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Electrochemical Methods for Human Growth Hormone Doping Detection



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# Electrochemical Methods for Human Growth Hormone Doping Detection

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biomedical Engineering

By

Michael Kai Tu

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ABSTRACT OF THE DISSERTATION

Electrochemical Methods

for

Human Growth Hormone Doping

Detection

by

Michael Kai Tu

Doctor of Philosophy in Biomedical Engineering

University of California, Los Angeles, 2015

Professor David T. Wong, Chair

Human Growth Hormone (GH) is produced by the anterior pituitary gland and promotes growth of tissue through direct uptake at target tissue sites, or alternatively, by regulating production of insulin-like growth factor-1. The World Anti-Doping Agency considers GH a performance enhancing substance, so the use of GH by athletes is prohibited in most sports. The current immunoassay for GH detection is suboptimal for routine screening of blood samples because of the large resources required for collecting, processing, transporting (blood must be shipped to testing sites at controlled temperatures), and testing blood samples.

This study details the development and testing of a novel 96-electrode high-throughput amperometric sensor system to measure GH isoforms in whole blood samples. This high-throughput biosensor system platform reduces the need for sample processing, requires less sample volume, and reduces assay time. The method selectively targets the 22kD and 20kD isoforms of GH, which will provide more statistical discriminatory power than the existing GH test. The present study demonstrate that the amperometric immunosensor assay configuration can detect GH isoforms in 25 µL of whole blood with high linearity ($R^2$ = 0.98) and a limit of quantitation of 50 pg/mL. This project also describes electrochemical tests to assess the validity of detecting GH from dried blood spots and saliva.

The dissertation of Michael Kai Tu is approved.

Yong Chen

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2015

Dedicated

To My

Father and Mother

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## Publications and Presentations

Tu, M., Wei, F., Yang, J., Wong, D. Detection of Exosomal Biomarker by Electric Field-induced Release and Measurement (EFIRM). ***J. Vis. Exp.*** (95), e52439, doi:10.3791/52439 (2015).

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Wei, F., Tu, Michael. SDx : Noninvasive Point of Care Saliva Diagnostic Platform – Poster presentation at the *California Nanosystems Institute* (3/2013)

Fung, A., Tu, M. MALiVA: A Chewable Rapid Diagnostic Test for Malaria Parasite Antigens in Saliva – Poster Presentation at *University of California Global Health Day (2010)*

M. Suh, L. S. Evangelista, C.-A. Chen, K. Han, J. Kang, M. K. Tu, V. Chen, A. Nahapetian, and M. Sarrafzadeh, “An automated vital sign monitoring system for congestive heart failure patients,” 2010, p. 108.

# Chapter 1 – Introduction

## 1 The Current State of Human Growth Hormone Doping Detection Tests

Human Growth Hormone is a polypeptide structure secreted by the anterior pituitary gland that promotes the growth of muscular and cartilage tissue. Growth hormone that is secreted by the pituitary gland comes in a variety of molecular isoforms: the most predominant form of Human Growth Hormone that is secreted is a monomeric unit with 22 kilodalton molecular weight, which constitutes more than 70% of net aggregate human growth hormone present[1]. The second most predominant form is a monomeric Growth Hormone unit that possesses a 20 kilodalton molecular weight[2], which is created by an alternatively spliced mRNA sequence. This 20 kilodalton form constitutes approximately 5%-10% of the growth hormone secreted by the human body[3]. The remaining portion of pituitary secreted growth hormone consists of a variety of oligomers and fragment growth hormone units.

Freely circulating growth hormone has two major pathways of initiating its physiological function: The first molecular pathway is by direct uptake into target tissue for the formation and production of cartilage and bone tissue[4]. Growth hormone may alternatively also act by also increasing the production of the insulin-growth factor 1 protein[5], another hormone that stimulates tissue growth.

The physiological function of human growth hormone in promoting tissue growth was elucidated in the early 1900s by efforts by Cushing, Evans, and Long[4]. The gradual increase in understanding of the physiological effects of growth hormone led to experimental medical usage of cadaver pituitary gland extract on individuals with growth defects. These efforts, while medically successful in promoting tissue growth, caused side effects because of viruses that

were transmitted in the pituitary extracts that were used for replacement therapy. These attendant problems with growth hormone replacement therapy were solved in the late 80s after the ascendency of molecular cloning made the production of recombinant growth hormone protein possible. The current medical intervention for pituitary dwarfism is the administration of regular doses of recombinant form of human growth hormone.

Attendant with the medical usage of recombinant growth hormone in treating conditions such as pituitary dwarfism, however, has been the associated off-label usage of the protein as a performance enhancing substance in athletics. This usage is prohibited by various sports regulatory agencies and governing bodies that wish to promote "clean competition", such as the World Anti-Doping Agency (WADA). These regulatory bodies periodically enforce the drug prohibition through random sample collection of blood samples. The collected samples are then sent to a properly credentialed central laboratory facility where the sample is processed and compared to preexisting diagnostic criteria. Failure to meet the diagnostic criteria limits set by the anti-doping regulatory body has the potential to disqualify the athlete from competition. The diagnostic criteria[6] for growth hormone doping is based on the levels of growth hormone isoforms present.

The existing workflow and procedure taken for the quantification and classification of athletes in regards to growth hormone doping is hindered by various logistical and practical factors that render the procedure particularly demanding of financial and personnel resources. The problems associated with growth hormone testing are:

A. **Personnel logistics:** In order to properly collect blood samples, it is necessary for proper sample collection to be made by personnel trained in blood drawing samples. This requires that the sample collector be specifically trained and credentialed for an intravenous blood draw procedure.

B. **Sample Collection and Transportation:** Approximately 5-10 mL of blood is drawn during the testing procedure, and rapid transportation at temperature controlled transportation of collected specimens is necessary in order to maintain sample integrity. This addition significantly adds to the sample collection cost, and athletes may object to the sample collection procedure because of the volume of blood taken.

C. **Equipment Needs for Processing:** Traditional methods of biodetection of specimens involve the centrifugation[25] of whole blood samples in order to remove interfering molecules that would hinder from biodetection. Since the existing method for Growth Hormone detection is through the traditional enzyme linked immunoabsorbant assay (ELISA) method, the collected whole blood samples must be subject to centrifugation technique to remove the red blood cells and render the specimen capable of chemical analysis. This method adds the need for additional equipment.

D. **ELISA based techniques:** The extant method of testing for growth hormone is only capable of testing for the 22kD recombinant growth hormone isoform and all pituitary forms of growth hormone. In this, a standard curve of 22 kilodalton recombinant growth hormone and a standard curve of all pituitary forms of growth hormone are compared to the levels of growth hormone in athlete samples. This method is not the most discriminatory method for testing for growth hormone, as studies have demonstrated that a more discriminatory method of doping detection is through a comparison of the 22kD and 20kD Growth Hormone ratios[1]. Furthermore, the existing standard assay takes place in a series of test tubes (which makes performing the assay cumbersome) instead of a compact biochemical format such as a 96-well microplate.

Because of the difficulties that are associated with traditional growth hormone detection methods, research efforts have been pursued to improve upon testing methods:

A. **Alternative methods of sample collection have been the subject of investigation.** Langkamp et al[7] have published a work wherein attempts to quantify growth hormone concentration in dried blood spots are made. Research has also been conducted for the development of urine based growth hormone tests as a methodology for reduced noninvasiveness for drug testing[8]. However, urine doping test methods are difficult for practical application because athletes have been known to utilize methods to circumvent urine testing, methods such as hiding protease granules in their urethra in order to degrade target analytes and render tests ineffective [9].

B. **Alternative biomarkers for growth hormone doping** such as insulin-growth factor 1 (IGF-1) [10] are currently being explored as a means for detecting growth hormone doping. This is advantageous because it seeks to allow detection of growth hormone without dealing with one of the most difficult parts of growth hormone tests: the clearance rate of growth hormone. This approach has promise, but it is still an open question whether IGF-1 can serve as a definitive marker for drug doping.

## *2 Amperometric Biosensors for Molecular Detection*

Amperometry is an experimental technique that involves the usage of electrodes in order to measure the electrical current flow in a solution. Amperometry as a technique has had a long history of being applicable to investigation and quantitation of biological systems, having its modern inception begun by Clark and Lyons[11] in 1962. Amperometric biosensors have a wide range of applications, having been used in bacterial detection in food[12], environmental monitoring[13], and glucose monitoring for diabetics[14]. Works have been produced showing that chronoamperometric biosensing methods can be also used for exploring the usage of non-invasive biofluids such as saliva[15] [16] and basic science mechanistic research [17]. Literature[12] indicates that the amperometric method may be advantageous to other techniques such as the

conductimetric and impedimetric approach because thermodynamic equilibrium does not need to be reached in amperometry (allowing for more rapid assay of a target analyte). The signal in an amperometric biosensor context is typically generated by an enzymatic component that will create oxidation and reduction reactions of a substrate.

One scheme of amperometric biosensing that has been developed and demonstrated to be effective for the quantification of biomarkers is in the work by Wei et al.[18] and Gau et al[19]., where the mechanism of generating measurable electrical current is a horseradish peroxidase enzyme that interacts with hydrogen peroxide ($H_2O_2$) and tetramethylbenzidine in a ping-pong reaction format. Gau[19] describes the action of the reaction utilized as consisting of three sequential steps:

$$Fe^{III}(Porphyrin)+H_2O_2 \rightarrow Fe^{IV}=O^{-II}(Porphyrin)^{\bullet+} + H_2O$$

$$Fe^{IV}=O^{-II}(Porphyrin)^{\bullet+} + AH_2 \rightarrow Fe^{IV}=O^{-II}(Porphyrin)H^{+} + AH^{\bullet}$$

$$Fe^{IV}=O^{-II}(Porphyrin)H^{+} + AH_2 \rightarrow Fe^{III}(Porphyrin) + AH^{\bullet} + H_2O$$

In this reaction configuration, the porphyrin structure present on the peroxidase enzyme is oxidized by the hydrogen peroxide, and then is reduced back to its original state through reacting twice with an organic mediator (here indicated by $AH_2$), which in work by Gau et al[19]. and Wei et al[20] is tetramethylbenzidine. In the ordinary course of the reaction the organic mediator would only exist in its oxidized form in the bulk solution. However, because of the application of a negative electrical bias at the interface of the electrode, the oxidized form of tetramethylbenzidine is replenished by the electrons at the electrode interface.

Because of the interactions that take place at the electrode interface in regenerating the organic mediator tetramethylbenzidine, the method utilized by Wei et al.[18] and Gau et al[19] takes advantage of this effect and measures the electric current flow during this process. The process

of tetramethylbenzidine regeneration is also dependent on the rate of peroxidase enzyme present in the bulk of the solution, the measured electrical current will be proportional to the peroxidase enzyme present in the electrode system. And by extension, since the peroxidase enzyme is proportional to the amount of target analyte captured by a capture probe, the measured current by the electrode is proportional to the amount of target analyte present. This techniques utilizing peroxidase and tetramethylbenzidine has been demonstrated to femtogram level sensitivities[20] for proteins and femtomolar level sensitivity for nucleic acids .

# 3 Low Voltage Electric Fields on Electrochemical Biosensors

Various electric-field driven methods have been proposed to enhance biosensing in electrochemical biosensors systems, methods such as electrothermal[21], electroosmotic, or dielectrophoretic[22] manipulation of proteins and biofluids. These methods utilize electric-field based phenomena to concentrate target analytes for enhancing sensor speed and sensitivity. While offering potential to enhance sensor performance, it has been noted that these techniques may have consequent side effects that would render them disadvantageous for point of care applications. Chang and Yeo[23], in their survey of the recent literature, state that high-voltage DC based electric field driven actuation can damage the structure of biomolecules, along with generating bubble and backflow pressure issues. Yuan and Wu[24] also note in a review that AC electric fields based can cause unwanted electrochemical side reactions and high-ionic strength environments (such as biofluids) may reduce the method's effectiveness.

Because of the potential disadvantages with existing approaches, alternative approaches exist that explore and apply low-voltage (<1.5 volts) electric fields as a method for enhancing biosensor function. This method uses low-voltages to facilitate immunocomplexing between a capture antibody and a target protein[15,20,18] . When applied to a protein target, the

method has been shown to allow for high sensitivity detection in <15 minutes[15,20,18] even in biofluids as complex as saliva or serum. The ability to apply low-voltage electric fields to perform a rapid, high-sensitivity protein assay is extremely promising. Low-voltage electric fields electrochemical assays would be practical for portable, low-cost, battery powered point-of-care diagnostic tools. The speed by which the assay could quantify multiple biomarkers in a patient would be extremely useful in helping clinicians make “chair-side” diagnoses, and avoid the need for follow-up appointments to discuss laboratory results.

While the low-voltage driven mechanism appears to be effective in a number of electrochemical protein immunoassays systems, there appears to be little development of methods to investigate the phenomena in a systematic and rapid manner.

# 4 Overview of Thesis

## 4.1 Problem Statement

Based on the existing state of growth hormone testing, there appears to be a clear need for an improved method for detecting human growth hormone for doping studies. Electrochemical biosensors have been demonstrated on numerous occasions to have the diagnostic potential to perform high-sensitivity detection of protein targets in complex biofluids. Furthermore, low-voltage electric field manipulation in biological samples has also been shown to be a highly effective technique for performing rapid (<10 minute) detection of protein targets and the ability to potentially enhance assay sensitivity.

The topic of this study seeks to investigate the hypothesis that electrochemical techniques can be used to improve on the traditional workflow for growth hormone detection. If electrochemical biosensors are to be a feasible means for improving the detection of growth hormone, a set of research questions must be addressed.

- Sensitivity of Electrochemical Biosensors: Will electrochemical techniques be able to perform a quantification of growth hormone isoforms at the sensitivity levels necessary as specified by the World Anti-Doping Agency? Will electrochemical techniques allow the achievement of levels necessary for the usage of more discriminatory biomarkers such as a 22 kilodalton/20 kilodalton biomarker ratio, as opposed to the traditional biomarker ratio test of 22kD/all forms of GH?
- Robustness of Electrochemical Biosensors: Are electrochemical techniques robust enough to perform detection of growth hormone in diagnostic mediums such as dried blood spots or whole blood samples? Will they be able to provide a superior method of sample collection and processing than the existing chemiluminescent ELISA method on serum?
- Electrochemical Platforms: Can electrochemical biosensors be adapted to a high-throughput fashion for growth hormone testing? Is it technically feasible to construct a high-throughput array for electrochemistry?

## *4.2 Overview of Dissertation*

For the investigation of these research questions, this dissertation is divided into chapters based on the different experiments that were conducted for investigating the applicability of electrochemistry to improving growth hormone detection.

Chapter One (this chapter) details the context of the dissertation. The existing state of growth hormone doping detection is described, and an overview of the key principles and research questions of the thesis are stated.

Chapter Two details the development of a high-throughput electrochemical assay system. The present assay for human growth hormone is a test-tube based antibody sandwich approach, which is very difficult to automate and makes the processing of a large quantity of

samples difficult. Existing electrochemical assay systems are also limited in their ability to process a large quantity of specimens (which would be needed for the screening of athletes for drugs). This chapter investigates a prototype proof-of-concept high-throughput method for conducting electrochemical assays.

Chapter Three discusses the development of a sensitive test for the 22 kilodalton and 20 kilodalton growth hormone isoforms in whole blood. Traditional growth hormone doping methods require that whole blood samples have their red blood cells removed before assaying. Furthermore, the assay protocol for growth hormone distinguishes growth hormone dopers from non-dopers based on the ratio of 22 kilodalton growth hormone to all pituitary forms of growth hormone. This section details whether a whole blood assay for the 22 kilodalton and 20 kilodalton isoforms can be developed using electrochemical techniques. This assay would be beneficial in removing the need for a processing step, and the usage of two specific isoforms could potentially make the test better able to distinguish between growth hormone dopers and non-dopers.

Chapter Four gives an overview of alternative methods for growth hormone test sample collection. In order to try to reduce the invasiveness of growth hormone tests, investigations were made into whether a dried blood spot approach or saliva based approach for growth hormone detection would be able to function as alternatives to the traditional detection methods involving intravenous blood draw.

Chapter Five describes the development of a high-throughput method for investigating low voltage electric field phenomena. A description is made of a control method for manipulating the individual applied electric fields in a 96-electrode array, and a basic screening study for electric field phenomena using the growth hormone assay and high-throughput platform developed in chapters two and three is described.

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# Chapter 2 - High-Throughput Electrochemical Array

## 1 Introduction

### 1.1 Overview

Inasmuch as electrochemical biosensors have precedence for being highly sensitive and robust methods of performing biodetection, a pivotal question that must be addressed is whether they can be adapted to handle the throughput required for a hospital laboratory or drug doping detection lab. Existing electrochemical platforms at best are only able to process 16 specimens in parallel, and this limited throughput may be impractical if hundreds of specimens must be tested in a very short period of time, a situation that realistically may happen in a hospital clinical laboratory or a drug doping detection lab. Developing a high-throughput biosensor platform that can accommodate for a large quantity of test specimens would be helpful in making electrochemical biosensors suitable for a clinical laboratory environment.

The practicality of electrochemical biosensors would also be increased if they could be easily adapted to automation in a clinical laboratory. Clinical laboratories often use automation platforms to ensure greater regularities on the performance of experimental protocols, lowering the coefficient of variation in tested samples and ensuring that tests are well within accepted quality standards. This usage of automation can also assist in lowering expenses by reducing manpower requirements. The current 16-electrode system for assay quantitation has automation tools available for use, but these automation tools would be difficult to integrate with existing clinical laboratory workflows.

For this work, exploratory studies are taken to see if a high-throughput electrochemistry biosensor system for molecular detection is technically feasible to develop. This high-throughput proof of concept, if validated, would be a key milestone in validating that electrochemical biosensors are capable of achieving the throughput necessary for wider application in a clinical laboratory context. The design of the high-throughput system is also meant to be easily integrated into existing laboratory automation tools such as liquid handlers and washers, and this will be evaluated. Achieving the goals of creating a high-throughput and easily automatable electrochemical system has the potential allow the proof-of-concept system to have a high uniformity and regularity that would be infeasible with smaller scale biosensors.

Development was made of a proof-of-concept high-throughput electrochemical system for molecular sensing by integrating a 96-electrode plate with a potentiostatic control system capable of performing electrochemical procedures functions on 96-channels simultaneously. The 96-electrode plate utilized in this study has been applied previously for impedance sensing applications of cardiomyocyte cell cultures[2], and in this line of investigation we will make tests to determine whether interfacing the 96-electrode microplate systems to a potentiostatic control system will allow the performance of molecular immunosensing using electrochemical techniques such as chronoamperometry and cyclic voltammetry. Based on the existing design of the electrochemical plate, a number of considerations must be made when trying to integrate the 96 electrode plate with an electrochemical potentiostatic control system for molecular biosensing:

A. **Electrode configuration:** Many electrochemical experiments typically use a three electrode setup: one “working electrode”, one “counter electrode”, and one “reference electrode”. This configuration is a well-known method for ensuring stability in an electrochemical reaction system because oxidation-reduction reactions can take place on the working and counter electrodes of the system, while the reference

electrode has a fixed potential that will remain independent and can be used to monitor the system while not having interference from current flow that can alter its potential. In the system utilized by the high-throughput system of this work, there is no distinct reference electrode utilized, and thus there is a risk of voltage drift due to the reactions taking place on the working and counter electrodes. Assessment is therefore necessary to see if generating measureable current flow in a biological system is possible with the two-electrode system.

B. **Effects on electrochemical performance:** Forster[3] notes that when a microelectrode structure is utilized for electrochemistry, there are a number of effects such as a reduced capacitance and a drop in ohmic resistance compared to traditional electrodes which are larger than a micrometer. Consequently, because microelectrode structures are known to differ from traditional electrodes, a study assessing assay performance on the 96-electrode array microelectrode structure would be necessary.

## *1.2 Comparison of Proof-of-Concept System to Existing Platforms*

The device that is developed for this study is distinct from existing platforms inasmuch as it allows for the simultaneous control and measurement of a 96-channel electrochemical plate in a parallelized fashion. Surveying the existing state of electrochemical systems for rapid electrochemical bioassay, there are systems that have been designed to connect to interface with 96-plate electrodes plates, but these configurations do not offer truly parallelized control of the 96-electrode system compared to the approach taken in this work.

An existing multichannel potentiostat system used for biological assay is the Genefluidics 16-electrode array system. This platform has been validated across a number of targets[4 1 5] , but the disadvantage of the system is that it only offers simultaneous readout of 16-channels at a time [1]. Additionally, the electrochemical instrumentation offers only a limited

applied bias voltage range (-1 to 1 volts) and a limited amount of electrochemical functions (only cyclic voltammetry and chronoamperometry). In an ideal biosensor platform, a wider range of voltages would be possible to be applied, and a larger array of electrochemical control methods could be applied such as differential pulse voltammetry[6] or impedance spectroscopy[7]. This is not attainable using the Genefluidics 96-electrode system.

The usage of a conventional potentiostat with multichannel multiplexing modules, such as an Autolab PG stat, while allowing access to a wide variety of electrochemical measurement methods such as cyclic voltammetry, chronoamperometry, and impedance spectroscopy, is limited by throughput. This system utilizes a number of multiplexing modules to allow switching to different channels for electrochemical measurements, but this multiplexing is actually a form which only a single channel of measurement and control at a time. This would be incompatible with the simultaneous readout of electrochemistry on multiple channels as would be needed for testing a large amount of clinical samples rapidly.

The closest hardware in comparison to the system prototyped in this study is the Dropsens DRP-96-Well Connector system, which was an adapter specifically designed for holding a 96-well plate electrode (also developed by Dropsens). This system is simply an adapter unit, and can be used with any potentiostat platform. This system is advantageous in that it is designed to be able to handle 96-electrodes, and can be used to interface with existing electrochemical equipment, meaning that it can run many electrochemical features beyond that of the Genefluidics platform. However, the adapter unit developed by Dropsens is only designed to interface a potentiostat system with 8 electrodes on the 96-well at a time, and furthermore, readout on a specific well requires that a mechanical dial be turned to manually select which electrode to measure.

The intent of the system that is proposed to be developed for this study is to develop a parallelized 96-electrode array system that will be a significant improvement compared to the existing state of the art in multiplexed electrochemical assays. Unlike the Dropsens and Autolab platforms, the system developed will be truly parallelized to read on 96-channels simultaneously and will not require the usage of mechanical switching in order to interface and access the 96-channels. Unlike the Genefluidics system, the prototype system developed in this work will also be capable of performing a wide variety of biodetection methods for electrochemical immunosensing, and goes beyond the 16 channels.

| **Requirement** | **Genefluidics Helios reader** | **Autolab PG Stat with multiplexing modules** | **Dropsens DRP-96-Well system** | **Proof-Of-Concept system developed for this project** |
|---|---|---|---|---|
| Electrodes Available | 16 electrodes | Up to 255 working electrodes, but only measurement on one electrode at a time | 96 Electrodes, but only 8 simultaneously | 96 electrodes in parallel, up to 256 electrodes in parallel possible. |
| Methods Available | CV, CA only | CV, CA, EIS, DPV, but only sequentially | Dependent on potentiostat system used, but only 8 at a time possible | CV, CA, DPV parallel, 96 at a time. EIS for sequential tests |

**Table 1. Comparison of different electrochemical techniques for quantitative assay, comparing existing platforms to the proof of concept system proposed in this work.**

# *2 Method*

## *2.1 Overview*

The high-throughput electrochemical system consisted of three subsystems, all of which were individually developed and integrated together into a working biosensor unit:

A. **Multichannel Potentiostat Subsystem:** In order to perform a high through-put detection system, it was necessary to develop a potentiostatic unit that was capable of rapidly performing multichannel electrochemical measurement in a multichannel fashion. This system would be able to robustly perform different electrochemical techniques such as cyclic voltammetry and chronoamperometry. In this phase, an

electrochemical potentiostat was integrated with add-on modules which would allow the multiplexing of multiple channels together.

