# Rat/Mouse Ghrelin (Active)

# 96-Well Plate

# Cat. # EZRGRA-90K

**RAT/MOUSE GHRELIN (ACTIVE) ELISA KIT**
**96-Well Plate (Cat. # EZRGRA-90K)**




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# RAT/MOUSE GHRELIN (ACTIVE) ELISA KIT

## I. INTENDED USE

This kit is used for the non-radioactive quantification of rat/mouse ghrelin (active) in serum and plasma. There is no cross reactivity to des-octanoyl-ghrelin. Circulating ghrelin is a multifunctional hormone produced primarily by the stomach. It consists of 28 amino acids and the n-octanoylation of serine3 position in the molecule is necessary for its bioactivity. Originally found as an endogenous ligand for the growth hormone secretagogue receptor in the pituitary gland, it distinguishes itself from the hypothalamic growth hormone-releasing hormone as another potent stimulator for growth hormone secretion. It is also an important orexigenic hormone in the regulation of energy homeostasis. One kit is sufficient to measure 38 unknown samples in duplicate.

***This kit is for Research Use Only. Not for Use in Diagnostic Procedures.***

## II. PRINCIPLES OF ASSAY

This assay is a Sandwich ELISA based on: 1) capture of ghrelin molecules (active form) in the sample by anti-ghrelin IgG and immobilization of the resulting complex to the wells of a microtiter plate coated by a pre-titered amount of anchor antibodies, 2) and the simultaneous binding of a second biotinylated antibody to ghrelin, 3) wash away of unbound materials, followed by conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetra-methylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured rat/mouse ghrelin (active form) in the unknown sample, the concentration of active ghrelin can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat/mouse ghrelin.

## III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

### 1. Microtiter Plate

Coated with pre-titered anchor antibodies.
Quantity: 1 Strip Plate
Preparation: Ready to use.
Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8 °C.

### 2. Adhesive Plate Sealer

Quantity: 2 sheets
Preparation: Ready to use.

### 3. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.
Quantity: 2 bottles containing 50mL each
Preparation: Dilute 1:10 with distilled or de-ionized water.

### 4. Rat/Mouse Ghrelin (Active) Standard

Rat/mouse Ghrelin (active) reference standard, lyophilized
Quantity: 1 bottle, 2mL/bottle upon hydration
Preparation: Hydrate thoroughly in distilled or de-ionized water immediately before use. Please refer to the analysis sheet for exact concentration. After hydration dilute with Assay Buffer according to § VIII. A.

### 5. Quality Controls 1 and 2

One vial each, lyophilized, containing rat/mouse ghrelin (active) at two different levels.
Quantity: 0.5 mL/vial upon hydration.
Preparation: Reconstitute each vial with 0.5 mL de-ionized water immediately before use. Aliquot unused portion in smaller quantity and freeze at -20$^{o}$C for later use. Avoid further freeze and thaw.

### 6. Matrix Solution

Processed serum matrix containing 0.08% Sodium Azide
Quantity: 1 mL/vial
Preparation: Ready to use.

## III. REAGENTS SUPPLIED (continued)

### 7. Assay Buffer

0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.05 % Triton X-100, 0.08% sodium azide, and 0.1% BSA.
Quantity: 15 mL/vial
Preparation: Ready to use.

### 8. Rat/Mouse Ghrelin (Active) Capture Antibody

Pre-titered capture antibody solution in buffer
Quantity: 3 mL/vial
Preparation: Mix 1:1 with Rat/Mouse Ghrelin (Active) Detection Antibody before use according to § VIII. C.

### 9. Rat/Mouse Ghrelin (Active) Detection Antibody

Pre-titered detection antibody solution in buffer
Quantity: 3 mL/vial
Preparation: Mix 1:1 with Rat/Mouse Ghrelin (Active) Capture Antibody before use according to § VIII. C.

### 10. Enzyme Solution

Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.
Quantity: 12 mL/vial
Preparation: Ready to use

### 11. Substrate

3, 3',5,5'-tetramethylbenzidine in buffer.
Quantity: 12 mL/vial
Preparation: Ready to use. Minimize the exposure to light.

### 12. Stop Solution

0.3 M HCl
Quantity: 12 mL/vial
Preparation: Ready to use.
[Caution: Corrosive Solution]

## IV. STORAGE AND STABILITY

Recommended storage for kit components is 2-8°C.

