Oxidative Stress and a High Fat Diet in Rats: An Intervention Study on the Effects of an Organometallic Compound on Enzyme Function, Inflammatory Markers, Endotoxins and Fasting Serum Glucose and Insulin Levels

by

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ABSTRACT

Cardiovascular disease has reached epidemic proportions resulting in its ranking as the number one cause of mortality in the Western world. A key player in the pathophysiology of vascular disease is oxidative stress due to free radical accumulation. This intervention study was conducted to evaluate any potential mediation of oxidative stress using a soil-derived organometallic compound (OMC) with suspected antioxidant properties. A 10-week study was conducted in male Sprague-Dawley rats (n = 42) fed either a high-fat diet (HFD) consisting of 60% kcal from fat or a standard Chow diet containing only 6% kcals from fat. Rats from each diet group were then subdivided into 3 subgroups (n = 6-10 each) that received 0.0 mg/mL, 0.6 mg/mL or 3.0 mg/mL OMC. Neither the diet nor OMC significantly changed protein expression of inducible nitric oxide synthase (iNOS) in isolated aortas. Plasma levels of the inflammatory marker, tumor necrosis factor alpha (TNFα) were below detection after the 10-week trial. Superoxide dismutase (SOD), a scavenger of the free radical, superoxide, was not significantly different following HFD although levels of SOD were significantly higher in Chow rats treated with 0.6 mg/mL OMC compared to HFD rats treated with the same dose ($p < 0.05$). Lipopolysaccharides (LPS) were significantly increased following 10 weeks of high fat intake ($p < 0.05$). This increase in endotoxicity was prevented by the high dose of OMC. HFD significantly increased fasting serum glucose levels at both 6 weeks ($p < 0.001$) and 10 weeks ($p < 0.025$) compared to Chow controls. The high dose of OMC significantly prevented the hyperglycemic effects of the HFD in rats at 10 weeks ($p = 0.021$). HFD-fed rats developed hyperinsulinemia after 10 weeks of feeding ($p = 0.009$), which was not prevented by OMC. The results of this study indicate that OMC

may be an effective strategy to help manage diet-induced hyperglycemia and endotoxemia. However, further research is needed to determine the mechanism by which OMC helps prevent hyperglycemia as measures of inflammation (TNFα) and vascular damage (iNOS) were inconclusive.

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CHAPTER 1

INTRODUCTION

Background. Cardiovascular disease (CVD) is on the rise in the United States (U.S.) at an epidemic rate. Rated as the leading cause of disease and mortality, heart disease accounts for 1 in 4 deaths and a total of 630,000 deaths per year in the U.S. Approximately $200 billion a year is spent in the U.S. for treatment and related costs of this disease (CDC, 2017). The human health and economic influence have warranted significant increases in research associated with oxidative stress and free radicals due to their implicated effects on the function of the endothelium and pathogenesis of many cardiovascular diseases.

According to mounting evidence, a major player in the role of cardiovascular dysfunction and disease is oxidative stress due to the abundance and accumulation of reactive oxygen species (ROS) (Taniyama 2003; Landmesser 2004; Madamanchi 2005; Tiefenbacher 2001). In particular, it is suggested that ROS have pathophysiological effects on vascular endothelial cells impairing their vasodilation responsiveness (Taniyama 2003). Taniyama et al. (2003) attribute this pathophysiology to the production of the ROS superoxide, O2-, due to its ability to rapidly generate other ROS and reduce the bioavailability of nitric oxide (NO).

Various enzymatic and oxidative processes generate a free radical cascade within the vasculature which impacts the bioavailability of NO, a key endogenous regulator of vasodilation. In opposition to this cascade, a variety of complimentary, endogenous antioxidant responses work to counteract the development of vascular pathogenesis (Taniyama 2003; Madamanchi 2005; Landmesser 2004). If the body cannot maintain

balance in these opposing processes, then accumulation of these free radicals creates a state of oxidative stress which, in turn, causes vascular dysfunction leading to diseases such as hypertension, diabetes, coronary artery disease and atherosclerosis (Taniyama 2003).

The addition of a specific, exogenous antioxidant can possibly provide attenuating properties against vascular damage. The results could provide a secondary defense against a variety of cardiovascular diseases.

Purpose of Study. The purpose of this study is to determine if a soil-derived organometallic compound (OMC) with suspected antioxidant properties, will attenuate oxidative stress and inflammation and improve glucose regulation as shown by measures of specific enzymes, inflammatory markers, endotoxins and fasting serum glucose and insulin concentrations in rats fed a high-fat diet (HFD). The OMC is a proprietary blend provided by Isagenix International, LLC. In as much as we know, this is the first study of its type. This study has been approved by the Institutional Animal Care and Use Committee of Arizona State University (Tempe, AZ, USA).

Research Aim and Hypothesis. The aim of this research study is to examine the effects of OMC on serum glucose and insulin concentrations, enzyme activity, inflammatory markers and endotoxins in relation to the mitigation of high-fat induced vascular dysfunction in a rat model.

> H_0 : The OMC will show no measurable differences in the expression of inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), lipopolysaccharides (LPS), tumor necrosis factor alpha (TNFα), or serum glucose and insulin levels than the vehicle, in this case, water.

Definition of Terms. The following terms in this thesis are defined by this author.

Bioavailability – the degree to which a substance is available for physiological use.

Low-density lipoprotein (LDL) – a form of cholesterol considered to be detrimental and a risk factor of heart disease when laboratory values are elevated.

NADPH oxidase 1 (NOX1) – a member of a family of enzymes found in vascular smooth muscle cells that is responsible for the generation of reactive oxygen species.

Oxidative stress – a condition of imbalance in the body due to overabundance of reactive oxygen species and inability of internal detoxifying mechanisms to match those levels.

Protein disulfide isomerase (PDI) – an enzyme that catalyzes protein folding and is positively correlated with elevated reactive oxygen species and disease states.

Reactive oxygen species (ROS) - a natural, toxic byproduct of normal metabolism resulting from the reduction of oxygen by addition of electrons.

Tetrahydrobiopterin (BH4) – a cofactor or "helper molecule" in the production of nitric oxide by the nitric oxide synthases.

Vasodilation – relaxation of smooth muscle cells in the lining of a blood vessel allowing a widening of the vessel and lowering of blood pressure.

Abbreviations. The following are abbreviations used in this thesis.

iNOS – inducible nitric oxide synthase

LPS – lipopolysaccharide

NO – nitric oxide, an endogenous vasodilator

O_2- - superoxide, a free radical

OMC – organometallic compound, the intervention

ROS – reactive oxygen species

Delimitations and Limitation. The nature of this study, the need for organ and vessel extraction necessitated a rat model as opposed to humans. Limitations of this study were small sample size, use of male rats, and individual housing. Use of this design may restrict the translation of benefits to a human population but may aid in directing further research.

CHAPTER 2

REVIEW OF LITERATURE

Cardiovascular Disease. Cardiovascular disease (CVD) is on the rise in the United States (U.S.) at an epidemic rate. Rated as the leading cause of disease, heart disease accounts for 1 in 4 deaths and a total of 630,000 deaths per year in the U.S. A total of approximately 735,000 people have a heart attack each year in the U.S. (CDC, 2017). Approximately $200 billion a year is spent in the U.S. for treatment and related costs of this disease (CDC, 2017). The most common form of heart disease is coronary artery disease. This form of heart disease is characterized by a build-up in the arteries of cholesterol and other substances, most often referred to as plaque. The process of this accumulation of plaque is referred to as atherosclerosis (CDC, 2017). Coronary artery disease is responsible for over 370,000 individual deaths in the U.S. annually. Several risk factors are associated with heart disease, such as, smoking, hypertension, high cholesterol, genetics, lifestyle, and age. Family history and age are two factors that cannot be controlled. 47% of Americans have at least one of these risk factors (Fryar and Chen, 2012). Hingorani et al. (2000) point to the role of acute inflammation and an acute cardiovascular event with the suspected precurser to a cardiac event being abnormal endothelium-dependent vasodilation. This study also indicated the role of mild inflammation in the interruption of vascular tone (Hingorani, 2000). Although there is an apparent connection between inflammation and vasodilation, the specific mechanism by which the disruption occurs still eludes understanding. The possibility of simultaneous mechanistic opponents to healthy vascular tone as cause for dysfunction exists. It is widely accepted that vascular inflammation plays a part in cardiovascular disease,

specifically in hypertension and atherosclerosis (Touyz, 2005, Griendling et al, 2003). Connection to vasomotion, vascular tone and the endothelium's role in regulation of these characteristics is suggested as a potential clinical indicator for assessing patients at risk. Inflammatory markers, such as C-Reactive protein, may aid in early detection of vascular degeneration and onset of disease (Landmesser, 2004). Epidemiological studies have implicated a correlation between potential of developing cardiovascular disease and inflammation, both acute and chronic (Maseri, 1997). The changes in vasculature endothelium due to inflammation show risk of a cardiovascular event or a slow progression toward atherosclerosis (Vallance, 1997). Both chronic low-grade inflammation and an acute inflammatory response have been associated with atherogenesis (Ridker, 1997). Aside from angiogenesis pathology there is another problem occuring due to radical oxygen species. Cell death, apoptosis, is induced by exposure to these free radicals that develop during these dysfunctional processes (Dimmeler S. , 2000). The death of cells in the endothelium contribute to the degenerative processes associated with endothelial dysfunction. Moreover, cell death can be initiated by several factors, including angiotensin II, oxidation of LDL and hyperglycemia (Taniyama, 2003).

Reactive Oxygen Species (ROS). The generation of ROS through several metabolic pathways and its accumulation lead to high levels of oxidative stress resulting in vascular dysfunction, in particular, a reduction of vasodilation (Sweazea, 2010). Due to the nature of ROS and the unpaired electrons they possess, these molecules scavenge electrons from other molecules within the vasculature. Some of these scavenging molecules include superoxide (O_2^-), peroxynitrite ($ONOO^-$), and lipid radicals (Cai,

2000). Generation of a free radical cascade that overwhelms the endogenous antioxidant counteractive measures causes a reduction in the bioavailability of nitric oxide (NO), a key regulator of vasodilation (Taniyama, 2003). Nitric Oxide plays a major role as an endothelium-derived vasodilator and is considered of great importance (Tiefenbacher, 2001). The pathogenesis of vascular dysfunction leads to diseases such as hypertension, diabetes, coronary artery disease and atherosclerosis (Niedowicz and Daleke, 2005). There is a general consensus that excess ROS can deluge the body's own endogenous antioxidant mechanisms resulting in pathophysiology. Some research depicts the role of inflammation in connection to hypertension, existing either directly from a known disease state, such as diabetes or as an indirect result of altered function of the vasculature (Androwiki, 2015, Simperova, 2016).

Mechanisms of Vascular Dysfunction. Various methodological approaches have been taken in order to examine the many mechanisms associated with endothelial dysfunction. According to Landmesser et al the process is complicated and a multi-stepped cascade of events. It is these factors that ultimately hinder endothelium-dependent vasomotion (Landmesser, 2004). Vaccine-induced inflammatory response was measured in one study to illustrate a connection to the various cytokines that are triggered and their impact on the vasculature (Hingorani, 2000). In another study, inflammatory markers such as inducible nitric oxide synthase (iNOS) were measured and compared to superoxide production and nitric oxide reduction. The superoxide production was specifically measured using a dye to examine and quantify fluorescense microscopically (Simperova, 2016). There are numerous ideas as to what actually produce the free radicals that are capable of oxidizing biomolecules due to the nature and complexity of

chemical reactions in the body. Madamanchi et al. (2005) suggest a critical role of NADPH as the culprit in atherogenic stress. Also noted in their research was the role of oxidized LDL and xanthine oxidase in free radical production. Madamanchi et al. (2005) goes further to point out even more areas of ROS production through deficiency of the cofactor 5,6,7,8-tetrahydrobiopterin (BH_4). In this case, this interrupts the normal production of NO thus reducing its bioavailability. At the same time this produces the free radical, superoxide. And he goes on further to point out the electron transport chain can become dysfunctional and also produce superoxide radicals (Madamanchi, 2005). Xanthine oxidase along with NADH/NADPH oxidases, peroxidases, arachidonic acid pathways and mitochondrial respiration to name a few are diagnosed as ROS producers by Cai and Harrison (Cai, 2000). In general, most researchers would agree that a depletion of nitric oxide plays a very important role in the reduction of vasomotion. These redox signalling processes, says Androwiki et al. (2015), lead to not only vascular dysfunction, but hypertension. Androwiki et al. (2015) goes on to suggest a strong correlation between the presence of protein disulfide isomerase (PDI) and ROS generation due to its regulation of these NADPH oxidases. This is especially noted in cases of hypertension. In the absence of PDI, it is supposed that angiotensin II can stimulate one of the NADPH enzymes called NOX1. This in turn initiates a rapid fire reaction producing ROS (Androwiki, 2015). Thus, there are numerous avenues of approach to the understanding of how ROS are formed. Many times this is a direct result of a chemical reaction and other times it is the result of lack of bioavailability of a cofactor or molecule. Whatever the case may be, conditions such as atherosclerosis develop due to these types of conditions (Tiefenbacher, 2001). Studies by Shinozaki et al.

(2004) suggest the mechanism which alters vasodilation is the reduction in NO due to the scavenger superoxide. Its role in electron exchange thus produces peroxynitrite ($ONOO^-$). Peroxynitrite in turn, reduces the availability of NO and strengthens the resistance of vasomotion (Shinozaki, 2004). These events have been shown to take place in condtions of diabetes and/or insulin resistant states.