B. **Disposable electrode plate subsystem:** Because of the high volume of samples that must be tested in a clinical laboratory test and the bio hazardous nature of the specimens tested, it is preferable that specimens be placed on disposable components that can easily be removed from the testing equipment. This is opposed to a design where the clinical specimens and the laboratory testing equipment are tightly integrated to the point where intense cleaning is necessary when new samples are processed, as is the case with microbead based assay systems[8] or surface plasmon resonance based biosensing techniques[9]. In this phase of the development, an electrode plate that fit these requirements was found and development work was made to interface it with the potentiostat unit.

C. **Peroxidase and Conducting Polymer Subsystem:** After determination of the basic functionality of the electrode plate and the potentiostat control system was performed, tests were also performed to test whether this system was capable of performing readout on biologically based oxidation-reduction reactions. This was done through a series of tests on peroxidase enzyme systems and conducting polymers that were previously known to work well for electrochemical biosensing[1].

## *2.2 Multichannel potentiostat subsystem development*

The first component of the high-throughput system is the potentiostatic control unit. The Iviumstat Compactstat made by Ivium Technologies was found to be able to perform a wide variety of electrochemical techniques such as cyclic voltammetry and chronoamperometry. More importantly, this CompactStat control system was capable of being interfaced with additional modules that allow for the multichannel multiplexing of electrochemical channels. Three MultiWE32 and a Compactstat control unit were acquired and interfaced together to make

a total of 96-electrodes to add-on to the system (Figure 1). This system, according to specifications delineated by the manufacturer, would allow for the sampling of all 96 channels at a sampling rate of 10 current measurements per second for each channel. Initial tests were run to validate that the system was capable of working by connecting the multiplexing modules to dummy cells, and performing measurement and characterized the effectiveness of the system by making electrochemical measurements based on the dummy cell performance.


Figure 1 Potentiostat units interfaced together. The electrochemical interface for this experimental setup utilizes three multiplexing modules on top of the CompactStat electrochemical potentiostat. These are interfaced together by a ribbon cable, and the multiplexing modules are connected to electrodes via a 37-pin D connector system located on the rear of the modules.

## *2.3 Disposable electrode plate subsystem development*

For the second component of the prototype, the disposable electrode plate, the prototype used an ACEA Biosciences E-Plate 96 as a staging unit for electrochemical experimentation. The rationale behind this decision was based on the electrode plates' high uniformity electrode structure, its plate design being compatible with the standard 96-well microtiter plate that is commonly used in biological assay work, and the fact that it has been validated in experimental biological work in other contexts[2].

The 96-well E-plate from ACEA Biosciences consists of a glass substrate with a complex gold microelectrode structure deposited on the surface. Following this deposition of the electrode structure on six different glass slides (each having 16 electrodes), the slides are bonded together and a plastic well covering is placed over the six slides to complete the mechanical housing of the electrochemical chips into a single 96-well plate. The electrodes can then be connected to electronic equipment through the copper contact pads and printed circuit board material located on the underside of the plate that also connect to the electrodes on the bottom side of the plate (Figure 2). Examination of the electrode structure through a digital voltmeter also reveals that in terms of the routing of the electrodes for an individual column, electrodes in rows A/B/C/D share a common counter electrode, and the electrodes in rows E/F/G/H in an individual column also share a common working electrode.

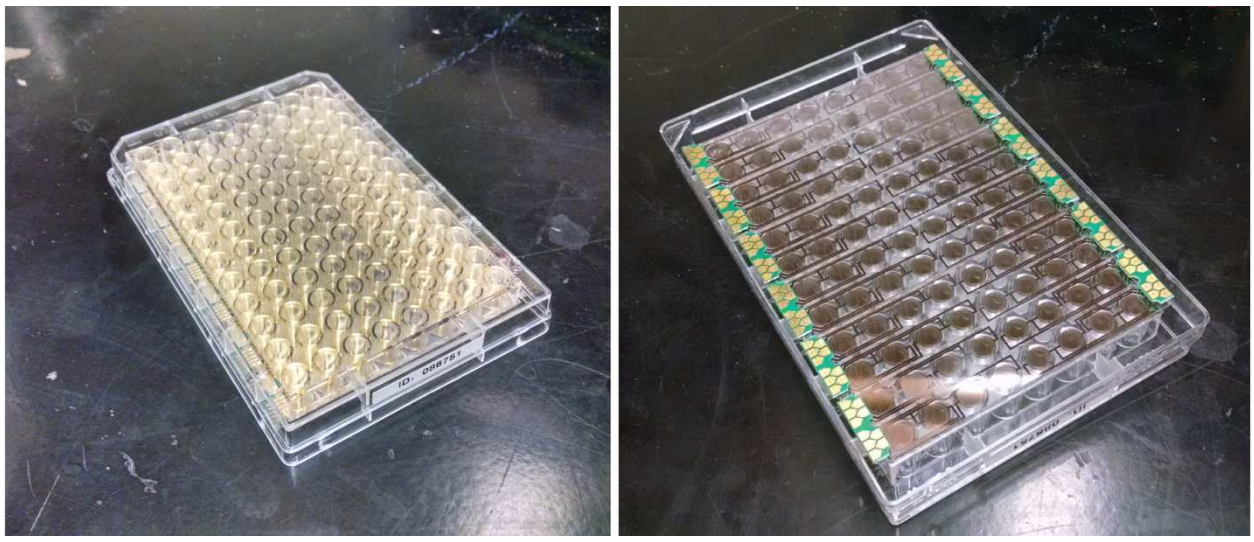

Figure 2. 96-Electrode plate available through ACEA Biosciences. Left figure is a top view of the footprint of the plate. Note how the plastic top creates 96 separate electrodes. Right figure is a view of the bottom of the electrode, showing the electrode structure with the printed circuit board components at the edges of the plate in order to serve as contact points between the electrodes and the electrochemical equipment.

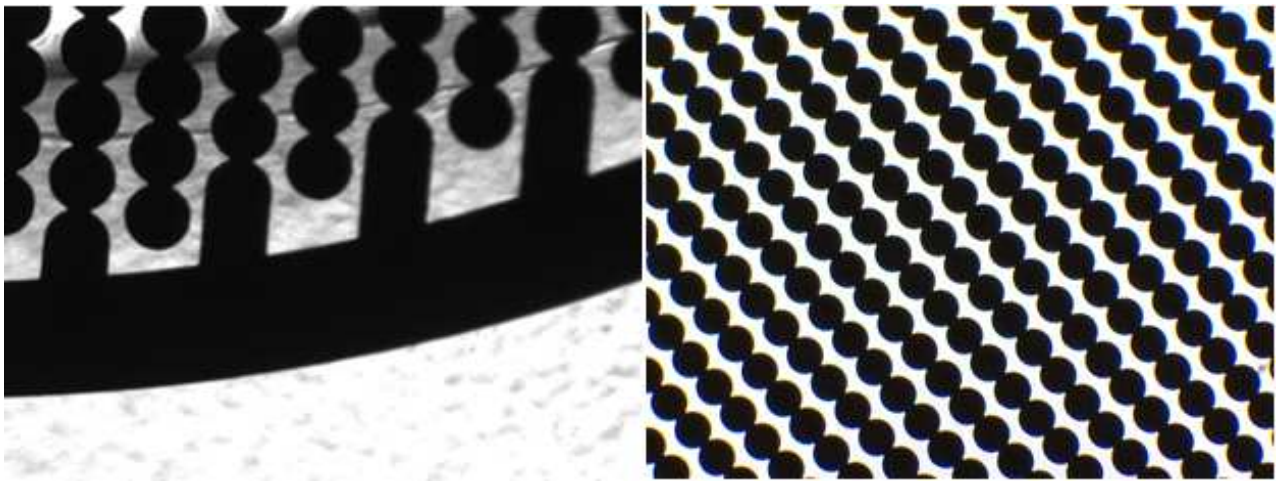

Figure 3 Electrode profile of electrodes taken with inverted microscope, demonstrating the intercalated electrode structure of the electrode in each well. The left figure illustrates the edges of the electrode structure and the differentiation between the working and counter electrodes of the system. The right figure shows the highly uniform nature of the working and counter electrodes placed in parallel in each individual well.

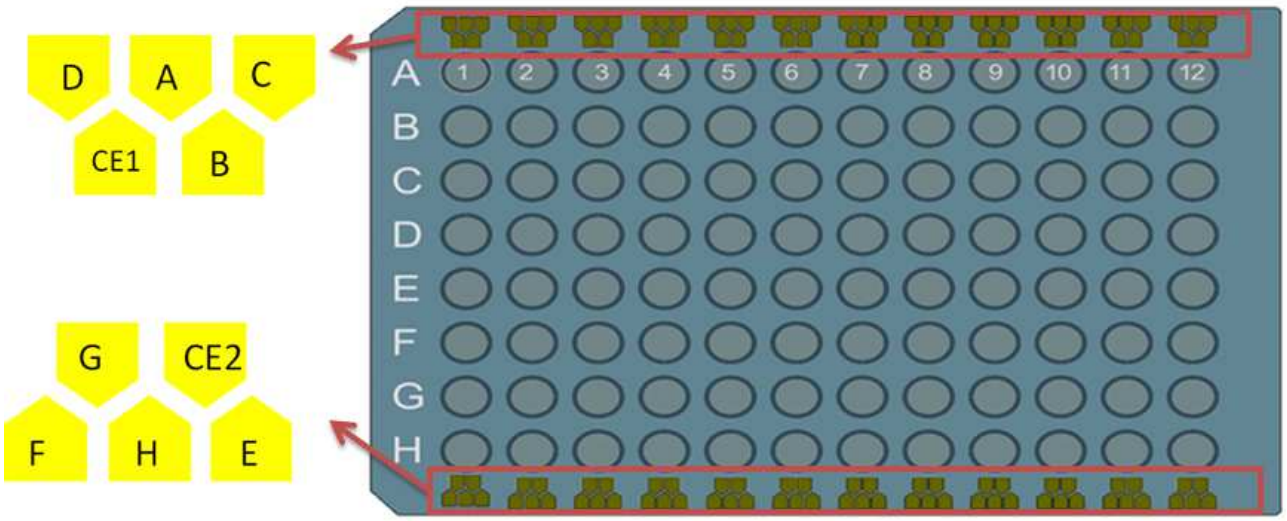


Figure 4 Schematic overview of the electrode structure and its correspondence with the geometry of the plate. In an individual column (numbered 1 through 12), the working electrodes in rows A,B,C, and D share a common counter electrode (CE1) and the working electrodes for row G, F, H, and E share a different common counter electrode (CE2).

After the routing of the electrodes on the 96-electrode was characterized, a design was made to connect the D-sub connector systems of the multiplexing potentiostat unit. This design

consisted of a housing manifold that was designed to enclose the electrode plate in a stable fashion but also be easily removed once electrochemical measurement was completed (**Figure 5**). The housing manifold also possessed a series of conducting pogo pins that connected the electrode system to a circuit board that was appropriately routed to connect to the 37-pin system of the multiplexed potentiostat. The completed design of the housing manifold interfaced with a total of three 37-pin D-sub connectors, each individual 37-pin D-sub connector connecting one of the three multiplexing modules on the CompactStat Potentiostat.

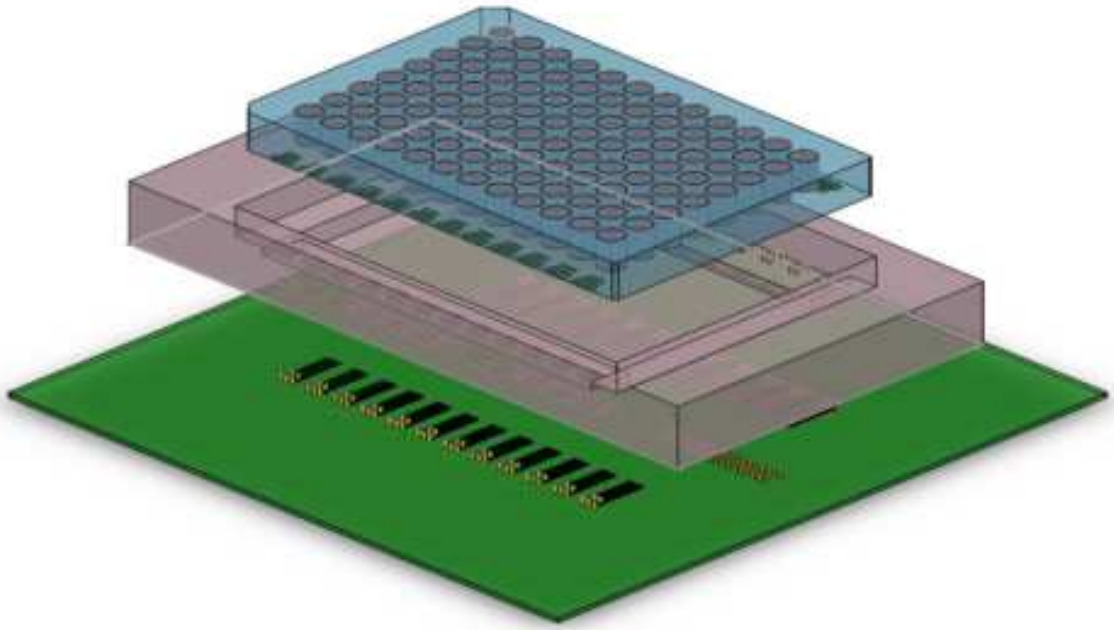

Figure 5. Schematic overview of integration of the electrode structure with the mechanical components. The topmost layer of the construction is the 96-electrode plate which will be placed on top of the mechanical manifold unit complete with pogo pin structures to interface with the electrodes present on the plate. The pogo pins of the middle layer will be interfaced with a printed circuit board on the bottom layer, the printed circuit board routing the connections of the pogo pins in a manner that will facilitate the connection of the 96-electrode plate with the potentiostatic control system (which in this case is an Iviumstat Compactstat).

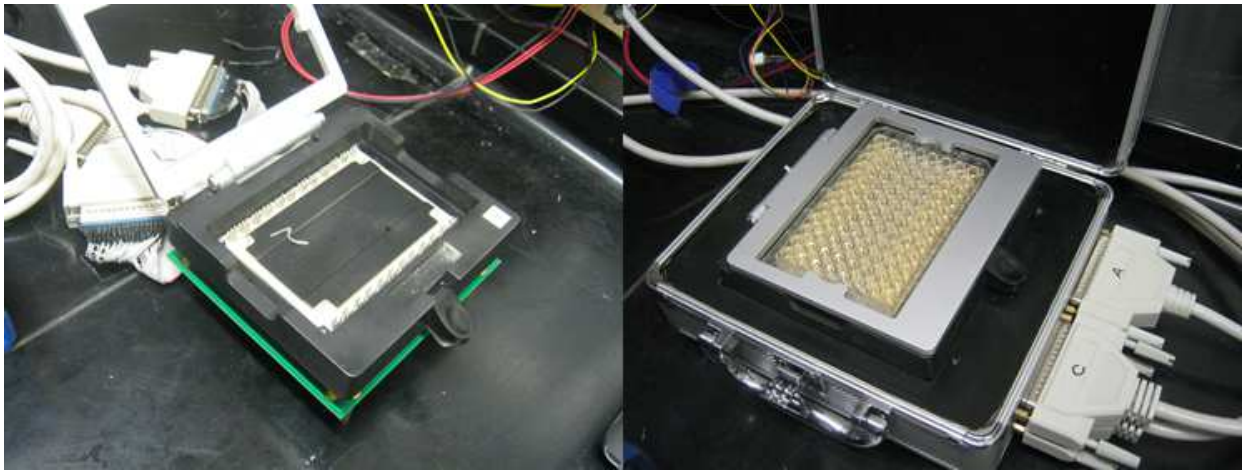

Figure 6. Completed prototype design of the 96-electrode interface unit to mechanically house the electrochemical electrode plate and interface the plate with a potentiostat. The left figure illustrations the open reader with pogo pins that will connect and interface with the 96-electrode plate. The right figure illustrates the interface unit with the 96-electrode locked in place and secured by a locking lid system. Three separate 37-pin D connectors are connected to the 96-well electrode plate for the interfacing with the potentiostat control system.

In order to verify the functionality of the potentiostat system and the electrode plate system, potassium ferricyanide standards were prepared and tested on the 96 electrode plate. Potassium ferricyanide is a commonly used standard for characterizing electrochemical experiments because it can be reversibly oxidized and reduced[10]. For the reduction of potassium ferricyanide, when a negative potential is applied, the electrons at the surface of the working electrode will contribute an electron to the iron and reduce the compound to $[Fe(II)CN_6]^{4-}$. When the electrode has a sufficiently positive enough potential, the electrode charge will drive the ferricyanide to donate an electron to the electrode, and thus the $[Fe(II)CN_6]^{4-}$ will be oxidized back to the state of $[Fe(III)CN_6]^{3-}$.

For the cyclic voltammetry experiments using electrochemical standards, 100 mM potassium ferricyanide standards in 1M KCl solution was made and 60 µL was pipetted to the surface of electrodes in the plate. A cyclic voltammetry protocol starting at a zero potential of +0V, and scanning through the ranges of +1.0 Volts to -1.0 Volts and a step rate of 50mV/second was run to assess the electrodes effects on oxidizing and reducing the potassium ferricyanide.

## *2.4 Peroxidase and Conducting Polymer Subsystem*

In the third phase of the test, a number of tests were made to validate the functionality of the electrode system and the potentiostat system and the suitability of the system for performing biosensing tests.

A. **Peroxidase and Hydrogen Peroxide in Bulk Solution:** Initial assessment of the electrode system to measure the peroxidase-based current generation when mixed with tetramethylbenzidine/hydrogen peroxide mixture was tested. For this protocol, approximately 20 mL of tetramethylbenzidine/hydrogen peroxide mixture (1 Step Ultra TMB from Fisher Scientific) was combined with 5 nanograms of a peroxidase enzyme conjugated to an antibody. This mixture was then added to the electrodes on the plate. This was a method of ensuring that real-time measurement was possible using the peroxidase-enzyme and tetramethylbenzidine/hydrogen peroxide reaction on all channels.

B. **Evaluation of electrochemical polymerization of pyrrole to surface of electrode:** Polypyrrole matrixes have a wide variety of application in biosensor applications [1] [11], and in order to assess the capabilities of similarly developing a polypyrrole biosensor on the surface of the 96-electrode system, a mixture of 1M pyrrole and 0.15M potassium chloride was applied to the surface of the gold electrodes and different electrochemical procedures were tested in order to test whether the distinct darkening of the electrode surface as commonly occurred in pyrrole polymerization would occur.

C. **Electropolymerized encapsulation of protein using pyrrole:** Following the test of whether polymerization could occur using the high-throughput system to electropolymerize pyrrole to the surface of the gold electrode, tests were performed to encapsulate an peroxidase-conjugated antibody in the polymer matrix and verify

whether a biological active component such as an enzyme would still be functional in the polymer matrix. This technique of immobilizing an enzyme in a polypyrrole matrix has been used for fabricating glucose sensors[11]. In this method, a monomer-protein mix consisting of 1M pyrrole, 0.15 M KCl, and 5 µg/mL peroxidase conjugated antibody was mixed together in solution, and a cyclic square wave electropolymerization was applied at the potentials of +1350mV for 1 second and +350 mV for 1 second for a variable number of cycles. Washoff of the unbound analyte was performed, and then the tetramethylbenzidine/hydrogen peroxide mixture was added to the surface. An amperometric current readout was then performed to evaluate the effect of the polymerization on the activity of the peroxidase enzyme and the current quantified.

D. **Evaluation using streptavidin doped in polypyrrole matrix:** Following the tests performed with polypyrrole and a peroxidase enzyme doped in the matrix, tests would be conducted to validate the integration of streptavidin protein into the polypyrrole matrix with electrochemical techniques. In this strategy, 1M pyrrole, 0.15 M KCl, and 10ug/mL biotinylated peroxidase were mixed together, and a cyclic square wave electropolymerization technique was applied, which applied a potential of +1350mV for 1 second and +350 mV for 1 second for a variable number of cycles. Washoff of the unbound analyte was performed, and then a mixture of 2 ng/mL biotinylated peroxidase was incubated on the surface of the electrode for 30 minutes, then a wash off of the unbound biotinylated peroxidase was performed. 60 µL of a tetramethylbenzidine/hydrogen peroxide mixture was then added to the surface of the electrode, and readout was performed by performing chronoamperometric measurement at -200mV for 60 seconds in order to quantify the measured signal of the reaction.

E. **Magnetic Bead Immunoassay Format:** As an additional method of validating that the electrochemical measurement high-throughput platform was able to read the oxidation and reduction of Tetramethylbenzidine/hydrogen peroxide on an immunoassay system, a microparticle based immunoassay was used to perform a quantitative assay. This microparticle assay would allow us to test an entire immunoassay system isolated from the 96-electrode plate (as there is uncertainty about the effect of the electrodes on the immunoassay based system), and comparison studies could also be made comparing the measurement of the chemiluminscent byproducts of the microparticle immunoassay with an electrochemical measurement of the oxidation-reduction of the microparticle.

For this procedure, a streptavidin coated microparticle is incubated with biotinylated 5802 antibody (which is specific for 22 kilodalton isoform of growth hormone). The antibody coated beads are prepared by mixing together 30 µg of Streptavidin coated T1 Dynabeads (life technology) is with 10 µg of Biotinylated 5802 antibody and incubated at room temperature for 6 hours on a magnetic bead rotator. Following this, the magnetic nanoparticles are triple washed with 500 µL PBS, and subsequently incubated with recombinant growth hormone standards in casein/PBS at different concentrations (10 ng/mL, 500 pg/mL, 250 pg/mL, and 0 pg/mL) for 30 minutes with shaking and rotation on a magnetic bead rotator. This standard solution is then removed and three 500 µL washoffs of PBS are again performed on the bead. After this PBS washoff, 250 µL of a 5801 peroxidase-antibody at 5 µg/mL are incubated with the bead and sample complex for 30 minutes at room temperature in order to make a complete bead immunoassay complex. A triple wash off is performed using 500 µL of PBS to remove excess casein/PBS and reporter antibody from the solution, and then 300 µL of Tetramethylbenzidine/$H_2O_2$ is incubated. In this reaction the concentration of color evolution should be proportional

to target of the analyte present, which is what is observed upon performing this experiment. This color evolution should be measurable through the usage of an automated plate reader, and the oxidation-reduction reactions between Tetramethylbenzindine/hydrogen peroxide can also be measured using the high-throughput device developed for this experiment.

This method is operationally more difficult than the proposed usage of a biosensor array directly on the electrode, but this step serves as a transitory evaluation before a full immunoassay is optimized and attempted on the 96-electrode array system. Comparison steps between electrochemical measurements and ELISA chemiluminescent color absorbance will allow us to characterize and make comparison measurements between chemiluminescent methods and electrochemical chronoamperometry, and determine if there is a linear relationship between the two by testing the same reaction using different methods. If this step is successful, it assists in validating whether a complete immunoassay is achievable on the 96-electrode high-throughput system.

# 3 Results

## 3.1 Multichannel Potentiostat Subsystem Validation

The Iviumstat Compactstat unit was acquired and three 32-electrode expansion units were connected to the Compactstat system. The ability of the electrochemical potentiostatic unit to sample on 96-channels was tested by placing a series of dummy cells and interfacing them with the 32-electrode units. These dummy cells consisting of a large quantity of 10kΩ resistor soldered to 37-pin D-sub connectors, which were used to interface with the potentiostat and provide each of the 96-channels of the system with a 10kΩ resistance (see Figure 7).