All components are shipped and stored at 2-8°C. Reconstituted standards and controls can be frozen for future use but repeated freeze thaws should be avoided. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

## V. REAGENT PRECAUTIONS

**1.** Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

**2. Hydrochloric Acid**

Hydrochloric Acid is corrosive, can cause eye and skin burns. Harmful if swallowed. Causes respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

**Note: See Full Labels of Hazardous components on next page.**

**Full labels of hazardous components in this kit:**

| Ingredient, Cat # | | Full Label | |
|---|---|---|---|
| Rat/Mouse Ghrelin (Active) Capture Antibody | E1090-C |  | **Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| Rat/Mouse Ghrelin (Active) Detection Antibody | E1090-D |  | **Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| Rat/Mouse Ghrelin (Active) Quality Controls 1 & 2 | E6090-K |  | **Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention. |
| Rat/Mouse Ghrelin (Active) Standard2 | E8090-K |  | **Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention. |
| Assay Buffer | EABGR |  | **Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| Stop Solution | ET-TMB |  | **Warning.** May be corrosive to metals. |
| 10X HRP Wash Buffer Concentrate | EWB-HRP |  | **Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water. |

## VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and pipette tips: 10µL ~ 20 µL or 20µL ~ 100 µL
2. Multi-channel Pipettes and pipette tips: 5 ~ 50 µL and 50 ~ 300 µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. De-ionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm and 590nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth
9. Pefabloc or AEBSF [4-(2-Aminoethyl)-benzenesulfonyl fluoreide], 100 mg/mL aqueous stock solution (store at -20ºC, minimize multiple freeze/thaw cycles) is recommended for Sample Collection and Storage.
10. 5N HCl, recommended for Sample Collection and Storage.

## VII. SAMPLE COLLECTION AND STORAGE

**The active ghrelin molecule is extremely unstable in serum/plasma and should be rigorously protected during blood sample collection. Ideally all samples should be processed as quickly as possible and kept on ice to retard the breakdown of active ghrelin. For maximum protection, we recommend addition of Pefabloc or AEBSF and acidification of all samples. Acidification will result in noticeable protein precipitation but does not affect the assay. However, if the presence of precipitates interferes with the sample pipetting accuracy, the sample should be centrifuged and the supernatant used for assay.**

1. To prepare serum, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Immediately add enough Pefabloc or AEBSF to a final concentration of 1 mg/mL. Let blood clot at room temperature for 30 min.
2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2ºC.
3. Transfer serum samples in separate tubes and acidify with HCl to a final concentration of 0.05N. Aliquot acidified serum in small quantities. Date and identify each sample.
4. Use freshly prepared serum or store samples at –20 ± 5º C for later use. Avoid multiple (> 5) freeze/thaw cycles.
5. To prepare plasma sample, whole blood should be collected into a centrifuge tube containing enough $K_3$ EDTA to achieve a final concentration of 1.735 mg/mL and treated with Pefabloc or AEBSF as described for serum, followed by immediate centrifugation. Acidify plasma samples with HCl to a final concentration of 0.05N. Observe same precautions in the preparation of serum samples.
6. If heparin is to be used as anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.
7. Avoid using samples with gross hemolysis or lipemia.

## VIII. REAGENT PREPARATION

### A. Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Rat/Mouse Ghrelin (Active) Standard with 2 mL of deionized water. Please refer to the analysis sheet for exact concentration. Invert and mix gently until completely in solution.

2. Label six tubes 1, 2, 3, 4, 5, and 6. Add Assay Buffer to each of the six tubes according to the volumes outlined in the chart below. Dilute the reconstituted standard stock according to the chart below. Vortex each tube briefly to ensure complete mixing.

   Note: Change tip for every dilution. Wet tip with standard before dispensing. Unused portions of reconstituted standard should be stored in small aliquots at ≤ -20°C. Avoid multiple freeze/thaw cycles.

| Volume of Deionized Water to Add | Volume of Standard to Add | Standard Concentration pg/mL |
|---|---|---|
| 2 mL | 0 | X (refer to analysis sheet For exact concentration) |

| Tube # | Volume of Assay Buffer to Add | Volume of Standard to Add | Standard Concentration (pg/mL) |
|---|---|---|---|
| 1 | 500 µL | 500 µL of reconstituted Standard | X/2 |
| 2 | 500 µL | 500 µL of Tube 1 | X/4 |
| 3 | 500 µL | 500 µL of Tube 2 | X/8 |
| 4 | 500 µL | 500 µL of Tube 3 | X/16 |
| 5 | 500 µL | 500 µL of Tube 4 | X/32 |
| 6 | 500 µL | 500 µL of Tube 5 | X/64 |

### B. Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Rat/Mouse Ghrelin (Active) Quality Control 1 and Quality Control 2 with 0.5 mL distilled or deionized water and gently invert to ensure complete hydration. Unused portions of the reconstituted Quality Controls should be stored in small aliquots at ≤ -20°C. Avoid further freeze/thaw cycles.

