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# HUMAN ACYLATED GHRELIN
# ENZYME IMMUNOASSAY KIT

catalogue # A05106

96 wells

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U.S. patent # 50 47 330
European patent # 89 139 552

# THE ENZYME IMMUNOASSAY KIT FOR THE DETECTION OF HUMAN ACYLATED GHRELIN HAS BEEN DEVELOPED AND VALIDATED BY SPI-BIO.

*For research laboratory use only.*
*Not for human diagnostic use.*

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November 05

## HUMAN ACYLATED GHRELIN EIA KIT

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

This kit contains:

- ☞ A covered 96 wells plate, pre-coated with anti-Ghrelin mouse monoclonal antibody, ready to use
- ☞ One vial of anti-acylated Ghrelin tracer, lyophilised
- ☞ Two vials of Human acylated ghrelin standard, lyophilised
- ☞ Two vials of Quality Control, lyophilised
- ☞ One vial of EIA buffer, lyophilised
- ☞ One vial of concentrated Wash buffer, liquid
- ☞ One vial of tween 20, liquid
- ☞ Two vials of Ellman's reagent, lyophilised
- ☞ One instruction booklet
- ☞ One template sheet
- ☞ One well cover sheet

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 32 samples in duplicate.

## PRECAUTIONS FOR USE

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

Each time a new pipet tip is used, aspirate a sample or reagent and dispense it back into the same vessel. Repeat this operation two or three times before distribution.

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.

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.

## PRINCIPLE OF THE ASSAY

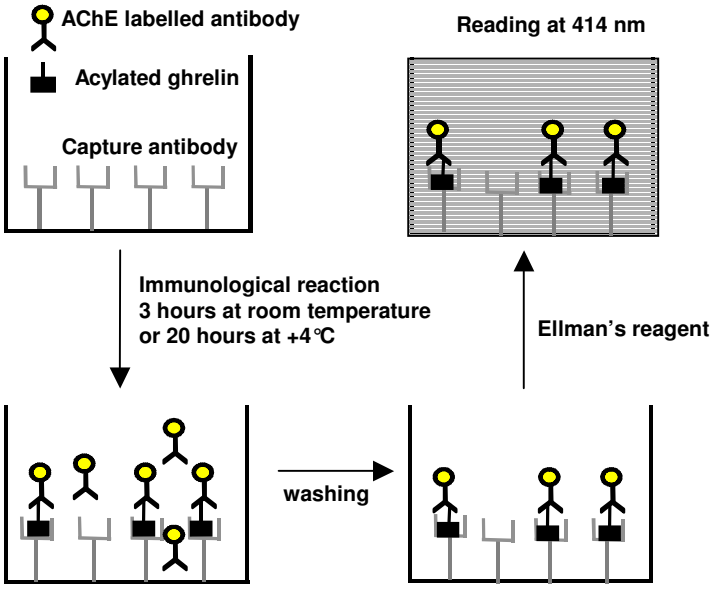
Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is synthesized principally in the stomach. It stimulates food intake and transduces signals to hypothalamic regulatory nucleid that controls energy homeostasis. The peptide consists of 28 amino acids, with a n-octanoylation of the serine-3 residue, which is necessary for the biological activity mentioned below. Ghrelin is present in the peripheral circulation under two forms: acylated and non-acylated. The human acylated ghrelin EIA kit specifically measures the acylated form of ghrelin.

This Enzyme Immunometric Assay (EIA) is based on a double-antibody sandwich technique. The wells of the plate supplied with the kit 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). The acetylcholinesterase (AChE) - Fab' conjugate which recognises the N-terminal part of acylated ghrelin is also added to the wells. This allows the two antibodies to form a sandwich by binding on different parts of the human acylated ghrelin.

The sandwich is immobilised on the plate so the excess reagents may be washed away. The concentration of the human acylated ghrelin is then determined by measuring the enzymatic activity of the immobilized AChE using the Ellman's Reagent. The AChE tracer acts on the Ellman's Reagent to form a yellow compound.