Research struggles with what is the clear indicator of vascular dysfunction due to the many avenues of the free radical cascade. It is clear, however, that radical oxygen species, despite how they were formed, have strong effects on the function of the vasculature. Deneau et al. (2011) point to yet another indicator of cause. It is suggested that the production of advanced glycation endproducts (AGE) as a result of a non-enzymatic glycation triggers an inflammatory response which can lead to complications in the vasculature and circulation in individuals with type 2 diabetes. Deneau et al. (2011) suggests the mitochondrial dysfunction in the diabetic condition prevents the production of the necessary extra energy it takes to overcome the AGEs and make repairs. Taniyama et al. (2003) consider discrepencies in understanding that result from the nature of enzymatic functions and multiple roles of different molecules in this cascade toward oxidative stress. In fact, it is pointed out that arteries show different responses to ROS than do the aorta. Also noted is the behavior of vasculature associated with angiotensin II and catalase. These factors have inhibited the exact determinant of vasomotor tone (Taniyama, 2003). It is not only the free radical cascade that produces the damage. The fact that the endogenous mechanisms of antioxidant systems are impaired contributes to this accumulation of highly reactive molecules. It is this accumulation more so than the actual production that effects the vasculature. Study after study show the inundation of

the endogenous system of repair prevents it from keeping up and thus allowing damage to the tissues. These ROS contribute to diseases such as atherosclerosis, mutagenesis, cancer and ageing. Barman et al. (2017) goes on to suggest that the contribution to developing oxidative stress can be due to glucose oxidation, protein glycation and the production of endproducts (Barman, 2017). The role of oxidative stress in hypertension is seemingly conclusive. The loss of vasomotion due to free radical scavenging and inactivation of NO increase vessel pressure. This hypertensive state further contributes to other states of disease, such as atherosclerosis. Diabetes on the other hand contributes to the NADPH oxidase activity and as a result leads to the inability of the vessels to relax (Taniyama, 2003). Sweazea et al. (2011) considered the role of increased adiposity from diet in cardiovascular disease. In a study of rats on a high fat diet, results indicated these animals developed hyperglycemia, increased fat mass, hypertension and oxidative stress (Sweazea et al., 2010; Sweazea and Walker 2011). Studies conducted by Sweazea et al. (2011) examined the effects of the diet on vasodilation and found that vasodilation was impaired as a result of oxidative stress, inflammation (Sweazea, 2010), and upregulated induced nitric oxide synthase (iNOS) activity (Sweazea and Walker, 2010) in the vasculature of the rats fed a high fat diet for 6 weeks. iNOS has been implicated in the production of superoxide radicals and therefore increasing the oxidative load on the body (Sweazea, 2011). The exact pathophysiological roles have yet to be determined (Stroes, 1998). It is clear that the common denominator in these pathophysiologies is directly related to ROS, however, it remains to be determined how exactly this takes place and what preventive measures can be applied to counteract the negative effects of ROS.

Therapeutic Intervention and Exogenous Supplements. Taniyama and Griendling (2003) refer to poor outcomes in trials involving exogenous supplementation as an antioxidant. Noting the diversity of pathways generating ROS, a more specific approach to supplementation formulation and use is advocated (Taniyama, 2003). Both the protagonist and antagonist relationships between nitric oxide (NO), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) form a very complex and not yet fully understood chain reaction. However, it is noted that there is a significant rationale to the presence, or lack thereof, and bioavailability of these molecules and the development of cardiovascular disease (Landmesser, 2004). Supplementation with a mineraloid substance has been evaluated and showed promise in regards to mitochondrial improvement in the diabetic cases studied (Deneau, 2011). The complexity of the state of disease exacerbates the negative effects of ROS production in most case studies in the literature. So, it is difficult to determine the mechanism involved. The counter measures of certain specific enzymes and also antioxidant vitamins have proven to be inhibitory to some extent in these processes (Dimmeler, 2000). Limitations have been presented in studies showing the lack of effectiveness of specific antioxidants due to their limited ability to scavenge free radicals. The endogenous mechanisms prove to be far superior in strength to any exogenous antioxidants tested. More clinical trials are necessary to establish a better understanding of the specific pathway (Taniyama, 2003). One study in particular, however, showed promising results in zinc supplementation. Zinc deficiency has been linked to development of oxidative stress or at least a sensitivity to its development. Barman et al. (2017) confirmed that partial prevention of oxidative stress was promoted by zinc supplementation in both aortic and hepatic tissues. The zinc

supplementation influenced the elevation of cardiac enzyme markers which are known to reduce the incidence of oxidative stress. Enzymatic reactions that are conducive to producing oxidative stress were reduced in activity (Barman, 2017). This discovery is suggestive of the mechanism by which the organometallic compound may act in my study. In another study, a mineraloid isolate showed promising results in its counter measures toward diabetes. Non-enzymatic glycation due to a diabetic state was inhibited and thus advanced glycation endproducts were less likely to be formed. This anti-glycation activity increased up to 48% in the rats that were studied (Deneau, 2011).

Measurement of Physiological Markers. Each respective study in this review approached its assessment through differing test models. Sweazea et al. (2010) used western blot on snap-frozen rat aortas to examine change in eNOS. Through a process of homogenization, protein separation and double incubations, the chemiluminescence shown as immunoreactive bands could be visualized and quantified. This group also measured ROS using a fluorescence technique as well. The results concluded that diets high in fat and sucrose diminish endothelial vasodilation due to the dependency on nitric oxide. Nitric oxide was scavenged by ROS lowering its bioavailability and thus resulting in dysfunction of the endothelium (Sweazea et al., 2010). These methods are somewhat time consuming but are very cost effective and easily quantifiable.

Mitochondrial activity and non-enzymatic glycation were measured by Deneau et al (2011). Mitochondrial activity was measured with the use of a kit which took three days to process and was easily read by spectrophotometry. The glycation process utilized the measure of fluorescence by wavelength through a micro-fluorometric analyzer. DNA assays were performed on fibroblasts incubated with the mineraloid solution and

electrophoresis was used to analyze the samples following protein extraction. The results suggest an improvement in mitochondrial metabolism and non-enzymatic glycation. Mitochondrial improvement was expressed in the upregulation of key genes that enhance metabolism. For these reasons, it is suggested that magnesium may play a role in this since it is high in concentration in the mineraloid substance. Further studies would be necessary to confirm these assumptions. Blood analysis and body weight were significantly improved in the diabetic mice of this study. Alkaline phosphatase, a sign of diabetes mellitus, was greatly decreased (Deneau, 2011). Landmesser et al. (2004) describe a process of flow-dependent dilation introduced by Celermajer et al (Celermajer, 1992) which can monitor the health of the endothelium and effects of various stimuli on vasodilation (Landmesser, 2004). The study performed by Simperova et al. (2016) measured superoxide production through fluorescense using the dye, dihydroethidium (DHE). This particular dye is often used in this type of assessment. This study measured superoxide activity using a standard assay kit. And again this group presented another western blot approach to assessing inducible nitric oxide synthase. In this study some concurrent and contradictory results were found. Fat mass and body weight declined in the obese mice just as suggested in clinical studies. However, one significant result was the fact that genestein supplementation promoted oxidative stress and inflammation which was contrary to their hypothesis. This correlates with higher levels of the radical, superoxide. iNOS, an infammatory marker, was also increased in the vasculature (Simperova, 2016). In a study designed to evaluate superoxide production by endothelial nitric oxide synthase, one group examined recombinant bovine eNOS using a kit assay and through electron spin resonance, a spin trapping process. This was further processed

and evaluated by microwave frequency measuring magnetic field. The effects of cofactors involved in eNOS activity were also measured using luminescence spectroscopy. The inhibition of both superoxide and NO could be assessed in this manner. As predicted, the superoxide radicals increased after treatment with the NADPH treatment. And the addition of tetrahydrobiopterin (BH_4) conclusively decreased superoxide production. Further conclusions included in this study show the heme domain, alone controls the production of eNOS-mediated superoxide radicals (Stroes, 1998). One limitation of the study is the use of isolated enzymes compared to experiments in living cells, not to mention the other risk factors associated with atherosclerosis. Barman et al. (2017) explored measuring plasma lipid profiles. Both methylation of fatty acids and triacylglycerols (TAGs) were measured using assays demonstrated in other literature. RNA isolation was performed to assess genetic upregulation. Western blot technique was used according to standard protocols to evaluate protein expression of oxidative stress markers. Results showed significant decreases in the size and weight of hearts in the rats supplemented. However, this is indicated only in the low-dose intervention group as no significant difference was exemplified in the high dose group. Zinc supplemented rats also showed drops in ROS and peroxide levels by as much as 25%. In comparison, the control groups showed much higher levels of both, as well as, higher levels of most all antioxidant enzymes. The rats given the supplement had much lower cholesterol and TAGs in the heart muscle. Phospholipid levels decreased in the intervention group by as much as 34%, a significant change. Apoptosis gene regulation was reduced by zinc supplementation, whereas, upregulation of beneficial gene sequencing was improved. While this study, in fact, offers lucrative evidence of success, it still necessitates further

examination on the effects against oxidative stress. The evidence depicting a definitive inverse reaction with the use of supplementation sets a clear course for future research. The results concluded in this study offer promising potential in exogenous supplemention advancements to counteract ROS. However, as with most studies in this review, the verdict is not yet in.

Hingorani (2000) demonstrated a vaccine induced inflammatory response using a salmonella typhi vaccine. Various cytokines, inflammatory markers, were measured by ELISA testing. Blood flow in the forearm in response to various vasodilators was measured using plethysmography. And the brachial artery dilation, both pre and post, was measured though the use of ultrasound. The results showed increased white cell count, an immune response which demonstrates inflammation. The cytokines were elevated. There was no change in heart rate or blood pressure or body temperature. Forearm blood flow increased as well. Vasodilation response was inhibited following the vaccination. The control group showed variable changes in dilator responses but no changes in any of the other assessments. The results of this study concluded that even though vasorelaxation was inhibited, it was only temporary in this case (Hingorani, 2000). It could be observed in this study that the temporary reaction was due to a temporary stimulus, the vaccine. Further study on subjects in pre-conditions of disease state may exhibit more long-term responses as they would be in a more compromised state. Despite the insight provided, this study did not provide an adequate model to make any promising conclusions. This study leaves the reader feeling somewhat incomplete.

CHAPTER 3

METHODS AND MATERIALS

Experimental Groups. Six-week old male, Sprague-Dawley rats (Envigo, Indianapolis, IN) were randomly separated into two groups and then randomly divided into 3 intervention subgroups. The Chow and HFD control subgroups contained 10 and 8 rats, respectively. The Chow and HFD intervention subgroups each contained 6 rats. Mean weight of rats at the start of the study was 150 grams. Male rats were preferred over female due to ovulation and higher risk for cardiovascular disease among female rats. Rats were housed individually to allow for the analysis of the fecal microbiome for a separate study. Visual, auditory and olfactory contact with other rats was provided. All cages were identical and in the same facility location. Environment was structured to provide 12:12h light-dark cycles (see Figure 1).

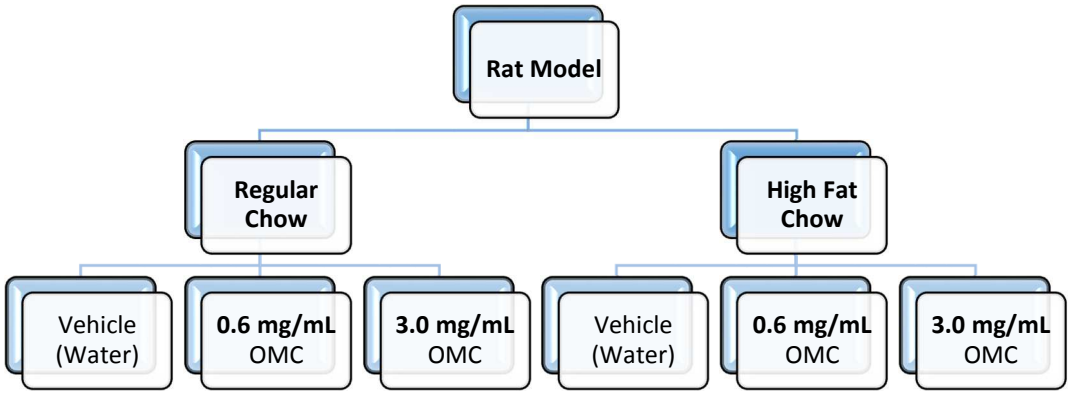

Figure 1. Experimental Design (n = 10 Chow, n = 8 HFD, n = 6 Chow and HFD intervention groups). OMC = organometallic compound, HFD = high fat diet.

One group of rats was fed a standard rodent chow diet comprised of 18.6% protein, 44.2% carbohydrates, and 6.2% fat kcal quantities (2018; Harlan Teklad;

Appendix A). The other group was fed a HFD comprised of 20% protein, 20% carbohydrates, and 60% fat kcal quantities (D12492; Research Diets, Inc.; Appendix B). Rats were fed the allocated diets for 10 weeks. Diets, water and cages were replaced every third day to maintain quality. Food and water was always readily available to rats. The OMC with suspected antioxidant properties was used as the intervention. Each of the 3 intervention subgroups from the chow and HFD groups were provided a vehicle (plain water), 0.6 mg/mL OMC intervention, or 3.0 mg/mL OMC intervention, respectively. The intervention was administered in their drinking water throughout the 10-week feeding study.

At the end of the 10-week study, rats were euthanized with an overdose of sodium pentobarbital (200mg/kg body mass, i.p.) and blood was collected by cardiac puncture for the analysis of plasma nitric oxide (total nitrates and nitrites). Following blood collections, the thoracic aortas were isolated and divided into two segments. One segment of the thoracic aorta was snap-frozen in liquid nitrogen for western blotting procedures to examine changes in the protein expression of iNOS and the other segment was embedded in optimal cutting temperature (OCT) compound prior to freezing for tissue cryosectioning and analysis of oxidative stress, specifically superoxide, in a future study.

Western Blots

The snap-frozen aortic tissue was used to measure total iNOS, an inflammatory marker that can overproduce NO. Procedures for western blot tests follow the laboratory protocol established by Dr. Karen Sweazea (Sweazea, Lekic, Walker 2010).

