Analysis of ghrelin concentrations in serum samples processed at one, two, and three hours after collection

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ANALYSIS OF GHRELIN CONCENTRATIONS IN SERUM
SAMPLES PROCESSED AT ONE, TWO, AND
THREE HOURS AFTER COLLECTION

by

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ABSTRACT

Analysis of Ghrelin Concentrations in Serum Samples Processed at One, Two, and Three Hours after Collection

by

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University of Nevada, Las Vegas

Obesity is becoming an epidemic of modern society. While the health-related consequences of obesity have been well studied, scientists continue to search for biological risk factors associated with this disease. Since the 1999 discovery of an appetite-stimulating hormone, ghrelin, researchers have further questioned the physiological interactions that lead to obesity. The fundus of the stomach, as well as the intestines and kidneys produce ghrelin before it circulates throughout the body and attaches to its receptor sites located within the section of the brain responsible for appetite-regulation. The hormone increases and decreases in a circadian rhythm that may dictate the routine human eating patterns of breakfast, lunch and dinner.

While the field of ghrelin research continues to grow as evidenced by the increasing number of published articles, the majority of the ghrelin research occurs in the laboratory setting. This environment has provided researchers with the preservation and storage techniques necessary to analyze ghrelin. For example, the current Millipore Human Ghrelin (Total) ELISA kit protocol for measuring ghrelin requires the collected blood be centrifuged thirty minutes after collection and immediately placed in a freezer.
This limitation alone may impede researchers wishing to conduct clinical research because these requirements are frequently not possible.

The objectives of this study were to determine the optimal storage time before processing blood samples collected in the clinical setting to ensure the preservation of ghrelin and to conclude whether a change in the manufacturer’s protocol was warranted.

By comparing blood samples processed according to the Millipore ELISA kit protocol to samples stored for one, two, and three hours, it was determined that mean ghrelin concentrations at thirty minutes, one hour and two hour were not significantly different, suggesting that the current Millipore ELISA protocol of thirty minutes can be extended for a period of up to two hours.

When the crude model was adjusted to include the variables time, waist circumference and exercise, the statistically significant variables in previous ANOVA models, the presence or absence of an exercise routine was found to have the strongest association to mean ghrelin concentrations. These findings will facilitate the experimental design of research aimed at studying ghrelin levels in individuals outside of the laboratory setting, and can also support new research analyzing the effect of exercise on ghrelin levels.
ACKNOWLEDGMENTS

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# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ iii

ACKNOWLEDGMENTS .................................................................................................................. v

LIST OF TABLES .......................................................................................................................... vii

CHAPTER 1   INTRODUCTION ................................................................................................. 1
  Purpose of the Study .................................................................................................................. 4
  Research Questions .................................................................................................................. 5

CHAPTER 2   REVIEW OF RELATED LITERATURE ............................................................ 7

CHAPTER 3   METHODOLOGY ............................................................................................... 10
  Collection of Data .................................................................................................................. 11
  Treatment of Data .................................................................................................................. 12

CHAPTER 4   FINDINGS OF THE STUDY .............................................................................. 14
  Analysis of Data ...................................................................................................................... 14
  Statistical Analysis of Research Questions ......................................................................... 19

CHAPTER 5   SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS .......................... 25
  Discussion of Results .............................................................................................................. 26
  Conclusions and Recommendation for Further Study ...................................................... 28

APPENDIX 1   IRB APPROVAL ............................................................................................. 30

APPENDIX 2   MILLIPORE HUMAN GHRELIN (TOTAL) ELISA
  KIT PROTOCOL ...................................................................................................................... 31

REFERENCES .............................................................................................................................. 51

VITA ............................................................................................................................................ 53
LIST OF TABLES

Table 1. Demographics of Participants ................................................................. 15
Table 2. Mean Ghrelin Concentrations at Four Time Intervals ......................... 17
Table 3. The Four Levels of Time: Baseline, 1, 2 and 3 Hours ......................... 19
Table 4. Crude Repeated Measures ANOVA ...................................................... 20
Table 5. Pairwise Comparison for the Crude ANOVA .................................... 20
Table 6. Correlations between Multicollinear Variables .................................. 21
Table 7. Between-Subject Effects for Waist Circumference in ANOVA ............ 22
Table 8. Between-Subject Effects for Weight in ANOVA ................................. 22
Table 9. Between-Subject Effects for Height in ANOVA ................................. 23
Table 10. Final Adjusted Repeated Measures ANOVA ..................................... 24
CHAPTER 1

INTRODUCTION

Obesity is the second leading cause of preventable death in the U.S. and it is estimated that almost 100 million adults in this country have a body mass index over 25 (CDC, 2002). Combating obesity has been a main objective of the CDC for several years. As part of the Healthy People 2010 initiative, the CDC hoped that obesity rates would decrease to 15% of the population in each state; however, no state met that objective (CDC, 2009). The causes of obesity can be subdivided into environmental and biological factors. A decrease in physical exercise combined with a high caloric intake accounts for the majority of obesity cases. However, biological factors such as metabolic disorders like Prader-Willi syndrome, and imbalances in the various proteins that surround food intake and digestion, if not managed properly, can also lead to obesity.

This disease is also of great concern for public health officials since it is often cited for the great number of associated co-morbidities. Cardiovascular disease, Type II diabetes, certain types of cancer, and sleep apnea can all result from being obese (Bray, 2004). The direct medical costs associated with obesity are staggering. The impact of obese patients seeking medical attention stresses the US healthcare system since an obese patient can require the care of numerous physicians, while being prescribed various medications. This utilization of the available healthcare resources due to obesity cost $147 billion in 2008 (Hammond, 2010). However, the consequences of obesity are not just limited to health related outcomes; it also places strain on other aspects of daily life.

In addition to the direct medical costs associated with this disease, obesity affects the nation by causing loss of productivity, human capital, and increasing the cost of
transportation (Hammond, 2010). While members of the population who are normal
weight may not currently be affected by the increasing levels of obesity in the nation, the
stresses that obesity places on the infrastructure of American lifestyles as well as the U.S.
healthcare system are serious consequences that were once overlooked.

Fortunately for those individuals seeking to lose weight, there are numerous
dietary, behavioral, pharmacological and surgical interventions that have proven to be
successful weight loss strategies. While all programs distinctly advertise themselves, the
basic premise of burning more calories than one consumes prevails. For individuals who
accept the challenge of taking strides toward a healthier lifestyle, weight loss and a
decrease in the risk factors for co-morbidities are the awards. However, once one reaches
an ideal weight, maintaining the new weight proves difficult. Even the most optimistic
data report that only twenty percent of those who lose ten percent or more of their initial
body weight do not regain that weight for at least one year (Wing, 2005). Since a large
proportion of people can not sustain their weight loss goals, investigating long-term
weight loss maintenance is critical to our nation’s health.

In 1999 researchers identified a hormone that may contribute to weight regain.
Ghrelin attaches to a region in the brain responsible for inducing feelings of hunger
(Wren, 2001) and the concentration of ghrelin peaks prior to the routine meal pattern of
breakfast, lunch and dinner (Cummings, 2001). Furthermore, circulating ghrelin levels
are suppressed in obese individuals and elevated in underweight individuals (Tschop,
2001; Shiiya, 2002). This inverse proportion of weight and ghrelin is intuitively logical.
An obese person does not need to consume a similar amount of calories as the
underweight individuals since the obese person has fat reserves which the body can
utilize for energy. However, when obese individuals lose weight, some data suggest that ghrelin levels may elevate to concentrations disproportionate to the individuals’ new weight (Hansen, 2002). This increase in ghrelin levels triggers a stimulus for food-intake that ultimately combats the positive results of weight loss. Although there is an emerging body of data evaluating ghrelin levels in weight-loss maintainers in controlled laboratory environments (Cummings, 2001; Cummings, 2002; Tanofsky-Kraff, 2007), descriptive data collected in the non-laboratory environment are limited.

