Ghrelin RIA

KIPMR90

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Human-Ghrelin-RIA

Radioimmunoassay with Coated Tubes for the Quantitative Detection of human Ghrelin

KIPMR90

IN VITRO DIAGNOSTIC USE

CE

en

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TECHNICAL FEATURES+APPLICATIONS

- analytical sensitivity 0.04 ng/ml
- Intra- and Inter Assay Variance < 10%
- Recovery of recombinant Ghrelin 97%
- Control Serum included
- For measurement in human serum

INTENDED USE

This radioimmunoassay kit is suited for measuring human Ghrelin in serum and EDTA-Plasma.

INTRODUCTION

Ghrelin is a 3.5 kDa protein of 28 amino acids and is serine octanylated. Bioactivity of this small peptide hormone depends on octanylation (1). It is mainly synthesized by stomach but also in duodenal and heart cells (2) and therefore indicates the relevance of the stomach as endocrine organ (3).

Ghrelin is able to cross the blood-brain barrier and is a natural ligand of growth hormone secretagogue receptor in pituitary and hypothalamus (4). Ghrelin exert influence on several neurological processes, for instance memory retention can be modulated by ghrelin (5) and Ghrelin secretion is influenced by sleep (6, 7). Not only growth hormone but several other hormones are influenced by Ghrelin e.g. ACTH, cortisol, prolactin (8, 9). It is also present in pancreatic islets and regulates insulin secretion (10-12). In women with polycystic ovary syndrome Ghrelin levels are decreased and highly correlated to insulin sensitivity (24), so there are several regulatory circles influenced by Ghrelin. Additionally to endocrine action Ghrelin exerts influence on immunological processes. In Human umbilical vein endothelial cells Ghrelin inhibits basal and TNF-alpha-induced cytokine release and mononuclear cell binding and in vivo endotoxin-induced proinflammatory cytokine production in rats was also inhibited by intravenous administrated Ghrelin (13).

Sites of Ghrelin synthesis as well as receptor location indicate a role for the hormone and the gut in appetite regulation (1) (23). So many Ghrelin receptors are present in the hypothalamic arcuate nucleus, a brain area important in food intake control. Several investigations demonstrate a circadian rhythm of Ghrelin secretion (14), controlled by ingestion. Shortly before food intake Ghrelin plasma concentration increases and decreases after finishing. In eating disorders Ghrelin levels reflect illness, so obesity suppresses Ghrelin concentration in blood (14) and in anorexia nervosa an increase of Ghrelin serum concentration can be detected (15-17). Ghrelin might act as counterpart to Leptin in the regulation of food intake and fat utilization, so in patients with primary biliary cirrhosis parallel to increasing Leptin levels Ghrelin serum concentration decreases (16). It also influences the adipogenesis negatively (18). A significant decrease of Ghrelin concentration is detected in elderly people (19). This could explain the anorexia of elderly people and offers a new target in anti-aging research.

Further investigation of Ghrelin serum concentration in chronic liver disease resulted in a correlation of Ghrelin concentration with several biochemical and clinical parameters: e.g. encephalopathy, anaemia, hypoglycaemia, renal dysfunction (20).

In Patients with the Prader-Willi-Syndrome show a nearly 5fold increase of serum Ghrelin, so Ghrelin may be involved in the pathogenesis of hyperphagia in PWS (21, 22).

So Ghrelin seems to be involved in the regulation of many physiological processes and its influence on many of these processes has not been investigated in detail. With this test system we provide an easy but reliable tool for the quantification of human Ghrelin in serum.

PRINCIPLE

For the Radioimmunoassay for the determination of human Ghrelin a polyclonal rabbit-antibody of high specificity is used. Ghrelin is measured quantitatively.

Calibrators are prepared from recombinant Ghrelin, ^{125}I-Tracer from a C-terminated peptide (AS 15 – 28) with presynthetised Tyrosine.

Calibration of the Assay

The assay was calibrated against the internal test of Medical School Hannover, Prof. Brabant.