Thoracic aorta segments were transferred to microcentrifuge tubes containing microbeads and ice-cold buffer containing 10mM Tris (pH 7.6), 1mM EDTA, 1% triton

X-100, 0.1% Na-deoxycholate, and 10uL protease phosphatase inhibitor (HALT, Cat. No. 1861281; Thermo Scientific, Waltham, MA) were added to the tubes to prevent protein breakdown. The samples were then homogenized using a BeadBug Benchtop homogenizer (3.0mm zirconium beads; Benchmark Scientific, Edison, NJ, USA). The resultant homogenate was then transferred to a new centrifuge tube and centrifuged at 4000g (relative centrifugal force) for ten minutes at 4˚C to remove any soluble debris. The supernatant was analyzed for total protein content using the Bradford method micro-assay protocol (Bio-Rad, Hercules, CA). Tissue sample proteins (25 μg/lane) were resolved by 7.5% Tris-HCL sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA) at 200V for a period of 35 minutes at room temperature. Gel electrophoresis was used to separate the proteins by molecular weight which were then transferred to Immuno-blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) at 100V for 90 minutes. The PVDF membranes were then incubated overnight at 4˚C in blocking buffer (100 mL Tween/Tris-buffered saline (TTBS) consisting of 3% BSA. Following overnight incubation, the membranes were washed in TTBS and incubated for 4 hours at room temperature with mouse monoclonal antibody for iNOS detection (1:1500; Cat. 610431; BD Transduction Laboratories, San Jose, CA). Rabbit polyclonal antibody to β-actin was used as the control (1:10,000; Cat. Ab8227; AbCam, Cambridge, MA). Tri-buffered saline (TBS) washes followed by exposure to chemiluminescence western blotting substrate for 1 minute demonstrated chemiluminescent signals (Thermo Scientific, Rockford, IL). The signals were then detected by exposure to x-ray film (Kodak X-OMAT, Thermo Fisher

Scientific, Pittsburgh, PA) and ImageJ software was used to analyze the densitometry of interest.

Tumor Necrosis Factor α

Tumor necrosis factor alpha (TNF α), a cytokine involved in systemic inflammation, was analyzed in plasma samples collected at 10 weeks using a Rat TNF alpha ELISA Kit (Cat. ER3TNFA, ThermoFisher Scientific, Rockford, IL) per manufacturer's protocol (see Appendix C).

Superoxide Dismutase

Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation or fractionation of the free radical superoxide, O_2^- into molecular oxygen or hydrogen peroxide. Plasma SOD activity at 10 weeks were analyzed using the Superoxide Dismutase Activity Assay Kit (Cat. 706002, Caymen Chemical, Ann Arbor, MI) per manufacturer's protocol (See Appendix D).

Lipopolysaccharides

Lipopolysaccharides (LPS), also known as endotoxins are found in the peptidoglycan layer of gram-negative bacteria. When released into the bloodstream, these toxins initiate an inflammatory response. Plasma analyses of LPS were performed using Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Cat. 88282, ThermoFisher Scientific, Rockford, IL) per manufacturer's protocol (See Appendix E).

Fasting Serum Glucose

Glucose is a 6-carbon, monosaccharide utilized as the body's primary energy source. Elevated serum levels have been linked to diabetes and hypertension. A colorimetric assay kit (Cat. 10009582, Caymen Chemical, Ann Arbor, MI) was used

according to manufacturer's protocol to quantitate fasting serum glucose levels after 6 and 10 weeks (See Appendix F).

Fasting Serum Insulin

Insulin is a hormone secreted by the pancreas as a blood glucose regulator. Lack of insulin or cellular resistance to insulin action is linked to diabetes and hypertension. Fasting serum insulin quantification was performed according to manufacturer's protocol using the Ultra-Sensitive Rat Insulin ELISA Kit (Cat. 90060, Crystal Chem USA, Downers Grove, IL; See Appendix G).

Statistics

Comparison within and between group data was performed for total iNOS protein expression as well as plasma SOD and LPS using two-way analysis of variance (ANOVA) with diet and dose of OMC as factors. Fasting serum glucose and insulin levels were analyzed by two-way RM ANOVA with diet and dose of OMC as factors comparing within and between group data. Tukey post-hoc analyses were used to confirm differences in statistically significant data. Means ± standard error of the mean was used to report the data. Sigmastat 3.0 (Systat Software, San Jose, CA) was used for all statistical analyses.

CHAPTER 4

RESULTS

Effects of OMC on iNOS expressions. There was no statistically significant difference between the Chow fed rats and HFD rats ($p = 0.752$). Two-way ANOVA showed no statistically significant difference between the HFD control rats, rats treated with 0.6 mg/mL OMC or HFD rats with 3.0 mg/mL OMC ($p = 0.708$).

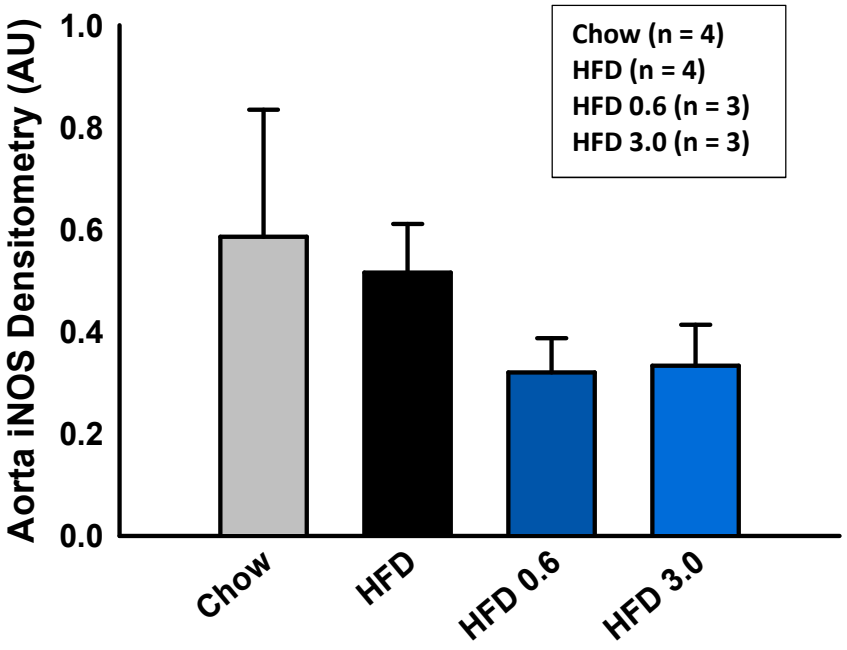

Figure 2. iNOS expression between respective HFD and Chow control groups ($p > 0.05$) and between HFD control and intervention groups ($p > 0.05$). iNOS = inducible nitric oxide synthase, HFD = high fat diet.

Effects of OMC on plasma TNFα inflammatory marker. Plasma TNFα values were below detection.

Effects of OMC on SOD levels in plasma at week 10. Two-way ANOVA analyses show HFD 0.6 mg/mL OMC resulted in significantly lower SOD concentrations

compared to Chow fed rats treated with 0.6 mg/mL OMC ($p < 0.05$). All other group comparisons showed no statistical significant difference ($p > 0.05$; Table 1).

Table 1: Plasma SOD Levels at Week 10

Plasma SOD Activity (U/mL)	**Mean ± SEM**	**(n)**
Chow	1.31 ± 0.16	(9)
Chow + 0.6 mg/mL OMC	1.62 ± 0.14	(6)
Chow + 3.0 mg/mL OMC	1.28 ± 0.16	(6)
HFD	1.17 ± 0.08	(8)
HFD + 0.6 mg/mL OMC	1.18 ± 0.09	(6) *
HFD + 3.0 mg/mL OMC	1.13 ± 0.16	(6)

Data expressed as mean ± SEM (n). Data analyzed by two-way RM ANOVA with Tukey posthoc analyses, * $p < 0.05$ vs. respective Chow. SOD = superoxide dismutase, HFD = high fat diet, OMC = organometallic compound.

Effects of OMC on plasma lipopolysaccharides (LPS). Data analyzed by two-way ANOVA showed significantly increased plasma LPS in HFD control compared to Chow control rats ($p < 0.05$). Additionally, 3.0 mg/mL OMC prevented the increase in plasma LPS in rats fed a HFD ($p < 0.05$). All other groups showed no statistical significance ($p > 0.05$; Table 2).

Table 2: Plasma LPS Levels at Week 10

Plasma LPS Concentration (EU/mL)	**Mean ± SEM**	**(n)**
Chow	0.395 ± 0.029	(6)
Chow + 0.6 mg/mL OMC	0.294 ± 0.026	(4)
Chow + 3.0 mg/mL OMC	0.400 ± 0.089	(6)
HFD	0.620 ± 0.061	(3) *
HFD + 0.6 mg/mL OMC	0.441 ± 0.075	(6)
HFD + 3.0 mg/mL OMC	0.303 ± 0.024	(6) #

Data expressed as mean ± SEM (n). Data analyzed by two-way RM ANOVA with Tukey posthoc analyses, * $p < 0.05$ vs. respective Chow, # $p < 0.05$ vs. respective HFD control. LPS = lipopolysaccharides, HFD = high fat diet, OMC = organometallic compound.

Effects of OMC on serum glucose levels. Two-way RM ANOVA analyses (see Table 3) showed significantly greater fasting serum glucose concentrations in HFD rats compared with Chow fed animals at week 6 ($p < 0.001$) and week 10 ($p = 0.025$). The high dose of OMC prevented hyperglycemia in HFD rats at 10 weeks ($p = 0.021$) and a tendency to decrease fasting serum glucose from week 6 to week 10 in HFD rats ($p = 0.085$). The low dose of OMC tended to lower serum glucose in HFD rats at 10 weeks ($p = 0.067$). There were no significant differences in serum glucose among any of the Chow groups ($p > 0.05$).

Table 3: Serum Glucose Levels at Week 6 and 10

	Week 6		**Week 10**	
Serum Glucose (mM/L)	**Mean ± SEM**	**(n)**	**Mean ± SEM**	**(n)**
Chow	6.88 ± 0.28	10	7.08 ± 0.17	10
Chow + 0.6 mg/mL OMC	7.43 ± 0.36	6	7.34 ± 0.30	6
Chow + 3.0 mg/mL OMC	6.73 ± 0.45	6	7.01 ± 0.17	6
HFD	8.83 ± 0.41 *	8	8.32 ± 0.33 *	8
HFD + 0.6 mg/mL OMC	8.01 ± 0.34	6	7.30 ± 0.04	6
HFD + 3.0 mg/mL OMC	7.91 ± 0.29	6	7.07 ± 0.19 #	6

Data expressed as mean ± SEM (n). Data analyzed by two-way RM ANOVA with Tukey posthoc analyses, * $p < 0.05$ vs. respective Chow, # $p < 0.05$ vs. respective HFD control. HFD = high fat diet, OMC = organometallic compound.

Effects of OMC on serum insulin levels. Two-way RM ANOVA analyses (see Table 4) showed significant hyperinsulinemia in the HFD rats at week 10 compared to Chow controls ($p = 0.009$). HFD rats treated with 0.6 mg/mL OMC tended to have a higher fasting serum insulin level at week 6 compared to the respective Chow rats, however, it was not statistically significant ($p = 0.064$). In addition, HFD rats tended to have higher fasting serum insulin concentrations at week 6 compared to Chow rats ($p = 0.066$). There were no significant effects of OMC on fasting insulin concentrations.

Table 4: Serum Insulin Levels at Week 6 and 10

	Week 6		**Week 10**	
Serum Insulin (mU/L)	**Mean ± SEM**	**(n)**	**Mean ± SEM**	**(n)**
Chow	12.9 ± 2.73	10	12.2 ± 1.27	10
Chow + 0.6 mg/mL OMC	14.7 ± 3.28	6	16.3 ± 2.99	6
Chow + 3.0 mg/mL OMC	12.4 ± 2.11	6	15.2 ± 1.69	6
HFD	29.2 ± 5.52	8	32.8 ± 3.80 *	8
HFD + 0.6 mg/mL OMC	34.6 ± 7.41	6	34.3 ± 6.55	6
HFD + 3.0 mg/mL OMC	30.3 ± 9.18	6	25.8 ± 5.36	6

Data expressed as mean ± SEM (n). Data analyzed by two-way RM ANOVA with Tukey posthoc analyses, * $p < 0.05$ vs. respective Chow, # $p < 0.05$ vs. respective HFD control. HFD = high fat diet, OMC = organometallic compound.

CHAPTER 5

DISCUSSION

Discussion of Results. Oxidative stress develops when oxidizing mechanisms in the body overwhelm antioxidant protective measures. This imbalance has potential to cause cellular damage in the vasculature (Stocker, 2004). Vascular damage sparks events that lead to cardiovascular diseases, such as atherosclerosis and hypertension (Schulze, 2005). The main culprit in the development of oxidative stress is free radicals. Free radicals are molecules containing unpaired electrons (Halliwell, 1999). Damage occurs when free radicals generate more free radicals from other molecules rather than pairing to form non-radical products (Schulze, 2005). Normal vascular processes are interrupted by oxidative stress, specifically synthesis of nitric oxide (NO) by nitric oxide synthases. NO is important in the vasodilation of vascular smooth muscle cells lining the blood vessels (Landmesser, 2004). Lack of vasodilation affects vascular tone which can lead to hypertension, a precursor to heart disease. Numerous physiological markers can assess the presence or potential of vascular dysfunction. Exogenous supplementations have been introduced as a therapeutic intervention for oxidative stress, but due to the complexity and nature of the mechanisms involved they are difficult to target with exogenous supplementation and often fall short (Conti, 2016).