Hosoda (2004) completed the first study aimed at characterizing ghrelin stability. While his sample size and experiments were limited, his findings have impacted the collection and handling of blood samples used to analyze ghrelin concentrations. Today, collecting blood samples in a laboratory setting specifies quick preservation including the addition of 0.1 N HCl to prevent ghrelin degradation, and storage as recommended by Hosoda (2004), and outlined by the ELISA protocols used to analyze ghrelin concentrations in humans.

Accurate evaluation of the impact of ghrelin on weight regain in the clinical setting requires a reliable method for the collection, processing, and storage of samples following blood collection. While blood may be immediately preserved, processed and/or frozen in laboratory studies, this is not the case with research occurring in the clinical setting. The existing Millipore Human Ghrelin (Total) ELISA kit protocol for measuring ghrelin concentrations require that blood be centrifuged thirty minutes after collection and immediately placed in a freezer. In clinical research, collection of the sample and transportation to a laboratory to process the sample can frequently not be completed within the time limits of the protocols. Thus current guidelines for processing samples
may be deterring researchers interested in evaluating ghrelin in non-laboratory settings, and ultimately stalling the future of translational research in this field.

An extensive search in literature databases has revealed that there are no published research reports that specifically evaluate stability of ghrelin in blood samples in the clinical setting. Measuring ghrelin concentrations in the clinical setting makes it virtually impossible to immediately preserve and store samples since the transportation of centrifuges and freezers are unrealistic. Because trends in ghrelin concentrations following attainment of goal weight will provide a foundation for interpreting the significance of post-weight loss ghrelin concentrations, it is critical to evaluate the accuracy of ghrelin concentrations measured in the clinical setting. To do this, ghrelin concentrations determined according to strict observance of the existing protocol will be compared to ghrelin concentrations measured at time periods longer than recommended.

Purpose of the Study

The objectives of this study are: (1) to analyze the serum ghrelin concentrations in blood after storage at 4°C for thirty minutes, one hour, two hours, and three hours and (2), to conclude based on the aforementioned findings whether a change in the manufacturer’s protocol is warranted.

If findings suggest that blood samples collected to determine ghrelin concentrations can indeed be stored for periods of time beyond the manufacturer’s recommendation of thirty minutes, the effects will be far-reaching. First, the ability to compare data collected in the clinical setting to published data obtained in the laboratory environment will confirm that ghrelin research is indeed translational. If findings differ between the two settings there exists the possibility that current ghrelin research is not
capturing the spectrum of both environmental and biochemical interactions that influence circulating ghrelin concentrations. Since no published studies have yet to investigate the effect of storage time on mean ghrelin concentrations as detected in an ELISA assay, this novel research will also lead to a more defined characterization of ghrelin stability. Additionally, positive findings related to increased storage time, may attract future researchers to this field that were once limited by storage time in research design.

While manufacturers of kit protocols, such as the one provided by Millipore, have scientific and legal reasons for not providing the scientists using the kits with a rationale for the processing guidelines, scientists need to understand how changes among the protocols may affect the outcome of a study. Since no studies have yet to determine the effect of time on mean ghrelin concentrations, it is unclear whether the few clinical ghrelin research studies are reporting skewed data since Type II error may have been inadvertently entered into the study design.

Research Questions

H₀: There will be no statistically significant differences in mean ghrelin concentrations among blood samples stored for one, two, or three hours after collection compared to the samples analyzed according to the Millipore protocol.

The aforementioned time intervals represent realistic storage times that a researcher may experience in the clinical setting. Factors such as transportation, access to a centrifuge, and the number of patients being evaluated are all concerns in constructing study designs.

H₀: The variables height, weight, and waist circumference will exhibit a positive, significant correlation to mean ghrelin concentrations.
It is expected to see that the variables which are well known for correlating to mean ghrelin concentrations as evidenced in previous, laboratory research will remain statistically significant in this research ($r > 0.5$, $p < 0.1$).
CHAPTER 2
REVIEW OF RELATED LITERATURE

Researchers first identified the natural ligand, ghrelin, for the growth hormone secretagogue receptor in 1999 (Kojima, 1999). The binding of ghrelin releases growth hormone into the body which then binds to receptors that regulate energy balance in the brain. Ghrelin has been identified as a hunger-stimulating hormone (Wren, 2001) and is now considered the most important and powerful orexigenic hormone currently recognized (Asakawa, 2001). Ghrelin is found in an active, acyl, and unactive, desacyl, form in the human body (Hosoda, 2000) and degrades into the desacyl form. Desacyl ghrelin is relatively stable, and can be exposed to various storage conditions including high pH and temperature, and remain stable (Hosoda, 2004). The majority of research (Hosoda, 2000; Cummings, 2001; Cummings, 2002) has quantified total ghrelin since Ariyasu (2002) reported a lack in marked discrepancies between desacyl and acyl forms.

In addition, it is the only orexigenic hormone that stimulates food ingestion, regardless of other physiologic or environmental cues for eating or satiety (Asakawa, 2001). Existing ghrelin research focuses on the biological effects of the hormone (Cummings, 2001; Cummings, 2002; Tschop, 2001; Tschop, 2002). Research performed by Cummings et al (2002) has provided insight into the factors that determine hunger. This hormone may also play an important role in understanding and combating the obesity epidemic since ghrelin concentrations are weight dependent (Tschop, 2001; Shiiya, 2002). Hansen (2002) concluded that when an obese person loses weight, post-weight loss ghrelin concentrations surpass ghrelin concentrations found in normal-weight individuals. This biological response to weight loss may prove instrumental in
understanding the difficulties of weight maintenance as well as the factors involved in weight gain.

However, to date, the majority of the ghrelin research has taken place in laboratory settings. While this setting allowed scientists to quickly process and quantify ghrelin samples (Cummings, 2001; Cummings, 2002; Natalucci, 2005), the research takes place under specific guidelines that are not applicable to everyday life. New translational research aims to investigate whether previously reported ghrelin trends (Cummings, 2001; Cummings, 2002) are observable in clinical settings. In stark contradiction to preprandial and postprandial ghrelin trends reported by Cummings et al (2001 & 2002), in unpublished, recently completed research, Sally Miller failed to find a statistically significant decrease between preprandial and postprandial ghrelin concentrations in the clinical setting. The inability to find a similar trend in subjects measured in two settings raises the issue of the importance of proper handling and processing techniques concerning ghrelin. Current ELISA manufacturer protocols for measuring ghrelin require that blood be centrifuged and frozen within thirty minutes of collection. In clinical research, this is frequently not possible.

Unlike aforementioned ghrelin research that occurred in a laboratory setting, blood samples collected in the clinical environment may sit for hours before processing can occur. Thus, it is imperative to understand how various processing methods can affect the stability of ghrelin.

In 2004, Hosoda revealed that ghrelin is most stable at pH 4 and recommended a 10% addition of 1 M HCl to the sample to achieve the greatest stability of the acyl side chain. In this literature review, the research lacked a comparison between 0.05 N HCl,
Millipore’s recommended acidification, and 0.1 N HCl which may lead to further preservation research.

Additionally, Hosoda (2004) recommends chilling and centrifuging the sample within 30 minutes. Groschl (2002) and Staes (2010) both report the stability of ghrelin at 4°C, a condition satisfied by placing collection tubes in an ice chest until processing can occur; however, guaranteed immediate access to a centrifuge in the clinical setting is unrealistic. While this previous research has provided an introduction into ghrelin stability, one must also consider the small sample sizes utilized in the studies. Hosoda (2004) only collected samples from three participants, while Groschl (2002) has a larger sample size of sixteen individuals. Groschl ultimately subdivided the participants to study different storage conditions.

