before distributing the reagents and samples, remove the buffer from the wells by inverting the plate and blot the last drops by tapping it on paper towels.

Plate set-up

A plate set-up is suggested hereafter.
The content 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	S7	S3	*	*	*	*	*	*	*	*	*
B	Bk	S7	S3	*	*	*	*	*	*	*	*	*
C	Bk	S6	S2	*	*	*	*	*	*	*	*	*
D	NSB	S6	S2	*	*	*	*	*	*	*	*	*
E	NSB	S5	S1	*	*	*	*	*	*	*	*	*
F	NSB	S5	S1	*	*	*	*	*	*	*	*	*
G	S8	S4	QC	*	*	*	*	*	*	*	*	*
H	S8	S4	QC	*	*	*	*	*	*	*	*	*

Bk : Blank
NSB : Non Specific Binding
* : Samples
S1-S8 : Standards 1-8
QC: Quality Controls

Pipetting the reagents

All samples and reagents must reach room temperature prior performing the assay.

Use new tips to pipet buffers, standards, samples, antibody and other reagents.

Before pipetting, equilibrate the pipette tips in each reagent. Do not touch the liquid already in the well when expelling with the pipette tip.

> *ELISA Buffer*

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

> *Acylated Ghrelin (human) 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.

> *Ghrelin Quality Control and Sample*

Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in ELISA Buffer.

▷ Incubating the plate

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

▷ Washing the plate

- Empty the plate by inverting it.
- Proceed with the following washing steps:
  - Wash each well 4 times with 300 µL of Wash Buffer;
  - Wash each well 1 time with 300 µL of Wash Buffer under slight agitation on an orbital shaker during 5 minutes;
  - Wash each well 5 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.

Pipetting the reagents

Acylated Ghrelin Tracer

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

Incubating the plate

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

Developing and reading the plate

- Reconstitute Ellman's reagent as mentioned in the Reagent preparation section.
- Empty the plate by inverting it. Proceed with the following washing steps:
  - Wash each well 4 times with 300 µL of Wash Buffer;
  - Wash each well 1 time with 300 µL of Wash Buffer under slight agitation on an orbital shaker during 5 minutes;
  - Wash each well 5 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 200µL of Ellman's reagent to each 96 well.
- Cover the plate with aluminum 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 405 nm or at 414 nm (yellow color) using spectrophotometer plate reader.

After addition of 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.

Assay procedure summary

Easy Sampling Enzyme Immunoassay Protocol (volumes are in µL)				
	Blank	NSB	Standard	Sample or QC
ELISA Buffer	-	100	-	-
Standard	-	-	100	-
Sample or QC	-	-	-	100
Cover plate, incubate 2 hours at 600rpm				
Wash plate x4, Wash & shake plate x1, Wash plate x5 Discard liquid from the wells				
Tracer	-	100	100	100
Cover plate, incubate 2 hours at 600rpm				
Wash plate x4, Wash & shake plate x1, Wash plate x5 Discard liquid from the wells				
Ellman's reagent	200			
Incubate with an orbital shaker in the dark at RT				
Read the plate at 405 nm or at 414 nm				

Data analysis

Make sure that your plate reader has subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate.

- Calculate the average absorbance for each NSB, standards and samples.
- For each standard, plot the absorbance (y axis) versus the concentration (x axis) graph. Draw a best-fit line through the points.
- To determine the concentration of 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 unknown samples (due notably to the minimal dilution for the assay 1/2).
- Samples with a concentration greater than 250 pg/mL must be re-assayed after dilution in ELISA Buffer.
- Most plate readers are supplied with curve-fitting software capable of graphing these data (logit/log or 4-parameter logistic fi t 4PL). It is highly recommended to use this software if available on the device. Refer to it for further information.

2 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 label of the QC vial)

Acceptable range

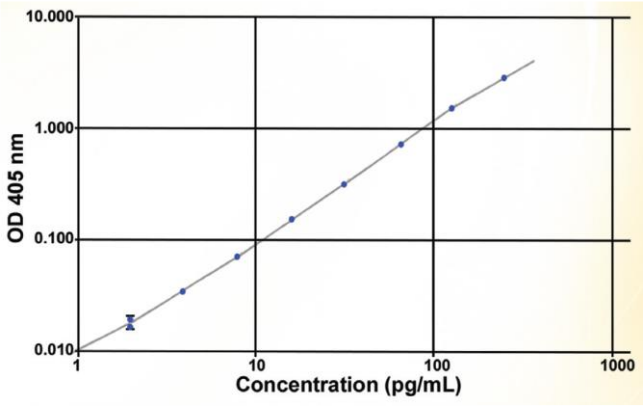
- Non-Specific Binding < 50 mA.U.
- Limit of detection in sample before dilution < 2 pg/mL.
- QC sample: ±25% of the expected concentration (see the label of the QC vial).

Typical results

The following data are for demonstration purpose only. Your data may be different and still correct. The data were obtained using all reagents as supplied in this kit under the following conditions: 60 minutes developing at room temperature, reading at 414 nm. A 5-parameter logistic fitting with a $1/Y^2$ was used to determine the concentrations.

Standard	Acylated Ghrelin (human) pg/mL	Absorbance (mA.U)
S1	250	2875
S2	125	1518
S3	62.5	741
S4	31.3	324
S5	15.6	163
S6	7.8	81
S7	3.9	43
S8	2.0	28
Blank	0.0	10

Typical Acylated Ghrelin (human) Easy Sampling standard curve



Troubleshooting

> *Absorbance values are too low:*

- organic contamination of water,
- one of the reagents was not properly 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 (Ellman's Reagent) or if it needs to be revealed again:***

- one only needs to wash the plate, add fresh Ellman's Reagent 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. If further information or explanation is needed, please contact Bertin Bioreagent Technical Support by phone on +33 (0)139 306 036, or by E-mail tech@bertin-bioreagent.com. Please have batch number of the kit (see outside the box) ready to provide to the technical support.

Bertin Bioreagent offers EIA Training kit #B05005. Feel free to contact our Technical Support. We are always happy to hearing from you.

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> Additional readings

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	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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Should you need help with an order, you can contact our customer service by emailing to order-life@bertin.fr

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