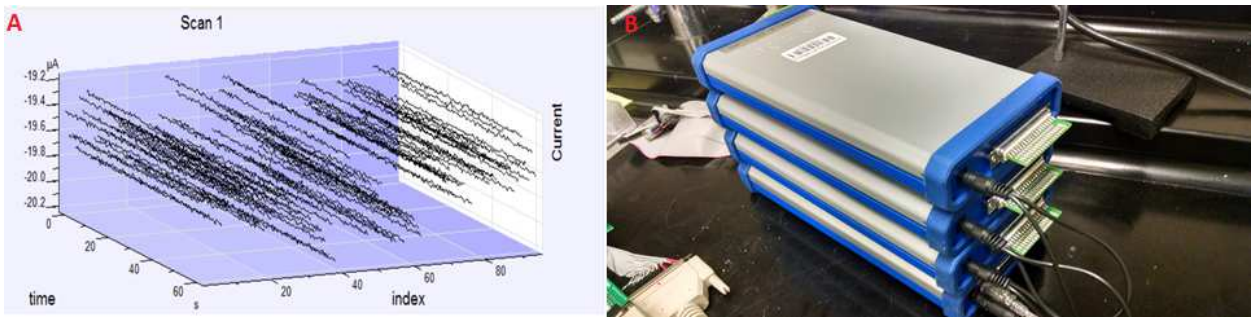


Figure 7. Part A is an example readout of electrochemical current readout across 96 channels using the dummy cells at a set potential of -200 mV. Part B is a picture of the potentiostat unit with dummy cells (10kΩ resistance) attached to the multiplexing electrode unit.

Based on Ohm's law (Voltage = Current x Resistance) and the usage of the 10kΩ dummy cell, a chronoamperometric readout with an applied voltage of -200mV should yield a measured current of approximately 20 µA. Initialization of chronoamperometric readout for 1 minute demonstrated that in performing readout on the 96-channels, the distribution of the readouts was relatively stable, with the sensor measuring between the range of 19.648 µA ± 0.212. Chronoamperometery readings were able to sample each channel at 10 samples per second (the recommended sampling for a multiplexed application), and the lowering of sampling rate did not seem to alter the performance of the dummy cell systems.

## *3.2 Disposable Electrode Plate Validation*

In order to test whether the electrode system that were fabricated were appropriate, a working solution of 100 mM potassium ferricynaide in 1 M potassium chloride was prepared and pipetted on the surface of the electrode. Following the pipetting of the standard liquid upon the electrode, a cyclic voltammetry procedure was run, initializing at a start potential of 0V and sweeping to the +1 V and the -1 V potential ranges. The sweeping rate for the cyclic voltammetry was 50 mV/second, a commonly used scanning rate. A hysteresis curve was observed for the cyclic voltammetry profile, indicating that the connectivity between the electrode and the potentiostat controller was electrically stable.

Observable in the profile of the cyclic voltammetry profile are peaks that are in electrochemistry associated with the redox and oxidation potentials of the solution present on the electrode. The peak observable in the +0V to +1V window of the cyclic voltammetry scan is associated with the oxidation that occurs to the potassium ferricyanide as it is oxidized at the interface of the electrode. A peak is also observed in the 0 to -1V window, which is associated with the reduction of the potassium ferricyanide as the electrode is slowly ramped to a negative potential, and the electrode is able to provide electrons for the reduction of the potassium ferricyanide.

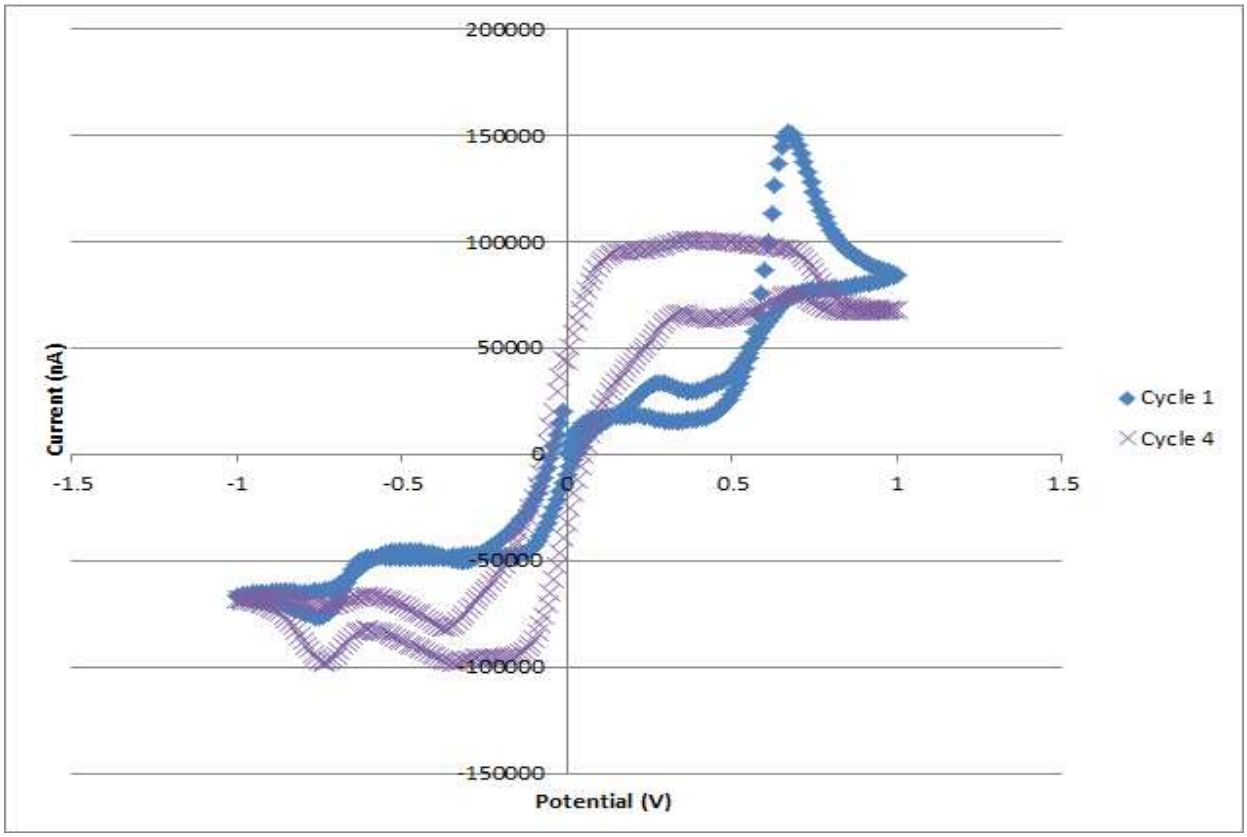


Figure 8. Cyclic voltammetry sweep of potassium ferricyanide (100mM in 1M potassium chloride) for 4 cycles. The blue trace indicates the voltammetry hysteresis curve is for the first cycle where cyclic voltammetry was initiated, while the purple curve shows output for the 4$^{th}$ cycle of the experiment, showing that there is a lowered amount of oxidation occurring by the 4$^{th}$ cycle.

In the analysis of the cyclic voltammetry data by this experiment, it was found that in the context of this experiment, the peak potentials in the first cycle indicate a peak potential at 670

mV for the positive potential range, and the peak potential in the negative range is -750 mV. The existence of these two peaks in the cyclic voltammetry profile indicates that the reaction is a reversible reaction, which is in accord with the known properties of potassium ferricyanide.

Further cycles of the cyclic voltammetry seem to indicate a shift away from oxidation into reduction. This can be observable in the data from the fourth cycle of cyclic voltammetry from the reduction of the oxidation peak in the 670 nm range and the growth of the oxidation peak in the -750 mV range. This reduction of the oxidation peak and increase of the reduction seems to indicate that the solution has been highly oxidized and the predominant form is $[Fe(III)CN_6]^{3-}$ ion.

Examination of the electrode structure following the application of the cyclic voltammetry experimentation found clear differences in the electrode. Compared to a bare gold electrode surface that has not had cyclic voltammetry of potassium ferricyanide applied to it, there is a clear coloration difference on the electrode surface. Furthermore, this coloration pattern is not a randomized pattern, but it is isolated to one of the electrode surface, an indicator that the cyclic voltammetry potential induced this effect.

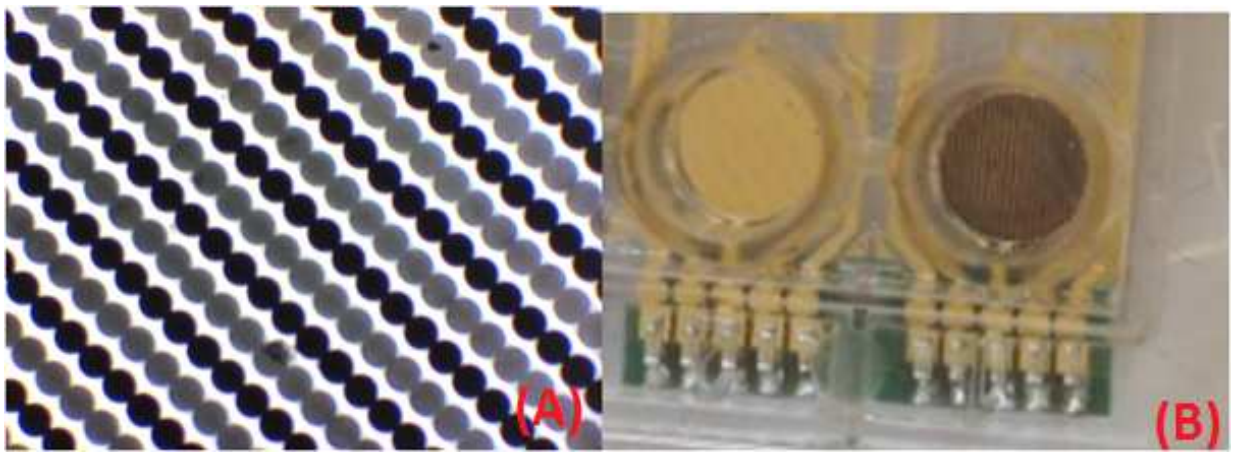


Figure 9. (A) Upon examination of the pattern of the microelectrode, it was clear that the coating can be made to specifically deposit on the working or counter electrode. (B) Comparison of an Au electrode where cyclic voltammetry procedure with potassium ferricyanide was performed with an Au electrode where solution as applied. The cyclic voltammetry protocol was demonstrated to leave a deep coloration on the surface of the electrode. **Peroxidase and Conducting Polymer Subsystem Validation Results**

### *3.2.1 Results of adding tetramethylbenzidine and hydrogen peroxide mixture to the 96-electrode systems*

A component of validation for the basic ability of the 96-electrode was to make a measurement of the electrochemical current in each channel individually. This was performed using a mixture of tetramethylbenzidine, hydrogen peroxide, and a peroxidase labeled antibody. This mixture, upon addition and mixture of the peroxidase labeled antibody, begins to evolve a reaction, which may visually observed by a distinct change of the tetramethylbenzidine/hydrogen peroxide mixture from a transparent solution to a blue colored solution, and electrochemically a current flow of the solution can also be measured. An electrochemical readout procedure was then initiated and measured using the proof-of-concept 96-electrode system and the solution pipetted to the electrode surface. As each column of the 96-electrode plate was filled with the mixture, one could observe (see Figure 10) an increase in measured signal at each interval of addition. Further tests where the mixture was added into one electrode at a time verified that the 96-electrodes possessed the ability to measure current on 96 channels rapidly in real time, and that there were no crosstalk problems between the individual electrodes. This test also functions as a preliminary validation that the tetramethylbenzidine/hydrogen peroxide and peroxidase antibody scheme would be performed using the 96-electrode system.

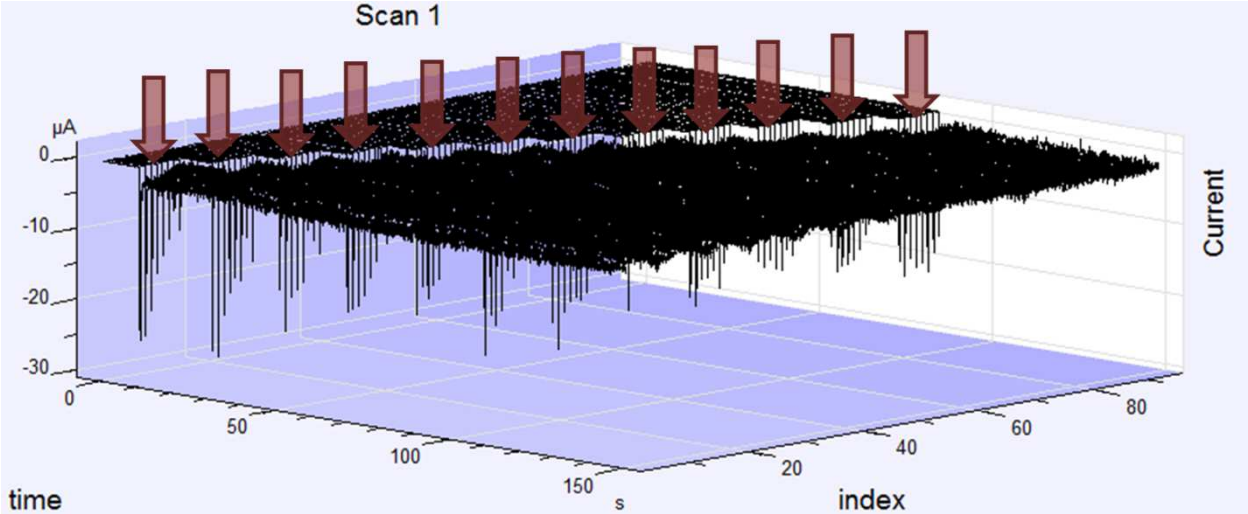


Figure 10 Testing current measurement for 96 electrode system. Tetramethylbenzidine/hydrogen peroxide mixture was mixed with a peroxidase enzyme on an antibody to generate oxidation-reduction reactions, and this reacting solution was then added to the surface of the electrode at approximately 10 second intervals. This test demonstrated clearly that the electrodes were able to make a real-time measurement of the current on the 96-electrodes. Red arrows indicate the points when solution was added to 8 electrodes.

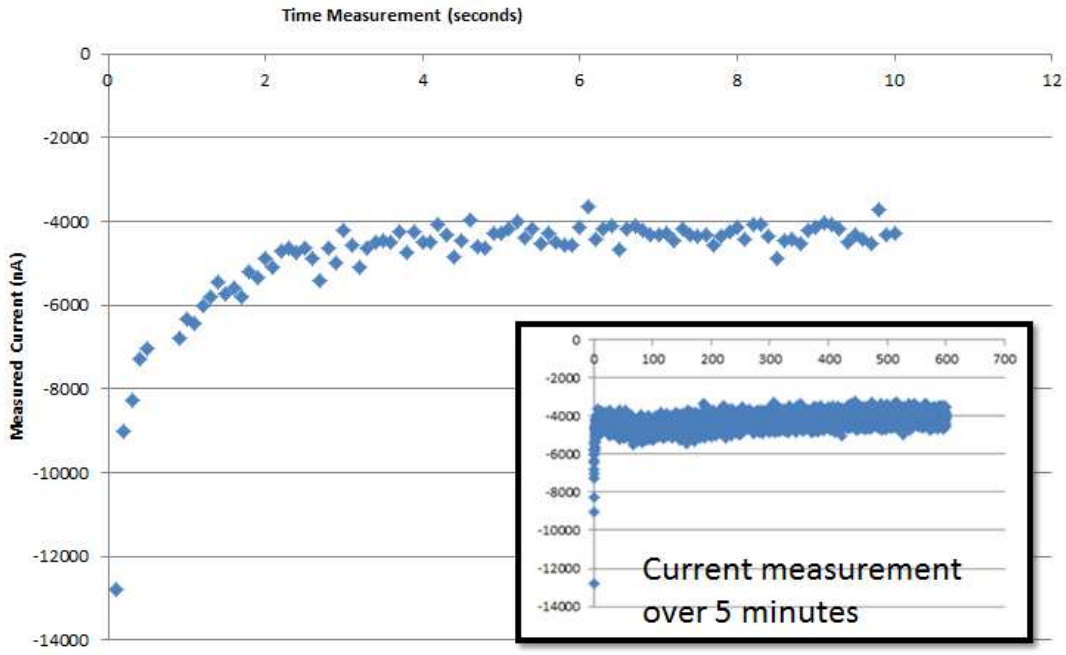


Figure 11. Measurement of electrochemical current over time using the peroxidase enzyme and tetramethylbenzidine/hydrogen peroxidase mixture. Large image shows the behavior of the reaction over a 12

second window, while the embedded subfigure shows the measurement of the electrochemical current over 5 minutes.

Following this real-time addition of the mixture, measurement of the current was made on all the 96 channels for a 5 minute interval (Figure 11). The electrochemical current measurement for the 5-minute interval shows an initial transient measurement of current that is extremely high, but in less than 3 seconds the signal stabilizes. This initial high magnitude current followed by steady state-behavior is ordinarily performance that is observed in electrochemical chronoamperometric measurement as predicted by the Cottrell equation[12], a well-known model which describes the behavior of current related to time in an electrode with a fixed potential.

### *3.2.2 Electropolymerization of Polypyrrole on Gold Electrode Using High Throughput Device*

Having verified that the cyclic voltammetry protocol (see 3.1) was able to be used for oxidation and reduction of a standard electrochemical solution, investigation was made into assessing the viability of electrodepositing of polypyrrole on the surface of the gold electrode. These preliminary protocols were made to determine if the microelectrode structure of the 96-well plate would allow for the high-throughput electropolymerization of pyrrole in order to create a pyrrole based strategy for immobilizing capture probes[11].

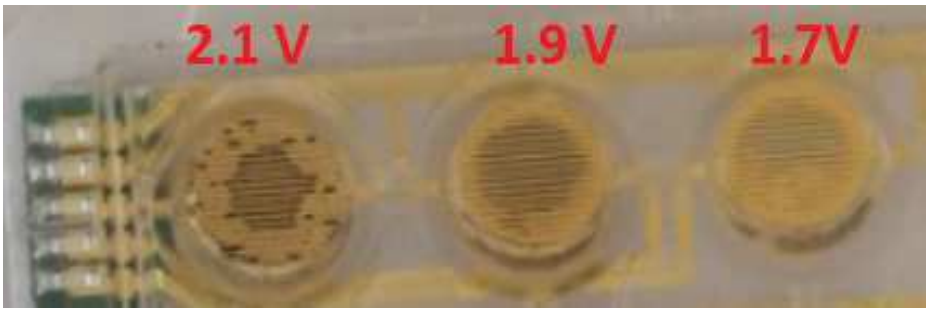


Figure 12. Polymerization pattern on gold electrode with different peak potentials during electropolymerization process.

The results of the experimental work suggested that the formation of the conducting polymer was highly dependent on the peak potential used in the formation of the pyrrole. Figure 12 demonstrates a cyclic voltammetry experiment with a zero potential of 0 V and sweeping to different peak potentials. The leftmost electrode on Figure 12 demonstrates a polymerization pattern on the electrodes when a peak potential of +2.1 volts was selected for the electropolymerization of pyrrole on the surface of the electrode, a pattern where coloration of the sensor is extremely dark but the formation pattern on the electrode surface is particularly uniform and coloration occurs in a random pattern. Lowering the peak potential to +1.9 V and +1.7 V in cyclic voltammetry led to a reduction in the randomness of the pyrrole deposition, and a more uniform centralization of pyrrole deposition at the center of the electrode. These initial results were also useful in verifying that deposition of polypyrrole was possible on the high-throughput electrode plate.

Following this validation based on the applied potential, tests were run to determine the effect of polymerization time on the formation of the polypyrrole layer. In this approach, a cyclic square wave was used for polymerization, an approach that had previously been applied with success to biosensor development[13]. The cyclic square wave was applied in increments of 10 cycles across a column of electrodes of the high-throughput 96-electrode plate. The results of the experimentation on the amount of cycles of +1350 mV for 1 second and +350 mV for 1 second demonstrate that the electropolymerization process is dependent on the amount of time the electropolymerization is applied to the surface of the electrode. This can be concluded from Figure 13B, as a clear gradient of darkening can be observed on the electrode surface as additional cycles of electropolymerization are initiated on the surface of the electrode. Figure 13A also demonstrates that this electropolymerization is primarily centralized around the center of the electrode well, and it does not appear on both the counter and working electrode, but only on one of the electrodes.

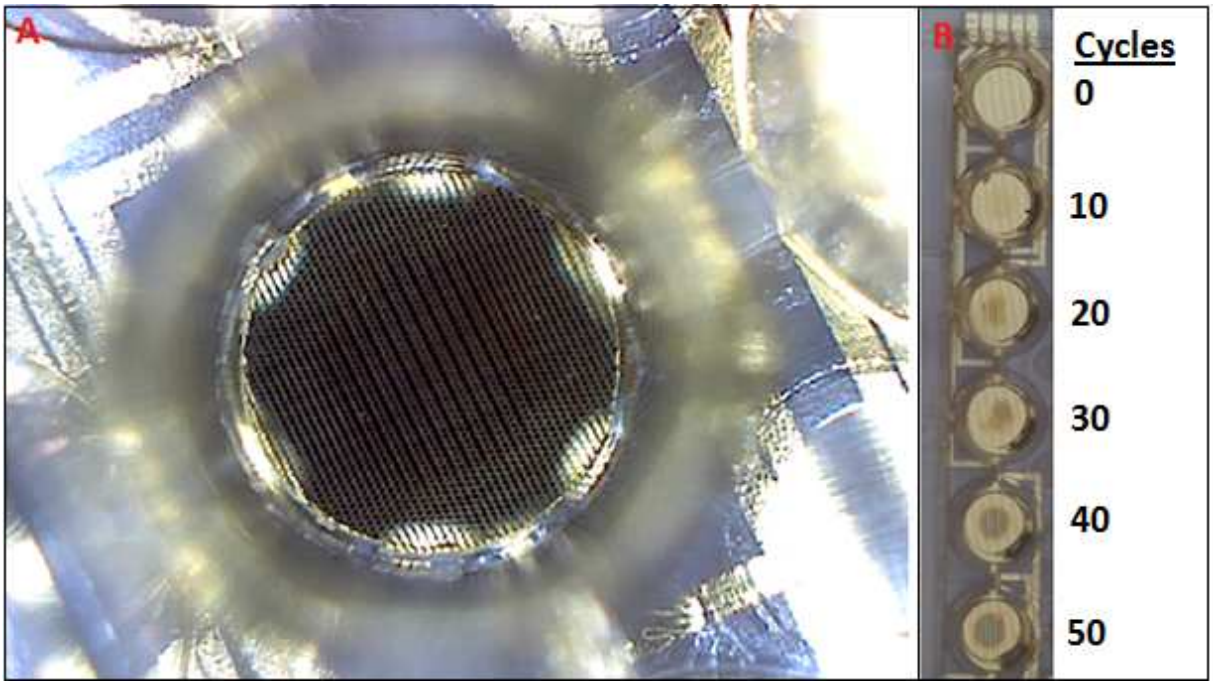


Figure 13. Electropolymerization of pyrrole of different cycle lengths. Section A shows a magnified image of the sensor surface of an electrode where the electropolymerization with pyrrole has been applied. The distribution of the pyrrole is concentrated on only one of the electrodes, which is as predicted. Section B illustrates the darkening of the electrode structure as increasing amounts of polymerization are applied to the electrode surface.

### *3.2.3 Validation of Pyrrole polymerization using peroxidase conjugated antibody*

Following the electropolymerization of a peroxidase-conjugated antibody to the surface of the electrode, the tetramethylbenzidine/hydrogen peroxide readout solution was added to the electrode and current readout was performed. When the solution is added to the sensor surface, one can observe the blue coloration that is consistent with tetramethylbenzidine that is being oxidized. Electrochemical measurement could be made by using a chronoamperometric method for 60 seconds, with averaging being performed on the last 10 seconds of the electrode as a final metric for experimentation.