While the initial research into the field of ghrelin has provided current research with important findings, there exists a myriad of research questions to be investigated. Based on the findings of this literature review, more research into the time-sensitive degradation of ghrelin performed using blood samples from a larger sample size is needed to better understand how to process collection tubes before centrifugation. If storage times do not significantly affect ghrelin concentrations over time, researchers may begin to expand ghrelin research to incorporate the clinical setting.
CHAPTER 3

METHODOLOGY

This research study involved the participation of human subjects, and as such the researcher applied for IRB approval to ensure the protection of research subjects throughout the research process. Subjects were assigned non-identifiable numbers, and the only forms that contained the subjects’ identities were stored in a separate location than the forms containing any identifying information such as date of birth and age. Obtaining subject consent implied informing the subjects of the voluntary nature of participation as well as the risks, compensation, and benefits of participation.

Recruitment for the research participants was limited to females aged 30 to 60 years. This demographic was necessary to complete other arms of the study that did not affect the outcome of the data reported in this thesis. This study included women within the 30 to 60 year old range, who were currently not pregnant, did not begin a weight loss program or started a new exercise schedule within the previous thirty days. Numerous factors such as the number of participants used in previous published literature related to ghrelin stability, and the financial constraints of the grant, limited the number of total research subjects to twenty six. The researcher did not use formal methods of recruitment, but rather relied on word of mouth and both inter-and intradepartmental emails toadvertize the study among the university campus. Additionally, professors within the University of Nevada, Las Vegas, School of Nursing informed students who met the inclusion and exclusion criteria. As all arms of the research were novel, the assumption was made that the demographics on the university campus were representative of the average female.
Collection of Data

Once a participant was consented and enrolled into the study as dictated by the IRB approval, she reported to the Bigelow Health Science building on the University of Nevada, Las Vegas campus in the morning following an overnight fast, which excluded the consumption of water since previous research has shown that water does not influence ghrelin concentrations in the body. The data of height, weight, waist circumference, last menstrual cycle, ethnicity, marital status, regular consumption of breakfast, medications, vitamins and/or supplements, and the presence or absence of an exercise routine was collected. To protect against variations in different instruments, the same Weight Watchers Conair WW70 Electronic Body Fat Scale was used to weight all research subject to one decimal point. Height and waist circumference were measured to the closest half inch.

Next, a certified and licensed phlebotomist used a combination 21 gauge needle and vacutainer to collect four, 2.5 mL blood samples into serum separator tubes. The phlebotomist selected a vein located within the nook of the elbow or the hand of the participant. Following venipuncture each blood sample was preserved with 1 mg/mL of Pefabloc in accordance with the Millipore Human Ghrelin (Total) ELISA kit recommendations to prevent ghrelin degradation. The tubes were labeled to include the participant’s identification number.

The first tube was stored for thirty minutes according to the Millipore protocol. The remaining three tubes were labeled “1hr,” “2hr,” and “3hr,” and stored in a cooler at 4°C for 1, 2, and 3 hours, respectively, before being centrifuged, acidified with 0.05 N HCl, and stored in accordance with the manufacturer recommendations. Processing of
the blood sample was delayed for a later day, but within the guidelines of protocol outlined by the manufacturer.

Treatment of Data

The ghrelin concentrations of each blood sample was found using the Millipore Human Ghrelin (Total) ELISA kit (Catalog# EZGRT-87K). The samples processed according to the Millipore protocol and the samples stored for one, two, and three hours were analyzed using the aforementioned ELISA assay. Samples collected from each participant at each time interval were measured in triplicate to increase the assay reliability of the study. The final step in the ELISA assay required the use of a spectrophotometer which analyzed the samples at different wavelengths to determine ghrelin concentrations in pg/mL. The software associated with the spectrophotometer used in this research produces the equation used to determine ghrelin concentration levels in each sample. The software then provided mean ghrelin concentrations (pg/mL) for each participant at each time interval. These data were analyzed using SPSS version 18.0 statistical programs to determine whether to reject or fail to reject the null hypotheses.

First, the variable of time was categorized as a factor, and divided into its four levels: baseline (30 minutes), 1 hour, 2 hour and 3 hours. Since time acted as the independent variable in this model, a repeated measures analysis of variance test was used to determine the effect of time on the dependent variable, mean ghrelin concentrations. Initially, time was entered as a main effect in the model, and a crude repeated measures analysis of variance was performed. Because the variable, time, was found to be significant, a Least Significant Difference post hoc test allowed the researcher to determine which time groups significantly differed from each other.
Next, the researchers ran an adjusted analysis of variance test using the variables that were assessed prior to the collection of the blood samples. The multicollinearity of the variables was determined using a correlation test within the SPSS version 18.0 software. Multicollinear variables (r > 0.5, p < 0.01) were entered into the adjusted repeated measures analysis of variance test separately. Again, time served as the independent variable, while mean ghrelin concentration acted as the dependent variable. All of the other variables collected were entered into the model as covariates. The significance variables found while running these adjusted models were incorporated into a final adjusted model to determine the statistically significant variables involved in predicting mean ghrelin concentrations.
CHAPTER 4

FINDINGS OF THE STUDY

In twenty six females aged 30 to 60 years, the effect of time played a statistically significant \( p < 0.1 \) role in determining mean ghrelin concentrations. A Least Significant Difference test determined that the mean ghrelin concentrations in samples stored for thirty minutes, one hour, and two hour differed statistically compared to the mean ghrelin concentrations recovered at the three hour storage time. However, when an adjusted model incorporating the variables height, weight, waist circumference, last menstrual cycle, ethnicity, marital status, consumption of breakfast, medications, vitamins and/or supplements, and the presence or absence of an exercise routine was ran, variables other than time were also significantly associated with mean ghrelin concentrations.

The final adjusted model included the statistically significant variables time, waist circumference and presence or absence of an exercise routine, but found that the presence or absence of an exercise routine was the only statistically significance association with mean ghrelin concentration.

Analysis of Data

Table 1 shows the demographics of the sample population. Caucasians represented 80.7% \( (n = 21) \) of the sample while three Hispanics constituted 11.5%, and the remaining two subjects were Asian American. Eighteen subjects identified as married, while five subjects were single, and three subjects were divorced. The majority of the sample \( (n = 17) \) associated into a pre-menopausal state, and 84.6% \( (n = 22) \) normally
Table 1.

Demographics and Characteristics of the Twenty-Six, Female Participants Aged 30-60 Years.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
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<td>100</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-45</td>
<td>14</td>
<td>53.8</td>
</tr>
<tr>
<td>46-60</td>
<td>12</td>
<td>46.2</td>
</tr>
<tr>
<td>Height (in)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>59-66</td>
<td>17</td>
<td>65.4</td>
</tr>
<tr>
<td>67-72</td>
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</tr>
<tr>
<td>Weight (lbs)</td>
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<td></td>
</tr>
<tr>
<td>110-140.9</td>
<td>6</td>
<td>23.1</td>
</tr>
<tr>
<td>141-175.9</td>
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<td>250-300</td>
<td>3</td>
<td>11.5</td>
</tr>
<tr>
<td>Waist Circumference (in)</td>
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<td></td>
</tr>
<tr>
<td>29-35</td>
<td>14</td>
<td>53.8</td>
</tr>
<tr>
<td>35.5-40</td>
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<td>23.1</td>
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<td>40-45.5</td>
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<tr>
<td>Marital Status</td>
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<tr>
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<tr>
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<tr>
<td>Pre-menopausal</td>
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</tr>
<tr>
<td>Yes</td>
<td>17</td>
<td>65.4</td>
</tr>
<tr>
<td>No</td>
<td>9</td>
<td>34.6</td>
</tr>
<tr>
<td>Normally eat breakfast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>22</td>
<td>84.6</td>
</tr>
<tr>
<td>No</td>
<td>4</td>
<td>15.4</td>
</tr>
<tr>
<td>Take medications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>46.2</td>
</tr>
<tr>
<td>No</td>
<td>14</td>
<td>53.8</td>
</tr>
<tr>
<td>Take vitamins or supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14</td>
<td>53.8</td>
</tr>
<tr>
<td>No</td>
<td>12</td>
<td>46.2</td>
</tr>
<tr>
<td>Routine Exercise schedule</td>
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<td></td>
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<tr>
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<td>13</td>
<td>50</td>
</tr>
<tr>
<td>No</td>
<td>13</td>
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</tr>
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</table>
ate breakfast. Half of the women did not have a routine exercise schedule (n=13), and less than half reported taking either medications or supplements.