The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of the human acylated ghrelin present in the well during the immunological incubation.

The principle of the assay is summarised below:




## MATERIALS AND EQUIPMENT REQUIRED

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

***FOR SAMPLE PREPARATION***

- ☞ Ethylenediaminetetra-acetic acid (EDTA)
- ☞ Potassium Phosphate buffer 0.1 M pH 7.4
- ☞ NaOH 10N
- ☞ p-hydroxymercuribenzoic acid (PHMB)
- ☞ HCl 1N

***FOR THE ASSAY***

- ☞ Precision micropipettes (20 to 1000 µL)
- ☞ Spectrophotometer plate reader (405 or 414 nm filter)
- ☞ Microplate washer (or wash-bottles)
- ☞ Microplate shaker
- ☞ Distilled or deionized water
- ☞ Polypropylene tubes

## SAMPLE 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 p-hydroxymercuribenzoic acid (1 mM in the final sample volume) to prevent the degradation of acylated ghrelin by protease. Samples are centrifuged at 3,500 rpm for 10 min at +4°C and then, supernatants are transferred in separate tubes. Add immediately 100 µL of 1N HCl per mL of collected plasma and centrifuge them at 3,500 rpm for 5 min at +4°C. Then, supernatants are transferred in separate tubes. Samples should be quickly assayed or stored at -20°C for later use. For the preparation of PHMB, we suggest preparing a 100 times concentrate solution (100 mM) in potassium phosphate buffer containing 1.2% NaOH 10N (v/v) and then, adding 10 µL of this solution per mL of blood.


For assaying the unacylated ghrelin, please refer to the section “Blood collection” of the protocol of the Human Unacylated Ghrelin EIA Kit #A05119.

### *SAMPLE PREPARATION*

Plasma samples may be directly assayed (without any extraction procedure) after being diluted at least to 1:5 in EIA buffer in order to avoid matrix effect.

## REAGENT PREPARATION

The coated plates and reagents are provided ready to use.

☞ EIA buffer
Reconstitute one vial with 50 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at 4°C: 1 month.

☞ Human acylated ghrelin standard
Reconstitute the vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 250 pg/mL. Prepare seven propylene tubes and add 500 µL of EIA buffer into each tube. Add 500 µL of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes. Thus, standard concentrations are: 250 (S1), 125 (S2), 62.5 (S3), 31.3 (S4), 15.6 (S5), 7.81 (S6), 3.91 (S7) and 1.96 pg/mL (S8), respectively.
Stability at -20°C: 1 week.

☞ Quality Control
Reconstitute the vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at -20°C: 1 week.

☞ Anti-acylated Ghrelin-AChE tracer
Reconstitute one vial with 10 mL of EIA 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 1 mL of concentrated Wash buffer to 400 mL with distilled or deionized water. Add 200 µL of tween 20 (use a magnetic stirrer to mix the contents).
Stability at 4°C: 1 week.

☞ Ellman's Reagent
Five minutes before use, reconstitute with 49 mL of distilled or deionized water and 1 mL of concentrated wash buffer. The tube contents should be thoroughly mixed.
Stability at 4°C and in the dark: 1 day.

## 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 and place the unused strips back in the packet (stored at 4°C). 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 dry by inversion on absorbent paper.

### *DISTRIBUTION OF REAGENTS AND SAMPLES*

A plate set-up is suggested on this page. The contents of each well may be recorded on the sheet provided with the kit.

### *PIPETTING THE REAGENTS*

Note that the first column should be left empty for blanking Ellman's reagent. All samples and reagents must reach room temperature prior performing the assay. Use different tips to pipet the buffer, standard, quality control, samples, tracer, and other reagents.

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

B : Blank
NSB : Non Specific Binding
S1-S8: Standard 1-8
* : Samples or Quality Controls

- EIA buffer:
  Dispense 100 µL to Non Specific Binding (NSB)wells.
- Human acylated ghrelin standard:
  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 EIA buffer.
- Anti-acylated Ghrelin-AChE tracer:
  Dispense 100 µL to each well, except Blank (B) wells

***INCUBATING THE PLATE***

Cover the plate with adhesive film and incubate for:

- 3 hours at room temperature,

or

- 20 hours at +4°C.