This study was conducted assuming the null hypothesis (H_o) which states the OMC will show no measurable differences in the expression of inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), lipopolysaccharides (LPS), tumor necrosis factor alpha (TNFα), or serum glucose and insulin levels than the vehicle, in this case, water. Failure to reject the null hypothesis on several measures exist, including

iNOS, TNFα, serum insulin concentration and SOD. iNOS presents as a defense mechanism during oxidative stress and increases intracellular nitric oxide to toxic levels. As hypothesized, there were no significantly measurable differences in the application of the OMC intervention on iNOS protein expression within the aorta. Control groups also show lack of significant differences in aorta iNOS protein expression. TNFα levels were below detection in the assay and were thus inconclusive. Fasting serum insulin concentrations show a tendency towards greater fasting serum insulin at week 6 and significantly greater insulin concentrations at week 10 in HFD control animals compared to the respective Chow control which is suggestive of the development of insulin resistance in the HFD group. Free fatty acids (FFAs) circulating in the blood stream have been linked to the development of insulin resistance and hyperinsulinemia (Zhang, 2014). Jucker et al. (1997) confirmed that HFD-induced insulin resistance impairs glucose uptake in rats which correlates with both fasting serum insulin and fasting serum glucose levels in this study. FFAs deploy inflammatory responses which further contribute to cardiovascular complications, including atherosclerosis (Hummasti, 2010). There was no exhibited effect of OMC on fasting serum insulin concentrations. The statistical difference in SOD between the respective 0.6 mg/mL OMC groups was most likely due to the rise in SOD levels observed in the Chow group. There was no significance within the HFD groups demonstrating that the OMC intervention had no impact. SOD as an endogenous, antioxidant defense mechanism is of great importance. The nature of this enzyme provides a natural protection against the dismantling of NO by the free radical, superoxide. Thus, it protects vasorelaxation response and inhibits vasoconstriction (Fukai, 2011). As stated previously, this protection is overwhelmed under oxidative

stress. Exogenous reinforcements are of considerable interest in preempting the disease process. Despite the findings of this study, consideration should be given to further research in antioxidant therapies.

Other markers examined in this study suggest a rejection of the null hypothesis (H_o). LPS levels show a significant impact in the HFD 3.0 mg/mL OMC as it prevented the increase of plasma lipopolysaccharides. In addition to this confirmation, the comparative control groups show the HFD significantly higher in LPS than the Chow indicating increased levels of endotoxins due to a high-fat diet. The findings are consistent with current research on a high-fat diet, inflammation and endotoxemia (Wellen, 2005). Endotoxicity has also been linked to insulin resistance (Mehta, 2010) which is indicated in this study by both increased lipopolysaccharides and higher serum insulin levels in the HFD rats. Results also show a positive effect of the high dose of OMC on endotoxicity. Research has shown that consumption of high fat or high sucrose diet leads to metabolic syndrome and diseases such as diabetes (Panchal, 2011). In cases of metabolic syndrome due to over-nutrition, development of endotoxemia can occur due to increased intestinal permeability (Frazier, 2011). Zhou et al. (2014) examined metabolic disease induced by a high-fat and high-sugar diet in a rat model and found LPS levels to be elevated along with blood glucose and insulin levels. Insulin resistance also showed significantly high levels (Zhou, 2014). Fasting serum glucose levels were significantly increased at both week 6 and week 10. Consistent with existing research (Wei, 2009), the control group comparison showed the HFD induced elevated glucose levels. The prevention of elevated serum glucose levels with the 3.0 mg/mL OMC intervention signified significant impact of the treatment on fasting glucose and thus

glucose regulation. The reduction of blood glucose levels is key in the prevention of the formation of advanced glycation products (AGEs) which are conducive to the progression of diabetes. Deneau et al. (2011) showed significant anti-glycation activity using a mineraloid substance with suspected antioxidant properties which is consistent with the current findings. The Deneau study showed various gene expressions, including SOD, involved in mitochondrial repair were also increased, enhancing antioxidant protection (Deneau, 2011). Other research also showed improvement of mitochondrial function linked to prevention of insulin resistance (Peterson, 2003). The tendency toward reduction of glucose levels with the lower dose of 0.6 mg/mL OMC are consistent with the high dose OMC intervention, however, they were not significant.

The organometallic compound in this study was composed of a tri-mineral blend whose exact mineral composition is unknown. It is known that vitamins and trace minerals such as copper, selenium, and zinc play key roles in many biological processes (Shils, 1999). And studies have indicated a relationship between micronutrients and antioxidant potential (Evans, 2001). Deneau et al. (2011) measured the upregulation of gene expression using a mineraloid substance, similar to OMC in this study, containing numerous trace minerals and found the copper/zinc-dependent superoxide dismutase gene to be noticeably higher. A 2009 survey of 300 registered dieticians examined the likelihood of recommending supplements and for what purpose (Dickinson, 2012). The Dickinson et al. (2012) study confirmed that supplement recommendation was prevalent for bone health, nutrient deficiency, and overall health and wellness. Studies have also shown a strong connection between heart failure and levels of selenium, copper and zinc (McKeag, 2012). Unfortunately, specific studies that examine the connection between

diet and health have been limited with their focus on one single nutrient and disregarding the interaction of a specific nutrient and foods (Movassagh, 2017). It is important to note that despite this narrow approach to studying micronutrients and health, strong evidence supports the important role of micronutrients in the prevention of disease. Copper, selenium and zinc have been implicated in the pathogenesis of diabetes (Ozenc, 2015). Zinc is vital to insulin synthesis and secretion (Victorinova, 2009). Deficiency of magnesium has been linked to insulin resistance and diabetic complications (Praveeena, 2013). Selenium is a component of the antagonist response to free radicals by exhibiting antioxidant properties and aiding other processes (Laclaustra, 2010). The role of selenium and zinc are important in the action of superoxide dismutase (SOD) and its ability to dismantle superoxide radicals (Ruiz, 1998). Forte et al. (2013) suggested a connection between copper levels and glucose intolerance. Copper deficiency in rats has also been indicated in the reduction of activity of Cu-dependent enzymes, such as SOD (Roughead, 1999). A study of Zucker diabetic rats by Kim et al. (2014) examined the decrease in SOD activity and found it due to glycosylation of the Cu/Zn enzyme (Kim, 2014). Selenium also has a significant role to play in antioxidant protection. Selenium contributes to physiologically protective measures by way of conversion into selenocysteine (Berry, 1993). Ruseva et al. (2012) suggest the importance of endothelial selenoproteins in vascular tone, superoxide/NO balance, apoptosis, and cellular adhesion expression. Sprague-Dawley rat aortas were examined in a study on selenium and its potential atheroprotective role (Stupin, 2017). Stupin et al. (2017) stated the illusory mechanism behind the protection of vascular function could not be determined.

This study provides supportive evidence of current thought on the relationship between HFD and hyperglycemia-induced cellular damage (Zhang, 2014). The generation of ROS and the increase in oxidative stress due to hyperglycemia has been noted in numerous studies (Wei 2009; Wang 2006). As much of the published research suggests, the mechanism behind antioxidant support in vascular health has not been concluded. It leaves research still pondering what may be the most effective approach to supplemental intervention in cardiovascular disease (Zureik, 2004). However, strong support is suggested for the use of exogenous mineral supplementation in the pathophysiology of cardiovascular disease. This study enforces the need to continue with further research in the area of exogenous supplementation and the examination of OMC and any potential of toxicity. The minerals suggested as possible components of OMC in this study each have toxicity levels as recommended by the U.S.D.A. (Food and Nutrition Board, 2018). Consideration should also take into account the difference in the physiology of rats and humans in regards to levels of toxicity. Minerals are necessary in the biology of humans and, therefore, pose no threat from that standpoint. However, consumption of minerals are only needed in minute doses.

CHAPTER 6

CONCLUSION

It is a fair prediction that studies such as these show significant promise in the application and use of exogenous supplementation as a possible preventive measure against free radical accumulation, oxidative stress and the resultant vascular damage. It is also safe to say that this type of intervention is necessary and therefore warrants further research into the applicability of exogenous supplementations. Further studies should be directed toward the specific biological mechanisms that are impeded or encouraged. This approach toward specificity could possibly have major implications in the decceleration of disease progress. Consideration must be given to the significance of results in regard to hyperglycemia and insulin resistance. It is important to note the impact of this organometallic compound on the reduction of lipopolysaccharides and levels of endotoxicity as well as prevention of hyperglycemia in animals fed a high fat diet. Current research is directed in large at the correlation between gut health and physiological status. The OMC in this study may play a key role in connecting the gut to vascular disease through regulation of endotoxins. Considering the overwhelming exposure to environmental toxins and negative factors that are uncontrollable, antioxidant measures must be introduced to attenuate the progression of existing diseases into epidemic proportions. It is also noteworthy that many of these studies are evaluated in the rat model. This has its own complications in the translation to human subjects. Yet, once established as promising in the rat model, these food items can easily be tested in human subjects since many of those showing positive results are already approved for human

consumption. This will translate into a much faster intervention against the underlying mechanisms behind cardiovascular disease.

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APPENDIX A

STANDARD RODENT CHOW DIET

2018S

ENVIGO

Teklad Global 18% Protein Rodent Diet (Sterilizable)

Product Description- 2018S is a fixed formula, autoclavable diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. 2018S does not contain alfalfa, thus lowering the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 150 to 250 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. 2018S is supplemented with additional vitamins to ensure nutritional adequacy after autoclaving. **Also available certified (2018SC), the diet most commonly fed in Envigo Rodent Colonies.**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, kaolin, menadione sodium bisulfite complex (source of vitamin K activity), magnesium oxide, vitamin E acetate, calcium pantothenate, thiamin mononitrate, manganous oxide, niacin, ferrous sulfate, zinc oxide, riboflavin, vitamin A acetate, pyridoxine hydrochloride, copper sulfate, vitamin B_{12} supplement, folic acid, calcium iodate, biotin, vitamin D_3 supplement, cobalt carbonate.

Standard Product Form: Pellet

Macronutrients		
Crude Protein	%	18.6
Fat (ether extract) [a]	%	6.2
Carbohydrate (available) [b]	%	44.2
Crude Fiber	%	3.5
Neutral Detergent Fiber [c]	%	14.7
Ash	%	5.3
Energy Density [d]	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.4
Glutamic Acid	%	3.4
Alanine	%	1.1
Glycine	%	0.8
Threonine	%	0.7
Proline	%	1.6
Serine	%	1.1
Leucine	%	1.8
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.0
Tyrosine	%	0.6
Methionine	%	0.6
Cystine	%	0.3
Lysine	%	1.1
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Vitamins		
Vitamin A [e,f]	IU/g	30.0
Vitamin D_3 [e,g]	IU/g	2.0
Vitamin E	IU/kg	135
Vitamin K_3 (menadione)	mg/kg	100
Vitamin B_1 (thiamin)	mg/kg	117
Vitamin B_2 (riboflavin)	mg/kg	27
Niacin (nicotinic acid)	mg/kg	115
Vitamin B_5 (pyridoxine)	mg/kg	26
Pantothenic Acid	mg/kg	140
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.15
Biotin	mg/kg	0.90
Folate	mg/kg	9
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.2
C18:2ω6 Linoleic	%	3.1
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	0.9
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	3.4
Other		
Cholesterol	mg/kg	--

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

0915

APPENDIX B

HIGH FAT CHOW DIET – D12492 (BLUE)

OpenSource DIETS®

Report ▶ Repeat ▶ Revise

Product Data - DIO SERIES DIETS

The "Original" High-Fat Diets for Diet Induced Obesity

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

Research Diets, Inc. formulated the "original" high-fat diet for diet induced obesity (DIO) studies in 1996. Today, our high-fat diets are the research standard for DIO mice and rats worldwide.

DIO Low-Fat Control Diets

Matched, Purified Ingredient Diet

We recommend that you use a matched, purified ingredient diet and not a grain-based 'chow' diet. There are many, many differences between purified diets and chow diets and these variables make it difficult to interpret your data from a study in which one group was fed a purified ingredient high-fat and the other a low-fat chow diet. Differences between your groups could be due to the level of fat, but could also be due to differences in fiber type and level, source of carbohydrate, and the presence or absence of plant chemicals (such as phytoestrogens), just to name a few.

See next page for low-fat control formulas.

(DIO) Formulas				
Product #	D12451		D12492	
	gm%	kcal%	gm%	kcal%
Protein	24	20	26	20
Carbohydrate	41	35	26	20
Fat	24	45	35	60
Total		100		100
kcal/gm	4.73		5.24	
Ingredient	gm	kcal	gm	kcal
Casein, 30 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	72.8	291	0	0
Maltodextrin 10	100	400	125	500
Sucrose	172.8	691	68.8	275
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	177.5	1598	245	2205
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Red Dye #40	0.05	0		
FD&C Blue Dye #1			0.05	0
Total	858.15	4057	773.85	4057

*Typical analysis of cholesterol in lard = 72 mg per 100 gram.
D12451 -
Cholesterol (mg)/4057 kcal = 167.8
Cholesterol (mg)/kg = 195.5
D12492 -
Cholesterol (mg)/4057 kcal = 216.4
Cholesterol (mg)/kg = 279.6

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APPENDIX C

PROTOCOL RAT TNF ALPHA ELISA KIT

INSTRUCTIONS

Thermo SCIENTIFIC

Rat TNFα ELISA Kit

ER3TNFA ER3TNFA2 ER3TNFA5

1482.5

Number	Description
ER3TNFA	**Rat Tumor Necrosis Factor Alpha (TNFα) ELISA,** sufficient reagents for 96 determinations
ER3TNFA2	**Rat Tumor Necrosis Factor Alpha (TNFα) ELISA,** sufficient reagents for 2 × 96 determinations
ER3TNFA5	**Rat Tumor Necrosis Factor Alpha (TNFα) ELISA,** sufficient reagents for 5 × 96 determinations

Kit Contents	ER3TNFA	ER3TNFA2	ER3TNFA5
Anti-Rat TNFα Pre-coated 96-well Strip Plate	1 each	2 each	5 each
Lyophilized Recombinant Rat TNFα Standard	2 vials	4 vials	10 vials
Standard Diluent	25mL	2 × 25mL	5 × 25mL
Pre-Treatment Buffer	6mL	2 × 6mL	5 × 6mL
Biotinylated Antibody Reagent	6mL	2 × 6mL	5 × 6mL
Streptavidin-HRP Reagent	14mL	2 × 14mL	5 × 14mL
30X Wash Buffer	50mL	2 × 50mL	5 × 50mL
TMB Substrate	13mL	2 × 13mL	5 × 13mL
Stop Solution, contains 0.16M sulfuric acid	13mL	2 × 13mL	5 × 13mL
Adhesive plate sealers	6 each	12 each	30 each

For research use only – not for use in diagnostic procedures.