While the between subject ghrelin concentrations differ by as much as 1,200 pg/mL, the within subject concentrations grouped at similar concentrations (Table 2). Since this analysis aimed to identify the effect of time on individual’s ghrelin concentrations, the between subject differences did not bias the analysis. Missing data points resulted when the ELISA assay did not detect ghrelin concentrations. As a result, subject number 23 was removed from the dataset, and the baseline mean concentration value of subject number 25 was substituted with the ghrelin concentration recovered at 1 hour.

The data in Table 2 are also displayed in the format of a bar graph in Figure 1. The mean ghrelin concentrations (pg/mL) found in Table 2 served as the template to perform the crude repeated measures analysis of variance to determine the effect of time on mean ghrelin concentrations recovered at the baseline, one hour, two hours, and three hours.
Table 2.

Ghrelin Concentrations (pg/mL) of Twenty-Five Female Participants Aged 30-60 Years Analyzed at 4°C at Four Time Points: 30 Minutes, 1 Hour, 2 Hour, and 3 Hour.

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1618.9</td>
<td>1109.0</td>
<td>1615.9</td>
<td>1119.8</td>
</tr>
<tr>
<td>2</td>
<td>995.1</td>
<td>1260.3</td>
<td>1032.7</td>
<td>391.4</td>
</tr>
<tr>
<td>3</td>
<td>686.7</td>
<td>756.3</td>
<td>615.9</td>
<td>529.3</td>
</tr>
<tr>
<td>4</td>
<td>381.1</td>
<td>681.9</td>
<td>353.3</td>
<td>383.0</td>
</tr>
<tr>
<td>5</td>
<td>479.9</td>
<td>575.4</td>
<td>528.1</td>
<td>472.1</td>
</tr>
<tr>
<td>6</td>
<td>1702.5</td>
<td>1852.6</td>
<td>1535.5</td>
<td>1423.3</td>
</tr>
<tr>
<td>7</td>
<td>568.9</td>
<td>507.9</td>
<td>566.1</td>
<td>526.2</td>
</tr>
<tr>
<td>8</td>
<td>688.9</td>
<td>841.9</td>
<td>882.3</td>
<td>722.4</td>
</tr>
<tr>
<td>9</td>
<td>665.8</td>
<td>775.4</td>
<td>839.5</td>
<td>956.0</td>
</tr>
<tr>
<td>10</td>
<td>1008.4</td>
<td>987.5</td>
<td>833.6</td>
<td>793.9</td>
</tr>
<tr>
<td>11</td>
<td>903.0</td>
<td>596.5</td>
<td>580.8</td>
<td>779.8</td>
</tr>
<tr>
<td>12</td>
<td>717.5</td>
<td>375.5</td>
<td>511.8</td>
<td>372.9</td>
</tr>
<tr>
<td>13</td>
<td>516.9</td>
<td>314.5</td>
<td>519.3</td>
<td>446.6</td>
</tr>
<tr>
<td>14</td>
<td>290.2</td>
<td>359.6</td>
<td>205.8</td>
<td>189.2</td>
</tr>
<tr>
<td>15</td>
<td>1155.4</td>
<td>1498.6</td>
<td>958.3</td>
<td>1205.6</td>
</tr>
<tr>
<td>16</td>
<td>187.4</td>
<td>380.7</td>
<td>285.8</td>
<td>170.2</td>
</tr>
<tr>
<td>17</td>
<td>984.7</td>
<td>1409.7</td>
<td>926.9</td>
<td>957.3</td>
</tr>
<tr>
<td>18</td>
<td>444.9</td>
<td>293.9</td>
<td>422.1</td>
<td>408.0</td>
</tr>
<tr>
<td>19</td>
<td>681.3</td>
<td>824.9</td>
<td>751.6</td>
<td>634.1</td>
</tr>
<tr>
<td>20</td>
<td>1025.4</td>
<td>1109.1</td>
<td>1244.6</td>
<td>1255.6</td>
</tr>
<tr>
<td>21</td>
<td>252.4</td>
<td>364.8</td>
<td>389.3</td>
<td>391.3</td>
</tr>
<tr>
<td>22</td>
<td>963.5</td>
<td>909.1</td>
<td>862.0</td>
<td>987.8</td>
</tr>
<tr>
<td>24</td>
<td>373.6</td>
<td>458.8</td>
<td>482.6</td>
<td>442.6</td>
</tr>
<tr>
<td>25</td>
<td>331.5</td>
<td>331.5</td>
<td>303.0</td>
<td>207.9</td>
</tr>
<tr>
<td>26</td>
<td>2771.1</td>
<td>2636.9</td>
<td>2537.1</td>
<td>1981.7</td>
</tr>
</tbody>
</table>
Figure 1. A bar graph depicting the mean ± 5% error bars ghrelin concentrations at each storage time intervals: baseline (30 minutes), one hour, two hour, and three hours.
Statistical Analysis of Research Questions

The data found in Table 2 served as the template to perform a crude repeated measures analysis of variance to determine the effect of time on mean ghrelin concentrations at baseline (30 minutes), one hour, two hours and three hours. Because subject number 23 did not have three out of the four data point, the data were excluded in the statistical portion of this research. Additionally, because a baseline point from subject 25 was not recovered, the mean ghrelin concentration found at the one hour storage time also served as the baseline value.

As seen in Table 3, this model yielded four levels related to the factor, time: baseline (30 minutes), one hour, two hour and three hours. Each factor contained the dependent variable of ghrelin concentration (pg/mL). A statistically insignificant Mauchly’s test of sphericity (p > 0.05) confirms that sphericity is maintained, and thus directs the researcher to the correct values to interpret. These data did not have a statistically significant Mauchly test of sphericity (Mauchly’s W = 0.630, p = 0.062).

Table 3.

The Categorization of the Four Levels of Time Generated by the Repeated Measures ANOVA, and the Mean Ghrelin Concentrations (pg/mL) Recovered at Each Level of Time with Their Respective Standard Deviations.

<table>
<thead>
<tr>
<th>Levels of time</th>
<th>Dependent Variable [ghrelin]</th>
<th>Mean ghrelin concentrations (pg/mL)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>baseline</td>
<td>815.8044</td>
<td>563.92793</td>
</tr>
<tr>
<td>2</td>
<td>1 hour</td>
<td>848.5118</td>
<td>558.86906</td>
</tr>
<tr>
<td>3</td>
<td>2 hour</td>
<td>791.3658</td>
<td>515.57120</td>
</tr>
<tr>
<td>4</td>
<td>3 hour</td>
<td>709.9261</td>
<td>440.04356</td>
</tr>
</tbody>
</table>
This information allowed the researcher to interpret the sphericity assumed column on the test of within subject effects which demonstrated in Table 3 that the various time points showed a statistically significant effect of time (Table 4; F= 3.643, p= 0.017).

Table 4.

<table>
<thead>
<tr>
<th>Source</th>
<th>F</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor 1</td>
<td>Sphericity Assumed</td>
<td>3.643</td>
</tr>
</tbody>
</table>

*Note. * = p ≤ 0.1

From this test, the Least Significant Difference (LSD) post-hoc test was used to determine which groups statistically differed from each other (Table 5).

Table 5.