PERFORMANCE CHARACTERISTICS

Sensitivity
The analytical **sensitivity** of the assay yields **40 pg/ml (about 10 pmol/L)** measured as 2x SD of zero calibrators.

Specificity
"This assay is specific for human Ghrelin. The antibody shows in addition cross-reactivity with: rabbit, cat, chicken, guinea pig, hamster, goat, sheep, rat, horse, donkey, pig, dog, rabbit, mouse and bovine Ghrelin. No cross-reactivity was found with other proteins such as insulin or GH."

Intra-Assay-Variation

	Number of determinations	Mean value [ng/ml]	VC%
Sample 1	6	1028.13	2.6
Sample 2	6	1275.95	4.0
Sample 3	6	1325	5.3

Inter-Assay-Variation

	Mean value (ng/ml)	Standard deviation (ng/ml)	VC% (%)
Sample 1	762	55.3	7.3
Sample 2	1085	52.5	4.8
Sample 3	790	65	8.2

Recovery
Serum spiking experiments with recombinant human Ghrelin yielded a recovery of 97% (± 2%).

SPECIMEN COLLECTION, PREPARATION, AND STORAGE

Serum/ EDTA-Plasma samples are suitable (inappropriate are Heparin- and Citrat-Plasma). An external sample preparation prior to assay is not required.

For testing of single blood samples, the specimens may be taken in the morning or early afternoon. For specific questions, the influence of food intake should be taken in consideration.

Samples should be handled as recommended in general: as fast as possible and chilled as soon as possible. In case there will be a longer period between the sample withdrawal and determination store the undiluted samples frozen at –20°C or below in tightly closable plastic tubes. Avoid on principal repeated freeze-thaw cycles of serum/plasma (if required, please subaliquote) although Ghrelin levels were found to be unaffected by few cycles (5x) in our experiments.

Because of the wide effective range of this RIA kit a preparative sample dilution is generally not necessary. For most of the determinations (serum or plasma samples, and no extreme values expected) **the use of undiluted samples 100 µl per tube**, should be appropriate**.** In case of extremely high Ghrelin levels the sample should and can be diluted in Assay Buffer, e.g. 1:10 at a concentration of 1000pg/ml the precision of this assay is maximal.

REAGENTS PROVIDED

Materials for 100 tubes

The reagents listed below are sufficient for 100 tubes including the calibrator curve.

ASS BUF	Assay Buffer (1 bottle, 30 ml, ready for use), for reconstitution of 1st antibody, tracer, NSB, CAL and control 1.
ANTISERUM	1st Antibody: rabbit-anti-ghrelin, reconstitute in 10.5 ml assay buffer. (1 bottle, 10.5 ml, lyophilized)
Ag 125I	Tracer: ^{125}I-Ghrelin; < 2,3 µCi or < 85 kBq, reconstitute in 10.5 ml assay buffer. (1 bottle, 10.5 ml, lyophilized, red coloured)
NSB	Rabbit IgG for non-specific binding (NSB) (1 vial, 1ml, lyophilized)
CAL N	Calibrators 1-6 (6 vials, 0.75 ml each, lyophilized), contain human recombinant Ghrelin. The calibration curve covers a range of 0.2-6.4 ng/ml Ghrelin. Please use 100 µl calibrator solution per tube. See exact values on the vial label
CONTROL 1	Control 1 (human serum). (1 vial, 750 µl each, lyophilized), reconstitute in 750 µl assay buffer. See exact values on the vial label

2nd Ab	2nd Antibody (anti-rabbit IgG), reconstitute in 1 ml assay buffer. Transfer dissolved material to reagent PEG immediately before use (ratio 1:56) to obtain the precipitating solution. Please mix only the required quantity of precipitating solution (500µl/vial). The rest can be frozen. The assay is unaffected by the possible occurrence of turbidity after adding 2nd Ab to reagent PEG. (1 vial, 1 ml, lyophilized)
PEG	Precipitation Reagent (1 vial, 55 ml, ready for use after adding reagent 2nd Ab)

MATERIALS REQUIRED BUT NOT PROVIDED

Precision pipettes (100 and 200µl) Micropipettes and multichannel pipettes with disposable plastic tips
Disposable polystyrene or polypropylene tubes. Conical tubes are highly recommended because of the small volume of the immunoprecipitates
Vortex-mixer
Centrifuge
Device to aspirate the fluid from the tubes (recommended because of the potential danger of radioactivity and infection by human samples)
Ice-Cold deionized water
Gamma Counter

REAGENT PREPARATION

In conducting the assay, follow strictly the test protocol. Room temperature incubation means: Incubation at 20 - 25°C.