Evaluation and averaging the current in the wells found a very consistent distribution of signal with similar conditions. As can be seen by the data of Figure 14, statistically significant

amount of signal is increased when 4 electropolymerization cycles are applied of the electrode mixture compared to the wells where the mixture solution was pipetted into the well but no electropolymerization was applied. Interestingly enough, in the intervals after 4 cycles, there is a clear observable trend in the decrease of the measured electrochemical signal as the amount of cycles of electropolymerization is applied. This may suggest that the excess electropolymerization cycles may cause the polypyrrole density to increase to the point where it will cause the steric hindrance of the peroxidase that is conjugated to the antibody, and thus hinder its ability to catalyze the peroxidase/tetramethylbenzidine reaction.

In this study, the peroxidase antibody in a PBS solution without any pyrrole was also pipetted to the surface of the electrode in order to make a comparison metric between the effects of the electropolymer in comparison to an approach such as the direct adsorption of antibody on the electrode surface. As the measurement of current indicates, it seems that the electropolymerization process for encapsulating the antibody leads to an over 2-fold increase in signal magnitude compared to the antibody based adsorption method. This is an encouraging note, but it must also be noted that in the development of electrochemical assay, the fact that a peroxidase antibody was incubated and adhered to the surface of the gold electrode in a quick amount of time, enough to yield a measurable signal, indicates that precautions must be made in the course of electrochemical biosensor development to make tests for non-specific binding of biomolecules to prevent them from adding to the noise of the signal.

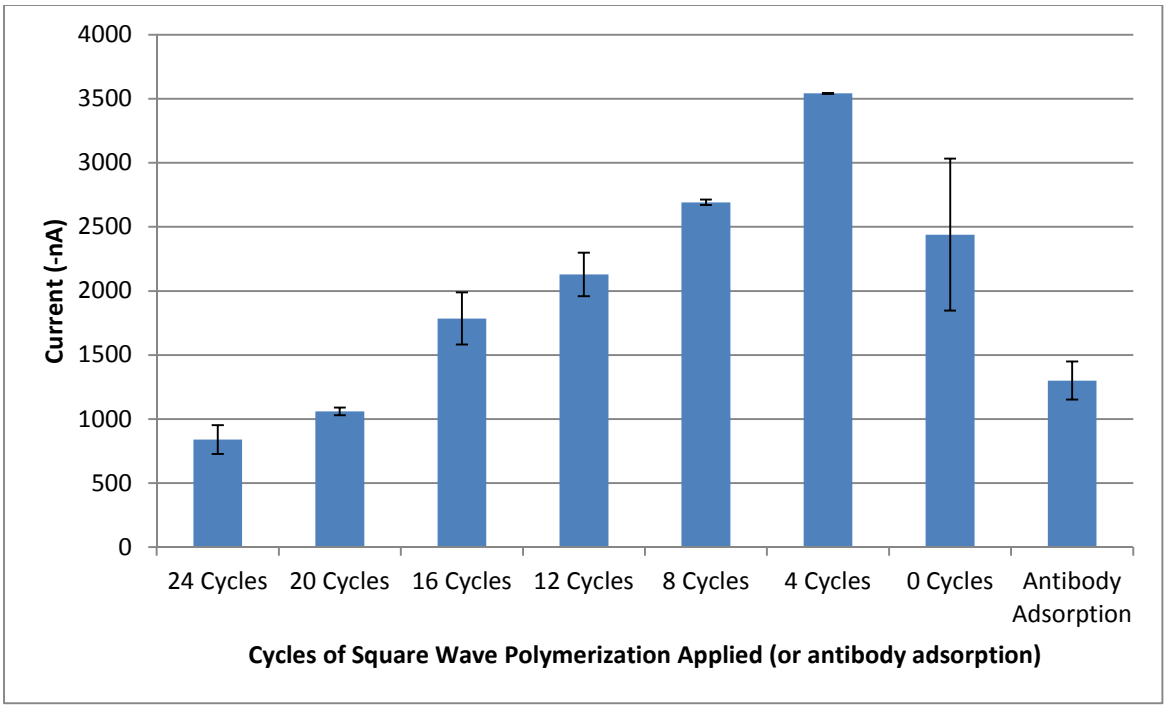


Figure 14. Current measurement of different polymerization cycles of peroxidase-antibody embedded in a polymer matrix. While there appear to be adsorption of the antibody on the gold electrode surface, results indicate a significant increase in measured peroxidase signal when the antibody is electropolymerized in the pyrrole

### *3.2.4 Polymerization of Streptavidin*

Following the validation of the ability of the pyrrole system to immobilize the peroxidase conjugated antibody inside the polymer matrix, experimental work was also conducted to determine if a streptavidin protein would be capable of being integrated into the polymer matrix. This was done by taking a mixture of pyrrole, KCl, and streptavidin, and electropolymerizing at different cycle lengths using the +1350mV for 1 second and +350 mV for 1 second cyclic square wave profile at different cycles. This step would be useful in developing a biosensor system because a streptavidin base would be useful in immobilizing any molecular probes or biomolecules that can be biotinylated. Different amounts of cycles of electropolymerization were applied on the surface of the electrode (including tests in wells where the monomer solution was applied but no electropolymerization was applied). Following wash off of unbound

monomer and incubation of a biotinylated peroxidase (and the wash off of the unbound biotinylated peroxidase), signal readout was performed on the electrode.

As the results of the experiment demonstrate (see Figure 15), there is an appreciable 30% increase in the signal observed when comparing the wells that had 2 cycles of electropolymerization applied compared to the wells where 0 cycles of electropolymerization were applied. No clearly discernable trend was observed when the amount of cycle of electropolymerization were increased using the streptavidin approach, but in general it seems that there is a slight decrease in average measured current when the cycle lengths are increased beyond 6 cycles. All the electropolymerized wells show a statistically significant improvement in signal levels compared to the wells where electropolymerization did not occur. This data seems to suggest that the streptavidin immobilization using pyrrole technique is effective in immobilizing protein.

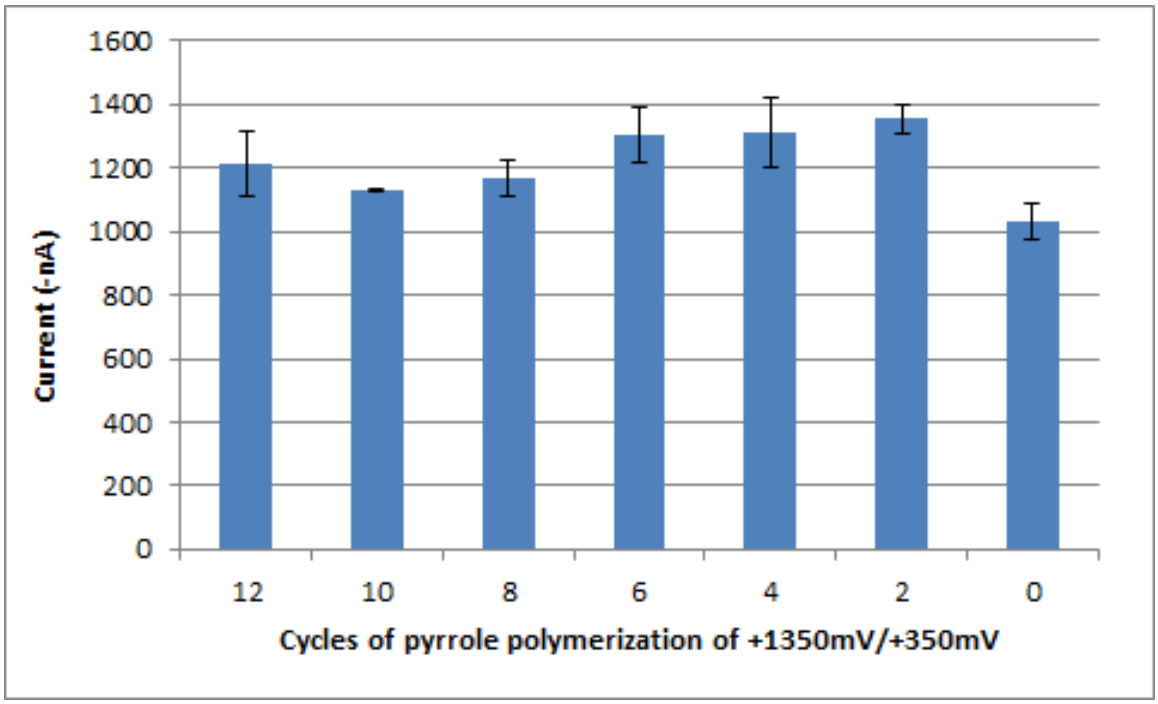


Figure 15. Comparison test of cycle lengths of streptavidin doped in a conducting polymer matrix. Optimization studies seem to indicate that 2 cycles of +1350/350mV is the optimal combination for the electropolymerization of streptavidin protein on the surface of the electrode

Just as in the case of the peroxidase-conjugated antibody polymerized in the bead, it must be noted that even the wells where no polymerization were applied seem to indicate that streptavidin still managed to passively adhere to the electrode . This consideration that nonspecific binding to the electrode occurs must be made in future development, and it may be appropriate to develop techniques to prevent nonspecific binding in order to improve electrode sensitivity.

### *3.2.5 Comparison Between Chemiluminscent ELISA and Electrochemical Quantification*

Validation of the applicability of a high-throughput electrochemical system for the biodetection of protein targets involved an experiment comparing electrochemical measurement and a chemiluminescent plate-reader measurements on the same microparticle based ELISA technique. This comparison is possible because both the electrochemical method and a chemiluminscent ELISA system utilize the byproducts of the oxidation-reduction reactions between tetramethylbenzidine, hydrogen peroxide, and peroxidase enzyme: the chemiluminscent ELISA technique utilizes the color change that occurs in solution during a reaction, and the electrochemical sensor utilizes the oxidation and reduction of tetramethylbenzidine to create a quantifiable measurement. Using an independent third method (ELISA on microparticles), the resulting solution was then evaluated for four concentrations of growth hormone in a casein/PBS buffer (10 ng/mL, 5 ng/mL, 2.5 ng/mL).

Taking the resulting mixture of tetramethylbenzidine, hydrogen peroxide, and the bead-sandwich, and testing for the absorbance of the solution at the 370nm and making electrochemical measurements of the same mixture on the 96-electrode plate, the results (Figure 16) of comparing the signal levels of the two methods show that the linearity between the averages of the measured signal have a high linearity ($R^2$ = 0.93). This data is suggestive that when taking identical measurements of the same sample, the electrochemical method and

the chemiluminscent absorbance measurement techniques correlate quite well, and the electrochemical measurement technique is very likely capable of performing quantitative measurements of a protein using an antibody sandwich technique.

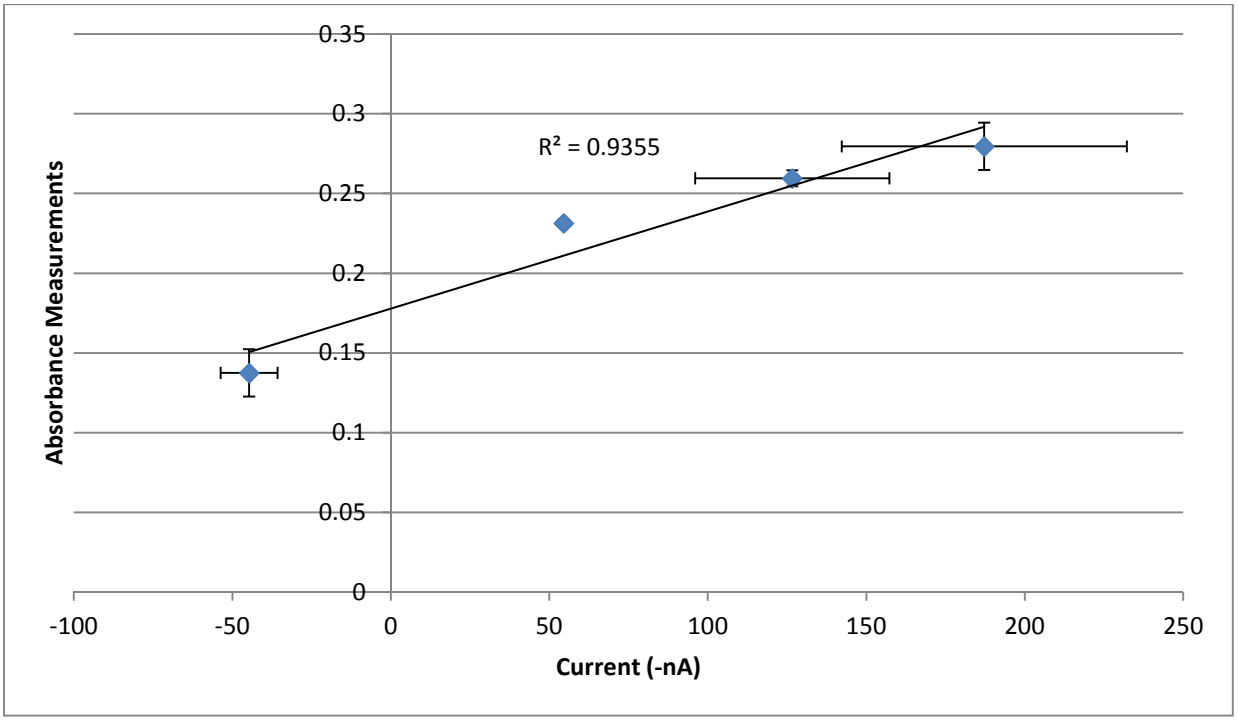


Figure 16. . Comparison study between chemiluminescent measurement of magnetic bead experiment and the electrochemical readout of experiment. There appears to be a correlation between the plate reader and electrochemical measurement.

# 4 Conclusion

What was herein outlined in this study was a description of the development of a high-throughput system for the electrochemical measurement of biomolecules. The existing precedence of biosensors for performing high sensitivity detection and the lack of a truly functional existing rapid high-throughput electrochemical biosensor system serves as a ground for the development work performed in this study. A description of the development method and prototyping method was described, along with the resultant experiments that attempt to validate

that the proof of concept system was functional and capable of being used for biosensor based experiments.

The results of this work show that a prototype system has been developed that is capable of performing parallelized readout of electrochemical current 96-channels at a rate of 10 samples per second. This ability to perform readout is advantageous in speed compared to existing techniques such as the chemiluminescent ELISA, which typically has a plate reader sequentially read samples, or the Lumina magnetic bead approach, which also reads the plates in a well sequentially. Experimental results seem to suggest that the performance of the prototype system correlates well with the results of plate-reader based systems.

Experimental work also seems to validate that the pyrrole based biomolecule immobilization strategy can be easily integrated into the high-throughput electrochemical platform, and data is highly suggestive that this pyrrole approach is able to be useful for enhancing the signal of an assay. This pyrrole immobilization technique is used in a wide variety of detection contexts, and its ability to be used on the high-throughput system suggests that the high-throughput will be able to perform quantitation for targets in both protein and nucleic acid based systems.

There are still some unknown questions remaining in the study. Experimental results demonstrated that adsorption of proteins occurred extremely quickly on the gold electrode plate (see Figure 15 and Figure 14), and further investigation is necessary in order to determine whether preventing adsorption of undesired proteins is possible. Furthermore, though the proof-of-concept system is able to perform these techniques, the present study did not comprehensively investigate other techniques commonly used in electrochemical biosensors such as differential pulse voltammetry and electrochemical impedance spectroscopy, and these

studies would be helpful supplements in assessing the capabilities of the electrochemical system.

The results herein demonstrated seem to validate the hypothesis that a rapid high-throughput electrochemical biosensor system for protein markers is possible. If developed further, it seems a high-throughput electrochemical system would be highly useful in a wide variety of laboratory contexts where accurate and rapid quantitation of biomarkers is necessary.

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# Chapter 3 – Whole Blood Growth Hormone Isoform Assay

## 1 Introduction

Due to the variability of human growth hormone in the body due to activity[1] and the wide variation of human growth hormone levels across ages and genders, it is inadequate to merely quantify total human growth hormone concentration in athletes to distinguish between doping and non-doping athletes. The existing gold-standard method for identification of growth hormone doping is through the testing of athlete serum samples and quantifying the different isoforms of growth hormone that are found. Currently, the standard isoform ratio test is the discrimination between the 22 kilodalton (the most abundant form that is produced by the human pituitary gland) and all forms of pituitary growth hormone expressed in the body. If the ratios between 22 kilodalton growth hormone and all other forms of human growth hormone are found to exceed certain natural limits[2] in the human body, it becomes probable that the athlete may be administrating growth hormone in an unauthorized fashion for enhancement of performance.

In terms of practically executing the collection and testing of growth hormone isoforms in athletes, there is a complex workflow that is necessary in order to collect samples and perform the tests. First, the athlete must subject himself to an intravenous blood draw from a trained phlebotomist. Following this intravenous blood draw, the blood sample must be spun and the red blood cells removed, and the resulting serum sample must be quickly transported at a controlled temperature environment to the testing facility where the isoform quantification test will be run. Failure to retain the sample at a specific temperature range can result in the

disqualification of the sample. After arrival at the site of testing, a trained technician must take the collected serum samples and perform a multistep ELISA assay in order to quantify the growth hormone isoform levels present in the serum. This multistep ELISA protocol is an assay that is performed without laboratory automation, requires a fair amount of dexterity to perform the washing and sample preparation within specified time limits (or else degradation of samples and reagents will occur), and requires two separate 2-hour long incubation times for the completion of the assay. The existing test for growth hormone isoform discrimination is performed in a series of test tubes that have been pre-coated with antibodies specific for growth hormone isoforms, as opposed to the conventionally used ELISA 96-microplate format which is able to hold all samples in a 130cm x 90cm footprint for washing and sample preparation. This multistep ELISA protocol that is performed across multiple tests tubes has a high chance of increasing variability and human error during the performance of the protocol:

In this section of my dissertation, I seek to make an assessment of whether a whole blood based electrochemical immunoassay system can improve and simplify growth hormone testing. Because electrochemistry is able to achieve high sensitivity detection of protein biomarkers, it can be hypothesized that it will allow scientists to quantify specific isoforms at sensitivities unachievable by existing assays for growth hormone. Specifically, electrochemical techniques may be sensitive enough to allow for the testing of the 22 kilodalton to 20 kilodalton growth hormone isoform ratio, as opposed to the method that compares the 22 kilodalton isoform to all pituitary forms of human growth hormone test. My study seeks to make a preliminary assessment of whether the 22 kilodalton to 20 kilodalton growth hormone isoform ratio test is feasible by combining electrochemical techniques with monoclonal antibody pairs that specifically target the 20 kilodalton or 22 kilodalton forms of growth hormone.

Another additional facet that we wish to explore in our platform development is whether the electrochemical method can allow for the assaying of protein targets without removing red-

blood cells. This removal of red-blood cells is generally considered useful to reduce the matrix effects that can negatively affect assay performance, but adds to the complexity of collecting and processing collected samples. This complexity is due to the fact that converting collected whole blood to serum requires the addition of a motorized rotator to separate out red blood cells using centrifugal force and a human operator to remove the serum that occurs when separating out red blood cells. Other systems have demonstrated that it is possible to make electrochemical quantitation of various analytes (such as uric acid[3] or TNF-α [4]) in whole blood situations, and this fact alongside the absence of a whole blood assay in growth hormone makes electrochemical investigation of whole blood assays a meritorious exploration. If experimental work validates that growth hormone is detectable in whole blood samples using electrochemical techniques, it will be a critical milestone in demonstrating that growth hormone detection methods can be improved and the collection process can be simplified.

# 2 Method

## 2.1 Workflow of Assay Development

The strategies and experiments that were pursued for the validation of a sensitive growth hormone assay for 22 kilodalton and 20 kilodalton growth hormone isoforms can be divided into two main phases:

A. **Initial Proof of Concept Phase:** Initial experimental work on the project utilized a Genefluidics 16-array Helios platform, which is a multichannel potentiostat system developed for the purpose of being a quantitative platform capable of performing detection for a variety of nucleic acid and protein based targets. Previous works have indicated that high sensitivities have been achievable using this platform on serum[5] and saliva[6] based biomarker targets using a conducting polymer strategy, and alternative

configurations of biosensor fabrication using a thiol-based techniques have also been used for bacterial detection[7]. Because the system has been well characterized and previously applicable to a wide variety of biomarkers, this platform was selected as an appropriate test platform for testing electrochemical approaches to detection of growth hormone in whole blood samples, and whether an isoform form based discriminatory test system could be developed. A number of tests and optimizations were conducted on factors such as antibody concentration, incubation time, and electrode coating layer in order to gain a preliminary assessment of whether whole blood assay with picogram level sensitivity and high linearity was tangibly achievable. The results of a proof of concept experiment using the 16-electrode platform would serve as a foundation of validating that the antibody pairs for 22 kilodalton and 20 kilodalton growth hormone were able to specifically identify their desired isoforms without interference.

B. **High-Throughput 96-electrode assay development phase:** Following assessment of the assay functionality using the 16-electrode array system and validation of the antibodies performance, steps would be taken in this second phase to determine if the working components of the 16-electrode array system would be adaptable to a microelectrode structure on a prototype 96-electrode array experimental system. This 96-electrode array was more in line with the desired end-product of a high-throughput automated electrochemical array system, but was relatively uncharacterized in performance, and thus initial tests on the 16-electrode system were necessary to separate prototype equipment technical performance from issues with the electrochemical antibody system. In this 96-electrode array assay development phase, optimizations on a variety of assay factors (antibody concentrations, incubation times, etc.) would be performed in a similar fashion to the 16-electrode system to make an exploration of whether the different electrode geometry and potentiostatic control equipment would positively or negatively affect performance of the assay.

## 2.2 Method of 16-Array Chip Electrochemical Biosensor for Growth Hormone Tests

The electrodes utilized on the initial first phase of this project are 16 bare gold electrodes that are easily interfaced with the Genefluidics Helios potentiostat reader through a holder unit. This electrode is a sheet of plastic with sputter deposited gold electrode to make up a three-electrode system of working, counter, and reference electrode. Using this electrode system connected to the Genefluidics Helios system, basic potentiostatic control and electrochemical readout could be performed.

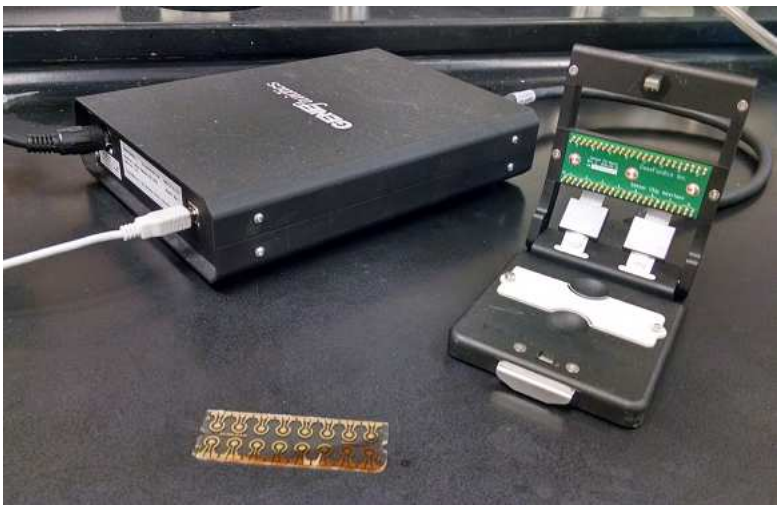


Figure 17. The Genefluidics 16-array electrochemical platform that was used in the initial phases of experimentation. This is a well-validated electrochemical system that has been widely applied to a variety of detection contexts, and serves as an initial first platform for evaluation of the electrochemical assay method prior to tests for adapting the electrochemical method to a more high-throughput context.