<table>
<thead>
<tr>
<th>Time level being analyzed</th>
<th>Compared to the time levels</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hour 30 minutes</td>
<td>30 minutes</td>
<td>.041*</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>.014*</td>
</tr>
<tr>
<td></td>
<td>2 hour</td>
<td>.063*</td>
</tr>
</tbody>
</table>

*Note: * = p ≤ 0.1

The small sample size combined with the conservative nature of this the LSD post hoc test justified interpreting significance at p < 0.1. Mean ghrelin concentrations (Table 3) in
the baseline, one hour, and two hour groups demonstrated statistical differences, \( p = 0.041, \ p = 0.014, \) and \( p = 0.063 \) respectively, from mean ghrelin concentrations in samples stored for 3 hours (Table 5).

To avoid entering mutilcollinearity into the model, a bivariate statistical correlation was analyzed for the four variables: exercise, height, weight and waist circumference. With \( r > 0.5 \) and \( p < 0.01 \), the correlations between height, weight and waist circumference were significant (Table 6). This finding supports performing three separate models to analyze the effect of height, weight and waist circumference. Next, the first of three adjusted models was analyzed using a repeated measure analysis of variance test to determine if any of the following variables significantly competed with the effect of time on mean ghrelin concentrations:

Table 6.

<table>
<thead>
<tr>
<th></th>
<th>Weight</th>
<th>Waist Circumference</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson Correlation (( r ))</td>
<td>1</td>
<td>.916**</td>
<td>.692**</td>
</tr>
<tr>
<td>Sig (( p ))</td>
<td></td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td><strong>Waist Circumference</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>.916**</td>
<td>1</td>
<td>.524**</td>
</tr>
<tr>
<td>Sig</td>
<td>.000</td>
<td></td>
<td>.007</td>
</tr>
<tr>
<td><strong>Height</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>.692**</td>
<td>.524**</td>
<td>1</td>
</tr>
<tr>
<td>Sig</td>
<td>.000</td>
<td>.007</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* \( ** = r > 0.5, \) and \( p \leq 0.01. \)
age, waist circumference, marital status, ethnicity, pre-menstrual status, ethnicity, breakfast consumers, users of prescribed medications and supplements and presence or absence of an exercise schedule in her lifestyle. Table 7 shows the test of between-subject effects which assesses the significance of each variable entered into the model. The

Table 7.

*The Test of Between-Subjects Effects Analyzes the Effect of Covariates on the Impact of Time in the Repeated Measures Analysis of Variance. Waist Circumference is the Only Statistically Significant Variable.*

<table>
<thead>
<tr>
<th>Variable</th>
<th>F</th>
<th>Sig (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference</td>
<td>3.213</td>
<td>0.095*</td>
</tr>
</tbody>
</table>

*Note. * = p ≤ 0.1

The small sample size of n= 25 and the exploratory nature of this research allowed the researcher to interpret significance at p < 0.1. Table 7 shows only one variable, waist circumference, to be significant (p = 0.095). Another repeated measures analysis of variance test was then performed removing the variable, waist circumference, and replacing it with the variable, weight (Table 8). None of the variable was statistically significant (p > 0.1).

Table 8.

*The Test of Between-Subjects Effects Analyzed the Effect of Potential Covariates on the Impact of Time in the Repeated Measures Analysis of Variance.*

<table>
<thead>
<tr>
<th>Variable</th>
<th>F</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>3.019</td>
<td>0.103</td>
</tr>
</tbody>
</table>
Lastly, researchers substituted the variable, height, in the repeated measures analysis of variance test to determine what effect, if any, it had on mean ghrelin concentrations measured at different time intervals. Table 9 shows that while the variable, height, did not impact the dependent variable (p = 0.953) the variable, exercise, demonstrated a significant p – value (p = 0.061).

Table 9.

The Test of Between-Subjects Effects Analyzes the Effect of Potential Covariate, Height, on the Impact of Time in the Repeated Measures Analysis of Variance.

<table>
<thead>
<tr>
<th>Variable</th>
<th>F</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence or absence of exercise routine</td>
<td>4.056</td>
<td>.061*</td>
</tr>
</tbody>
</table>

* = p ≤ 0.1

With the findings that two variables had a statistically significant impact (p <0.1) on the dependent variable, mean ghrelin concentration, an adjusted model was used to determine the effect of waist circumference and exercise on time when entered in to a repeated measures ANOVA.

The test of within-subject effect in Table 10 shows that the variable, time, is no longer a significant variable in the model (F= 1.013, p = 0.392). While both waist circumference and exercise proved significant predictors during the modeling process, the between-subjects effects in Table 10 shows that only the variable, exercise, remained statistically significant in the adjusted model (F = 4.725, p = 0.041).
Table 10.

The Adjusted Repeated Measures Analysis of Variance Test of Within-Subject Effects Measured the Effect of Time on Mean Ghrelin Concentrations (pg/mL) while the Between-Subjects Effect Analyzed the Association between Potential Covariates on Mean Ghrelin Concentrations (pg/mL).

<table>
<thead>
<tr>
<th>Test of Within-Subjects Effects</th>
<th>Between-Subject Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>F</td>
</tr>
<tr>
<td>Time</td>
<td>1.013</td>
</tr>
<tr>
<td>Sphericity Assumed</td>
<td></td>
</tr>
</tbody>
</table>

*Note. * = \( p \leq 0.1 \)
CHAPTER 5
SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

The statistical technique used to analyze the mean ghrelin concentration over the four storage periods of 30 minutes, one hour, two hour and three hours concluded that there were statistically significant ($p < 0.1$) differences among the mean ghrelin concentrations. In the crude model the null hypothesis was rejected and a post-hoc test utilizing least significant difference (LSD) was implemented to determine which of the time periods were statistically significant. Ultimately, the mean ghrelin concentrations recovered at the three hour storage time were concluded to be statistically less than the thirty minute, one hour and two hour mean ghrelin concentrations.

The final adjusted model revealed that exercise had the strongest association with mean ghrelin concentrations even when adjusted for time and waist circumference. The impact of exercise on mean ghrelin concentration plays a more significant role than time if it is entered as a covariate in the model.

The two objectives of this study included (1) analyzing ghrelin levels in blood samples allowed to sit for thirty minutes, one hour, two hours and three hours before processing according to a manufacturer’s ELISA kit and (2) concluding based on the aforementioned findings whether a change in the manufacturer’s protocol is warranted. The results that suggest the mean ghrelin concentrations recovered after thirty minutes do not differ statistically between samples stored for two hours accomplished this goal. This research can serve as an initial argument for amending storage protocols within the ELISA assay; however, future studies will need to be conducted before changes to the existing protocol are considered.
Discussion of Results

The demographics of the participants of this study were 80.8% Caucasian (n = 21), 11.5% Hispanic (n = 3) and two subjects or 7.7% of the sample was Asian American (Table 1). The majority of the participants were married (n = 18), while only 3 subjects were divorced, and 5 were single (Table 2). The ELISA assay did not detect ghrelin concentrations for four data points. Because three of the missing data points were found among the samples provided by one subject, 23, the data did not contribute to the statistical analysis of the data. For the last missing data point, the baseline mean concentration value for subject number 25 was replaced with the mean ghrelin concentration recovered at 1 hour. The increase at 1 hour among some of the samples was an unexpected phenomenon. While one can only hypothesize about the increase in ghrelin concentrations during this time interval, it is most likely the result of assay variability, and perhaps human error involved in loading the 96 well plates. The mean ghrelin concentrations and their standard deviations (Table 3) were depicted in a bar graph (Figure 1), to allow for a visual representation of the task of determining if the mean ghrelin concentrations were far enough apart to state that the mean ghrelin concentrations statistically differed at various points in time.

When the data were entered in to the statistical software for analysis, the program categorized the independent variable, time, in to four levels: baseline, 1 hour, 2 hour and three hour (Table 3). Overall, the findings of the crude repeated measures ANOVA found time to be a significant variable in mean ghrelin concentrations (Table 4). A Least Significant Difference post hoc test revealed that mean ghrelin concentrations among the
thirty minute, one hour and two hour time intervals did not differ by a statistically significant amount (Table 5).