Reagents with different lot numbers should not be mixed. All reagents are stable unopened until the expiry date, if stored in the dark at 2° - 8°C (see label).

Control 1, antiserum, 2nd antibody, tracer, NSB and CAL have to be reconstituted in **Assay Buffer.** It is recommended to keep the reconstituted reagents at room temperature for 30 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer. Transfer reconstituted 2nd antibody into the precipitation reagent. The assay is unaffected by the possible occurrence of turbidity after add in 2nd antibody to precipitation reagent.

The shelf life of the components after opening is not affected, if used appropriately. Reconstituted Components should be stored at -20°C (or below). Repeated freeze-thaw cycles have to be avoided.

Before use, all kit components should be brought to room temperature, if nothing different is indicated. **Precipitates, possible in buffers, should be dissolved before use through mixing and warming**.

WARNINGS AND PRECAUTIONS

For in-vitro diagnostic use only. For professional use only.
Possession and use of the kit is subject to the regulations of the national nuclear regulatory authorities.

Reagents with different lot numbers should not be mixed.

Reagents contain as preservative Kathon CG (containing 0.001615% of the mixture 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one). It can cause sensitisation by skin contact. (R 43 and S 26-36/37/39-45).

Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.

Before use, all kit components should be brought **to room temperature at 20 - 25°C,** if not indicated differently. Precipitates in buffers should be dissolved before use by thorough mixing and warming. **Temperature WILL affect** the assay. However, values for the patient samples will not be affected.

Caution: This kit contains material of human and/or animal origin. Source human serum for the Control Serum provided in this kit was tested by FDA recommended methods and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV) antibody. No known test methods can offer total assurance of the absence of infectious agents; therefore all components and patient's specimens should be treated as potentially infectious.

Radioactivity - Before ordering or using radioactive materials, it is necessary to take the appropriate actions to ensure compliance with national regulations governing their use. Local rules in each establishment, which define actions and behaviour in the radioactivity working areas, should also be adhered to. The advice given here does not replace any local rules, instructions or training in the establishment, or advice from the radiation protection advisers. It is important to follow the code of good laboratory practice in addition to the specific precautions relating to the radionuclide I-125 used.

Iodine-125 has a radioactive half-life T1/2 of 60 days and emits 35.5 keV gamma radiation, 27 – 32 keV x-rays and no beta radiation. Shielding is effective done by lead, first half value layer is 0.02 mm lead, reduction to 10 % is made by 0.2 mm.

To reduce the radiation dose time spent handling radioactivity should be minimized (plan ahead), and distance from source of radiation should be maximized (doubling the distance from the source quarters the radiation dose).

Formation of aerosols, e.g. by improper opening and mixing of vials or pipetting of solutions which may cause minute droplets of radioactivity become airborne, is a hazard and should be avoided.

Solutions containing iodine should not be made acidic, because this might lead to the formation of volatile elemental iodine.

As some iodo-compounds can penetrate rubber gloves, it is advisable to wear two pairs, or polyethylene gloves over rubber.

For cleaning of contaminated areas or equipment, the Iodine-125 should be rendered chemically stable by using alkaline sodium thiosulphate solution together with paper or cellulose tissue.

General first aid procedures:
Skin contact: Wash affected area thoroughly with water. Discard contaminated cloths and shoes.
Eye contact: In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes. In order to assure an effectual rinsing spread the eyelids.
Ingestion: If swallowed, wash out mouth thoroughly with water. Immediately see a physician.
Do not eat, drink or smoke in these areas.
Never pipette the materials with the mouth.
Spilled material must be wiped off immediately and should become disinfected. Clean contaminated areas and equipment with a suitable detergent.