The long immunological reaction allows the increase of assay sensitivity: 0.3 pg/mL versus 0.8 pg/mL for short immunological reaction.

***DEVELOPING AND READING THE PLATE***

Reconstitute Ellman's Reagent as indicated in reagent preparation section. Wash each well five times with the wash buffer (300 µL/well), slightly shake the plate for 5 minutes (with the orbital shaker) and then rewash 5 times with the wash buffer (300 µL/well). Remove the liquid from the wells by inverting the plate. Dry by inversion on absorbent paper.

Dispense 200 µL of Ellman's Reagent to the 96 wells. Incubate the plate in darkness at room temperature. Optimal development is obtained using an orbital shaker. The plate should be read between 405 and 414 nm: 30 minutes for long immunological reaction (20 hours at +4°C) and 30 to 60 minutes for short immunological reaction (3 hours at room temperature) after adding the Ellman's reagent.

| Enzyme Immunoassay Protocol (Volumes are in µL) | | | | | |
|---|---|---|---|---|---|
| | Blank | Non Specific Binding | Standard | Sample |
| Buffer | - | 100 | - | - |
| Standard | - | - | 100 | - |
| Sample | - | - | - | 100 |
| Tracer | - | 100 | 100 | 100 |
| Cover plate, incubate at room temperature during 3 hours or at +4°C during 20 hours | | | | |
| Wash the strips 5 times, slightly shake them 5 min, rewash them 5 times and remove the liquid from the wells | | | | |
| Ellman's reagent | 200 | 200 | 200 | 200 |
| Incubate with an orbital shaker in the dark at room temperature | | | | |
| Read the plate between 405 and 414 nm | | | | |

### *DATA ANALYSIS*

Make sure that your plate reader has substracted the absorbance readings of the blank well (absorbance of Ellman's reagent) 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.
- Plot the absorbance for each standard (Y axis) versus the concentration (X axis ) using a 4-parameter logistic fitting or cubic spline fitting.
- 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. Do not forget to integrate the dilution factor of your samples (due notably to the minimal dilution for the assay 1:5 and the addition of HCl 1N).
- Samples with a concentration greater than 250 pg/mL should be re-assayed after dilution in EIA buffer.
- Most plate readers are supplied with curve-fitting software capable of graphing this type of data. If you have this type of software, we recommend using it. Refer to it for further information.

## TYPICAL DATA

### *EXAMPLE DATA*

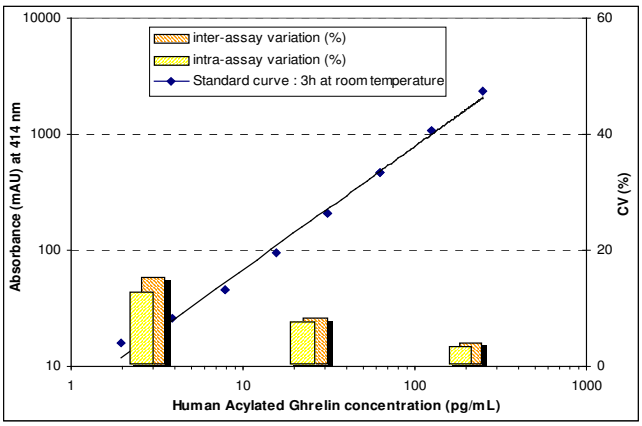
The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 30 minutes developing at room temperature for long immunological reaction (20h at +4°C) and 60 minutes developing for rapid immunological reaction (3h at RT), reading at 414 nm. A 4-parameter logistic fitting was used to determine the concentrations.