Next, the multicollinearity of the variables weight, height, waist circumference and exercised were assessed. Because the variables waist circumference, weight and height were found to be multicollinear ( $r > 0.5$, p < 0.01 ) in Table 6, these three variable were entered in to the adjusted repeated measures ANOVA found in Tables 7, 8, and 9, respectively. Only the variable, waist circumference, proved to be statistically significant (Table 7). However, Table 9 which analyzed the effect of height on the model showed the presence or absence of an exercise routine to be a statistically significant variable (p < 0.1). When the three significant variables time, waist circumference and the presence or absence of an exercise routine were entered in to the adjusted repeated measures ANOVA (Table 10), the presence or absence of an exercise routine proved to have the most significant association with mean ghrelin concentrations.

While the findings of this study will benefit many researchers wishing to expand the current realm of ghrelin research, there were some limitations that affected the final outcome.

A study can only analyze as many subjects as the funding can support, and this study is a testament to those financial restrictions. While a sample size of twenty five subjects was larger than any previously reported sample size used to study ghrelin stability, it may not be large enough to determine the actual trends of ghrelin degradation over time.

Additionally, the sample size is too small to make generalizations about the overall population; however, the findings supported here provide an initial step in
learning more about the effects of storage conditions of this appetite stimulating hormone.

Participants were asked to complete this study while in a fasted state, and it is possible that ghrelin concentrations could not be recovered in subject number 23 due to consumption of food or beverages before the blood collection. In a more ideal setting, the researcher would be able to confirm that food intake does not occur before taking samples to ensure that ghrelin concentrations can be detected.

Furthermore, while the goal was to centrifuge and acidify blood samples at the indicted times, there were occasions when the processing of samples was delayed by five to ten minutes. In future studies, the ability to be precise while processing the sample should be taken in to account. In addition, the need to minimize human error and increase assay validity as other aspects to be considered in future studies.

Conclusions and Recommendations for Further Study

The crude repeated measures ANOVA showed that mean ghrelin concentrations at thirty minutes, one hour and two hour did not differ statistically. This finding could allow future researchers wishing to observe ghrelin in the non-traditional, clinical setting, to go in to the field to collect samples from a broad range of subjects. Prior to these findings, researchers were limited to just thirty minutes to collect and preserve the sample, as well as transport the specimen to a centrifuge to be processed. The crude findings alone will positively impact the field of translational ghrelin research.

With increased storage times scientists studying ghrelin can design and implement research in the clinical setting. Collecting data in this environment will give scientists greater insight into the effect ghrelin has on individuals acting in their routine settings.
The ability to perform translational research will positively impact the current knowledge base regarding ghrelin, and its effect on obesity. Ultimately, future research on ghrelin could prove instrumental in helping individuals maintain weight loss, prevent obesity, and reduce the risk of co-morbidities related to this epidemic.

The field of research related to ghrelin is vast, and results of new experiments are continually being published. In this specific area a similar study could be expanded to include both female and male participants over the age of 18, and process samples more frequently, such as every fifteen minutes, instead of the thirty and sixty minute intervals used in this research. Additionally, an increased sample size may elucidate the effect of variables that researchers expected to be significant, such as waist circumference, and weight. As researchers learn more about the optimal preservation of ghrelin, they can continue to lift design limitations that have previously constricted research of this hormone.
APPENDIX 1

IRB APPROVAL

UNLV
UNIVERSITY OF NEVADA LAS VEGAS

Biomedical IRB – Expedited Review Approval Notice

NOTICE TO ALL RESEARCHERS:
Please be aware that a protocol violation (e.g., failure to submit a modification for any change) of an IRB approved protocol may result in mandatory remedial education, additional audits, re-consenting subjects, researcher probation, suspension of any research protocol at issue, suspension of additional existing research protocols, invalidation of all research conducted under the research protocol at issue, and further appropriate consequences as determined by the IRB and the Institutional Official.

DATE: January 19, 2011
TO: Dr. Sally Miller, Physiological Nursing
FROM: Office for the Protection of Research Subjects
RE: Notification of IRB Action by Charles Raemussen/Dr. Charles Raemussen, Co-Chair
Protocol Title: A Comparison of Exercise Induced Cholecystokinin Suppression to Postprandial Ghrelin Suppression in Women Aged 30-60
Protocol #: 1012-3666
Expiration Date: January 18, 2012

This memorandum is notification that the project referenced above has been reviewed and approved by the UNLV Biomedical Institutional Review Board (IRB) as indicated in Federal regulatory statutes 45CFR46.110 - Cat. 2 and UNLV Human Research Policies and Procedures.

The protocol is approved for a period of 12 months and expires January 18, 2012. If the above-referenced project has not been completed by this date you must request renewal by submitting a Continuing Review Request form 30 days before the expiration date.

PLEASE NOTE:
Upon approval, the research team is responsible for conducting the research as stated in the protocol most recently reviewed and approved by the IRB, which shall include using the most recently submitted Informed Consent/Assent forms and recruitment materials. The official versions of these forms are indicated by footer which contains approval and expiration dates.

Should there be any change to the protocol, it will be necessary to submit a Modification Form through ORI - Human Subjects. No changes may be made to the existing protocol until modifications have been approved by the IRB. Modified versions of protocol materials must be used upon review and approval. Unanticipated problems, deviations to protocols, and adverse events must be reported to the ORI – HS within 10 days of occurrence.

If you have questions or require any assistance, please contact the Office of Research Integrity – Human Subjects at IRB@unlv. edu or call 895-2794.
APPENDIX 2

MILLIPORE HUMAN GHRELIN (TOTAL) ELISA KIT PROTOCOL

HUMAN GHRELIN (TOTAL) ELISA KIT
96-Well Plate (Cat. # EZGRT-89K)

I. Intended Use 2
II. Principles Of Procedure 2
III. Reagents Supplied 3
IV. Storage and Stability 4
V. Reagent Precautions 5
VI. Materials Required But Not Provided 5
VII. Sample Collection And Storage 6
VIII. Reagent Preparation 7
IX. Assay Procedure 8
X. Microliter Plate Arrangement 11
XI. Calculations 12
XII. Interpretation 12
XIII. Graph of Typical Reference Curve 13
XIV. Assay Characteristics 14
XV. Normal Range of Total Ghrelin Levels in Human Blood 17
XVI. Post-prandial Attenuation of Total Ghrelin Levels 18
XVII. Correlation Graph 19
XVIII. Quality Controls 20
XIX. Troubleshooting Guide 20
XX. Replacement Reagents 20
XXI. Ordering Information 21
HUMAN GHRELIN (TOTAL) ELISA KIT

I. INTENDED USE

This kit is used for the non-radioactive quantification of total human ghrelin (both intact and des-octanoyl forms) in serum and plasma. 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 hypotalamic 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 39 unknown samples in duplicate.

This kit is for research purpose only.

II. PRINCIPLES OF ASSAY

This assay is a Sandwich ELISA based on: 1) capture of human ghrelin molecules (both active and des-octanoyl forms) in the sample by anti-human 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'-tetramethylbenzidine. 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 total human ghrelin in the unknown sample, the concentration of total ghrelin can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human 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 50 mL each
   Preparation: Dilute 1:10 with distilled or de-ionized water.

4. **Human Ghrelin (Total) Standard**
   Human Ghrelin (total) reference standard, 5 ng/mL, lyophilized
   Quantity: 1 bottle, 5 ng/mL after reconstitution with appropriate amount of water.
   Preparation: Hydrate thoroughly in distilled or de-ionized water immediately before use. Please refer to the analysis sheet for exact amount of water to be used since it will be lot dependent. After hydration dilute with Assay Buffer according to § VIII. A.

5. **Human Ghrelin (Total) Quality Controls 1 and 2**
   One vial each, lyophilized, containing human ghrelin (total) 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°C for later use. Avoid further freeze and thaw.