The handling of radioactive and potentially infectious material must comply with the following guidelines:
The material should be stored and used in a special designated area.
Do not eat, drink or smoke in these areas.
Never pipette the materials with the mouth.
Avoid direct contact with these materials by wearing laboratory coats and disposable gloves.
Spilled material must be wiped off immediately. Clean contaminated areas and equipment with a suitable detergent.
Unused radioactive material and radioactive waste should be disposed according to the recommendations of the national regulatory authorities.

ASSAY PROCEDURE
NOTES: All determinations (Calibrators, Control 1 and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.
When performing the assay, the Calibrators, Control 1 and the samples should be pipette as fast as possible.

Flow Chart of Assay Protocol

Tube Nr. :	Contents	Assay Buffer µl	Calibrators, Control 1, Samples µl	NSB µl	Antiserum µl	Tracer µl	Precipitating solution µl
1,2	Total Counts	-	-	-	-	100	-
3,4	NSB	100	-	100	-	100	500
5,6	B_0 (zero calibrator)	100	-	-	100	100	500
7-18	Calibrators 1-6	-	100	-	100	100	500
19,20	Control	-	100	-	100	100	500
21,22	Sample 1	-	100	-	100	100	500
23,24	Sample 2	-	100	-	100	100	500
etc.							

1) Labelling of the assay tubes should be done in the following order (duplicates):
   1, 2 total counts (**TC**),
   3, 4 non specific binding (**NSB**)
   5, 6 zero calibrator (**B_0**),
   7-18 **Calibrators** 1 to 6
   19, 20 Control **C1**
   21,22 etc. **samples**.
2) Add **100 µl** of Assay Buffer to tubes 3, 4 and 5,6.
3) Add **100 µl** of **Calibrators 1-6** to tubes 7-18:
   7, 8 Calibrator 1 (200 pg/ml)
   9, 10 Calibrator 2 (400 pg /ml); etc. up to 18.
4) Add **100 µl** Control **C1** to tubes 19, 20.
5) Add **100 µl** of **sample** to tubes 21, 22, etc.
6) Add **100 µl NSB** to tubes 3 and 4.
7) Add **100 µl** of Antiserum (1st. Antibody), beginning with tube 5.
8) Mix tubes with a Vortex-Mixer and incubate **overnight** at 2-8°C (**at least 20 h, maximal 24 h**).
9) Add **100 µl** of Tracer to all tubes.
   Seal tubes 1 and 2 (total counts) with a stopper and set aside until step 16 (steps 10 to 15 are not performed on the tubes 1 and 2).
10) Mix the remaining tubes with a Vortex-Mixer and **incubate overnight (at least 16 h, maximal 20 h) at 2-8°C**.
11) Add **500 µl** of cooled (2-8°C) precipitating solution beginning with tube 3.
12) Mix tubes with a Vortex-Mixer and incubate for precipitation for **1 h at 2-8°C**.
13) Add **1** ml **ice-cold water**.
14) Centrifuge at 2-4°C at **3000 x g for 20 min**.
15) Aspirate the supernatant. In order not to destroy or aspirate the small precipitate a rest of approx. 2 mm supernatant should be left over the precipitate. (Tip: Add limit stop to the aspirate needle). Depending on laboratory equipments and common laboratory practice supernatant can also be decanted carefully.
16) Count the radioactivity of all tubes in Gamma-Counter.

Extended washing procedure for increased precision:

After the Incubation of 1 hour (step 12) centrifuge the tubes (see step 14) and aspirate the supernatant (see step 15). Add directly 1 ml ice-cold water. This should not be done too vigorously in order to keep the precipitate intact. **Do not mix again!** Centrifuge the tubes once again at 3000g for 5 min, aspirate the supernatant and count the radioactivity of all tubes in the gamma-counter.
This extended procedure results in a somewhat higher precision bound up with higher work expenditure. The higher precision may be relevant only in special cases.