Storage: For maximum stability, store in a non-defrosting -20°C freezer and refer to the expiration date for frozen storage on the label. Alternatively, store at 2-8°C and refer to the expiration date for refrigerated storage. Once thawed, store at 4°C until the expiration date for refrigerated storage. Kit is shipped on dry ice.

Table of Contents

Introduction

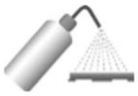
The Thermo Scientific Rat TNFα ELISA Kit is for measuring rat TNFα in serum, EDTA plasma and culture supernatant.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce

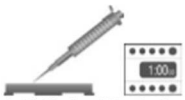
Procedure Summary

1. Add 50µL of Pre-Treatment Buffer to each well.

2. Add 50µL of Standards or samples to each well in duplicate.* Cover plate and incubate at room temperature (20-25°C) for 1 hour.



3. Wash plate THREE times.



4. Add 50µL of Biotinylated Antibody Reagent to each well. Cover plate and incubate at room temperature for 1 hour.

5. Wash plate THREE times.

6. Add 100µL of Streptavidin-HRP Reagent to each well.

7. Cover and incubate plate at room temperature for 30 minutes.

8. Wash plate THREE times.

9. Add 100µL of TMB Substrate to each well.

10. Develop plate in the dark at room temperature for 10 minutes.

11. Stop reaction by adding 100µL of Stop Solution to each well.

12. Measure the absorbance on a plate reader at 450nm minus 550nm. Calculate results.

* Always dilute serum and EDTA plasma samples 1:1 before testing.

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-1000µL and plastic pipettes to deliver 5-15mL
- A glass or plastic 2L container to prepare Wash Buffer
- A squirt wash bottle or an automated 96-well plate washer
- 1.5mL polypropylene or polyethylene tubes to prepare standards – do not use polystyrene, polycarbonate or glass tubes
- Disposable reagent reservoirs
- A standard ELISA reader for measuring absorbance at 450nm and 550nm. If a 550nm filter is not available, the absorbance can be measured at 450nm only. Refer to the instruction manual supplied with the instrument being used.
- Graph paper or a computerized curve-fitting statistical software package

Precautions

- **All samples and reagents must be at room temperature (20-25°C) before use in the assay.**
- Review these instructions carefully and verify all components against the Kit Contents list (page 1) before beginning.
- Do not use a water bath to thaw samples. Thaw samples at room temperature.
- When assaying culture medium, prepare the standard curve and sample dilutions using the same medium used to culture cells. For example, if RPMI with 10% fetal calf serum (FCS) was used to culture cells, then use RPMI with 10% FCS to dilute standard and samples. For best results, use a culture medium that contains a carrier protein such as FCS. Lack of a carrier protein in the media or addition of other compounds may compromise assay results. If alternative media must be

used, prepare two standard curves: one with Standard Diluent and one with the alternative media. If the two curves have OD values within 10% of the mean for both curves, then perform the assay with Standard Diluent. If the OD values of the two curves are not within 10% of the mean for both curves, then perform the assay with the alternative media. If the alternative media has significant effects on the assay, consider using different media.

- To avoid cross-contamination, always use a new disposable reagent reservoir and new disposable pipette tips for each transfer. Also use a new adhesive plate cover for each incubation step.
- Once reagents have been added to the plate, DO NOT let the plate DRY at any time during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can become contaminated thereby causing assay variability.
- Vigorous plate washing is essential.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- When completed with kit, discard unused ELISA components. Do not mix reagents from different kit lots.
- Do not use glass pipettes to measure TMB Substrate Solution. Take care not to contaminate the solution. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain antibiotics and preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.

Additional Precautions for the 2- and 5-plate Kits

- Dispense, pool and equilibrate to room temperature only the reagent volumes required for the number of plates being used. Do not combine leftover reagents with those reserved for additional plates.

Sample Preparation

Sample Handling

- Serum, EDTA plasma and culture supernatants may be tested in this assay.
- For each well, 50µL of EDTA plasma, culture supernatant or 1:1 diluted serum is required.
- Store samples to be assayed within 2 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C.
- Avoid repeated freeze-thaw cycles when storing samples.
- Test samples and standards must be assayed in duplicate each time the assay is performed.
- Gradually equilibrate samples to room temperature before beginning assay. Do not use a heated water bath to thaw or warm samples.
- Mix samples by gently inverting tubes.
- If samples are clotted, grossly hemolyzed, lipemic or microbially contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret results with caution.

Sample Dilution

- **Serum and EDTA plasma samples must first be diluted 1:1 before testing.** To prepare a 1:1 dilution, add 100µL of sample to 100µL of Standard Diluent in a separate tube and mix well. Alternatively, after adding the Pre-Treatment Buffer to the wells add 25µL of Standard Diluent to the appropriate sample wells and then add 25µL of sample. Tap the plate gently to mix. Either method produces a final 1:1 dilution of the sample in each well.
- If the rat TNFα concentration possibly exceeds the highest point of the standard curve (i.e., 2500pg/mL), prepare one or more five-fold dilutions of the test sample. When testing **culture supernatants**, prepare the serial dilutions using culture medium (see Precautions Section). When testing **serum or plasma**, prepare the serial dilution using the Standard Diluent provided. For example, prepare a five-fold dilution by adding 50µL of test sample to 200µL of appropriate diluent. Mix thoroughly between dilutions before assaying.

Reagent Preparation

For procedural differences when using partial plates, look for **(PP)** throughout this instruction booklet.

Wash Buffer

- **(PP)** When using partial plates, store the Wash Buffer at 2-8°C.
- **Note:** Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer that has become visibly contaminated during storage.

1. Label a clean glass or plastic 2L container "Wash Buffer." The 30X Wash Buffer may have a cloudy appearance.
2. Add the entire contents of the 30X Wash Buffer (50mL) bottle to the 2L container and dilute to a final volume of 1.5L with ultrapure water. Mix thoroughly.

Standards

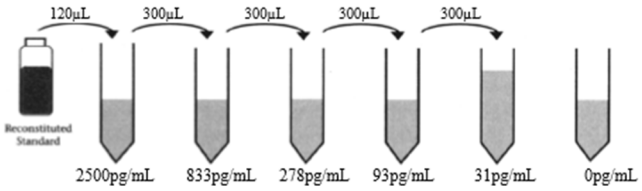
- **(PP)** Reconstitute and use one vial of the lyophilized Standard per partial plate.
- Prepare Standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.

1. For **culture supernatant** samples, reconstitute standard with ultrapure water to the volume indicated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the sample culture medium to prepare Standard Curve dilutions (see Precautions Section).

   For **serum** or **plasma** samples, reconstitute standard with ultrapure water to the volume indicated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the Standard Diluent provided to prepare standard curve serial dilutions.

   When testing **serum** or **plasma** and **cell culture supernatant** samples on the same plate, validate the media to establish if the same standard curve can be used for both sample types. Prepare a standard curve (including a zero) using culture medium to dilute the standard. Use medium containing serum or other protein to maximize stability of the rat TNFα. Perform this curve in parallel with a standard curve prepared with Standard Diluent. If the OD values of the two curves are within 10% of the mean for both curves, then perform the assay with Standard Diluent, whether testing culture supernatant, plasma or serum samples.
2. Label six tubes, one for each standard curve point: 2500, 833, 278, 93, 31, and 0pg/mL. Prepare an initial 1:6 dilution followed by 1:3 serial dilutions for the standard curve as follows:
3. Pipette 600µL of appropriate diluent into each tube.
4. Pipette 120µL of the reconstituted standard into the first tube (i.e., 2500pg/mL) and mix.
5. Pipette 300µL of this dilution into the second tube (i.e., 833pg/mL) and mix.
6. Repeat serial dilutions (using 300µL) three more times to complete the standard curve points.

Standard Dilutions Schematic



Assay Procedure

A. Sample Incubation

- **(PP)** Determine the number of strips required. Leave these strips in the plate frame. Tightly seal the remaining unused strips in the foil pouch with the desiccant provided and store at 2-8°C. After completing the assay, retain the plate frame for the second partial plate. When using the second partial plate, place the reserved strips securely in the plate frame.
- Use the Data Template provided to record locations of the zero standard, rat TNFα standards and test samples. Perform five standard points and one blank in duplicate with each series of unknown samples.

1. Add 50µL of the Pre-Treatment buffer to each well.
2. Add 50µL of the reconstituted standard or diluted sample to each well in duplicate. Mix by gently tapping the plate several times.

   Note: All serum and EDTA plasma samples must be diluted 1:1 before testing (see the Sample Dilution Section). If the TNFα concentration in any test sample is expected to exceed the highest point on the standard curve (i.e., 2500pg/mL) refer to the Sample Dilution section.
3. Add 50µL of Standard Diluent to all wells that do not contain standards or samples.
4. Carefully cover plate with an adhesive plate cover. Ensure that all edges and strips are sealed tightly by running your thumb over the edges and down each strip. Incubate for one (1) hour at room temperature (i.e., 20-25°C).
5. Carefully remove the adhesive plate cover. Wash plate THREE times with Wash Buffer using the procedure described in the Plate Washing Section (Section B).

B. Plate Washing

Note: Automated plate washers may produce sub-optimal results. For best results, perform a manual wash the first time the assay is performed. Automated plate washing may require validation to determine the number of washes necessary to achieve optimal results; typically, 3-5 washes are sufficient.

1. Gently squeeze the long sides of plate frame before washing to ensure all strips securely remain in the frame.
2. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of THREE washes. Blot plate onto paper towels or other absorbent material.

   Note: For automated washing, aspirate all wells and wash with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material. The number of washes necessary may require optimization.

C. Biotinylated Antibody Reagent Incubation

- If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the Biotinylated Antibody Reagent.

1. Add 50µL of Biotinylated Antibody Reagent to each well.
2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 1 hour at room temperature.
3. Carefully remove the adhesive plate cover, discard plate contents and wash THREE times as described in the Plate Washing Section.

D. Streptavidin-HRP Reagent Incubation

- If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the Streptavidin-HRP Reagent.

1. Add 100µL of Streptavidin-HRP Reagent to each well.
2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 30 minutes at room temperature.

3. Carefully remove the adhesive plate cover, discard plate contents and wash THREE times as described in the Plate Washing Section (section B).

E. Substrate Incubation and Stop Step

- Use new disposable reagent reservoirs when adding TMB Substrate Solution and Stop Solution.
- Dispense from bottle ONLY the amount required, 100µL per well, for the number of wells being used. Do not use a glass pipette to measure the TMB Substrate Solution.
- **(PP)** Do not combine leftover substrate with that reserved for the second partial plate. Take care not to contaminate remaining TMB Substrate Solution.
- If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the TMB Substrate Solution and when adding the Stop Solution.

1. Pipette 100µL of TMB Substrate Solution into each well.
2. Allow the color reaction to develop at room temperature in the dark for 10 minutes. Do not cover plate with a plate sealer. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
3. After 10 minutes, stop the reaction by adding 100µL of Stop Solution to each well.

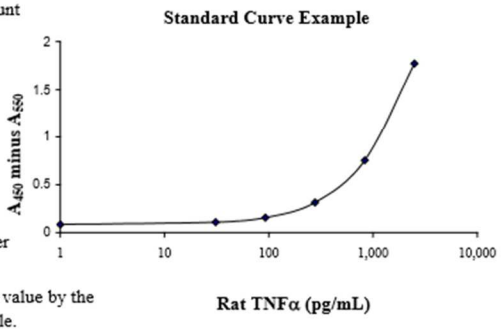
F. Absorbance Measurement

Note: Evaluate the plate within 30 minutes of stopping the reaction.

Measure absorbance on an ELISA plate reader set at 450nm and 550nm. Subtract 550nm values from 450nm values to correct for optical imperfections in the microplate. If an absorbance at 550nm is not available, measure the absorbance at 450nm only. When the 550nm measurement is omitted, absorbance values will be higher.

G. Calculation of Results

- The standard curve is used to determine rat TNFα amount in an unknown sample. Generate the standard curve by plotting the average absorbance obtained for each Standard concentration on the vertical (Y) axis vs. the corresponding TNFα concentration (pg/mL) on the horizontal (X) axis.
- Calculate results using graph paper or curve-fitting statistical software. Determine the TNFα amount in each sample by interpolating from the absorbance value (Y-axis) to TNFα concentration (X-axis) using the standard curve. For best results, use a four-parameter curve fit.



- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate pg/mL of TNFα in the sample.
- Absorbance values obtained for duplicates should be within 10% of the mean value. Carefully consider duplicate values that differ from the mean by greater than 10%.

Performance Characteristics

Sensitivity: < 15pg/mL
The sensitivity or lower limit of detection (LLD)[1] was determined by assaying 24 replicates of zero and the standard curve. The mean signal of zero + 2 standard deviations read in dose from the standard curve is the LLD.

Assay Range: 31-2500pg/mL
Suggested standard curve points are 2500, 833, 278, 93, 31, and 0pg/mL.

Specificity: The antibodies utilized in this ELISA are specific for the measurement of natural and recombinant rat TNFα. They do not cross-react with human TNFα, human TNFβ or porcine TNFα. Mouse TNFα cross-reacted weakly (< 3%).

Calibration: The standard in this ELISA is calibrated to an internal rat TNFα reference standard.

Precision/Reproducibility:

Intra-assay CV: < 10% (Table 1)
Inter-assay CV: < 10% (Table 2)

Table 1. Intra-assay results using the rat TNFα ELISA kit.

Mean (pg/mL)	%CV	n
2022	3.2	24
912	4.2	24
148	4.0	24

Table 2. Inter-assay results using the rat TNFα ELISA kit.