6. **Human Ghrelin (Total) Matrix**
   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, 0.09% sodium azide, and 0.1% BSA.
   Quantity: 15 mL/vial
   Preparation: Ready to use.

8. Human Ghrelin (Total) Capture Antibody
   Pre-titered capture antibody solution in buffer
   Quantity: 3 mL/vial
   Preparation: Mix thoroughly with Human Ghrelin (Total) Detection
   Antibody before use according to § VIII. C.

9. Human Ghrelin (Total) Detection Antibody
   Pre-titered detection antibody solution in buffer
   Quantity: 3 mL/vial
   Preparation: Mix thoroughly with Human Ghrelin (Total) 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

   All components of the kit should be stored at 4°C. Prepare and use standard/GC
   solutions within a day after reconstitution and aliquot in smaller quantity and store
   at ≤-20°C for later use, if necessary. Avoid further freeze/thaw cycles. 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
Sodium Azide has been added to certain reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper pluming to form explosive metal azides. On disposal, flush with large volume of water to prevent azide build up.

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.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and pipette tips: 10 µL ~ 20 µL or 20 µL ~ 100 µL
2. Multi-channel Pipettes and pipette tips: 5 ~ 50 µL and 50 ~ 300 µL Buffer and Reagent Reservoirs
3. Vortex Mixer
4. De-ionized Water
5. Microtiter Plate Reader capable of reading absorbency at 450 nm and 590nm
6. Orbital Microtiter Plate Shaker
7. Absorbent Paper or Cloth
8. Peptide or AEDSF [4-(2-Aminoethyl)-benzenesulfonyl fluoride], 100 mg/mL aqueous stock solution (store at -20°C, minimize multiple freeze/thaw cycles) is recommended for use in Sample Collection and Storage.
9. 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 AEB3F and acidification of all samples. Neat samples without such treatment exhibit ~30% (range 20% ~ 60%) less total ghrelin content then samples that have been protected. 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 Vacutainer® serum tube that contains no anti-coagulant. Immediately add enough AEB3F 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 ± 2°C.

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 ± 5°C for later use. Avoid multiple (> 5) freeze/thaw cycles.

5. To prepare plasma sample, whole blood should be collected into Vacutainer® EDTA-plasma tubes and treated with AEB3F 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 Human Ghrelin (Total) Standard with the amount of distilled or deionized water specified in the data sheet supplied with this kit to give a final concentration of 5 ng/mL (or 5000 pg/mL) of Total Ghrelin Standard. Invert and mix gently until completely in solution.

2. Label five tubes with the additional concentrations of standards to be prepared: 100 pg/mL, 200 pg/mL, 500 pg/mL, 1000 pg/mL, and 2000 pg/mL. Add Assay Buffer to each of the five tubes according to the volumes outlined in the chart below. Dilute the reconstituted 5 ng/mL 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 standard should be stored in small aliquots at ≤ -20°C. Avoid multiple freeze/thaw cycles.

<table>
<thead>
<tr>
<th>Concentration of Standards</th>
<th>Volume of 5 ng/mL Stock to Add</th>
<th>Volume of Assay Buffer to Add</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 pg/mL</td>
<td>0.020 mL</td>
<td>0.980 mL</td>
</tr>
<tr>
<td>200 pg/mL</td>
<td>0.040 mL</td>
<td>0.960 mL</td>
</tr>
<tr>
<td>500 pg/mL</td>
<td>0.100 mL</td>
<td>0.900 mL</td>
</tr>
<tr>
<td>1,000 pg/mL</td>
<td>0.200 mL</td>
<td>0.800 mL</td>
</tr>
<tr>
<td>2,000 pg/mL</td>
<td>0.400 mL</td>
<td>0.600 mL</td>
</tr>
<tr>
<td>5,000 pg/mL</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>

B. Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Human Ghrelin (Total) 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 Human Ghrelin (Total) Capture Antibody (3mL) and Human Ghrelin (Total) Detection Antibody (3mL) at a 1:1 ratio and invert to mix thoroughly.
IX. HUMAN GHRELIN (TOTAL) 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 deionized 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/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.

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.
IX. **HUMAN GHRELIN (TOTAL) ELISA ASSAY PROCEDURE (continued)**

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.)

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. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well.
### Assay Procedure for Human Ghrelin (Total) ELISA Kit (Cat. #EZGRT-59K)

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
<th>Step 5</th>
<th>Step 6</th>
<th>Step 7</th>
<th>Step 8</th>
<th>Step 9</th>
<th>Step 10-12</th>
<th>Step 13</th>
<th>Step 14-15</th>
<th>Step 16</th>
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<tbody>
<tr>
<td>Water</td>
<td>20 μl</td>
<td>30 μl</td>
<td>--</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1, D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
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<tr>
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<tr>
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<tr>
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**X. MICROTITER PLATE ARRANGEMENT**

**Human Ghrelin (Total) ELISA**

<table>
<thead>
<tr>
<th></th>
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<th>2</th>
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<tbody>
<tr>
<td>A</td>
<td>Blank</td>
<td>1000 pg/mL</td>
<td>QC2</td>
<td>Etc.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Blank</td>
<td>1000 pg/mL</td>
<td>QC2</td>
<td>Etc.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>100 pg/mL</td>
<td>2000 pg/mL</td>
<td>Sample 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>100 pg/mL</td>
<td>2000 pg/mL</td>
<td>Sample 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>200 pg/mL</td>
<td>5000 pg/mL</td>
<td>Sample 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>200 pg/mL</td>
<td>5000 pg/mL</td>
<td>Sample 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>500 pg/mL</td>
<td>QC1</td>
<td>Sample 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>500 pg/mL</td>
<td>QC1</td>
<td>Sample 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

EZGRT-89K  Rev. 06/11/2009  Millipore
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 100 pg/mL Total Ghrelin (20 µL sample size).

4. The appropriate range of this assay is 100 pg/mL to 5,000 pg/mL Total Ghrelin (20 µL sample size). Any result greater than 5,000 pg/mL in a 20 µL sample should be diluted using matrix solution and the assay repeated until the results fall within range.
XII. GRAPH OF TYPICAL REFERENCE CURVE

![Graph of Typical Standard Curve]

For Demonstration Only – Do not use for calculations

XIV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Total Ghrelin that can be detected by this assay is 100 pg/mL when using a 20 µL sample size.
XIV. ASSAY CHARACTERISTICS (continued)

B. Specificity

- Human Ghrelin (Active) 80%
- Des-Octanoyl Human Ghrelin 100%
- Canine Ghrelin (Active) 70%
- Porcine Ghrelin (Active) 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%
- Human GIP (3-42) 0%
- Human insulin 0%
- Human Leptin 0%
- Human GLP-1 0%
- Human C-peptide 0%
- Human Amylin 0%
- Glucagon 0%

- Rat/Mouse Ghrelin (Active) 52%*
- Des-Octanoyl Rat/Mouse Ghrelin 54%*

* Purified ghrelin only. This kit should not be used for ghrelin assay in rat/mouse serum or plasma.