### C. Preparation of Capture and Detection Antibody Mixture

Prior to use, combine the entire contents of Rat/Mouse Ghrelin (Active) Capture Antibody (3 mL) and Rat/Mouse Ghrelin (Active) Detection Antibody (3 mL), or at a 1:1 ratio, and invert to mix thoroughly.

## IX. RAT/MOUSE GHRELIN (ACTIVE) ELISA ASSAY PROCEDURE

**Pre-warm all reagents to room temperature immediately before setting up the assay.**

1. Dilute the 10X concentrated HRP wash buffer 10 fold by mixing the entire contents of both buffer bottles with 900 mL de-ionized or glass distilled water.

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and fill each well with 300 µL diluted Wash Buffer. Decant wash buffer and remove the residual amount by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate using this procedure 2 additional times. **Do not let wells dry before proceeding to the next step.** If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.

3. Add 20 µL Matrix Solution to Blank, Standards and Quality Control wells (refer to § X. for suggested well orientations).

4. Add 30 µL Assay Buffer to each of the Blank and sample wells.

5. Add 10 µL Assay Buffer to each of the Standard and Quality Control wells.

6. Add in duplicate 20 µL Ghrelin Standards in the order of ascending concentrations to the appropriate wells.

7. Add in duplicate 20 µL QC1 and 20 µl QC2 to the appropriate wells.

8. Add sequentially 20 µL of the unknown samples in duplicate to the remaining wells**.**

9. Transfer the Antibody Solution Mixture (1:1 mixture of capture and detection antibody) to a buffer or reagent reservoir and add 50 µL to each well with a multi-channel pipette.

10. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

## IX. RAT/MOUSE GHRELIN (ACTIVE) ELISA ASSAY PROCEDURE (continued)

11. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.

12. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

13. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 min on the micro-titer plate shaker.

14. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid.

15. Wash wells 6 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

16. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake in the plate shaker for approximately 5 - 20 minutes. Blue color should be formed in wells of Ghrelin standards with intensity proportional to increasing concentrations of Ghrelin.

    **Note:** Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

17. Remove sealer and add 100 µL Stop Solution **[CAUTION: CORROSIVE SOLUTION]** and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read absorbance at 450 nm and 590nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well.

## Assay Procedure for Rat/Mouse Ghrelin (Active) ELISA Kit (Cat. # EZRGRA-90K)

| | Step 1 | Step 2 | Step 3 | Step 4-5 | Step 6-8 | Step 9 | Step 10-12 | Step 13 | Step 14-15 | Step 16 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Well # | Dilute both bottles of 10X HRP Wash Buffer with 900 mL de-ionized water. | Wash plate 3X with 300 µL diluted HRP wash buffer. Remove residual buffer by tapping smartly on absorbent towels | Matrix Solution | Assay Buffer | Standards/QCs/ Samples | Capture/ Detection Ab. Mixture | Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer. | Enzyme Solution | Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 6X with 300 µL Wash Buffer. | Substrate | Seal, Agitate, Incubate 15 minutes at Room Temperature. | Stop Solution | Read Absorbance at 450 nm and 590 nm. |
| A1, B1 | | | 20 µL | 30 µL | -- | 50 µL | | 100 µL | | 100 µL | | 100 µL | |
| C1, D1 | | | 20 µL | 10 µL | 20 µl of Tube 6 Std | | | | | | | | |
| E1, F1 | | | 20 µL | 10 µL | 20 µl of Tube 5 Std | | | | | | | | |
| G1, H1 | | | 20 µL | 10 µL | 20 µl of Tube 4 Std | | | | | | | | |
| A2, B2 | | | 20 µL | 10 µL | 20 µl of Tube 3 Std | | | | | | | | |
| C2, D2 | | | 20 µL | 10 µL | 20 µl of Tube 2 Std | | | | | | | | |
| E2, F2 | | | 20 µL | 10 µL | 20 µl of Tube 1 Std | | | | | | | | |
| G2, H2 | | | 20 µL | 10 µL | 20 µl reconstituted Standard | | | | | | | | |
| A3, B3 | | | 20 µL | 10 µL | 20 µL of QC 1 | | | | | | | | |
| C3, D3 | | | 20 µL | 10 µL | 20 µL of QC 2 | | | | | | | | |
| E3, F3 | | | -- | 30 µL | 20 µL of Sample 1 | | | | | | | | |
| G3, H3 Etc. | | | -- | 30 µL | 20 µL of sample 2 | | | | | | | | |