Mean (pg/mL)	%CV	n
2021	2.9	4
898	3.8	4
142	4.7	4

Recovery: Cytokine recovery was determined by spiking recombinant rat TNFα into serum and plasma samples collected from apparently healthy rats and a Standard Diluent control buffer (Table 3).

Table 3. Spike and recovery results using the rat TNFα ELISA Kit.

	Mean Percent Recovery	
Spike Level	Serum	Plasma
High	102	118
Medium	78	95
Low	75	84
Mean	**85**	**99**
Range	**73-105**	**68-119**

Cited Reference

1. *Immunoassay: A Practical Guide*, ed. Chan and Perlstein, Eds. (1987). Academic Press: New York, p.71.

APPENDIX D

PROTOCOL SUPEROXIDE DISMUTASE ACTIVITY ASSAY KIT

Superoxide Dismutase Assay Kit

Item No. 706002

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 Well Quantity/Size	480 Well Quantity/Size
706001	Assay Buffer (10X)	1 vial/5 ml	1 vial/20 ml
706003	Sample Buffer (10X)	1 vial/5 ml	1 vial/10 ml
706004	Radical Detector	1 vial/250 µl	2 vials/250 µl
706005	SOD Standard	1 vial/100 µl	2 vials/100 µl
706006	Xanthine Oxidase	3 vials/150 µl	6 vials/150 µl
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	5 plates
400012	96-Well Cover Sheet	1 cover	5 covers

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888
Fax: 734-971-3641
Email: techserv@caymanchem.com
Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring an absorbance at 440-460 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism.[1]

$$2O_2^{\bullet-} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$$

Three types of SODs have been characterized according to their metal content: copper/zinc (Cu/Zn), manganese (Mn), and iron (Fe). SOD is widely distributed in both plants and animals. It occurs in high concentrations in brain, liver, heart, erythrocytes, and kidney. In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial MnSOD, and extracellular SOD.[2] Extracellular SOD is found in the interstitial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph, and synovial fluid.[3,4]

The amount of SOD present in cellular and extracellular environments is crucial for the prevention of diseases linked to oxidative stress. Mutations in SOD account for approximately 20% of familial amyotrophic lateral sclerosis (ALS) cases. SOD also appears to be important in the prevention of other neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases.[5,6] The reaction catalyzed by SOD is extremely fast, having a turnover of 2×10^9 $M^{-1}sec^{-1}$ and the presence of sufficient amounts of the enzyme in cells and tissues typically keeps the concentration of superoxide ($O_2^{\bullet-}$) very low.[1] However, in a competing reaction, nitric oxide (NO) reacts with $O_2^{\bullet-}$ with a rate constant of 6.7×10^9 $M^{-1}sec^{-1}$ to form the powerful oxidizing and nitrating agent, peroxynitrite.[7] Under conditions in which SOD activity is low or absent (*i.e.*, SOD mutation) or which favor the synthesis of µM concentrations of NO (*i.e.*, ischemia/reperfusion, iNOS upregulation, etc.), NO out-competes SOD for superoxide, resulting in the formation of peroxynitrite. The presence of nitrotyrosine as a "footprint" for peroxynitrite, and hence the prior co-existence of both $O_2^{\bullet-}$ and NO, has been observed in a variety of medical conditions, including atherosclerosis, sepsis, and ALS.[7]

About This Assay

Cayman's Superoxide Dismutase Assay Kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (see scheme 1, below). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD). The assay provides a simple, reproducible, and fast tool for assaying SOD activity in plasma, serum, erythrocyte lysates, tissue homogenates, and cell lysates. Mitochondrial MnSOD can be assayed separately following the procedure outlined under Sample Preparation (see page 8).

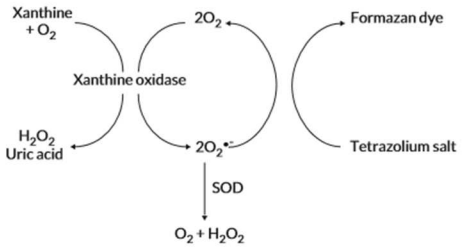

Figure 1. Scheme of the Superoxide Dismutase Assay

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Assay Buffer (10X) - (Item No. 706001)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water for assaying 96 wells. Prepare additional Assay Buffer as needed. This final Assay Buffer (50 mM Tris-HCl, pH 8.0, containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 0.1 mM hypoxanthine) should be used to dilute the radical detector. When stored at 4°C, this diluted Assay Buffer is stable for at least two months.

2. **Sample Buffer (10X) - (Item No. 706003)**

   Dilute 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water for assaying 96 wells. Prepare additional Sample Buffer as needed. This final Sample Buffer (50 mM Tris-HCl, pH 8.0) should be used to prepare the SOD standards and dilute the xanthine oxidase and SOD samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least six months.

3. **Radical Detector - (Item No. 706004)**

   The vials contain 250 µl of a tetrazolium salt solution. Prior to use, transfer 50 µl of the supplied solution to another vial and dilute with 19.95 ml of diluted Assay Buffer. Cover with tin foil. The diluted Radical Detector is stable for two hours. This is enough Radical Detector for 96 wells. Prepare additional detector as needed. Store unused Radical Detector at -20°C.

4. **SOD Standard - (Item No. 706005)**

   The vials contain 100 µl of bovine erythrocyte SOD (Cu/Zn). Store the thawed enzyme on ice and see Standard Preparation on page 14 for preparing the standard curve. Store unused enzyme at -20°C. The enzyme is stable for at least two freeze/thaw cycles.

5. **Xanthine Oxidase - (Item No. 706006)**

   These vials contain 150 µl of Xanthine Oxidase. Prior to use, thaw one vial and transfer 50 µl of the supplied enzyme to another vial and dilute with 1.95 ml of Sample Buffer (dilute). Store the thawed and diluted xanthine oxidase on ice. The diluted enzyme is stable for one hour. This is enough Xanthine Oxidase for 96 wells. Prepare additional Xanthine Oxidase as needed. Do not refreeze the thawed enzyme. Any unused enzyme should be thrown away.

Sample Preparation

The procedures listed below for tissue homogenates and cell lysates will result in assaying total SOD activity (cytosolic and mitochondrial). To separate the two enzymes, centrifuge the 1,500 x g supernatant at 10,000 x g for 15 minutes at 4°C. The resulting 10,000 x g supernatant will contain cytosolic SOD and the pellet will contain mitochondrial SOD.[8] Homogenize the mitochondrial pellet in cold buffer (*i.e.*, 20 mM Hepes, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). If not assaying on the same day, freeze the samples at -80°C. The samples will be stable for at least one month.

The addition of potassium cyanide to a final concentration of 1-3 mM in the assay will inhibit both the Cu/Zn-SOD and extracellular SOD, resulting in the detection of only Mn-SOD activity.[3,9]

Samples can be assayed in the absence of Xanthine Oxidase to generate a sample background. This sample background absorbance should be subtracted from the sample absorbance generated in the presence of Xanthine Oxidase thus correcting for non-SOD generated absorbance.

Tissue Homogenate

1. Prior to dissection, either perfuse or rinse tissue with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose per gram tissue.
3. Centrifuge at 1,500 x g for five minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation at 1,000-2,000 x g for 10 minutes at 4°C. For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Homogenize or sonicate the cell pellet in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose.
3. Centrifuge at 1,500 x g for five minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month. Plasma should be diluted 1:5 with Sample Buffer before assaying for SOD activity.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in four times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month. The erythrocyte lysate should be diluted 1:100 with Sample Buffer before assaying for SOD activity.

Serum

1. Collect blood without using an anticoagulant such as heparin, citrate, or EDTA. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.
3. Serum should be diluted 1:5 with Sample Buffer before assaying for SOD activity.

Tissue Homogenization using the Precellys 24 Homogenizer

- Prior to dissection, either perfuse or rinse tissue with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots.
- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of issues in liquid nitrogen is preferred.
- Add cold 20 mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210 mM mannitol, and 70mM sucrose.
- Homogenize the tissue sample using the Precellys 24 according to appropriate settings:
- Spin the tissue homogenates at 10,000 x g for 15 minutes at 4°C.
- Collect supernatant and assay samples according to the kit booklet protocol. Samples may need to be diluted appropriately for assay and should be normalized using a protein assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of SOD standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
B	B	B	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
C	C	C	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
D	D	D	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
E	E	E	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
F	F	F	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
G	G	G	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
H	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41

A-G = Standards
S1-S41 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 230 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 25°C.
- All reagents except samples and Xanthine Oxidase must be equilibrated to room temperature before beginning the assay.
- It is recommended that the samples and SOD standards be assayed at least in duplicate.
- Monitor the absorbance between 440-460 nm using a plate reader.

Standard Preparation

Dilute 20 µl of the SOD Standard (Item No. 706005) with 1.98 ml of Sample Buffer (dilute) to obtain the SOD stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of SOD stock and Sample Buffer (dilute) to each tube as described in Table 1 below.

Tube	SOD Stock (µl)	Sample Buffer (µl)	Final SOD Activity (U/ml)
A	0	1,000	0
B	20	980	0.005
C	40	960	0.010
D	80	920	0.020
E	120	880	0.030
F	160	840	0.040
G	200	800	0.050

Table 1. Superoxide Dismutase standards

Performing the Assay

1. **SOD Standard Wells** - add 200 µl of the diluted Radical Detector and 10 µl of Standard (tubes A-G) per well in the designated wells on the plate (see **Sample plate format**, Figure 2, page 12).
2. **Sample Wells** - add 200 µl of the diluted Radical Detector and 10 µl of sample to the wells. *NOTE: If using an inhibitor, add 190 µl of the diluted Radical Detector, 10 µl of inhibitor, and 10 µl of sample to the wells. The amount of sample added to the well should always be 10 µl. Samples should be diluted with Sample Buffer (dilute) or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to fall within the standard curve range.*
3. Initiate the reactions by adding 20 µl of diluted Xanthine Oxidase to all the wells you are using. Make sure to note the precise time you started and add the Xanthine Oxidase as quickly as possible. *NOTE: If assaying sample backgrounds, add 20 µl of Sample Buffer instead of Xanthine Oxidase.*
4. Carefully shake the 96-well plate for a few seconds to mix. Cover with the plate cover.
5. Incubate the plate on a shaker for 30 minutes at room temperature. Read the absorbance at 440-460 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample. If assayed, subtract sample background absorbance from the sample.
2. Divide standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbances to yield the linearized rate (LR) (*i.e.*, LR for Std A **=** Abs Std A/Abs Std A; LR for Std B **=** Abs Std A/Abs Std B).
3. Plot the linearized SOD standard rate (LR) (from step 2 above) as a function of final SOD Activity (U/ml) from Table 1. See Figure 3 (on page 18) for a typical standard curve.
4. Calculate the SOD activity of the samples using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity is standardized using the cytochrome c and xanthine oxidase coupled assay.

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{sample LR - y-intercept}}{\text{slope}}\right) \times \frac{0.23\text{ ml}}{0.01\text{ ml}}\right] \times \text{sample dilution}$$

Performance Characteristics

Precision:

When a series of 60 SOD standard measurements were performed on the same day, the intra-assay coefficient of variation was 3.2%. When a series of 60 SOD standard measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.7%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0.005-0.050 units/ml SOD.

Representative Superoxide Dismutase Standard Curve

The standard curve presented here is an example of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use this one to determine the values of your samples.

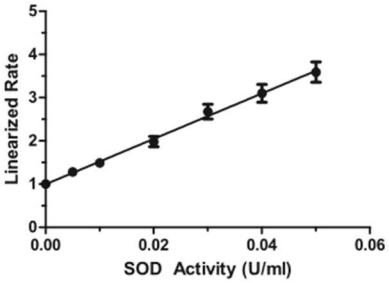

Figure 3. Superoxide Dismutase standard curve

RESOURCES

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris	No
	Borate	Yes
	HEPES	No
	Phosphate	No
Detergents:	SDS (0.1%)	Yes
	Triton X-100 (≤0.1%)	No
	Polysorbate 20 (0.1%)	Yes
	CHAPS (0.1%)	Yes
Proteases/Inhibitors/ Chelators:	Antipain (0.1 mg/ml)	Yes
	PMSF (1 mM)	Yes
	Leupeptin (1 mg/ml)	Yes
	Trypsin (0.1 mg/ml)	Yes
	Chymostatin (0.1 mg/ml)	Yes
	EGTA (≤1 mM)	No
	EDTA (≤1 mM)	No
Solvents:	Ethanol (10 µl)	No
	Methanol (10 µl)	Yes
	Dimethyl Sulfoxide (10 µl)	Yes
Others:	Glutathione (≤1.5 mM)	No
	Glycerol (≤1%)	No
	BSA (≤1%)	No
	Dithiothreitol (3 mM)	Yes

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No activity was detected in the sample	SOD activity was too low or sample was too dilute	Concentrate the samples using an Amicon concentrator with a molecular weight cut-off of 10,000 and re-assay
No color development in any of the wells	The radical detector was not added to the diluted Assay Buffer	Dilute the radical detector with diluted Assay Buffer and re-assay

References

1. Malstrom, B., Andreasson, L., and Reinhammer, B.*in* The Enzymes. Boyer, P., editor. XIIB, Academic Press, New York, **533** (1975).
2. Sandstrom, J., Nilsson, P., Karlsson, K., *et al.* 10-Fold increase in human plasma extracellular superoxide dismutase content caused by a mutation in heparin-binding domain. *J. Biol. Chem.* **269(29),** 19163-19166 (1994).
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6. Liu, D. The roles of free radicals in amyotrophic lateral sclerosis. *J. Mol. Neuro.* **7**, 159-167 (1996).
7. Beckman, J.S. and Koppenol, W.H. Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol.* **271**, C1424-C1437 (1996).
8. Mattiazz, M., D'Aurelio, M., Gajewski, C.D., *et al.* Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *J. Biol. Chem.* **277(33),** 29626-29633 (2002).
9. MacMillan-Crow, L.A., Crow, J.P., Kerby, J.D., *et al.* Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. USA* **93**, 11853-11858 (1996).