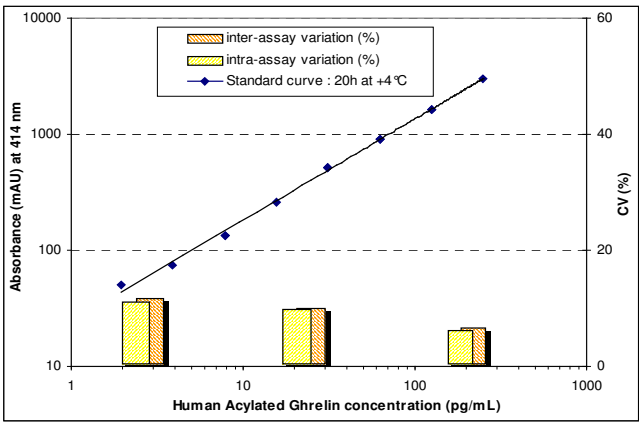
| | Absorbances (mAU) | |
|---|---|---|
| Ghrelin standard (pg/mL) | Short immunological reaction (3h RT) | Long immunological reaction (20h +4°C) |
| 250 | 2372 | 2996 |
| 125 | 1071 | 1637 |
| 62.5 | 464 | 913 |
| 31.3 | 211 | 522 |
| 15.6 | 96 | 260 |
| 7.81 | 46 | 134 |
| 3.91 | 26 | 74 |
| 1.95 | 16 | 50 |

### *ACCEPTABLE RANGE*

- Non Specific Binding < 50 mAU
- Limit of detection in the sample before dilution: 1.5 pg/mL for long immunological reaction and 4 pg/mL for short immunological reaction.
- Limit of quantification in the sample before dilution: 10 pg/mL.

## HUMAN ACYLATED GHRELIN STANDARD CURVE




## ASSAY VALIDATION AND CHARACTERISTICS

The Enzyme Immunometric Assay of human acylated ghrelin has been validated for its use in buffer and in plasma (without extraction but diluted at least 1:5). A sigmoidal 4-parameter logistic fitting was used to determine the concentrations.

☞ The limit of detection, calculated as the concentration of acylated ghrelin corresponding to the NSB average (n = 8) plus three standard deviations is 0.3 pg/mL and 0.8 pg/mL respectively for long and short immunological reaction. Due to the minimal plasma dilution (1:5), the limit of detection in the samples are 1.5 pg/mL (20h at +4°C) and 4 pg/mL (3h at RT), respectively.

☞ Intra-assay & inter-assay variations and recovery:

| QC levels | Theoretical concentrations in diluted QC (pg/mL) | Mean of observed concentrations (pg/mL) | Intra-assay (CV%) | Inter-assay (CV%) | Recovery (%) | Confidence intervalle ($\alpha$ = 0.05) |
|---|---|---|---|---|---|---|
| Incubation 20 hours at +4°C | | | | | | |
| QC1 | 2 | 1.83 | 10.3 | 10.9 | 91.4 | 91.4 ± 4.6 |
| QC2 | 25 | 25.8 | 8.10 | 8.30 | 103 | 103 ± 3.5 |
| QC3 | 200 | 219 | 5.50 | 5.90 | 110 | 110 ± 2.9 |
| Incubation 3 hours at room temperature | | | | | | |
| QC1 | 2 | 2.29 | 11.80 | 14.40 | 115 | 115 ± 9.5 |
| QC2 | 25 | 27.0 | 6.20 | 6.70 | 108 | 108 ± 3.4 |
| QC3 | 200 | 217 | 2.9 | 3.4 | 109 | 109 ± 2.1 |

The intra-assay and inter-assay variations were studied on 30 human plasma (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 EIA buffer before assay. Replicate samples (n=6) at each of the three validation levels were analysed along with the calibration curve for a total of 5 independent runs.

☞ Matrix variability:

| Matrix | Theoretical concentration (pg/mL) | Acylated ghrelin measured (pg/mL) | Recovery (%) | Mean of recovery (%) |
|---|---|---|---|---|
| 1 | 25 | 25.9 | 104 | 106 |
| 2 | | 25.2 | 101 | |
| 3 | | 26.7 | 107 | |
| 4 | | 27.2 | 109 | |
| 5 | | 27.1 | 108 | |

Five individual lots of human plasma samples were tested. Validation samples (n=3) were prepared five times, concentrated in each matrix (free of ghrelin) and then diluted to 1:5 in order to obtain a final concentration of 25 pg/mL. QC were analysed against a calibration curve derived from a pool of human plasmas.