For the initial interactions of the fabrication of the first coating layer of the 16-array chip, first a plastic well with an adhesive layer is applied and compressed to isolate the electrode on the 16-electrode chip and prevent cross-contamination during electrochemical procedures. Following this, a preparation of a mixture of 0.1M pyrrole monomer (Sigma Aldrich), 0.15M potassium chloride (Mettler Toledo), and streptavidin dendrimer (UltraAmp$^{TM}$ from Genisphere) is diluted in distilled water and vortexed together to disperse the monomer and dendrimer in solution. Following the mixture of this monomer-dendrimer mix, 60uL of this solution is pipetted

to the individual electrodes present on the Genefluidics well (the electrodes are kept separate with a plastic well divider that is applied to the 16-array chip). The electrode is connected to the Genefluidics potentiostat unit, and an application of +350mV for 1 second and +950mV for 1 second is applied for a total of 5 cycles, the purpose of which is to facilitate a reaction that electropolymerizes the pyrrole into a polymeric layer of polypyrrole on the surface of the gold electrode, with the streptavidin dendrimer structure intercalated and trapped within the polypyrrole matrix. Unbound analytes are then triple washed using distilled water.

Various different coatings besides the streptavidin dendrimer based approach were also tested:

1. Streptavidin-coated nanoparticles method: This protocol modified the above protocol by replacing the streptavidin dendrimer in the polymer mixture with $3.5x10^7$ streptavidin coated gold beads of different diameters. This approach was a candidate approach that was considered because of its potential to have more streptavidin present on the electrode compared to the dendrimer approach
2. Streptavidin-Adsorption: This method simply incubated 10ug/mL solution of streptavidin on the surface of the gold electrode for room temperature. This approach was a candidate approach that was considered for increasing the streptavidin density on the gold electrode compared to the dendrimer approach.

Following the application and optimization of the initial coating layer, a biotinylated antibody which is specific to the targeted isoform of human growth hormone[8] is diluted in phosphate buffered saline solution and applied to the surface of the electrode, and incubated at room temperature for 120 minutes to facilitate binding of the streptavidin units on the dendrimer and the biotinylated antibody in order to form a capture antibody layer. The antibody that has not bound to the dendrimer is then triple washed with distilled water. The antibody pairs used for

detection of the 22 kilodalton form of growth hormone is the 5802/5801 antibody pair commercially available from Medix Biochemica. This antibody pair has been previously applied[8] [9] to the study of growth hormone levels and is characterized to have high specificity for the 22 kilodalton growth hormone isoform. The antibody pair used for the 20 kilodalton growth hormone is the 1c72/5cr antibody pair previously validated by Wu et al[10] to be specific for the 20 kilodalton isoform of growth hormone. This antibody was acquired by material transfer agreement from one of the co-authors of Wu et al[10].

Following this creation of a capture antibody layer, the capture and detection of the target analyte by the biosensor can occur. Standards of 22 kilodalton (Life Technologies) human growth hormone in Casein/PBS (Thermofisher) were incubated on the sensor surface at room temperature minutes to allow the antibody to capture the growth hormone present in the standards. The unbound analytes are then triple washed with distilled water, and a mixture of Peroxidase-Conjugated Antibody (Conjugated with Dojindo Technologies Amine-Peroxidase kit) specific to the targeted isoform of human growth hormone diluted in Casein/PBS is applied to the surface and incubated to complete an antibody sandwich. The unbound analytes are again rinsed off using distilled water, and a mixture of tetramethylbenzidine/$H_2O_2$ (1-Step$^{TM}$ Ultra TMB from Fisher Scientific) is applied to the surface of the electrode for reading at -200mV for 60 seconds, averaging the last 10 seconds of the current readout of the 16 channels as a quantifiable metric for the level of biomarkers.

Figure 18. Photograph of the 16-array Genefluidics array electrode with a plastic well (in green) separating the individual electrodes. Whole blood samples are being incubated on the surface of the electrode chip, where upon completion of incubation wash off blood will be performed with distilled water. The remaining portions of the assay after this sample incubation step are the incubation of a detector antibody in casein/PBS, the washoff of unbound detector antibody, and pipetting of tetramethylbenzidine/$H_2O_2$

## *2.3 Method of 96-Electrode Plate for Growth Hormone Tests*

For these experimental investigations into whether a whole blood growth hormone isoform test can be performed, the electrochemical footprint was a 96-electrode array from ACEA Biosciences, which was utilized and interfaced with an Ivium Technologies Compactstat with three MultiWE32 electrodes to perform simultaneous readout on the 96-electrode channels. The electrode plate was interfaced with the potentiostat control system through a custom fabricated connector unit that consisted of a mechanical manifold to lock the plates to a series of conducting pogo pins, and a printed circuit board that properly connects the working and counter electrodes of the 96-electrode plate to the multiWE32 electrodes.

For the initial coating layer of the electrode, a streptavidin polymer approach was taken by incubating and vortexing together 10 µg of streptavidin protein (Pierce Biotechnologies) and 5uL of reagent grade pyrrole monomer (Sigma Aldrich) with density of 0.967 g/mL to arrive at final concentration of 0.1M polypyrrole, and 0.15M KCl (Mettler Toledo). 35uL of this monomer-protein mixture was placed on the surface of each well of the 96-electrode array, and electropolymerization procedures were applied by applying +350mV for 1 second alternating with +950mV for 1 second for 4 cycles, totaling a procedure of 8 seconds in length on the 96-electrodes. Distilled water was used to rinse off the unbound analytes.

Following the creation of this initial coating layer on the electrode, a biotinylated growth hormone specific antibody diluted in PBS was applied to the surface of the electrode at room temperature and incubated for 120 minutes at room temperature. A distilled water rinse off of unbound biotinylated antibody was performed after this incubation. Just as in the 16-electrode system, the antibody pairs used for detection of the 22 kilodalton form of growth hormone were the 5802/5801 antibody pair commercially available from Medix Biochemica, and the antibody pair used for the 20 kilodalton growth hormone were the 1c72/5c4 antibody pair acquired by material transfer agreement from Wu et al[10].

For the capture of the target analyte and readout, standards of 22 kilodalton human growth hormone (Life Technologies) diluted in whole in sheep blood was incubated on the surface of the electrode for room temperature for 30 minutes. After a wash off of the sample, a detector antibody conjugated to peroxidase in casein/PBS was incubated on the electrode surface for 30 minutes at room temperature. Following this, a Tetramethylbenzidine/$H_2O_2$ mixture was pipetted on the electrode surface, and a current measurement was made for 60 seconds. The results of the experimental were evaluated through the usage of a chronoamperometric readout at -200mV for 60 seconds, and averaging the current of the last 10 seconds.

Just as in the development of the 16-electrode array system assay of human growth hormone, a number of different optimizations and tests have been conducted in order to test the enhancement of the 96-electrode array system. Integrations of the 96-electrode plate with automation tools such as a Beckman Coulter Biomek 3000 liquid handling platform and an Amersham Biosciences Biotrak II Plate Washer was performed in order to increase the uniformity of experiments performed.

# 3 Results

## 3.1 Tests Using 16-Electrode Array

### 3.1.1 16-Electrode Chip Optimization Studies and Initial Results

Initial optimization studies for the concentration of growth hormone capture and detectors antibody was performed. In the capture antibody optimization experiment (Figure **19**), different serial dilutions of biotinylated capture antibody were applied to the surface of the electrode surface and each antibody concentration was incubated with a positive control of 500 pg/mL of growth hormone in casein/PBS, while a negative control of only casein/PBS without any protein present was also incubated on the surface of the electrode. Based on the results of the experiment, it appears that if the biotinylated antibody incubated on the surface of the electrode had a concentration below 6.25 µg/mL concentration, there was a significant drop off in the observed positive growth hormone signal. For the evaluation of the signal of the casein/PBS only negative control, it seems that the negative control sample is relatively stable. The fact that there does not appear to be a dramatic increase in the observed signal after the concentration is above 6.25 µg/mL may suggest that the electrode has been saturated with biotinylated capture antibody, and addition of higher concentrations of antibody would not significantly increase the assay performance.

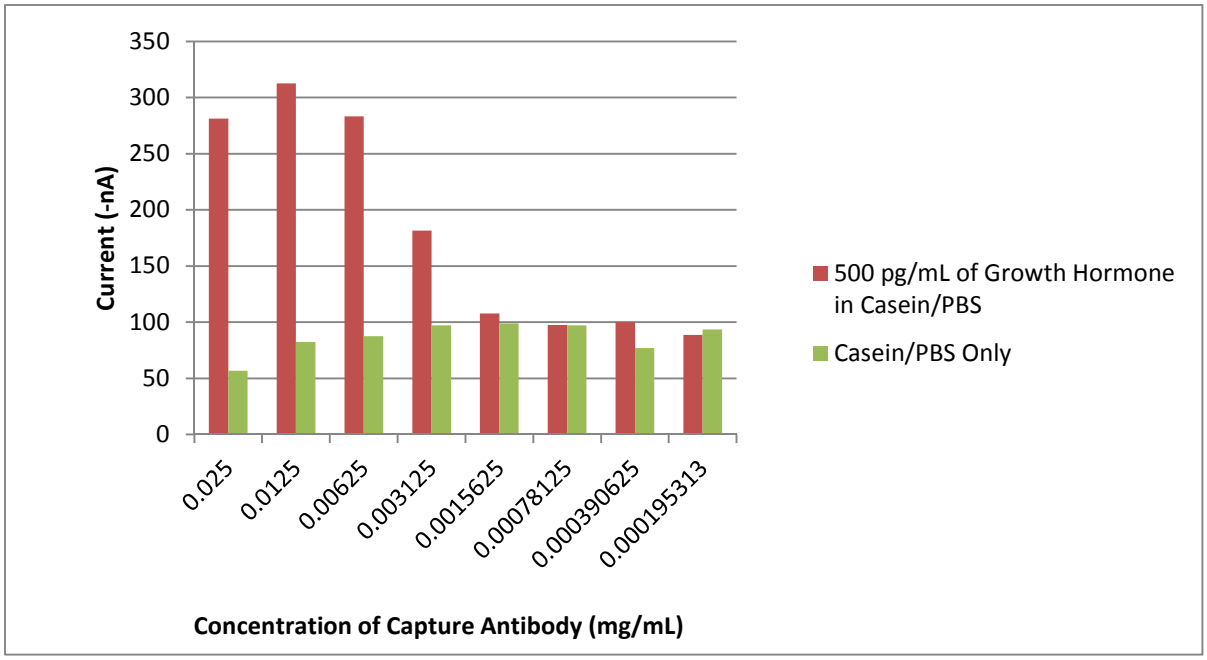



Figure 19 Experimental optimization of the capture concentration, testing the positive and negative controls on different concentrations of biotinylated 5802 antibody. 8 concentrations were tested on a 16 electrode chip, a positive control sample (500 pg/mL of casein/PBS) and a negative control (casein/PBS only) was tested for each concentration.

Similarly, the peroxidase-conjugated detector antibody optimization experiment (Figure 20) shown below compares different concentrations of detector antibody and their resulting signals on an electrode where positive controls of 500 pg/mL of growth hormone in casein/PBS and negative controls have already been incubated. Based on this data, a significant signal drop off occurs when the concentration of the peroxidase conjugated reporter antibody is lowered below the concentration of 1 µg/mL. There is also an appreciable improvement of signal when the detector antibody concentration is increased to 5 µg/mL, but also a corresponding increase in the variability of the capture and detector can be observed. Based on the results of this experiment, it seemed that a concentration of 5 µg/mL was the optimal concentration for the preparation of the growth hormone.

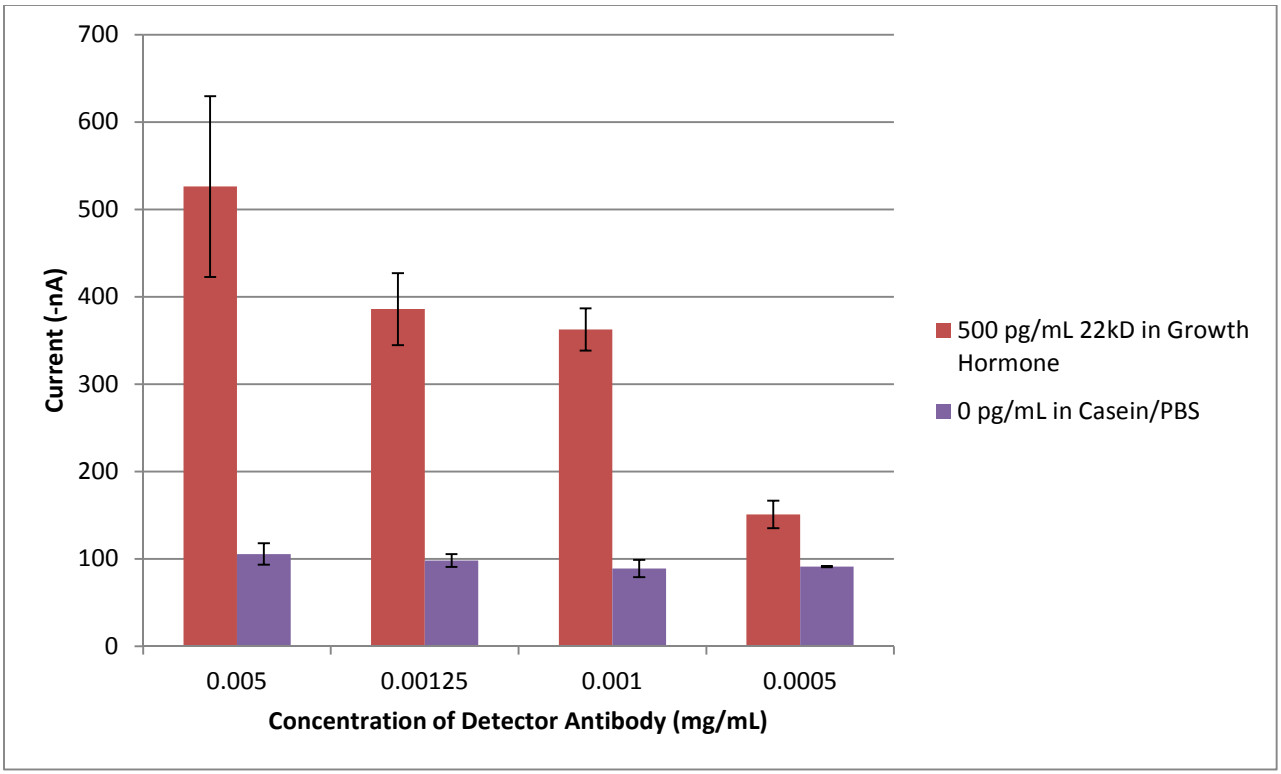



Figure 20 Experimental optimization of the peroxidase conjugated detector antibody 5801. Positive and negative controls were done in repeats on a single electrode chip for each detector antibody.

Experimental work was also conducted on determining the optimal incubation time for the growth hormone sample on the electrode chip. In these experiments, the 16-electrode chips were prepared with biotinylated antibody as a capture layer, and then positive controls of 500 pg/mL of growth hormone diluted in casein/PBS and negative controls of casein/PBS only were applied on the capture layers for different amounts of time, where after incubation time of the sample occurred the unbound sample was washed off and the detector solution was applied on all 16-electrodes for an equal amount of time. The study of incubation times revealed that in the range of 5 minutes to 40 minutes, the observed signal was found to generally increase over time, but after the 120 minute mark there was no highly appreciable elevation in the signal of the positive control as time progressed. One can observe, however, that the variability of the

positive control seemed to significantly elevate in the sample points that exceeded 120 minutes of incubation. It is of particular interest to note that while this increase of variability and signal occurs, the negative control appeared to be stable, with no significant differences in the blank values as time progressed.

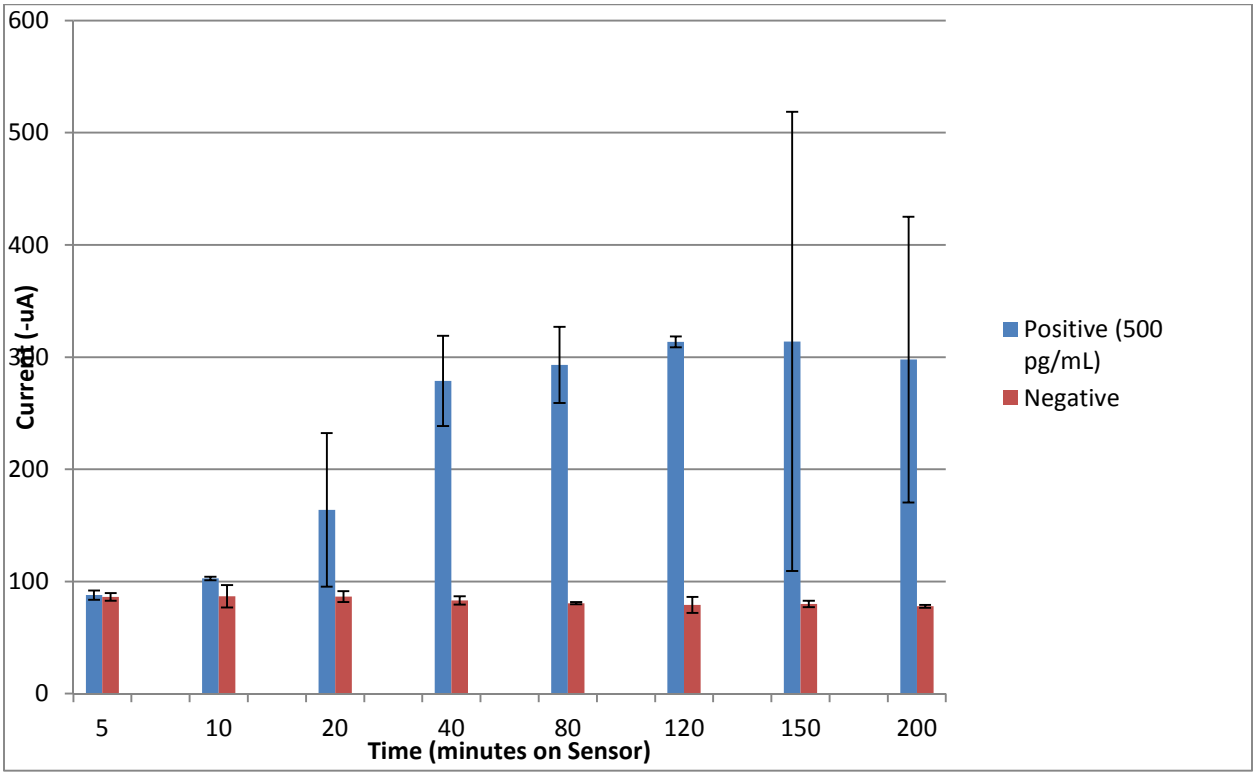



Figure 21. Experiment on two chips comparing signal measurement with different incubation times of growth hormone positive control on 16-electrode chip. Results indicate that no significant increase in mean signal is observable after the 120 minute mark, and there appears to be a high variability in the assay following 120 minutes of sample incubation.

### *3.1.2 16-Electrode System Linearity and Sensitivity in Buffer and Whole Blood*

After evaluation of techniques and comparing the streptavidin adsorption approach on a series of standards for human growth hormone, the results (Figure 22) seem to indicate this

calibration curve of different concentrations of growth hormone was able to achieve a linearity of $R^2 = 0.98$ for the 22 kilodalton growth hormone protein standards diluted in casein/PBS. Results of the figure below are duplicates of each protein standard ranging from 500 pg/mL to 0.5 pg/mL. In the result displayed in Figure 22, the results of performing an assay on the concentrations of 500, 50, 5, and 0.5 pg/mL even seem to suggest that the assay is sensitive to below 5 pg/mL in the casein/PBS buffer based preparation of growth hormone samples. These results that are acquired validate the hypothesis that a sensitive picogram level quantification of growth hormone is achievable using electrochemical techniques.

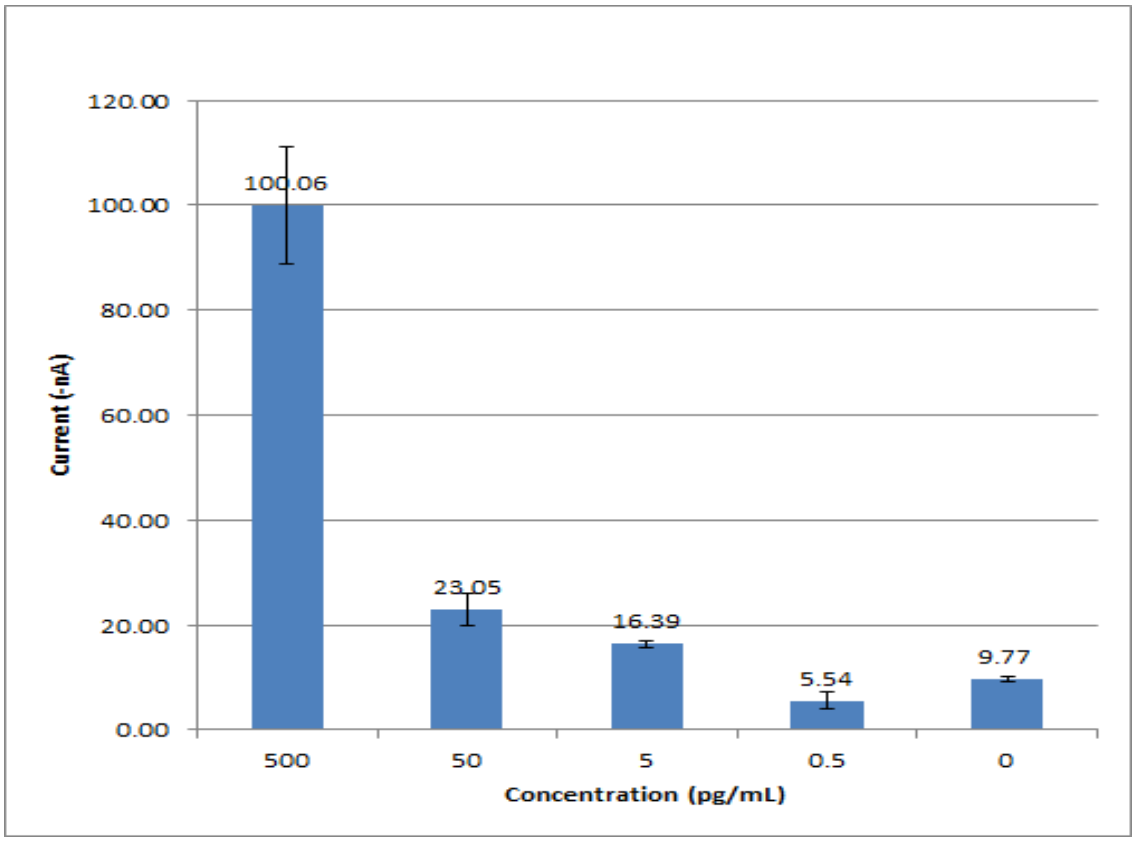



Figure 22. Plot of 22 kilodalton growth hormone standards in casein/PBS on the 16-electrode. This test was performed using duplicates of each protein standard on the 16-electrode tray.

Following initial assessment of the 16-electrode chip strategy using growth hormone standards diluted in casein/PBS, attempts were made to test if growth hormone could be detected if protein standards were prepared in whole sheep blood. The matrix of whole blood is

considerably more complex in composition than a casein/PBS standard and performance evaluations were necessary to see if assay performance decreases in whole blood. These tests of standards of growth hormone in sheep blood (Figure 23) tested on the electrochemical chip indicated that picogram level sensitivities for human growth hormone were achievable. The assay of whole blood seemed to have problems with the highest value (500 pg/mL) being unusually lower in signal than the 404 pg/mL tested standard, and overall the assay possessed linearity ($R^2$ = 0.75), but not to the degree observed in the buffer based test of growth hormone. Generally the standards were able to be distinguished from each other, except in the case of the 212 pg/mL sample which had an unusually high variability.