APPENDIX E

PROTOCOL CHROMOGENIC ENDOTOXIN QUANTITATION KIT

INSTRUCTIONS

Pierce LAL Chromogenic Endotoxin Quantitation Kit

Pub. No. MAN0016039
Rev B.0
Pub. Part No. 2162445

88282

Number	Description
88282	**Pierce LAL Chromogenic Endotoxin Quantitation Kit,** sufficient reagents to perform 50 assays of standards and samples in a microplate (i.e., 50 wells total)
	Kit Contents:
	***Escherichia coli* (*E. coli*) Endotoxin Standard (011:B4),** lyophilized, 1 vial, 15-40 endotoxin units (EU)/mL upon reconstitution
	Limulus Amebocyte Lysate (LAL), lyophilized, 2 vials, 1.4mL/vial upon reconstitution
	Chromogenic Substrate, lyophilized, 1 vial, 6.5mL/vial upon reconstitution
	Endotoxin-free Water, 1 vial, 30mL
	Storage: Upon receipt store at 4°C. Product shipped with an ice pack.

Introduction

The Thermo Scientific™ Pierce™ LAL Chromogenic Endotoxin Quantitation Kit is an efficient, quantitative endpoint assay for the detection of gram-negative bacterial endotoxins. Bacterial endotoxin catalyzes the activation of a proenzyme in the modified Limulus Amebocyte Lysate (LAL). The activated proenzyme then catalyzes the splitting of *p*-Nitroaniline (pNA) from the colorless substrate, Ac-Ile-Glu-Ala-Arg-pNA; the activation rate is proportional to the sample endotoxin concentration. After stopping the reaction, the released pNA is photometrically measured at 405-410nm. The correlation between absorbance and endotoxin concentration is linear in the 0.1-1.0EU/mL range. The developed color intensity is proportional to the amount of endotoxin present in the sample and can be calculated using a standard curve.

Important Product Information

- Accurate pipetting is critical for maintaining consistent results. A repetitive pipettor can aid in normalizing volumes between samples. Ensure pipetting order and rate of reagent addition remain consistent from well to well and row to row.
- All materials (e.g., pipette tips, glass tubes, microcentrifuge tubes and disposable 96-well microplates) must be endotoxin-free.
- Adjust the sample pH to 6-8 using endotoxin-free 0.1M NaOH or 0.1M HCl. Avoid pH-electrode contamination of the sample by testing the pH of a small sample taken from the bulk sample.
- Components of undiluted serum interfere in the assay. Serum samples must be diluted 50- to 100-fold to be compatible. The serum must be completely free of RBCs, and the diluted sample may need to be heat-shocked (70°C for 15 minutes).
- To stop all bacteriological activity, store samples to be tested at 2-8°C for <24 hours or -20°C for >24 hours.
- Maintaining the correct temperature is critical for reproducibility. Use a proper heating block at 37°C±1°C. Do not use a cabinet-style incubator to perform the assay.
- Endotoxin adheres to glass and plastic surfaces; before pipetting, vortex solutions to ensure the correct endotoxin concentrations are measured.
- Glass tubes are preferred for making standard stock solutions; however, polystyrene or polypropylene microcentrifuge tubes (1.5mL) may also be used. When using microcentrifuge tubes, dedicate the bag of tubes for the assay and follow aseptic techniques.
- If the test sample endotoxin concentration is >1.0EU/mL, dilute the sample five-fold in endotoxin-free water. Re-test.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | Thermofisher.com

- Assay inhibition occurs when substances in the test sample interfere with the LAL reaction. In the chromogenic assay, this inhibition results in a lower final absorbance, indicating lower levels of endotoxin than what may be present in the test sample. Determine the lack of product inhibition for each sample undiluted or at an appropriate dilution. See the Additional Information Section for more details.
- Some LAL-reactive glucans (LRGs) may result in false positive signal in the LAL assay.[1] In samples where LAL-reactive glucans may be present, researchers may want to compare cellulase-treated samples to untreated samples to determine contribution of signal resulting from LRGs.
- Samples turning yellow after addition of the Stop Reagent (25% acetic acid) or possessing significant initial color may require special attention. See the Additional Information Section for more details.
- The kit reagents are "matched" to comply with Food and Drug Administration (FDA) requirements for endotoxin testing. Each LAL lot is tested for functionality using the United States Reference Standard EC-6. The LAL lot is then matched to a lot of our Control Standard Endotoxin (CSE) by testing in parallel with the Reference Standard Endotoxin (RSE). The RSE/CSE correlation assay determines the potency of each CSE lot when used with each matching LAL lot.

Additional Materials Required

- Disposable endotoxin-free glass tubes or 1.5mL microcentrifuge tubes
- Disposable endotoxin-free pipette tips
- Disposable endotoxin-free 96-well microplates
- Heating block at 37°C±1°C
- Pipettor
- Repetitive pipettor (optional)
- Microplate reader
- 25% acetic acid (Stop Reagent)

Material Preparation

Note: Equilibrate all reagents to room temperature before use.

A. Endotoxin Standard Stock Solutions

1. Each *E.coli* Endotoxin Standard vial contains ~15-40EU of lyophilized endotoxin; the actual vial concentration is printed on the label. Reconstitute by adding 1mL of room temperature endotoxin-free water to make Endotoxin Standard Stock. For example, a vial with a concentration of 26EU, when reconstituted with 1.0mL of endotoxin-free water, will yield a concentration of 26EU/mL. Vortex the solution vigorously for at least 15 minutes on a high speed vortex mixer before use.

   Note: Store lyophilized *E.coli* Endotoxin Standard at 2-8°C. Reconstituted stock solution is stable for 4 weeks at 2-8°C. Prior to subsequent use, warm the solution to room temperature and vigorously mix for 15 minutes. (This is important since the endotoxin adheres to sides of the glass vial.)
2. Prepare Standard Stock Solutions from the Endotoxin Standard Stock using the dilutions and procedures in Table 1.

Table 1. Dilutions and procedures for preparing Standard Stock Solutions.

Vial	Volume of Endotoxin Standard Stock (mL)	Volume of vial A (mL)	Endotoxin-free Water (mL)	Final endotoxin concentration (EU/mL)
A	0.05	–	(X-1)/20*	1.00
B	–	0.25	0.25	0.50
C	–	0.25	0.75	0.25
D	–	0.10	0.90	0.10

*X = endotoxin concentration of the *E.coli* Endotoxin Standard supplied with the kit; refer to the Certificate of Analysis to get the lot-specific concentration.

A. Prepare a solution containing 1.0EU/mL of endotoxin standard by diluting 0.05mL of the Endotoxin Standard Stock with [(X-1)/20]mL of endotoxin-free water, where X equals the endotoxin concentration of the vial (e.g., if X= 26EU/mL, then dilute 0.05mL of this stock with (26-1)/20, or 1.25mL, of endotoxin-free water). **Vigorously vortex the solution for ≥1 minute before proceeding**.

B. Transfer 0.25mL of the 1.0EU/mL Standard vial A into a tube containing 0.25mL of endotoxin-free water to prepare 0.5EU/mL Standard Stock Solution and **vortex the solution vigorously for 1 minute before use.**

C. Transfer 0.25mL of 1.0EU/mL Standard vial A into a tube containing 0.75mL of endotoxin-free water to prepare 0.25EU/mL Standard Stock Solution and **vortex the solution vigorously for 1 minute before use.**

D. Transfer 0.1mL of 1.0EU/mL Standard vial A into a tube containing 0.90mL of endotoxin-free water to prepare 0.1EU/mL Standard Stock Solution and **vortex the solution vigorously for 1 minute before use.**

B. Limulus Amebocyte Lysate (LAL)

1. The LAL reagent contains lyophilized lysate prepared from the circulating amebocytes of the horseshoe crab *Limulus polyphemus*. **Reconstitute immediately before use** with 1.4mL of endotoxin-free water and swirl gently to dissolve the powder. If more than one vial is required, pool two or more vials before use. **Avoid foaming; do not vortex the solution**.

   Note: Store lyophilized LAL protected from light at 2-8°C. Reconstituted LAL reagent is stable for 1 week at -20°C or colder if frozen immediately after reconstitution. Upon thawing, the reconstituted LAL may be used only one time; once thawed, gently swirl the reagent to mix before adding to samples.

C. Chromogenic Substrate

1. Each vial contains ~7mg of lyophilized substrate. Reconstitute the Chromogenic Substrate by adding 6.5mL of endotoxin-free water to yield a final concentration of ~2mM.

   Note: Store lyophilized Chromogenic Substrate protected from light at 2-8°C. Reconstituted Chromogenic Substrate is stable for 4 weeks when stored at 2-8°C. **Prior to use, warm up sufficient substrate solution for the assay to 37°C±1°C.**

Microplate Assay Procedure

Note: Equilibrate all reagents to room temperature before use.

1. Pre-equilibrate the microplate in a heating block for 10 minutes at 37°C±1°C.

   Note: Do not use cabinet-style incubator to perform the test.

2. With the microplate maintained at 37°C±1°C, carefully dispense 50µL of each standard or unknown sample replicate into the appropriate microplate well.

   Note: Each series of determinations must include duplicate runs of a blank and the four endotoxin standards; the blank contains 50µL of endotoxin-free water. If reaction inhibition is possible, see the Additional Information Section.

3. At time T=0, add 50µL of LAL reagent to each well using a pipettor. Begin timing as the LAL is added. Once the LAL has been added into all plate wells, briefly remove from the heating block and gently tap several times to facilitate mixing. Cover the plate with the lid and return to heating block to incubate at 37°C±1°C for 10 minutes.

   Note: Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row.

4. After exactly T=10 minutes, add 100µL of Chromogenic Substrate solution (prewarmed to 37°C±1°C) to each well. Once the substrate solution has been added into all plate wells, briefly remove from the heating block and gently tap several times to facilitate mixing. Cover the plate with lid and return to heating block to incubate the plate at 37°C±1°C for 6 minutes.

   Note: Pipette the substrate solution in the same manner as in step 3. Maintain a consistent pipetting speed.

5. At T=16 minutes, add 100µL of Stop Reagent (25% acetic acid). Once the stop reagent was added into all plate wells, remove the plate from heating block and gently tap several times to facilitate mixing.

   Note: Maintain the same pipetting order as in steps 3 and 4.

6. Measure the absorbance at 405-410nm on a plate reader.

7. Subtract the average absorbance of the blank replicates from the average absorbance of all individual standards and unknown sample replicates to calculate mean Δ absorbance.

8. Prepare a standard curve by plotting the average blank-corrected absorbance for each standard on the y-axis vs. the corresponding endotoxin concentration in EU/mL on the x-axis. The coefficient of determination, r^2, must be ≥0.98.

9. Use the formulated standard curve (linear regression) to determine the endotoxin concentration of each unknown sample (Figure 1).

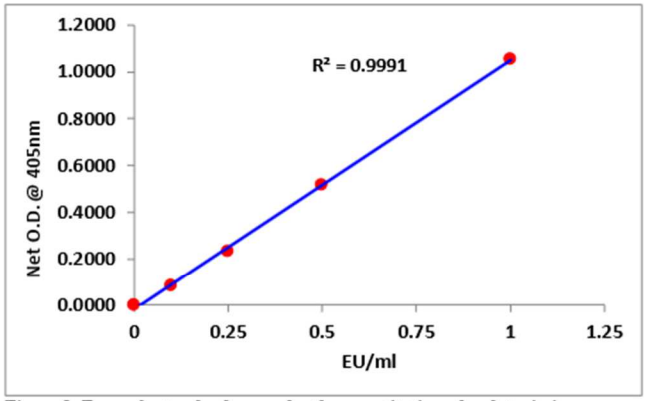

Figure 1. Example standard curve for the quantitation of endotoxin in a chromogenic assay.

Troubleshooting

Problem	Possible Cause	Solution
Non-linear standard curve	Standard Stock Solutions were not mixed well	Vortex the Endotoxin Standard Stock for 15 minutes before each use.
		Vortex all Standard Stock Solutions for 1 minute before each use.
	Pipetting order and rate of reagent addition was irregular	Ensure pipetting order and rate of reagent addition remain consistent from well to well and row to row.
		Use a repetitive pipettor.
	Incubation times were not followed	Strictly adhere to the incubation times.
		Start the timer at the point of adding reagent into the first well.
Higher absorbance in blank than standards	Materials (e.g., tips, vials, microplates) were contaminated	Use endotoxin-free materials.

Additional Information

A. Product Inhibition/Enhancement

Product inhibition occurs when substances in the test sample interfere with the LAL reaction. In the chromogenic assay, this inhibition results in a lower, final absorbance, indicating lower levels of endotoxin than what may be present in the test sample. Determine the lack of product inhibition for each sample undiluted or at an appropriate dilution.

To verify the lack of product inhibition, spike an aliquot or dilution of a test sample with a known amount of endotoxin (e.g., 0.5EU/mL). Assay the spiked sample and the unspiked samples to determine the respective endotoxin concentrations. The difference between the two calculated endotoxin values should equal the known concentration of the spike ±25%.

B. Example for Determination of a Non-inhibitory Dilution

Table 2. Example study for determining inhibition of a sample.

Sample dilution	Spiked[§] concentration (EU/mL)	Unspiked concentration (EU/mL)	Difference
1:10	0.28	0.18	0.10 = Inhibitory
1:20	0.36	0.11	0.25 = Inhibitory
1:40	0.50	< 0.1	0.50 = Non-inhibitory

[§] Spiked concentrations should all show a value of 0.50EU/mL The values of 0.28 and 0.36 are indicative of inhibition at the respective dilutions.

Samples showing inhibition on the LAL reaction may require further dilution to overcome the inhibitory effects. Once the non-inhibitory dilution is determined, the exact dilution can be found by testing two-fold dilutions near that dilution. The degree of inhibition or enhancement is dependent on the product concentration.

Beta glucans are polymers of D-glucose found in fungi and plant cell walls with >1000-fold less LAL reactivity than lipopolysaccharides. Inhibition and false-positive colors can occur in samples contaminated with beta glucans. Use appropriate beta glucan blockers if any contamination is possible.