C. Precision

Intra and Inter-Assay Variations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Ghrelin (pg/mL) Mean, n = 6</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1, serum</td>
<td>384.6</td>
<td>1.26</td>
<td>7.81</td>
</tr>
<tr>
<td>#2, serum</td>
<td>904.5</td>
<td>0.90</td>
<td>6.28</td>
</tr>
<tr>
<td>#3, serum</td>
<td>1,522.4</td>
<td>0.99</td>
<td>6.18</td>
</tr>
<tr>
<td>#4, plasma</td>
<td>272.1</td>
<td>1.76</td>
<td>7.74</td>
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<tr>
<td>#5, plasma</td>
<td>868.4</td>
<td>1.11</td>
<td>5.18</td>
</tr>
<tr>
<td>#6, plasma</td>
<td>1,346.7</td>
<td>1.91</td>
<td>6.53</td>
</tr>
</tbody>
</table>

The assay variations of Human Ghrelin (Total) ELISA kits were studied on three fasting human serum and plasma samples with varying concentrations of endogenous ghrelin. 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 Total Human Ghrelin in Assay Samples

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>Ghrelin Spiked, pg/mL</th>
<th>Serum Ghrelin</th>
<th>Plasma Ghrelin</th>
<th>Recovery Rate</th>
<th>Recovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pg/mL</td>
<td>pg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (Basal)</td>
<td>101</td>
<td>115</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>296</td>
<td>312</td>
<td>79%</td>
<td>79%</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>868</td>
<td>877</td>
<td>77%</td>
<td>76%</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>1,528</td>
<td>1,571</td>
<td>71%</td>
<td>73%</td>
</tr>
<tr>
<td>1</td>
<td>0 (Basal)</td>
<td>397</td>
<td>296</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>250</td>
<td>662</td>
<td>553</td>
<td>103%</td>
<td>102%</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1,536</td>
<td>1,291</td>
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<td>99%</td>
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<tr>
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<td>95%</td>
</tr>
<tr>
<td>2</td>
<td>0 (Basal)</td>
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<td>712</td>
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<tr>
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<td>250</td>
<td>1,072</td>
<td>913</td>
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<td>81%</td>
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<td>1,708</td>
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<td>2,564</td>
<td>2,408</td>
<td>86%</td>
<td>85%</td>
</tr>
<tr>
<td>MEAN ± S.D.</td>
<td>250</td>
<td>91.3 ± 12.0%</td>
<td>87.3 ± 12.7%</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>1,000</td>
<td>88.0 ± 11.5%</td>
<td>86.7 ± 11.6%</td>
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<tr>
<td></td>
<td>2,000</td>
<td>85.3 ± 14.0%</td>
<td>84.3 ± 11.0%</td>
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</tr>
<tr>
<td>(n = 3)</td>
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</tr>
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<td>1</td>
<td>0 (Basal)</td>
<td>124</td>
<td>85</td>
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<td>--</td>
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<td>312</td>
<td>--</td>
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<td>106%</td>
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<td></td>
<td>1,000</td>
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<td>1,600</td>
<td>114%</td>
<td>129%</td>
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<tr>
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<td>2,677</td>
<td>2,887</td>
<td>114%</td>
<td>129%</td>
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<td>250</td>
<td>1,155</td>
<td>992</td>
<td>108%</td>
<td>116%</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>2,031</td>
<td>1,910</td>
<td>115%</td>
<td>121%</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>3,086</td>
<td>3,057</td>
<td>110%</td>
<td>118%</td>
</tr>
<tr>
<td>MEAN ± S.D.</td>
<td>250</td>
<td>101.0 ± 10.4%</td>
<td>115.7 ± 3.50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>107.3 ± 12.4%</td>
<td>118.7 ± 11.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>104.7 ± 12.9%</td>
<td>112.3 ± 15.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Varying amounts of active or des-octanoyl human ghrelin were added to 3 post-prandial human serum and plasma samples and the ghrelin content of each sample was assayed by Human Ghrelin (Total) ELISA. The recovery rate = [(Observed ghrelin concentration after spike – Basal ghrelin level) / spiked ghrelin concentration] x 100%.
### E. Linearity of Sample Dilution

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>Volume Assayed</th>
<th>Serum Ghrelin</th>
<th>Plasma Ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/mL</td>
<td>% of Expected</td>
<td>pg/mL</td>
</tr>
<tr>
<td>1</td>
<td>20 μl</td>
<td>719</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>15 μl</td>
<td>511</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>10 μl</td>
<td>352</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>5 μl</td>
<td>192</td>
<td>107%</td>
</tr>
<tr>
<td>2</td>
<td>20 μl</td>
<td>1,100</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>15 μl</td>
<td>796</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>10 μl</td>
<td>520</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td>5 μl</td>
<td>276</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>20 μl</td>
<td>421</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>15 μl</td>
<td>300</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>10 μl</td>
<td>200</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>5 μl</td>
<td>113</td>
<td>107%</td>
</tr>
<tr>
<td>MEAN ± S.D. (n = 3)</td>
<td>20 μl</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>15 μl</td>
<td>95.3 ± 0.6%</td>
<td>94.7 ± 3.2%</td>
</tr>
<tr>
<td></td>
<td>10 μl</td>
<td>95.7 ± 2.1%</td>
<td>99.0 ± 8.5%</td>
</tr>
<tr>
<td></td>
<td>5 μl</td>
<td>104.7 ± 4.0%</td>
<td>116.7 ± 17%</td>
</tr>
</tbody>
</table>

Fasting serum and plasma samples from 3 individuals were assayed at 20, 15, 10 and 5 μL each for total 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 TOTAL GHRELIN LEVELS IN HUMAN BLOOD

Human Ghrelin (Total) ELISA:
Correlation Between Serum and Plasma Concentrations

Fasting and post-prandial serum and plasma samples from 5 individuals were assayed for total ghrelin by ELISA and the paired results are analyzed by linear regression analysis.
XVI. POST-PRANDIAL ATTENUATION OF TOTAL GHSRELIN IN BLOOD

Post-meal Attenuation of Total Ghrelin Level in Blood

Fasting and 1-hour postprandial serum and plasma from 5 individuals were assayed for total ghrelin by ELISA.
XVII. CORRELATION GRAPH

Millipore RIA Cat. # GHRT-89HK vs ELISA Cat. # EZGRT-89K

Total Human Ghrelin Immunoassays:
Correlation Between RIA and ELISA

\[ Y = 1.37(X) - 877 \]
\[ r = 0.943 \]
\[ n = 13 \]

13 Fasting human serum samples collected with AEBSF & HCl treatment are assayed for total ghrelin level by RIA (Linco Cat. # GHRT-89HK) and ELISA (EZGRT-89K). 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 Millipore website www.millipore.com/bmia.

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

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Cat. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microtiter Plates</td>
<td>EPRAM</td>
</tr>
<tr>
<td>10X HRP Wash Buffer Concentrate (50 mL)</td>
<td>EWB-HRP</td>
</tr>
<tr>
<td>Human Ghrelin (Total) Standard</td>
<td>E8089-K</td>
</tr>
<tr>
<td>Human Ghrelin (Total) Quality Controls 1 and 2</td>
<td>E6089-K</td>
</tr>
<tr>
<td>Human Ghrelin (Total) Matrix Solution</td>
<td>EMTX-GT</td>
</tr>
<tr>
<td>Ghrelin ELISA Assay Buffer</td>
<td>EABGR</td>
</tr>
<tr>
<td>Human Ghrelin (Total) Capture Antibody</td>
<td>E1089-C</td>
</tr>
<tr>
<td>Human Ghrelin (Total) Detection Antibody</td>
<td>E1089-D</td>
</tr>
<tr>
<td>Enzyme Solution</td>
<td>EHRP</td>
</tr>
<tr>
<td>Substrate</td>
<td>ESS-TMB2</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>ET-TMB</td>
</tr>
</tbody>
</table>
XXI. ORDERING INFORMATION

A. To place an order:
For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:
1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:
Toll Free US (866) 441-8400
(636) 441-8400

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to human or animals. All products are intended for in vitro use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.
REFERENCES


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University of Nevada, Las Vegas

Christine Elisabeth Prato

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University of Nevada, Reno

Bachelor of Arts, Spanish, 2009
University of Nevada, Reno

Special Honors and Awards:
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Community Health Sciences, UNLV
Phi Kappa Phi
Alpha Zeta
Millennium Scholarship
Phi Theta Kappa

Thesis Title: Analysis of Ghrelin Concentrations in Serum Samples Processes at One,
Two, and Three Hours after Collection

Thesis Examination Committee:
Chairperson, Mark Buttner, Ph. D.
Committee Member, Sheniz Moonie, Ph. D.
Committee Member, Timothy Bungum, Dr.P.H
Graduate Faculty Representative, Sally Miller, Ph. D.