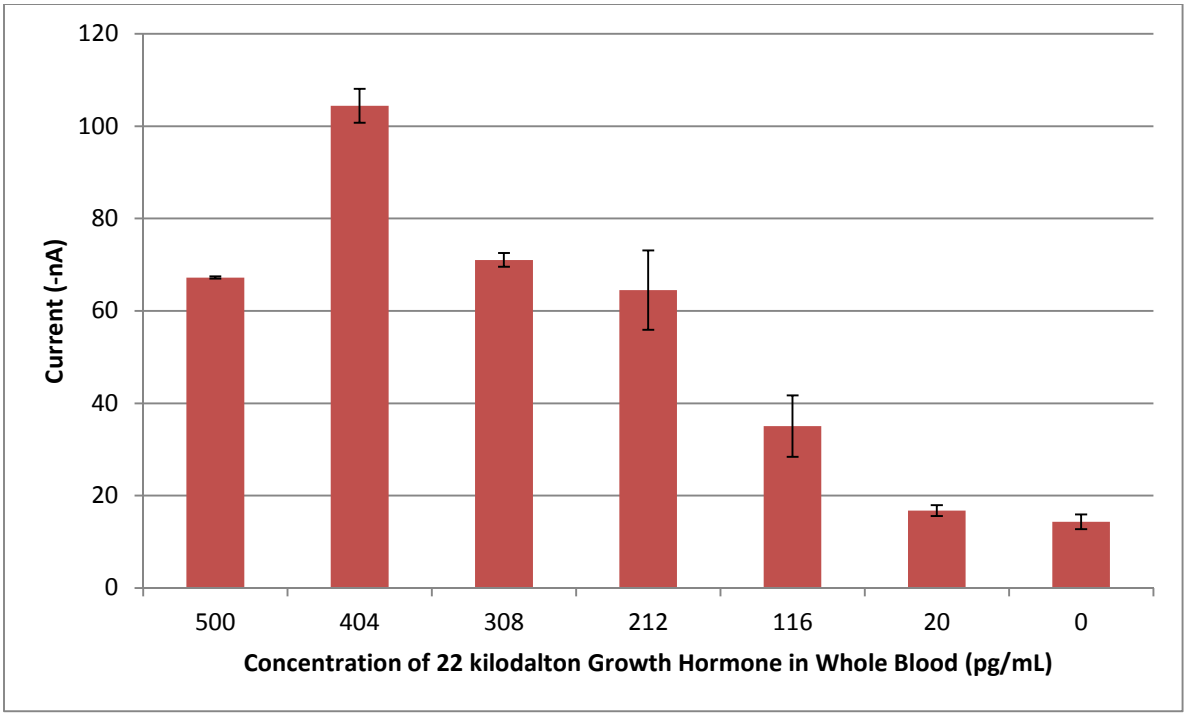



Figure 23. Current repeats of 22 kilodalton GH Standards diluted in whole blood with the streptavidin protein coating approach using the 16-electrode system. While a calibration curve is observable in whole blood, it appears that the sensor possesses effects where the highest value of 500 pg/mL(which is located on the leftmost portion of the 16-electrode chip) is lower than the 404 pg/mL protein standard in whole blood.

### *3.1.3 Gold Nanoparticle Based Strategy for Electrode Coating Results*

Different initial electrode coating layers were evaluated for the 16-array chip to assess if there were more optimal ways to enhance the sensitivity of the assay system. Figure 24 illustrates experimental work that was conducted on streptavidin-coated gold nanoparticles mixed with pyrrole in this solution. In this experiment, the different diameter nanoparticles doped in the polymer matrix were incubated with duplicate samples of a high concentration of growth hormone (500 pg/mL) and a negative control of casein/PBS also was incubated on the gold electrode surface. These optimization experiments show that the gold nanoparticle based strategy is able to capture growth hormone. Overviewing the data seemed to suggest that the 20nm diameter for a streptavidin gold nanoparticle was able to yield the best signal to noise ratio. No discernable trend between bead diameter and signal was yielded from the experimental data for the positive control, however there was a noticeable increase in the average signal of the negative control as the diameter was decreased. Based on the signal that was observed in this experimental result, the 20 nanometer diameter gold nanoparticle coated with a streptavidin protein was selected because it had the highest positive control to background ratio.

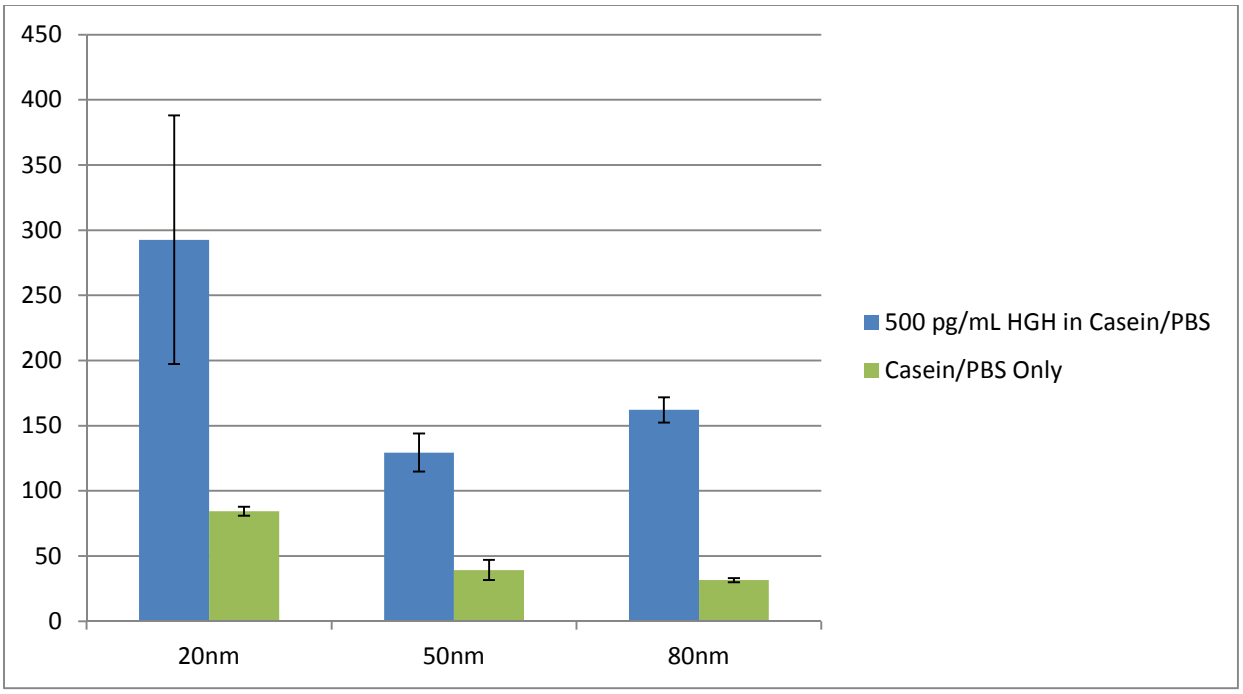



Figure 24. Testing of 500 pg/mL of growth hormone in casein/PBS and negative control of casein/PBS on a pyrrole streptavidin-coated nanoparticle capture system. Different diameters of streptavidin-coated gold nanoparticles were evaluated for performance.

The approach for the gold nanoparticle seemed to be inferior in performance compared to the streptavidin based approach.. This conclusion is reached based on the results of both the buffer and blood based experiments with the gold nanoparticles:

1. In the buffer-based system (Figure 25), while the linearity in the assay ($R^2$ = 0.96) appears to be excellent, the achievable sensitivity of the gold nanoparticle based approach is not as high as the adsorbed streptavidin approach for detection, where sensitivity levels were indicated by the data to be below 5 pg/mL. It is also of note to also observe that just as in the streptavidin adsorption calibration curve experiment (Figure 23), the value for 500 pg/mL (which is on the edge of the plate) is not as high as expected when compared to the 404 pg/mL standard.
2. In tests of the blood based system (Figure 26), no discernable trend is observed between the standards and the signal, with the lack of linearity ($R^2$ = 0.25) in this

case showing that the bead based signal, while it is able to work in a casein/PBS based context, does not yield appreciable signal for the whole blood-based system of testing.

Because of these results, the streptavidin coated nanoparticle based approach as a candidate approach for detection of growth hormone did not emerge as the best candidate for creating a sensitive linear assay for growth hormone isoforms in whole human blood samples, and has this approach was discarded.

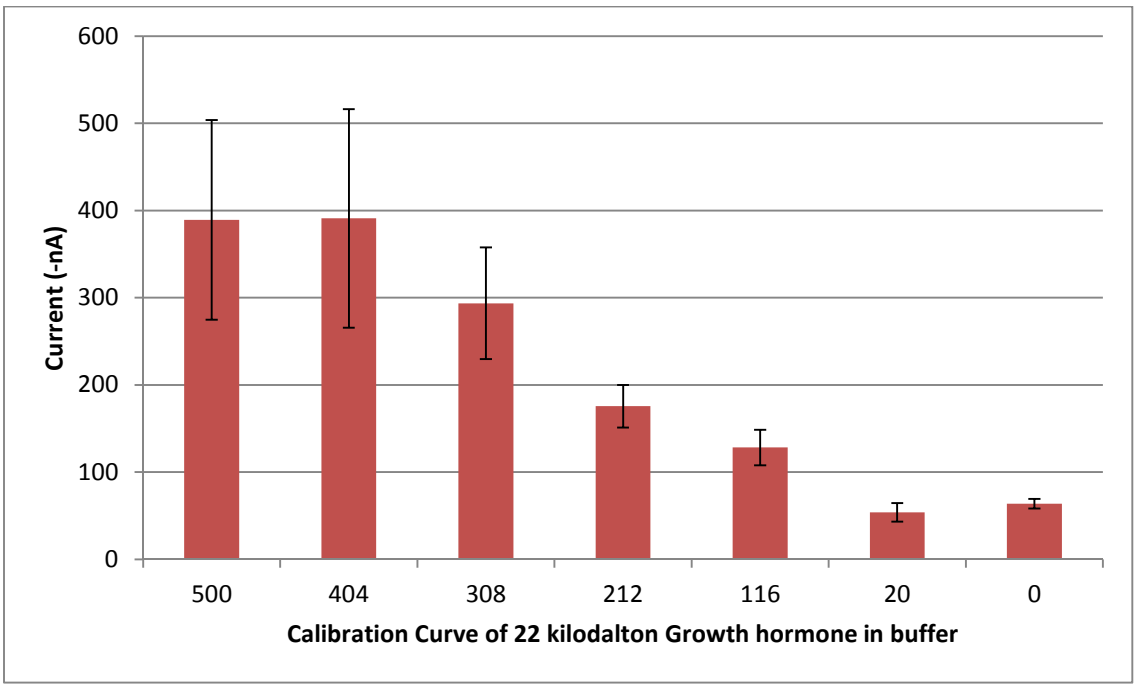



Figure 25. Buffer Calibration curve of streptavidin doped nanoparticles in polypyrrole matrix for immobilization of biotinylated growth hormone specific antibody. While it seemed that there was a discernable trend, there was high variability in the assay, as can be observed for the 500,404, and 308 pg/mL standards not having a statistically significant difference from each other. The assay appears to have high linearity ($R^2$ = 0.96).

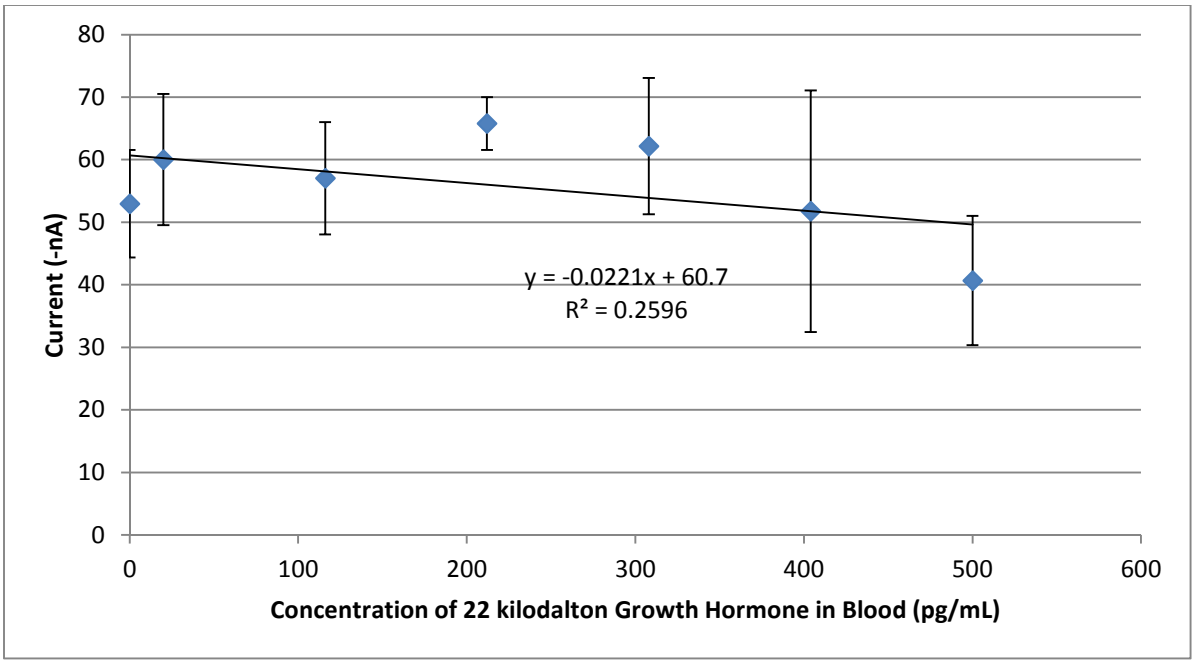



Figure 26 Streptavidin coated gold nanoparticle with blood performing calibration curve. Duplicates were tested for each concentration of human growth hormone in blood. Four samples were tested for the blank value (blood with no growth hormone spiked in).

### *3.1.4 Summary of Optimization Experiments with the 16-Electrode Chip*

Besides these tests of capture antibody, detector antibody, incubation times, and capture layer configuration, further optimization tests were also run regarding the washing off of unbound analytes during the assay procedure. While in the case of Wei et al[6] there was the application of nitrogen gas drying to the treatment of the electrode chip following wash off with distilled water, in the case of this study it was found that drying significantly lowered the observable signal.

| Factor | Tested Condition | Best Candidate |
|---|---|---|
| Initial Electrode Layer | Streptavidin dendrimer<br>Gold nanoparticle<br>Streptavidin protein | Streptavidin protein |
| Capture antibody | 25ug/mL to 0.195 µg/mL range | 6.25 µg/mL |
| Washing | Drying chip or non-chip drying | Non-drying chip |
| Detector antibody | Dilutions in 500 µg/mL to 0.5 µg/mL | 5 µg/mL |
| Incubation Time | Incubation times tested from 5 minutes to 200 minutes | 120 minutes |

**Table 2. Factors examined using the 16-array Genefluidics chip, the factors tested, and the conclusions reached.**

### *3.1.5 Variability Issues with 16-Electrode Array Chip*

Though experimental work was highly suggestive that the 16-electrode array and electrochemical technique was able to perform highly sensitive and specific quantitation of human growth hormone, these tests were hindered by the fact that reproducibility was particularly difficult. This is demonstrated most clearly in Figure 27, which shows the assay for human growth hormone standards in blood across three different chip experiments. Figure 27 demonstrates that where 150 pg/mL of growth hormone was tested, either the value is lower than the non-edge wells (such as the 124/pg/mL), or it possesses higher variability. It is also notable to observe that the 72 pg/mL concentration has the most consistent value and a low coefficient of variation.

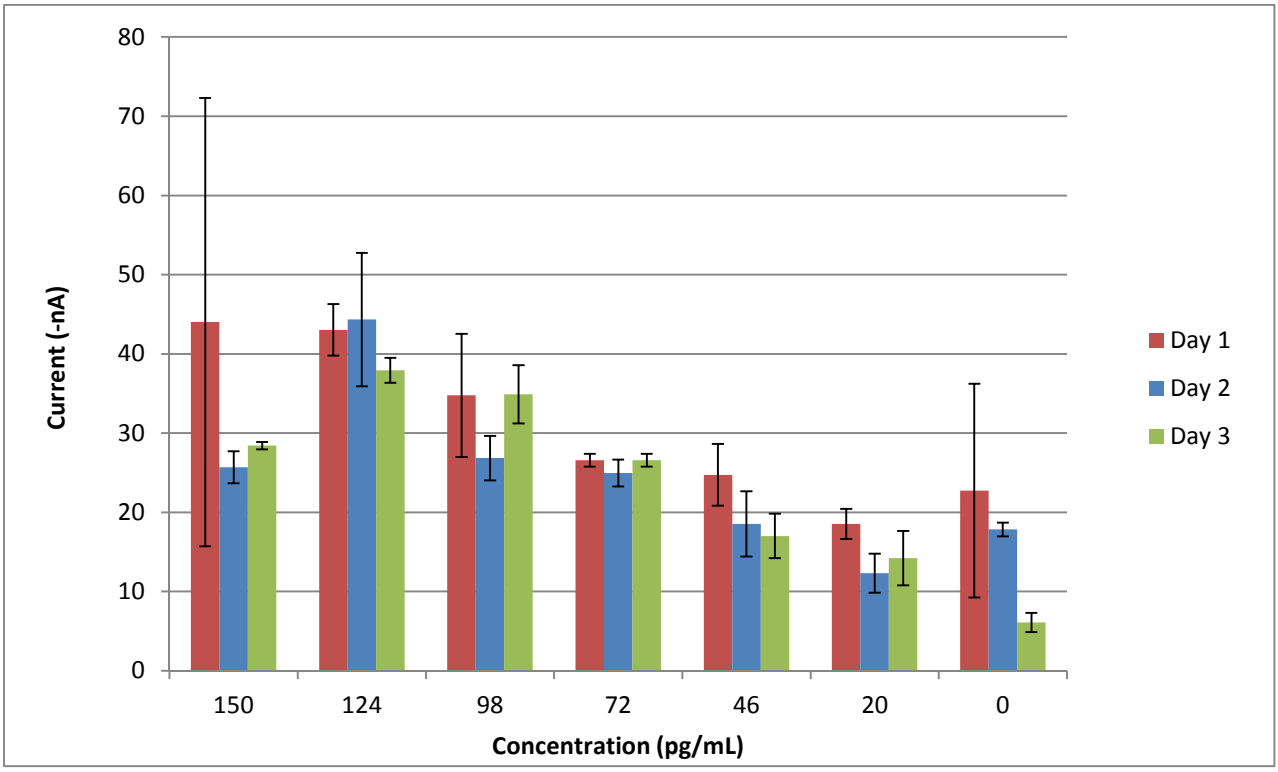



Figure 27 Comparison of calibration curve for growth hormone standards (22 kilodalton) in blood on three chips. Data shows that high variability exists in the assay, distorting the results. In day 2 and 3, the highest value (150pg/mL), which is located at the leftmost portion of the chip, is unusually low compared to the higher valued 124 pg/mL. It is of note to see that the center of the chip, where 72 pg/mL was tested, possesses the least amount of variability across three experimental runs.

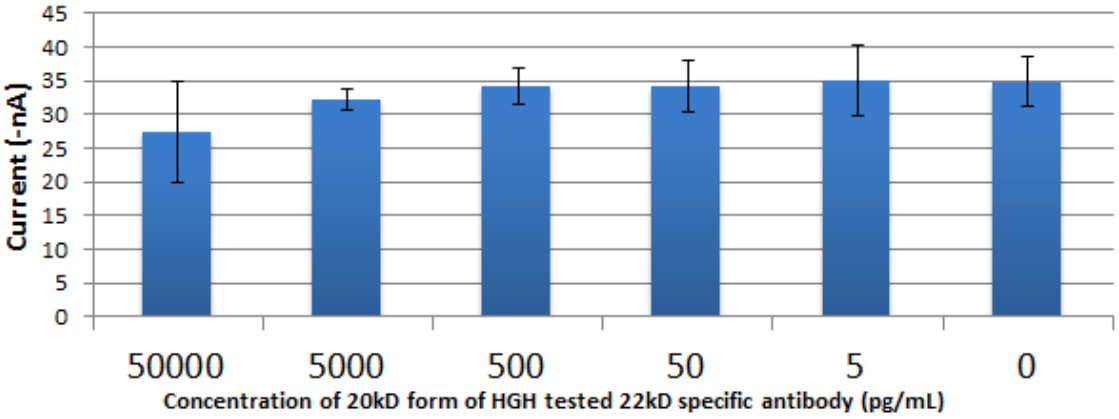
An analysis of the results of the different experiments and troubleshooting for the casual reason behind this variability led to the conclusion that the most likely cause of this variability between different runs of the same experiment is that the Genefluidics 16-electrode chip possesses some form of variability. The 150 pg/mL value, which is unusually lower than expected when compared to the 124 pg/mL value, was tested in all of the experimental runs on the leftmost part of the 16-well electrode chip. The blank 0 pg/mL growth hormone whole blood standard, located on the rightmost part of the electrode chip, also displays a high amount of variability in the runs, sometimes being lower than 20 pg/mL (day 3), sometimes being higher (day 2), sometimes possessing abnormally high variability (day 1). Furthermore, the case for the 16-electrode chip being a hindrance to the electrode may also be supported by the fact that the 72 pg/mL, which displays the most consistency and a low CV compared to the edge values, is located towards the center section of the chip.

### *3.1.6 Specificity Tests Using 16-Electrode System*

While the existing literature from Wu and Bidlingmaier et al[10] and Hashida et al[8] suggests that the growth hormone antibody pairs possess high specificity in the assay procedure, because the electrochemical system differs in mechanism and may affect the properties of the antibodies present, it was deemed of merit to perform basic specificity studies of the antibody pairs using the electrochemical platform. These tests for specificity were performed at a juncture where assay optimization tests of the 22 kilodalton and 20 kilodalton specific antibody pairs (5802/5801 and 1c72/5c4, respectively) had verified basic functionality and basic calibration curves that were achievable with buffer base protein standards.

For the performance of the specificity tests, standard curves for both the 20 kilodalton and the 22 kilodalton growth hormone isoforms were performed in a range from 50 ng/mL to 5 pg/mL. Antibodies were coated on the electrode surface and incubated with the protein standards of a different isoform of growth hormone (that is, the 22 kilodalton growth hormone

protein was incubated with 20 kilodalton specific antibodies, and the 20 kilodalton growth hormone protein was incubated with 22 kilodalton specific antibodies). Following incubation washoff of unbound analytes, peroxidase conjugated reporter antibodies were incubated on the surface, the unbound reporter was washed off with distilled water, and readout was performed by measuring electrical current after applying a tetramethylbenzidine/$H_2O_2$ mixture.