C. Colored Samples

In the chromogenic assay, samples turning yellow after addition of the Stop Reagent or possessing significant initial color (e.g., tissue culture media) may require special attention. To determine if a sample's intrinsic color will alter the absorbance readings, construct a mock reaction tube by adding 50µL of sample, 150µL of endotoxin-free water and 50µL of Stop Reagent with no incubation. Read the absorbance at 405-410nm. If the absorbance is significantly greater than the absorbance of endotoxin-free water, then the intrinsic color will alter the correct sample absorbance readings. In such cases, include appropriate controls in the assay.

Related Thermo Scientific Products

88270	**Pierce High Capacity Endotoxin Removal Resin,** 10mL
88271	**Pierce High Capacity Endotoxin Removal Resin,** 100mL
88272	**Pierce High Capacity Endotoxin Removal Resin,** 250mL
88273	**Pierce High Capacity Endotoxin Removal Spin Column, 0.25mL,** 5 columns
88274	**Pierce High Capacity Endotoxin Removal Spin Column, 0.50mL,** 5 columns
88276	**Pierce High Capacity Endotoxin Removal Spin Column, 1mL,** 5 columns
20339	**Detoxi-Gel™ Endotoxin Removing Gel**
20344	**Detoxi-Gel Endotoxin Removing Columns**
89896	**Pierce Centrifuge Columns, 2mL,** 25/pkg
89897	**Pierce Centrifuge Columns, 5mL,** 25/pkg
89898	**Pierce Centrifuge Columns, 10mL,** 25/pkg
23225	**Pierce BCA Protein Assay Kit**
22660	**Pierce 660nm Protein Assay Kit**

Reference

[1]Roslansky, P.F. and Novitsky, T.J. (2016). Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. *J Clin Microbiol* **54** (5). Jcm.asm.org/content/29/11/2477.short

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APPENDIX F

PROTOCOL GLUCOSE COLORIMETRIC ASSAY KIT

Glucose Colorimetric Assay Kit

Item No. 10009582

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
10010098	Glucose Assay Standard	1 vial/300 µl
700003	Sodium Phosphate Assay Buffer	1 vial/10 ml
10010100	Glucose Colorimetric Enzyme Mixture	4 vials
400014	96-Well Solid Plate (Colorimetric Assay)	2 plates
400012	96-Well Cover Sheets	2 covers

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888
Fax: 734-971-3641
Email: techserv@caymanchem.com
Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure absorbance between 500-520 nm
2. Adjustable pipettes and a multichannel or repeating pipette
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Glucose, a monosaccharide (or simple sugar), is the most important carbohydrate in biology. Transported *via* the blood stream, it is the primary source of energy for the body's cells. Glucose levels are tightly regulated in the human body. Failure to maintain blood glucose in the normal range leads to conditions of persistently high (hyperglycemia) or low (hypoglycemia) blood sugar. Diabetes mellitus, characterized by persistent hyperglycemia, is the most prominent disease related to improper blood sugar regulation.

The determination of glucose levels in blood is critical in the control of diabetes. A dinitrosalicylic acid (DNS) assay has been available since 1955 but more recently, several enzymatic assays using either hexokinase-glucose-6-phosphate dehydrogenase or glucose oxidase-peroxidase for glucose quantification have been developed.[1-3] The nonenzymatic assay quantitates all reducing sugars whereas the enzymatic assay is specific for glucose, allowing for more accurate quantification.

About This Assay

Cayman's Glucose Colorimetric Assay Kit provides a simple, reproducible, and sensitive tool for assaying glucose in plasma, serum, and urine. In this assay, glucose is oxidized to δ-gluconolactone with concomitant reduction of the flavin adenine dinucleotide (FAD)-dependent enzyme glucose oxidase (see Figure 1 on page 7; reaction 1). The reduced form of glucose oxidase is regenerated to its oxidized form by molecular oxygen to produce hydrogen peroxide (reaction 2). Finally, with horseradish peroxidase as a catalyst, hydrogen peroxide reacts with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminoantipyrine (also called 4-aminophenazone) to generate a pink dye with an optimal absorption at 514 nm (reaction 3).[4]

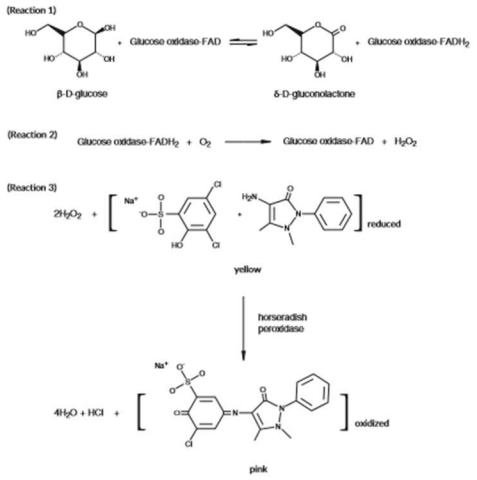


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Glucose Assay Standard - (Item No. 10010098)**

   This vial contains 300 µl of 1,000 mg/dl glucose. It is ready to use as supplied to prepare the standard curve. Sufficient Standard is provided to prepare four standard curves.

2. **Sodium Phosphate Assay Buffer - (Item No. 700003)**

   This vial contains 10 ml of 250 mM sodium phosphate, pH 7.2. Mix the contents of the vial with 40 ml of HPLC-grade water. This solution is used to prepare the Glucose Standards and for dilution of the Enzyme Mixture. The diluted Buffer is stable for three months at 4°C.

3. **Glucose Colorimetric Enzyme Mixture - (Item No. 10010100)**

   This vial contains a lyophilized enzyme mixture. Reconstitute 1 vial with 6 ml of diluted Assay Buffer and mix well. This reconstituted solution is now ready to use in the assay. The reconstituted solution is stable for at least one hour when stored at 4°C. One vial of the Enzyme Mixture is sufficient to assay 60 wells.

Sample Preparation

Plasma

Typically, normal human plasma has glucose concentrations in the range of 70-110 mg/dl.[5]

1. Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. If not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month when stored at -80°C.
3. Dilute plasma 1:5 with diluted Assay Buffer before assaying.

Serum

Typically, normal human serum has glucose concentrations in the range of 70-110 mg/dl.[5]

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month when stored at -80°C.
4. Dilute serum 1:5 with diluted Assay Buffer before assaying.

Urine

Typically, normal human urine has glucose concentrations in the range of 1-15 mg/dl.[5]

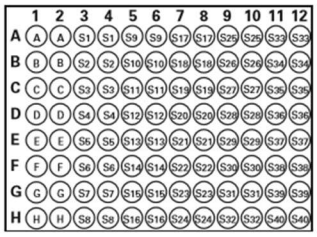
1. Urine does not require any special treatments. If not assaying the same day, freeze at -80°C.

NOTE: Glucose values from urine samples can be standardized using Cayman's Creatinine (urinary) Assay Kit (Item No. 500701).

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of glucose standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 18).



A-H = Standards
S1-S40 = Sample wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 200 µl in all wells.
- The incubation temperature is 37°C.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the standards and samples be assayed at least in duplicate.
- Monitor the absorbance at 500-520 nm using a plate reader.

Standard Preparation

Mix 50 µl of the 1,000 mg/dl Glucose Standard (Item No. 10010098) with 450 µl of diluted Assay Buffer to make a 100 mg/dl stock. Label eight clean glass test tubes or polystyrene tubes A-H. Add the amount of Glucose Standard and Assay Buffer to each tube as described in Table 1. The diluted Glucose Standards are stable for two hours at room temperature.

Tube	Glucose Stock (µl) (100 mg/dl)	Assay Buffer (µl)	Glucose Concentration (mg/dl)
A	0	200	0
B	5	195	2.5
C	10	190	5
D	15	185	7.5
E	20	180	10
F	30	170	15
G	40	160	20
H	50	150	25

Table 1. Preparation of Glucose Standards.

Performing the Assay

1. Glucose Standard wells - Add 85 µl of diluted Assay Buffer and 15 µl of each Standard (tubes A-H) to two wells (see suggested plate configuration, Figure 2, page 10).
2. Sample wells - Add 85 µl of diluted Assay Buffer and 15 µl of sample to two wells.
3. Initiate the reaction by adding 100 µl of Enzyme Mixture to all standard and sample wells.
4. Cover the plate with the plate cover and incubate for 10 minutes at 37°C.
5. Remove the plate cover and read the absorbance at 500-520 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample.
2. Subtract the absorbance value of the standard A (0 mg/dl) from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of the concentration of glucose (see Table 1, page 12).
4. Calculate the concentration of glucose for each sample from the standard curve. An example of the glucose standard curve is shown in Figure 3, see page 15.

$$\text{Glucose (mg/dL)} = \left[\frac{\text{(Corrected absorbance) - (y-intercept)}}{\text{Slope}}\right] \text{x dilution}$$

Performance Characteristics

Sensitivity:

When a series of thirty-six human serum and urine samples were assayed on the same day, the intra-assay coefficient of variation was 4.6% and 8.1%, respectively. When a series of thirty-six human serum and urine samples were assayed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 1.7% and 11.3%, respectively. The lower limit of detection (LLOD) for this assay is approximately 0.23 mg/dl.

Representative Glucose Standard Curve

The standard curve presented here is an example of the data typically produced with this kit. Your results may vary, and therefore should not be directly compared to these samples. You must run a new standard curve.

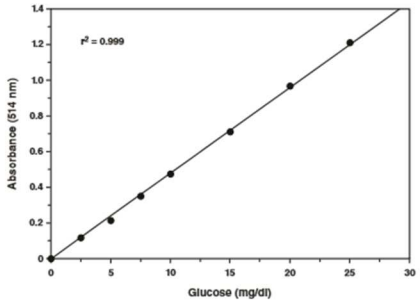


Figure 3. Glucose standard curve

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No glucose was detected in the sample and standard wells	Enzyme Mixture was not prepared or stored correctly	Prepare fresh Enzyme Mixture and re-assay
Sample absorbance values are above highest point in standard curve	Glucose concentration was too high in the samples	Dilute samples with assay buffer and re-assay; *NOTE: Remember to account for the dilution factor when calculating glucose concentration*

References

1. Bernfeld, P. In amylase, α and β, Chapter 17, *in* Methods in Enzymology. Colowick, S.P. and Kaplan, N.O., editors, 1, Academic Press, New York, 149-158 (1955).
2. Carroll, J. A colorimetric serum glucose determination using hexokinase and glucose-6-phosphate dehydrogenase. *Biochem. Med.* **4**, 171-180 (1970).
3. Sugiura, M. and Hirano, K. A new colorimetric method for determination of serum glucose. *Clin. Chim. Acta* **75**, 387-391 (1977).
4. Frost, L.D. Glucose assays revisited: Experimental determination of the glucose concentration in honey. *Chemical Educator* **9(4)**, 239-241 (2004).
5. Slein, M.W. and Bergmeyer, H.U. Methods of Enzymatic Analysis 117-123 (1963).

APPENDIX G

PROTOCOL RAT INSULIN ELISA KIT ASSAY

CATALOG# 90060

ULTRA SENSITIVE RAT INSULIN ELISA KIT

Catalog number

Ultra Sensitive RatInsulin ELISA.....90060

Intended use

A high quality enzyme immunoassay for the quantification of rat insulin in fluid, plasma, and serum.

Test principle

Crystal Chem's Ultra Sentitive Rat Insulin ELISA Kit is based on a sandwich enzyme immunoassay using only a 5 µL sample to produce same day results. The kit can be run using an ultrasensitive low range, wide range, or high range screening method to yield a wide dynamic range with just one kit.

Specifications

Sample Types	Serum, Plasma, and Fluid
Assay Time	Same Day Procedure
Range	Low Range: 0.1-6.4 ng/mL Wide Range: 0.1 - 12.8 ng/mL High Range: 1 - 64 ng/mL
Sample Size	5 µL
Sensitivity	0.05 ng/mL
Precision	CV < 10%

Specificity

RatInsulin.....100%
Mouse Insulin.....100%*
Human IGF-I.....Not detected
Human IGF-II.....Not detected

*Can vary from lot to lot. See insert in kit.

Highlights

- ✓ Kits use only 5 µL sample
- ✓ Very sensitive (0.05 ng/mL)
- ✓ Run different ranges using the same kit
- ✓ Works with multiple sample types
- ✓ Complete the full test in < 3.5 hours

Summary of protocol

Add 95 µL diluent with 5 µL sample
↓
Incubate 2 hours at 4 °C
↓
Wash plate
↓
Add 100 µL conjugate solution
↓
Incubte 30 minutes at room temperature
↓
Wash plate
↓
Add 100 µL substrate solution
↓
Incubate for 40 minutes (low/wide range)
or 10 minutes (high range)
↓
Add 100 µL stop solution
↓
Measure OD at 450/630 nm

See kit insert or email us for a complete protocol

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europe@crystalchem.com

EASY ONLINE ORDERING AT CRYSTALCHEM.COM

APPENDIX H

IACUC PROTOCOL APPROVAL

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 312
Tempe, Arizona 85287-6111
Phone: (480) 965-6788 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**17-1563R**
Protocol Title:	**Exploration of the metabolic and vascular protective effects of an organometallic complex (OMC)**
Principal Investigator:	**Karen Sweazea**
Date of Action:	**2/23/2017**

The animal protocol review was considered by the Committee and the following decisions were made:

The protocol was approved.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree.

Total # of Animals:	**60**	
Species:	**Rats**	**Pain Category: C**
Protocol Approval Period:	**2/23/2017 – 2/22/2020**	
Sponsor:	**Isagenix International LLC**	
ASU Proposal/Award #:	**FP00010367; FP00010473**	
Title:	**Exploration of the metabolic and vascular protective effects of an organometallic complex (OMC)**	

Signature: _____ for C. Miller Date: 2/27/2017
IACUC Chair or Designee

Cc: IACUC Office
IACUC Chair