## X. MICROTITER PLATE ARRANGEMENT

Rat/Mouse Ghrelin (Active) ELISA

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blank | Tube 3 Std | QC1 | Etc. | | | | | | | | |
| B | Blank | Tube 3 Std | QC1 | Etc. | | | | | | | | |
| C | Tube 6 Std | Tube 2 Std | QC2 | | | | | | | | | |
| D | Tube 6 Std | Tube 2 Std | QC2 | | | | | | | | | |
| E | Tube 5 Std | Tube 1 Std | Sample 1 | | | | | | | | | |
| F | Tube 5 Std | Tube 1 Std | Sample 1 | | | | | | | | | |
| G | Tube 4 Std | Reconstituted Standard | Sample 2 | | | | | | | | | |
| H | Tube 4 Std | Reconstituted Standard | Sample 2 | | | | | | | | | |

## XI. CALCULATIONS

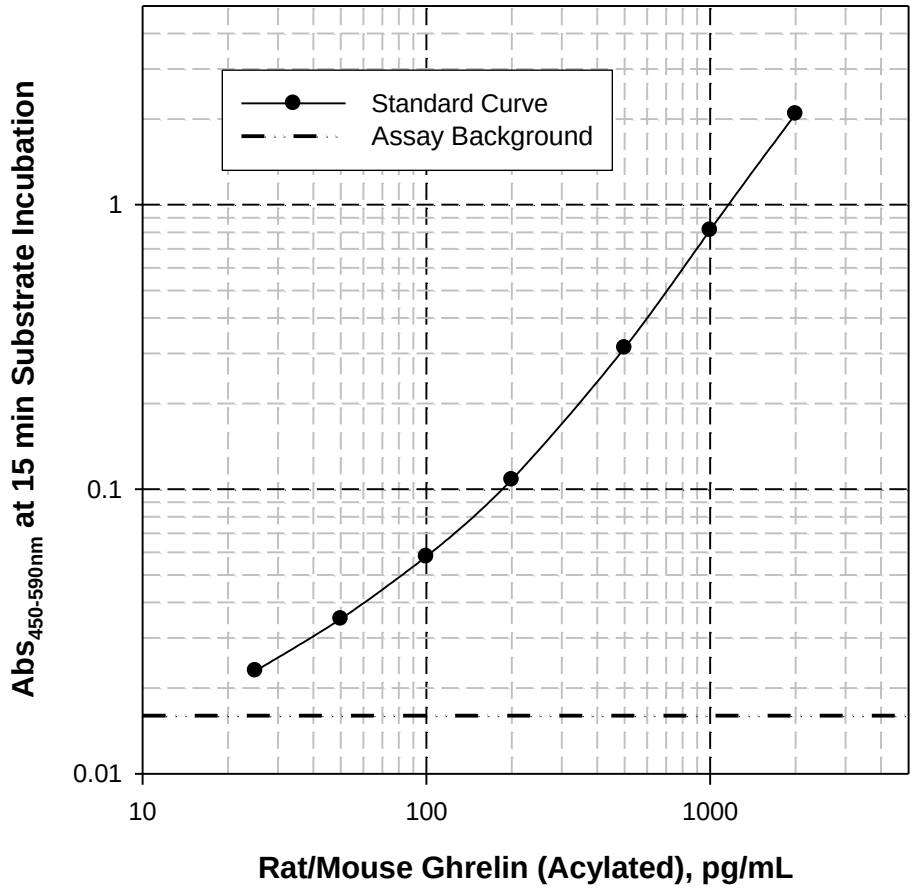
Graph a reference curve by plotting the absorbance unit of 450nm, less unit at 590nm, on the Y-axis against the concentrations of Ghrelin standard on the X-axis. The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

**Note:** When sample volumes assayed differ from 20 μL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 μL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 μL, compensate the volume deficit with matrix solution.