☞ Dilution test:

| Samples | Dilution Factor | Acylated Ghrelin measured (pg/mL) | Corrected concentrations (pg/mL) | Recovery (%) | Mean of recovery (%) |
|---|---|---|---|---|---|
| 1 | 1:5 | 27.4 | 137 | - | 86.0 |
| | 1:10 | 13.4 | 134 | 97.8 | |
| | 1:20 | 6.46 | 129 | 94.2 | |
| | 1:25 | 4.30 | 108 | 78.8 | |
| | 1:50 | 2.00 | 100 | 73.0 | |
| 2 | 1:5 | 17.3 | 86.5 | - | 86.9 |
| | 1:10 | 10.4 | 104 | 120 | |
| | 1:20 | 3.65 | 73.0 | 84.4 | |
| | 1:25 | 2.51 | 62.8 | 72.6 | |
| | 1:50 | 1.22 | 61.0 | 70.5 | |
| 3 | 1:5 | 24.2 | 121 | - | 94.6 |
| | 1:10 | 12.6 | 126 | 104 | |
| | 1:20 | 5.57 | 111 | 91.7 | |
| | 1:25 | 4.28 | 107 | 88.4 | |
| | 1:50 | 2.27 | 114 | 94.2 | |

Three human plasma samples were diluted to 1:5. Afterwards, four independent dilutions (n=3) were performed and analysed against a calibration curve.

☞ Stability test (freezing/thawing):

| Samples | Reference value (pg/mL) | 1 cycle (pg/mL) | 2 cycles (pg/mL) | 3 cycles (pg/mL) | Mean of recovery (%) |
|---|---|---|---|---|---|
| 1 | 186 | 127 | 162 | 163 | 81.0 |
| 2 | 66.2 | 71.3 | 67.0 | 73.0 | 106 |
| 3 | 70.8 | 53.8 | 59.0 | 67.0 | 84.7 |
| 4 | 120 | 82.7 | 113 | 95.0 | 80.8 |
| 5 | 176 | 141 | 158 | 149 | 84.8 |

Four human plasma samples (n=3) were analysed just after collection and dilution to 1:5 before the assay (reference value) and after 1, 2 and 3 freeze/thaw cycles.

☞ Cross-reactivity:

| | |
|---|---|
| - Ghrelin (Rat): | 118 % |
| - Ghrelin (Des-Octanoyl-Ser$^3$) (Human): | <0.001 % |
| - Ghrelin (Des-Octanoyl-Ser$^3$) (Rat): | <0.001 % |
| - 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 % |

## ASSAY TROUBLE SHOOTING

☞ Absorbance values too low: incubation in wrong conditions (time or temperature) or reading time too short or Human acylated ghrelin standard, or anti-acylated Ghrelin tracer or Ellman's reagent have not been dispensed.

☞ NSB value too high: contamination of NSB wells with Human acylated ghrelin standard, or inefficient washing.

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

These are a few examples of trouble shooting that may occur. If you need further explanation, SPI-BIO will be happy to answer any questions or information about this assay. Please feel free to contact our technical support staff by letter, phone (33 (0)1 39 30 62 60), fax (33 (0)1 39 30 62 99) or E-mail (sales@spibio.com), and be sure to indicate the batch number of the kit (see outside the box).

SPI-BIO proposes a training workshop in EIA practice & theory. This workshop is given twice a year. For further information, please contact our Customer Relation Representative (33 (0)1 39 30 62 60).

## BIBLIOGRAPHY

☞ **Grassi J. & Pradelles Ph.**
Compounds labelled by the acetylcholinesterase of *Electrophorus Electricus*. Its preparation process and it use as a tracer or marquer in enzymo-immunological determinations.
*United States patent, N°1,047,330. September 10, 1991*