CALCULATION OF RESULTS

Establishing the Calibration Curve

Calibrator	B_0	1	2	3	4	5	6
ng/ml	0	0,2	0,4	0,8	1,6	3,2	6,4
pg/ml	0	200	400	800	1600	3200	6400
pmol/l	0	59	119	237	475	949	1899

1. Calculate the average counts of each pair of tubes.
2. Subtract the average of NSB (NSB, Tubes 3 and 4) from the mean counts of the Calibrators, controls and samples. This corresponds to the corrected B values.
3. The corrected value of the zero calibrator (tubes 5 and 6) equals B_0.
4. Calculate the percent bound (%B/B_0): $\%B/B_0 = B/B_0 \times 100$ %
5. Plot %B/B_0 versus the calibrator concentrations on a semi-logarithmic or logit-log paperrespectively or per computer analysis.
6. For quality control calculate the percentage of %NSB/TC:
   NSB / TC (average counts of tubes 3 and 4 / average counts of tubes 1 and 2) x 100 %. It should be: %NSB/TC < 5%
7. Quality control, Calculate the %B_0/TC:
   B_0 (s*ee step 3.) / TC (total counts) x 100 %.*
   It should be: %B_0/TC > 20%

Evaluation of sample concentrations:

Read the Ghrelin concentration value (abscissa) corresponding to the %B/B_0-Value of the sample as in the example given below:

Example:

Average counts of the tubes 3 and 4 (NSB): 482 cpm

Average counts of the tubes 5 and 6 (Zero calibrator, B_0): 8927 cpm

Average counts of the tubes 19 and 20 (Control CS): 4794 cpm

$$\%B/B_0 = \frac{Sample - NSB}{B_0 - NSB} \times 100\% = \frac{4794 - 482}{8927 - 482} \times 100\% = 51.1\%$$

For a 51.1% value on the y-axis (ordinate) corresponds in this example abscissa value of 1055 pg/ml.
Multiplication of this value determined graphically or by aid of a computer program with the dilution factor gives the ghrelin concentration of the sample.
If it is preferred to express the results as pmol/l, the values given as pg/ml have to be divided by 3.371 to obtain pmol/l.

Example: 1055 pg/ml : 3.371 = 313 pmol/l.

Concentration of the control:
The control should fit with the labelled concentration range. The measured Ghrelin concentration are only valid, if the measured value of the control is in the labelled concentration range. Otherwise process analysis is required and depending on the results the measured values are accepted or not.

EXPECTED NORMAL VALUES

Concentration of Ghrelin in human sera varied from minimal 300 pg/ml (90 pmol/L) to maximal 4000 pg/ml (1200 pmol/L) so far. Whereas in most of the samples the concentration ranged between 600 pg/ml (180 pmol/L) and 1400 pg/ml (420 pmol/L).

52 sera or plasma from each healthy male and female blood donors, age 20 to 65 years, were measured regarding their Ghrelin concentration with DIAsource Ghrelin Ria KIPMR90. However, there is no information available regarding the nutritional status of the blood donors. The results in pg/ml are presented in the table 3.
Therefore valid reference values should be generated by a well designed study and the values presented here can only be taken as initial estimation.

Table 3: Ghrelin serum concentration of male and female blood donors.

	Male	Female
n	52	52
Mean	**801.3 pg/ml**	**1141.9 pg/ml**
Standard Deviation	179.1	570.9
Min	511.0 pg/ml	454.4 pg/ml
Max	1,555.2 pg/ml	2,565.7 pg/ml
Median	770.5 pg/ml	1,032.3 pg/ml

LITERATURE

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SUMMARY – Ghrelin R90

Reconstitution of the **Reagents**		
Antiserum	in Assay Buffer	**10.5 ml**
Tracer	in Assay Buffer	**10.5 ml**
NSB	in Assay Buffer	**1 ml**
Calibrators (1-6)	in Assay Buffer	**750 µl**
Control 1	In Assay Buffer	**750 µl**
2nd Antibody	in Assay Buffer Mix solution with **PEG.** Mix only the required quantity of precipitating solution.	**1 ML** **(1 ml 2nd Ab+ 55 ml PEG)** or **Ratio (1:56)**
Serum or plasma **samples** can be used **undiluted.**		