## XII. INTERPRETATION

1. The assay will be considered accepted when all Quality Control values fall within the calculated QC range. If any QCs fall outside of the control range, review results with a supervisor.
2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.
3. The theoretical minimal detecting concentration of this assay is 8 pg/mL Active Ghrelin (20 μl sample size).
4. The appropriate range of this assay is 25 pg/mL to 2,000 pg/mL Active Ghrelin (20 μl sample size). Any result greater than 2,000 pg/mL in a 20 μL sample should be diluted using matrix solution and the assay repeated until the results fall within range.

## XIII. GRAPH OF TYPICAL REFERENCE CURVE




**For Demonstration Only - Do not use for calculations**

## XIV. ASSAY CHARACTERISTICS

### A. Analytical Sensitivity

The lowest level of Active Ghrelin that can be detected by this assay is 8 pg/mL when using a 20 µL sample size.

## XIV. ASSAY CHARACTERISTICS (continued)

### B. Specificity

| | |
|---|---|
| Rat/Mouse Ghrelin (Active) | 100% |
| Des-Octanoyl Rat/Mouse Ghrelin | 0% |
| Canine Ghrelin (Active) | 111% |
| Porcine Ghrelin (Active) | 98% |
| Human Ghrelin (Active) | 53% |
| Des-Octanoyl Human Ghrelin | 0% |
| Motilin Related Peptide (Human, Rat/Mouse) | 0% |
| PYY 3~36 (Human, Mouse, Porcine) | 0% |
| NPY (Human/Rat) | 0% |
| Pancreatic Polypeptide (Human, Rat) | 0% |
| Human GIP (1~42) | 0% |

### C. Precision

Intra and Inter-Assay Variations

| Sample | Active Ghrelin (pg/mL) Mean, n = 6 | Intra-assay CV (%) | Inter-assay CV (%) |
|---|---|---|---|
| Rat serum 1 | 61 | 4.90 | 9.81 |
| Rat serum 2 | 206 | 1.08 | 2.10 |
| Rat serum 3 | 1,046 | 1.00 | 1.40 |
| Mouse serum 1 | 54 | 7.09 | 9.26 |
| Mouse serum 2 | 195 | 1.60 | 3.41 |
| Mouse serum 3 | 1,036 | 0.27 | 1.56 |
| Rat Plasma 1 | 90 | 0.57 | 4.90 |
| Rat Plasma 2 | 231 | 1.27 | 3.27 |
| Rat Plasma 3 | 982 | 0.65 | 1.15 |
| Mouse Plasma 1 | 58 | 5.56 | 4.70 |
| Mouse Plasma 2 | 210 | 2.70 | 4.08 |
| Mouse Plasma 3 | 1,035 | 0.86 | 1.23 |

Serum or plasma samples from rats and mice are pooled and treated with AEBSF and HCl, then divided into 3 aliquots each. Various amounts of rat/mouse ghrelin are added to the aliquots to create low, intermediate and high levels of ghrelin samples for precision tests. Intra-assay variations were calculated from results of six duplicate determinations in one assay. Inter-assay variations were calculated from results of six separate assays with duplicate samples in each assay.

## XIV. ASSAY CHARACTERISTICS (continued)

### D. Spike Recovery Rate of Active Rat/Mouse Ghrelin in Assay Samples

| Sample I.D. | Active Ghrelin Spiked, pg/mL | Serum Ghrelin | | Plasma Ghrelin | |
|---|---|---|---|---|---|
| | | pg/mL | Recovery Rate | pg/mL | Recovery Rate |
| Rat | **0 (Basal)** | 0 | -- | 53 | -- |
| | **50** | 60 | 96% | 106 | 106% |
| | **200** | 200 | 94% | 252 | 100% |
| | **1,000** | 885 | 102% | 1,019 | 97% |
| Rat | **0 (Basal)** | 0 | -- | 103 | -- |
| | **50** | 36 | 72% | 154 | 102% |
| | **200** | 172 | 86% | 285 | 91% |
| | **1,000** | 813 | 81% | 973 | 87% |
| Rat | **0 (Basal)** | 168 | -- | 181 | -- |
| | **50** | 218 | 100% | 233 | 104% |
| | **200** | 356 | 94% | 374 | 97% |
| | **1,000** | 1,070 | 90% | 1,100 | 92% |
| Mouse | **0 (Basal)** | 92 | -- | 53 | -- |
| | **50** | 129 | 74% | 109 | 112% |
| | **200** | 269 | 89% | 263 | 105% |
| | **1,000** | 965 | 87% | 1,073 | 102% |
| Mouse | **0 (Basal)** | 67 | -- | 116 | **--** |
| | **50** | 125 | 116% | 174 | 116% |
| | **200** | 296 | 115% | 317 | 101% |
| | **1,000** | 1,156 | 109% | 1,079 | 96% |
| Mouse | **0 (Basal)** | 136 | -- | 213 | -- |
| | **50** | 193 | 114% | 259 | 92% |
| | **200** | 359 | 112% | 396 | 92% |
| | **1,000** | 1,166 | 103% | 1,112 | 90% |
| **Mean ± S.D. (n = 6)** | **50** | | **95.3 ± 19%** | | **105.3 ± 8.4%** |
| | **200** | | **98.3 ± 12%** | | **97.7 ± 5.4%** |
| | **1,000** | | **95.3 ± 11%** | | **94.0 ± 5.4%** |