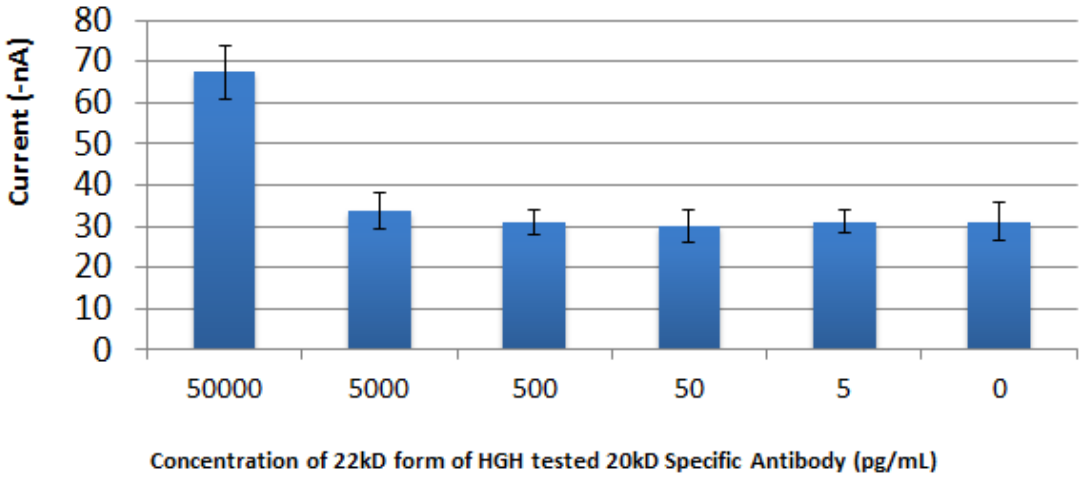




Figure 28 Tests using mismatched antibody pairs and protein targets (22 kilodalton growth hormone on 20 kilodalton specific antibodies, and 20 kilodalton growth hormone on 22 kilodalton specific antibodies). This mismatched antibodies and target proteins was tested using the 16-electrode Genefluidics electrode chip with antibody capture layer on repeats of each concentration in a casein/PBS buffer. As expected, the signal for the 22 kilodalton protein on the 20 kilodalton specific antibodies and the signal for the 20 kilodalton protein on the 22 kilodalton specific antibodies is near to the background signal, indicating that the antibodies would create signal in response to the incorrect isoforms.

If the antibodies were to perform specifically, incubating an isoform of growth hormone that is not meant for an antibody pair, it would yield signal that is very low and close to the background signal. This is what is observed in the results of testing the 20 kilodalton protein on the 22 kilodalton specific antibodies and testing the 22 kilodalton protein on 20 kilodalton specific antibodies, where all the samples are not significantly different from each other for all concentrations ranging from 50 ng/mL to 0 pg/mL. The sole exception for this signal is generated for a high (50 ng/mL) amount of 22 kilodalton protein for the 20 kilodalton specific growth hormone antibody. These tests seem to suggest that for the majority of cases the antibody specificity is preserved when the antibodies are used in an electrochemical detection system, and no specificity issues should be of concern unless an extremely large amount of 22 kilodalton growth hormone is incubated with the 20 kilodalton specific antibody pairs, an extremely large amount that is physiologically unlikely based on our knowledge of the bio-distribution of growth hormone in circulation.

## *3.2 Tests Using 96-Electrode Array*

### *3.2.1 Capture and Detector Concentration Antibody Optimization on 96-Electrode Array*

Following initial validation of the antibodies using the well characterized 16-electrode Genefluidics electrode biosensor platform, incremental steps were taken to determine if the assay antibody system was adaptable to the proposed high-throughput 96-electrode array system. The previous studies performed using the Genefluidics platform proved that the biotinylated capture antibodies and peroxidase-conjugated detector antibodies were functionally capable of being used for electrochemical procedures, but it was unknown if the electrode geometry and potentiostatic equipment of the 96-electrode system would allow the assays sensitivity and functionality to be retained.

The first phase of tests on the 96-electrode system of the project involved a preliminary assessment determining if a pair of candidate antibodies (5801 and 5802 commercially available antibodies for 22 kilodalton growth hormone that have been previously validated[8], and 1c72 and 5c4 acquired from Wu and Bidlingmaier et al.[10] for the 20 kilodalton growth hormone) was able to effectively capture the target proteins (22 kilodalton and 20 kilodalton growth hormone) and the optimal concentrations of each antibody to use in a study. These antibodies had been initially validated using the 16-electrode chip, but reoptimization of antibody concentrations was considered to be necessary because it was thought that 96-electrode structure of the plate would have significant differences in surface area available and potentially affect steps such as signal measurement and electropolymerization.

These optimization tests were done by studying the capture and antibodies in a "checkerboard design" for assay optimization. This method consists of testing different concentrations of capture and detector antibodies across the plate, with a decreasing gradient of capture antibody across the columns of the 96-well plate, and decreasing gradient of detector antibody across the rows of the plate. The effectiveness of the different combinations of capture and detector concentrations is tested by comparing the signal levels of a positive control (consisting of 500 pg/mL of human growth hormone in casein/PBS) and the negative control (consisting of just casein/PBS). The goal of this approach is to compare the positive control signal and the negative control signal and find the appropriate capture and detector levels that minimize background signal and still have high signal levels.

Based on the observed results (Figure 29 and Figure 30) of performing checkerboard based assays for the antibody pairs specific to 20 kilodalton and 22 kilodalton growth hormone, the electrochemical assay format developed using the 16-electrode array system also was able to perform well on the 96-electrode system. As expected for a working assay configuration, one is able to observe differentiation between the positive controls of 500 pg/mL Growth Hormone in

Casein/PBS and the negative controls of Casein/PBS only. These checkerboard titrations of different concentration also appear to possess reasonable trends, as when the capture antibody concentration is lowered there is also a lowering of the signal of the positive control, and when there is a lowering of the detector antibody concentration there is a lowering of the positive signal.

Based on a comparison of the positive control to negative control signals for the checkerboard assay design, it was determined that the optimal concentration range for the 22kD growth hormone was 0.00625 mg/mL for biotinylated capture antibody (5802) and 0.001 mg/mL for the peroxidase-conjugated detector antibody (5801). For the 20kD specific antibody pairs, it was determined that an optimal range was 0.0125 mg/mL for biotinylated capture antibody (1c72), and 0.001 mg/mL for the peroxidase conjugated detector antibody (5c4). In the analysis, it is inadequate to merely look at the positive signal of the different capture and detector antibody concentrations, because on occasion an increased signal of positive control also is correlated with an increased signal of the negative control.

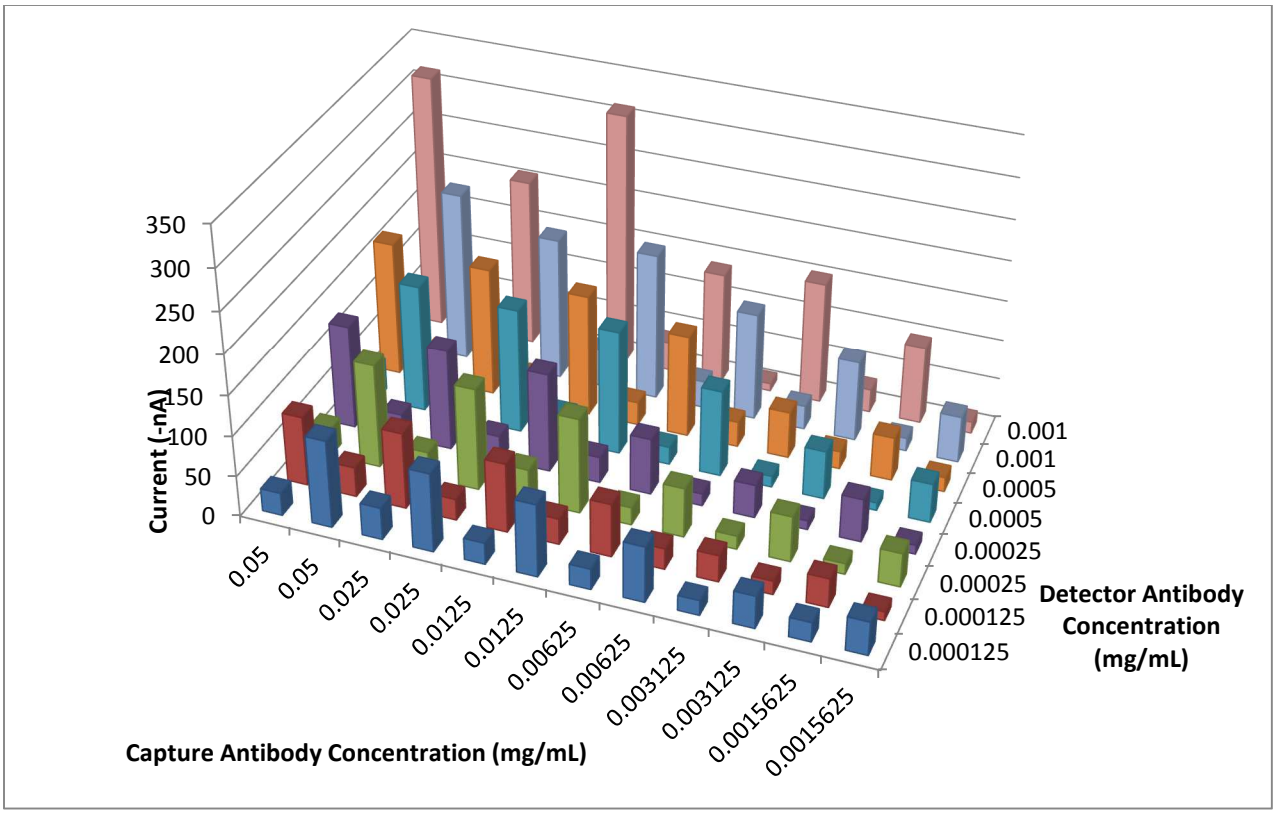



Figure 29 Checkerboard assay generated for the 22 kilodalton growth hormone isoform specific antibodies (Biotinylated 5802 and Peroxidase Conjugated 5801). A checkerboard pattern of a positive control (500 pg/mL of 22 kilodalton Growth Hormone diluted in Casein/PBS) and negative control (Casein/PBS) was incubated on the 96 electrode plates. Different concentrations of capture and detector antibodies were tested across the columns and rows of the 96-electrode plate.

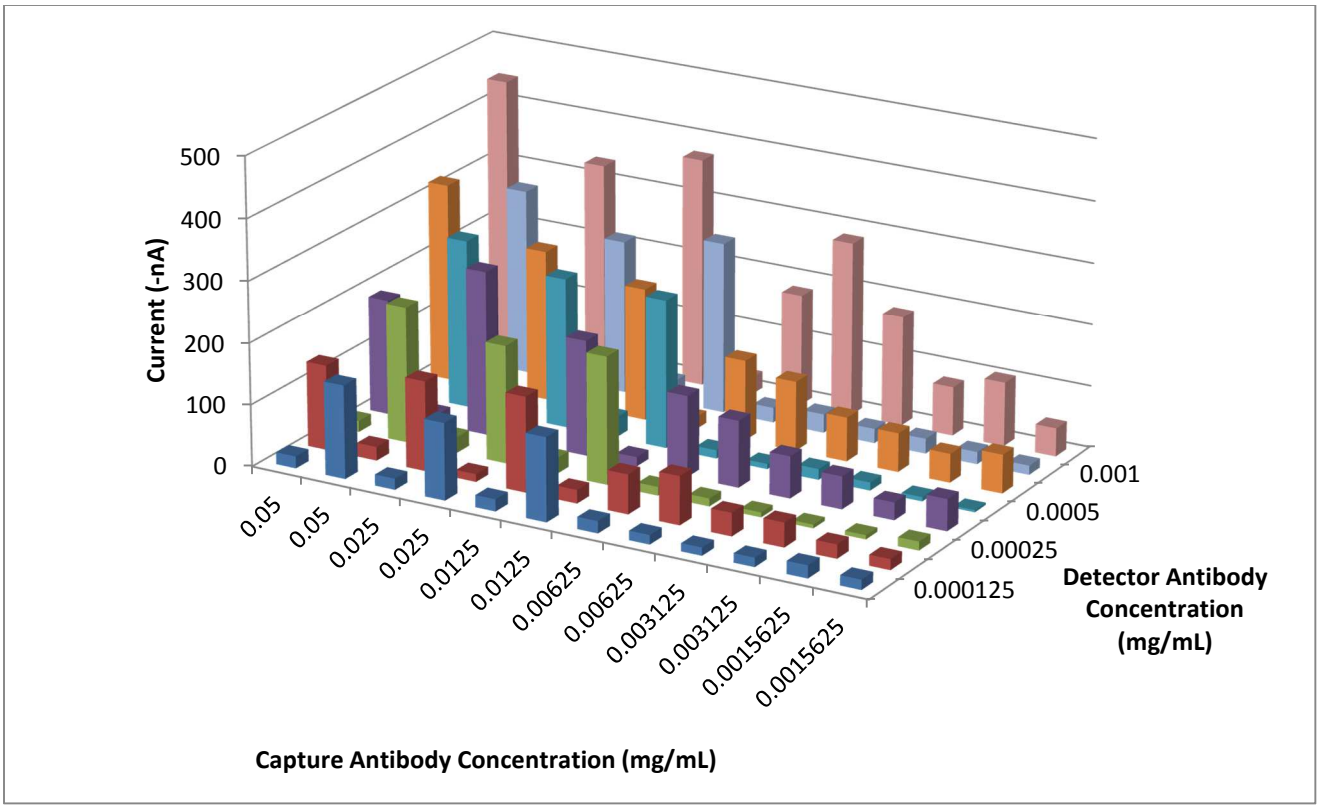



Figure 30 Checkerboard assay generated for the 20 kilodalton growth hormone isoform specific antibodies (Biotinylated 1c72 and Peroxidase Conjugated 5c4). A checkerboard pattern of a positive control (500 pg/mL of 20 kilodalton growth hormone diluted in casein/PBS) and negative control (casein/PBS only) was incubated on the 96 electrode plates. Different concentrations of capture and detector antibodies were tested across the columns and rows of the 96-electrode plate.

### *3.2.2 Initial Calibration Curves in Blood and Buffer for 96-Electrode Array*

Attending optimization work of the capture and detector antibody concentrations is the need to perform calibration curves by testing the antibody with different concentrations of human growth hormone in buffer to assess the ability to preserve the results of the 16-well electrode proof of concept system. Just as in the standard curve experiments of the 16-electrode system, the key metrics considered in this portion of the study are the assay linearity and sensitivity.

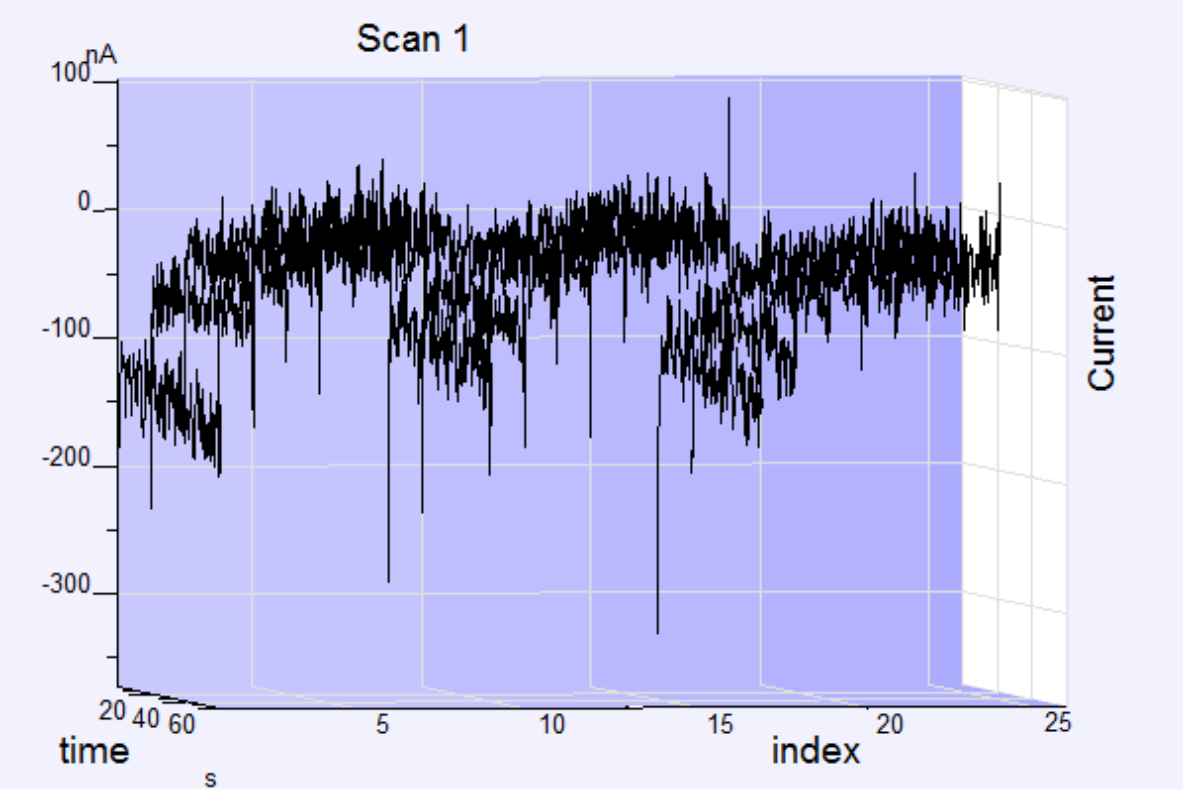



Figure 31. Chronoamperometric current readout using the IVIUM 96-Electrode Potentiostat. This figure illustrates the current readout when a calibration curve test is run on the electrode plate. In this figure, 24 electrodes (3 columns of growth hormone standards) are displayed. Three gradients of high average electric current readout to low average electric current readout can be observed in the signal, corresponding to the pattern of how growth hormone was pipetted into each of the three columns.

Similar to the Genefluidics 16-array system, it can be hypothesized that for the 96-electrode system the measured current from the potentiostatic system will be proportional to the amount of protein target present in a complete assay system. For the evaluation and quantification of the results, sampling occurred on all 96 channels at an interval of 10 samples per second per channel, and the applied voltage was -200 millivolts. The recorded current for the last 10 seconds of a 60 second sampling time is averaged, and these averaged values are the used as a single quantifiable metric for evaluating the current readout of each channel.

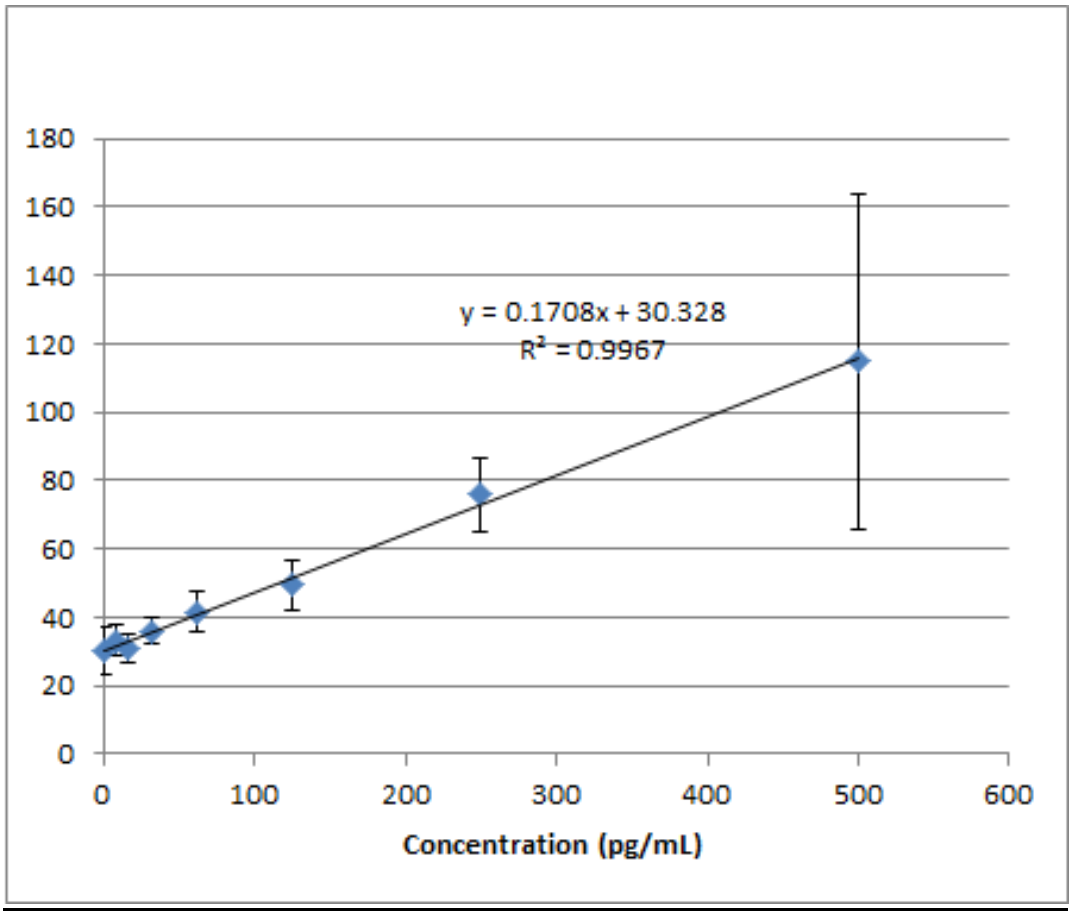



Figure 32. Initial calibration curve for 22 kilodalton growth hormone assay in the 96-electrode plate using a streptavidin coated 96-electrode plate coupled with biotinylated growth hormone specific antibody for capture (5802 antibody) and peroxidase-conjugated reporter antibody (5801 antibody). These initial assays possess a high linearity, but the replicate samples still display a high degree of variability.

Initial calibration curves using a buffer based system (Figure 32) seemed to indicate linearity ($R^2$ = 0.99) in the performance of the assay. These tests are highly suggestive of the effectiveness of the assay and a linear relationship between the target growth hormone present and the measured signal. And further tests were conducted to evaluate the effectiveness of the test on whole blood samples spiked with growth hormone. But attendant with the promising results regarding the linearity of the assay using the 16-electrode system is the reality that the data seemed to show that there was high variability in the assay which made it difficult to determine if the different concentrations were statistically different from each other. This variability is most noticeable at the 500 pg/mL reading. Based on the fact that this was an initial

reading, it was reasoned that this may possibly be due to an unoptimized wash method, as the construction of the 96-electrode plate system has differences with the 16-electrode array, and the 96-electrode array is difficult to wash because it has a deep well from where rinsing and removing dirty liquid in the wash is difficult to perform.

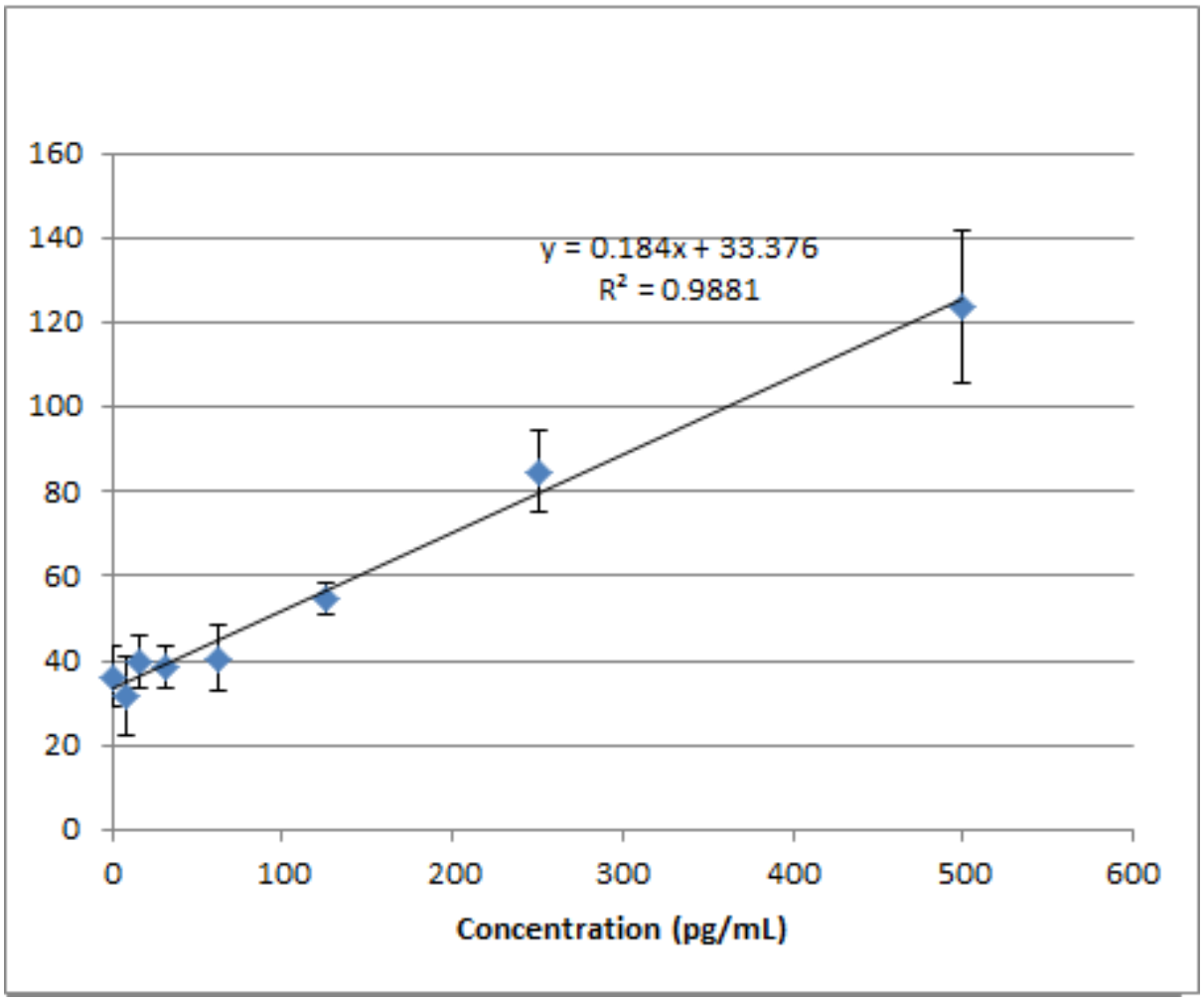



Figure 33. Calibration curve run with 22 kilodalton growth hormone standards prepared in whole sheep blood. This assay demonstrated excellent linearity in assay performance, a positive indicator of the 96-electrodes viability for being able to perform a high-throughput electrochemical method for growth hormone detection in whole blood samples. The lower range of samples (below 100 pg/mL) were difficult to distinguish from the negative control.