		Double Determinations	**Addition of Reagents [µl]**		
Tube Nr. :	**Contents**	**Assay Buffer µl**	**Calibrators, Control, Samples µl**	**NSB µl**	**Antiserum µl**
1,2	Total Counts	-	-	-	-
3,4	NSB	100	-	100	-
5,6	B_0 (zero calibrator)	100	-	-	100
7-18	Calibrators (1-6)	-	100	-	100
19,20	Control 1	-	100	-	100
21,22	Sample 1	-	100	-	100
23,24	Sample 2	-	100	-	100
etc.					

Mix all tubes with a Vortex-mixer.
Incubation overnight (at least 20 h, maximal 24 h) **at 2-8°C**
Add **100 µl** Tracer to all tubes.
Seal the tubes 1 and 2 (total counts) with a stopper and do not **remove until the step 16 is reached.**
Mix the remaining tubes with a Vortex-Mixer.
Incubation overnight (at least 16 h, maximal 20 h) **at 2-8°C**
Add **500 µl** precipitating solution (2-8°C), beginning with the tube 3.
Mix tubes with a Vortex mixer.
Incubation 1 h at **2-8°C**
Add **1 ml ice-cold water**, beginning with the tube 3.
Centrifugation at **2-4°C, 3000 x g, 20 min**
Aspirate the supernatant (a small amount of approx. 2 mm of supernatant should be left on the intact precipitate).
Count the **radioactivity** of all the tubes in a Gamma-Counter.

Revision Date: 2010-11-17

	Used symbols
	Consult instructions for use
	Storage temperature
	Use by
LOT	Batch code
REF	Catalogue number
CONTROL	Control
I V D	In vitro diagnostic medical device
	Manufacturer
	Contains sufficient for <n> tests
WASH SOLN CONC	Wash solution concentrated
CAL 0	Zero calibrator
CAL N	Calibrator #
CONTROL N	Control #
Ag 125I	Tracer
Ab 125I	Tracer
Ag 125I CONC	Tracer concentrated
Ab 125I CONC	Tracer concentrated
	Tubes
INC BUF	Incubation buffer
ACETONITRILE	Acetonitrile
SERUM	Serum
DIL SPE	Specimen diluent
DIL BUF	Dilution buffer
ANTISERUM	Antiserum
IMMUNOADSORBENT	Immunoadsorbent
DIL CAL	Calibrator diluent
REC SOLN	Reconstitution solution
PEG	Polyethylene glycol
EXTR SOLN	Extraction solution
ELU SOLN	Elution solution
GEL	Bond Elut Silica cartridges
PRE SOLN	Pre-treatment solution
NEUTR SOLN	Neutralization solution
TRACEUR BUF	Tracer buffer
	Microtiterplate
Ab HRP	HRP Conjugate
Ag HRP	HRP Conjugate
Ab HRP CONC	HRP Conjugate concentrate
Ag HRP CONC	HRP Conjugate concentrate
CONJ BUF	Conjugate buffer
CHROM TMB CONC	Chromogenic TMB concentrate
CHROM TMB	Chromogenic TMB solution
SUB BUF	Substrate buffer
STOP SOLN	Stop solution
INC SER	Incubation serum
BUF	Buffer
Ab AP	AP Conjugate
SUB PNPP	Substrate PNPP
BIOT CONJ CONC	Biotin conjugate concentrate
AVID HRP CONC	Avidine HRP concentrate
ASS BUF	Assay buffer
Ab BIOT	Biotin conjugate
Ab	Specific Antibody
SAV HRP CONC	Streptavidin HRP concentrate
NSB	Non-specific binding
2nd Ab	2nd Antibody
ACID BUF	Acidification Buffer