Varying amounts of active rat/mouse ghrelin were added to individual serum and plasma samples from rat and mouse and the ghrelin content of each sample was assayed by Rat/mouse Ghrelin (Active) ELISA. The recovery rate = [(Observed ghrelin concentration after spike – Basal ghrelin level) / spiked ghrelin concentration] x 100%.

## XIV. ASSAY CHARACTERISTICS (continued)

### E. Linearity of Sample Dilution

| Sample I.D. | Volume Assayed | Serum Ghrelin | | Plasma Ghrelin | |
|---|---|---|---|---|---|
| | | pg/mL | % of Expected | pg/mL | % of Expected |
| Rat | **20 µL** | 212 | **100%** | 221 | **100%** |
| | **15 µL** | 153 | 96% | 159 | 96% |
| | **10 µL** | 100 | 94% | 110 | 100% |
| | **5 µL** | 54 | 102% | 57 | 103% |
| Rat | **20 µL** | 640 | **100%** | 607 | **100%** |
| | **15 µL** | 460 | 96% | 448 | 98% |
| | **10 µL** | 304 | 95% | 300 | 99% |
| | **5 µL** | 159 | 99% | 153 | 101% |
| Rat | **20 µL** | 1,288 | **100%** | 1,220 | **100%** |
| | **15 µL** | 920 | 95% | 912 | 100% |
| | **10 µL** | 606 | 94% | 611 | 100% |
| | **5 µL** | 302 | 94% | 309 | 101% |
| Mouse | **20 µL** | 192 | **100%** | 219 | **100%** |
| | **15 µL** | 140 | 97% | 159 | 97% |
| | **10 µL** | 98 | 102% | 104 | 95% |
| | **5 µL** | 50 | 104% | 45 | 82% |
| Mouse | **20 µL** | 567 | **100%** | 621 | **100%** |
| | **15 µL** | 416 | 98% | 457 | 98% |
| | **10 µL** | 286 | 101% | 304 | 98% |
| | **5 µL** | 151 | 107% | 142 | 92% |
| Mouse | **20 µL** | 1,067 | **100%** | 1,297 | **100%** |
| | **15 µL** | 808 | 101% | 961 | 99% |
| | **10 µL** | 547 | 103% | 635 | 98% |
| | **5 µL** | 286 | 108% | 323 | 100% |
| **MEAN ± S.D. (n = 6)** | **20 µL** | | **100%** | | **100%** |
| | **15 µL** | | **97.2 ± 2.1%** | | **98.0 ± 1.4%** |
| | **10 µL** | | **98.2 ± 4.3%** | | **98.3 ± 1.9%** |
| | **5 µL** | | **102.3 ± 5.2%** | | **96.5 ± 8.1%** |

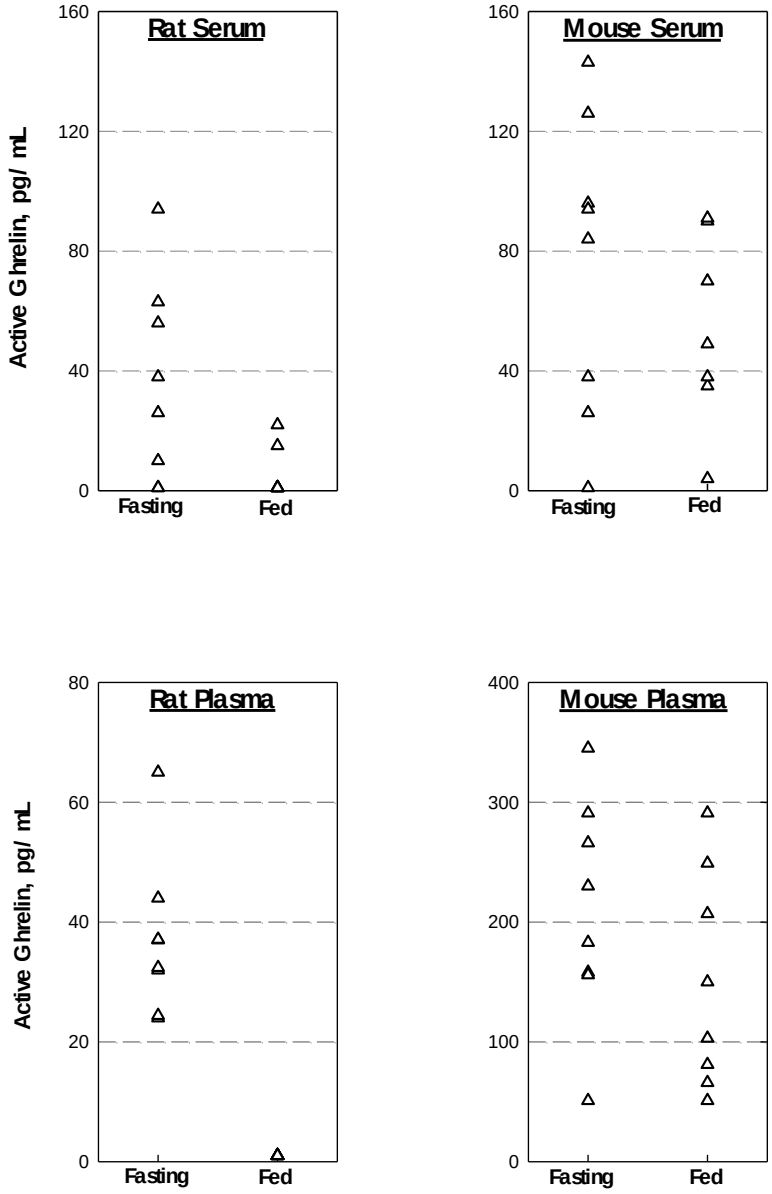
Spiked and pooled samples were assayed at 20, 15, 10 and 5 µL each for active ghrelin by ELISA. Measured ghrelin levels are corrected for various dilution factors and then divided by levels found at 20 µL sample size to obtain the % of expected values.

## XV. NORMAL RANGE OF ACTIVE GHRELIN LEVELS IN RAT/MOUSE BLOOD

The normal range of active ghrelin in 24-hour fasted rat (Sprague Dawley) and mouse (CD-1) blood is ~120 pg/mL and 600 pg/mL, respectively. The levels are lower in non-fasted animals.

## XVI. POST-PRANDIAL ATTENUATION OF ACTIVE GHRELIN IN BLOOD

**Effect of Fasting on Serum/ Plasma Ghrelin (Active) Levels**

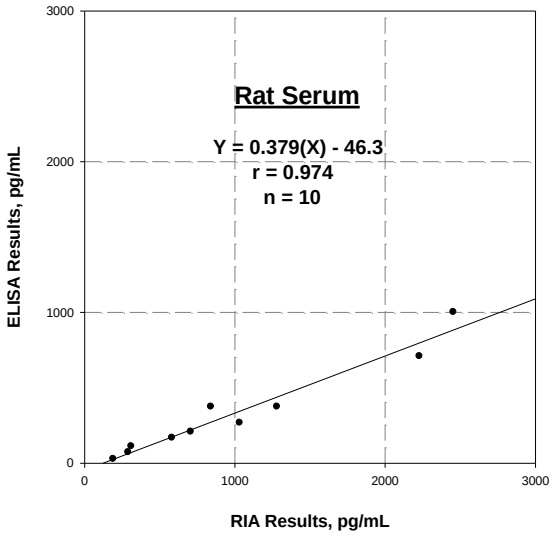


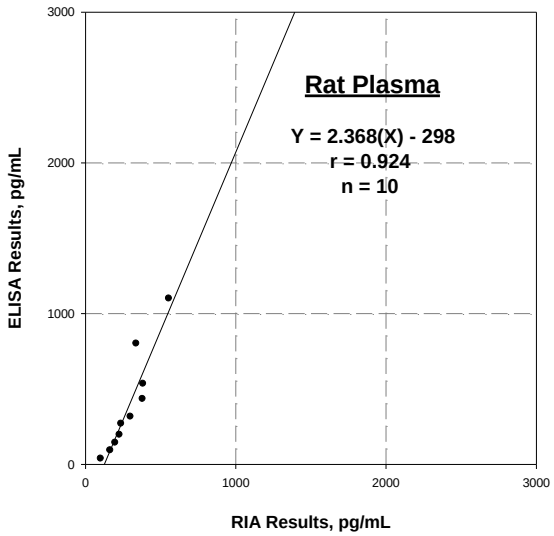

Each group contains 8 animals, either fed *ad lib* or 24-hour fasted before blood collection. All blood samples are treated immediately with 1 mg/ ml AEBSF and processed for serum/ plasma isolation. Resulting serum/ plasma samples are acdified to 0.05N HCl and stored at -20°C before ELISA assay.

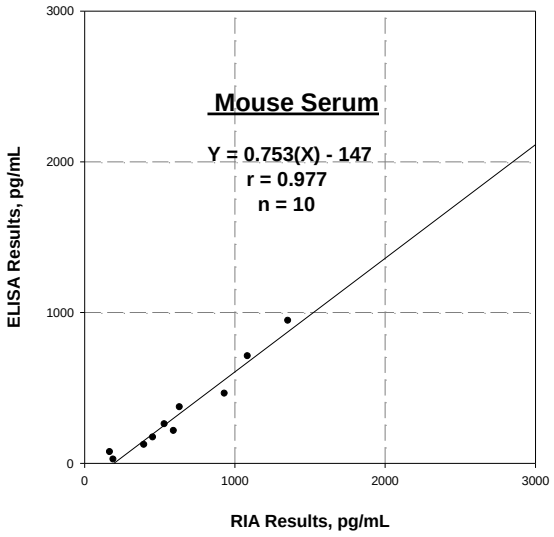
## XVII. CORRELATION GRAPH

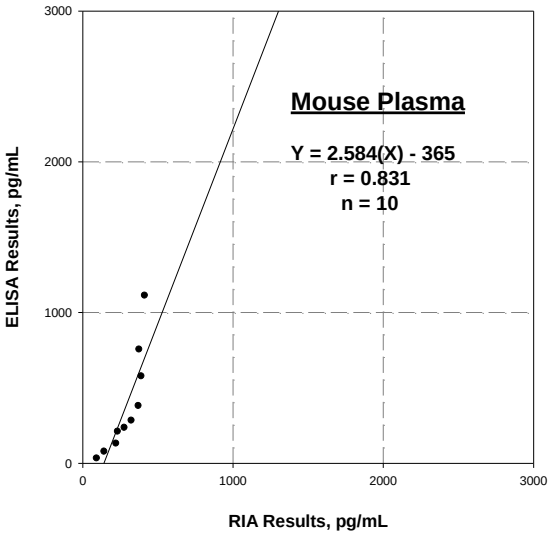
### EMD Millipore RIA Cat. # GHRA-88HK vs ELISA Cat. # EZRGRA-90K

**Pooled neat serum or plasma sample from rats/mice are thawed, treated with 1 mg/mL AEBSF followed by acidification with 0.05N HCl, spiked with rat/mouse ghrelin at levels from 50~1,000 pg/mL, and then assayed for active ghrelin by ELISA and RIA. Paired results are analyzed by linear regression analysis.**




## XVIII. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the EMD Millipore website emdmillipore.com using the catalog number as the keyword.

## XIX. TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with HRP Wash Buffer or 3) overexposure to light after substrate has been added.

## XX. REPLACEMENT REAGENTS

| Reagents | Cat. # |
|---|---|
| Microtiter Plates | EPDAR |
| 10X HRP Wash Buffer Concentrate | EWB-HRP |
| Rat/Mouse Ghrelin (Active) Standard | E8090-K |
| Rat/Mouse Ghrelin (Active) Quality Controls 1 and 2 | E6090-K |
| Matrix Solution | EMTX-GA |
| Assay Buffer | EABGR |
| Rat/Mouse Ghrelin (Active) Capture Antibody | E1090-C |
| Rat/Mouse Ghrelin (Active) Detection Antibody | E1090-D |
| Enzyme Solution | EHRP-88 |
| Substrate | ESS-TMB2 |
| Stop Solution | ET-TMB |

## XXI. ORDERING INFORMATION

To place an order or to obtain additional information about our immunoassay products, please contact your Customer Service or Technical Support Specialist.

Contact information for each region can be found on our website:

emdmillipore.com/contact

### Conditions of Sale

For Research Use Only. Not for Use in Diagnostic Procedures.

### Safety Data Sheets (SDS)

Safety Data Sheets for EMD Millipore products may be ordered by fax or phone or through our website at emdmillipore.com/msds.