Initial calibration curves using preparation of growth hormone standards diluted into whole human blood (Figure 33) also demonstrated that the 96-electrode configuration of the assay was able to perform quantification in whole blood. This conclusion is inferred from the fact

that the linearity of the whole blood based standard curve is extremely high ($R^2$ = 0.98). An interesting secondary result in comparing the calibration curve of the growth hormone in whole blood (Figure 33) against the calibration curve of the growth hormone in buffer system (Figure 32) is that that there was high concordance between the readings of the buffer-based system and the whole blood based system, with very little difference in the absolute signal readings. While there are still many uncharacterized features of the 96-electrode system, this data may be suggestive that the system has a particularly large robustness for performing assay in a complex matrix like whole blood. These results were exciting indicators that the 96-electrode system could be easily adapted to perform a functional growth hormone assay in blood, but further optimization appears to be necessary. Just as before in the buffer-based system, the blood-based system seemed to possess a great amount of variability, and a large amount of the standards (particularly at the lower range below 100 pg/mL) were not statistically significant from each other. Just as in the case of the buffer-based assay, it was hypothesized that a cause of variability in the assay was because hand-washing was used for all 96-electrodes, and that the usage of automated washing would facilitate the reduction of assay variability.

### 3.2.3 Automated Washing Optimization of 96-Electrode Array

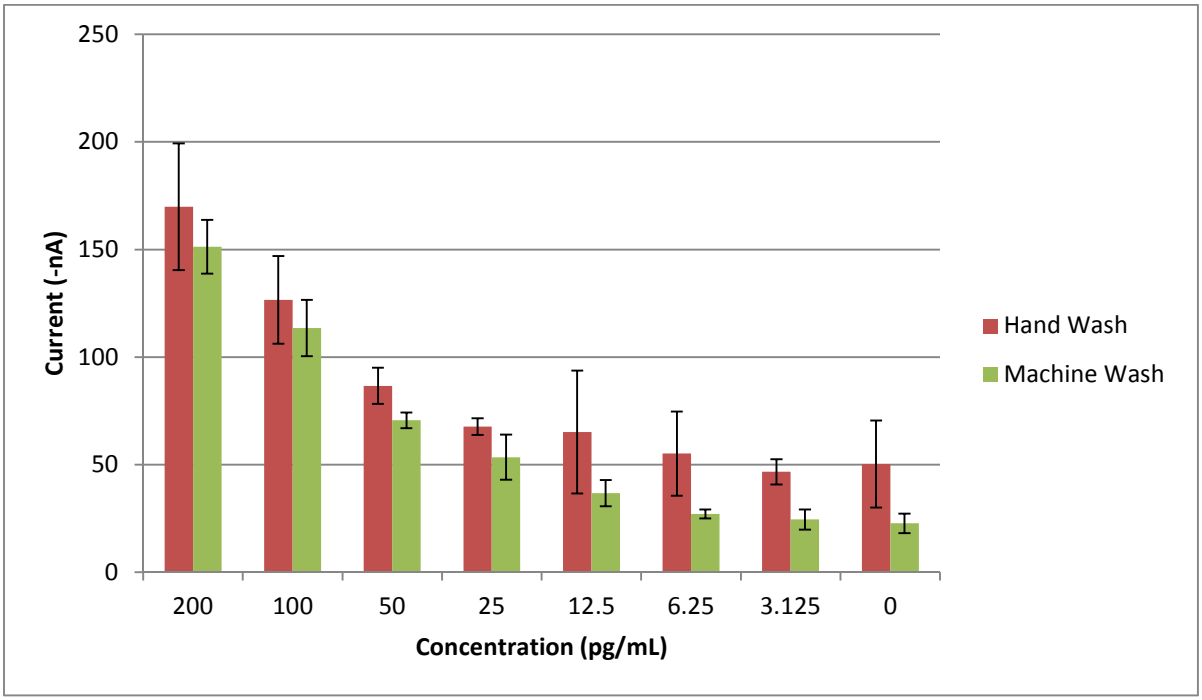



Figure 34. Comparison study between the usage of hand-washing and machine washing for 22 kilodalton growth hormone detection protocol using the 96-electrode plate.

Tests were conducted on the whole blood based assay of growth hormone comparing the usage of an automated platform for washing and aspirating of samples wells for the 96-electrode plate, against the usage of a hand-wash-based multichannel system for washing and aspirating the electrode wells. These tests were conducted performing the assay in the standard format of electropolymerizing streptavidin to the surface, applying a capture antibody layer of biotinylated growth hormone specific antibody, incubating a standard curve of growth hormone in whole blood, incubating a detector antibody, and performing electrochemical current readout with a tetramethylbenzidine/$H_2O_2$ mixture. In between the different steps of the electrochemical assay, washoff was performed on the same plate using the hand wash and the automated plate washer, with each method being used on half of the plate.

It was found in evaluation that a machine-wash-based system was superior in ensuring consistent wash, as indicated by the fact that the lower ranges of the machine wash system are

difficult to differentiate from each other. Observing the data (Figure 34), one can see that the variability of the assay is clearly lower in the machine-washed-based technique compared to the hand-wash-based technique, particularly at the concentrations of 0 pg/mL, 12.5 pg/mL, 6.25 pg/mL, 200 pg/mL, and 100 pg/mL.

### *3.2.4 Incubation Time Evaluation on 96-Electrode Array*

Tests were also conducted to evaluate the effects of incubation times on the standard curve using the 20 kilodalton growth hormone prepared in sheep blood at the nanogram range, which allowed a visible test of the effect of binding time against the concentration of target analytes. These studies demonstrate that while in the binding of the growth hormone in the higher ranges of an assay there was little difference between the 5 minute sample and the 30-minute incubation of sample, at the lower ranges for both a sample and detector incubation, a longer incubation time of 30-minutes appeared to have an elevated signal compared to the 5-minute incubation time. However, it seems that incubation times also potentially have the effect of elevating the variability of assay signal.

A notable result in these studies of the signal and observed current readout In the 20 kilodalton growth hormone tests is that for the majority of the experiments, the 250 to the 31.25 ng/mL range have no significant difference. This may be attributable to limitations in the electronics of the potentiostatic control system, or possibly due to a maximal saturation of the antibody immunosensor when 31.25 ng/mL or greater concentration is placed on the sensor surface for the 20 kilodalton growth hormone isoform.

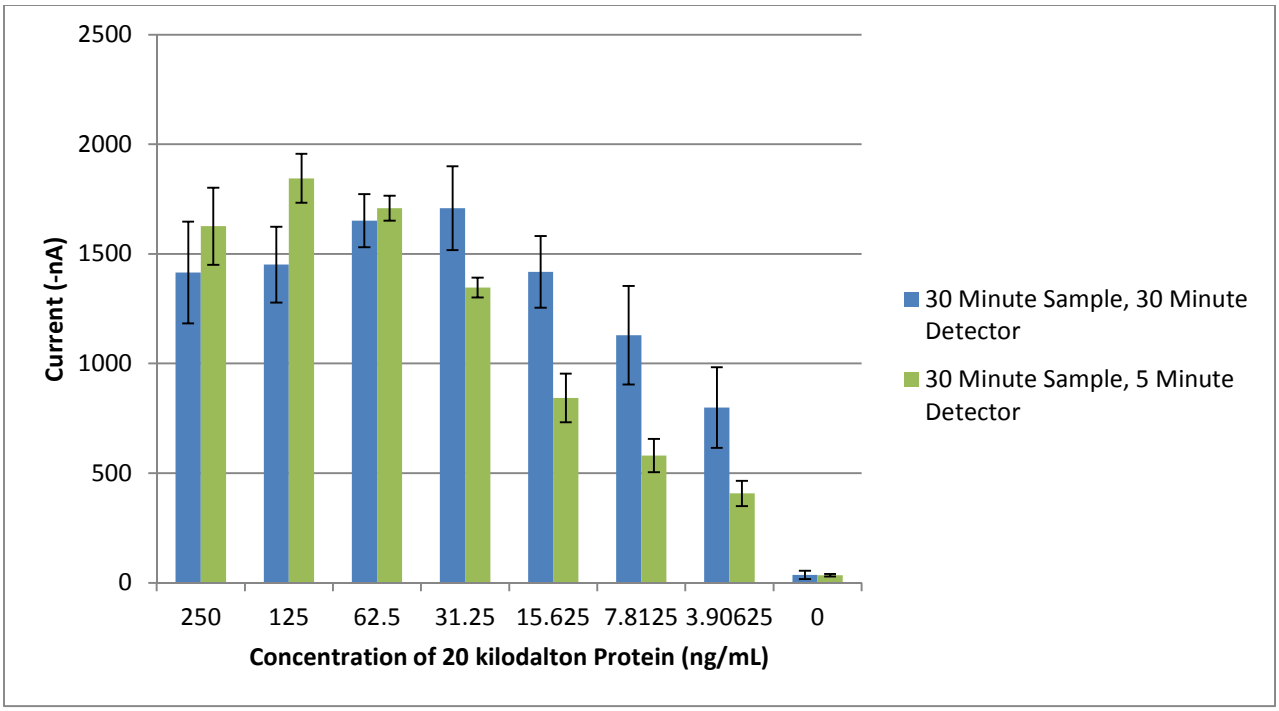



Figure 35 Incubation time and effect on electrode calibration curve for assay performance. The results of this experiment compare the incubation times of the sample incubation and detector antibody incubation for the 20 kilodalton growth hormone and evaluating the effects of time of incubation against the calibration curve.

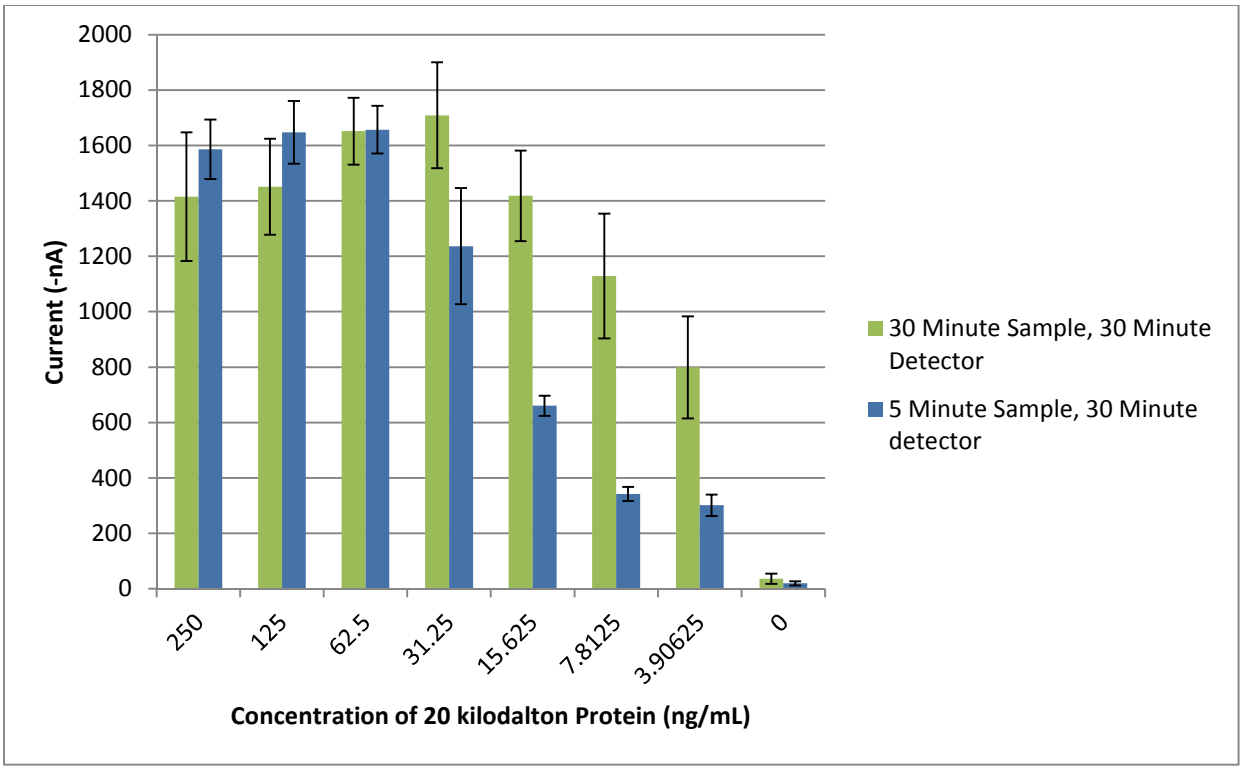



Figure 36 Incubation time for sample incubation times for the 20 kilodalton growth hormone on the electrochemical method.

### *3.2.5 Linearity and Sensitivity (22 kilodalton) for 96-Electrode System*

Following optimization studies of the antibody concentrations for the 22 kilodalton antibodies, standard curves using growth hormone standard concentration in whole blood were made. Each standard in the assay was tested with six replicates. Figure 37 shows a calibration curve test where growth hormone was incubated for 30 minutes on the surface of a streptavidin-polymer and biotinylated capture coated electrode, unbound analytes and red-blood cells were washed off with an automated plate washer, and an additional incubation of peroxidase-conjugated detector was incubated for 30 minutes before a final wash off and application of Tetramethylbenzidine/hydrogen peroxide for current measurement.

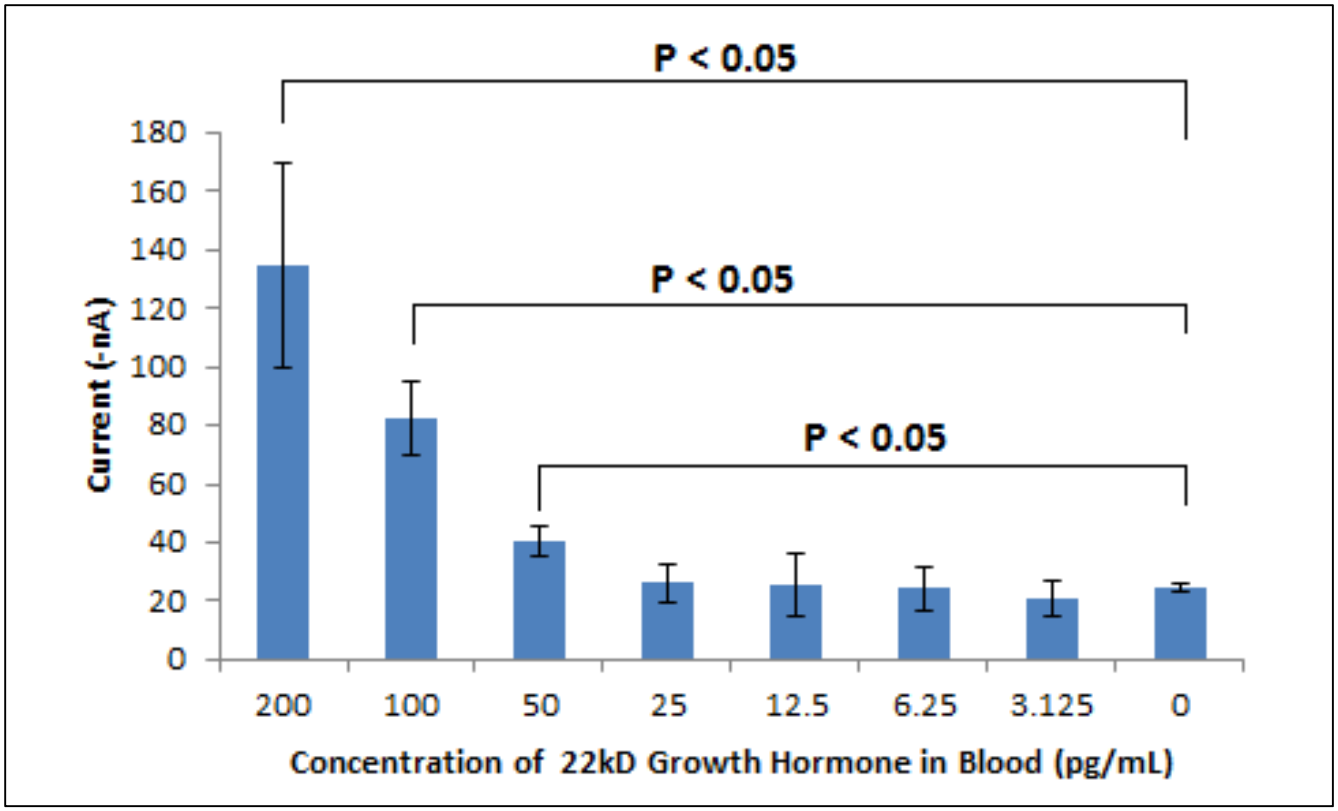



Figure 37. Plot of 22 kilodalton growth hormone standards in whole blood. Tests were performed on a 96-well plate electrode system with six repeats of 22 kilodalton growth hormone in whole blood. The blank sample had one outlier removed (> 2 Standard Deviations)

As indicated by the above figures, growth hormone in whole blood samples using the electrochemical format appears to have high linearity ($R^2$ = *0.98*) and sensitivity levels that appear to meet existing requirements for GH assay (50 pg/mL). This data is highly suggestive that detection of growth hormone in whole blood at the sensitivity levels requisite for anti-doping testing is achievable using electrochemical detection techniques. Based on these six replicates, it is most appropriate to state that 50 pg/mL is the existing sensitivity of the growth hormone assay. However, based on the results of previous calibration curve tests on the 96-well electrode (Figure 34) with standards done in duplicate, there may be grounds to reason that sensitivity lower than 50 pg/mL is achievable with further optimization work .

### *3.2.6 Linearity and Sensitivity (20 kilodalton) for 96-Electrode System*

Similar to the tests of the 22 kilodalton growth hormone isoform, tests were run using the 1c72/5c4 antibody pair with 20 kilodalton growth hormone standards in whole sheep blood. Figure 38 shows a calibration curve test where 20 kilodalton growth hormone was incubated for 30 minutes on the surface of a streptavidin-polymer and biotinylated capture coated electrode, unbound analytes and red blood cells were washed off with an automated plate washer, and an additional incubation of peroxidase-conjugated detector was incubated for 30 minutes before a final wash off and application of tetramethylbenzidine/hydrogen peroxide for current measurement.

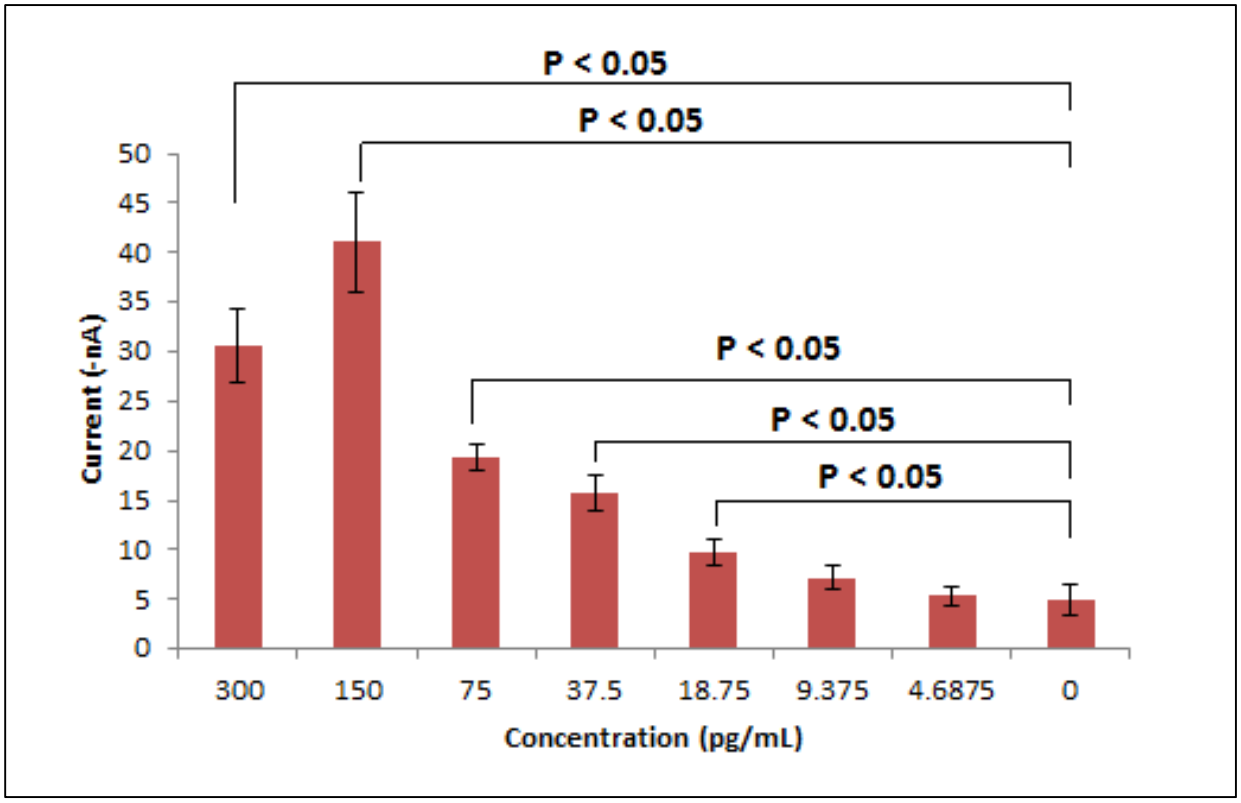



Figure 38 Plot of 20 kilodalton growth hormone in whole blood to demonstrate sensitivity. Analysis is across six repeats per sample, with edges removed from analysis to avoid potential assay edge effects. The 300 pg/mL reading is suspected to be due to plate edge effects.

The results of a whole blood assay test of the 20 kilodalton growth hormone isoform seem to suggest that the assay is able to create a functional calibration curve, however one major fault discovered in this portion of the assay is that there may be edge effects present in the assay that require troubleshooting. If edges are removed in the analysis, results of the 20 kilodalton isoform of growth hormone spiked in whole blood samples show that the assay performs with acceptable linearity ($R^2$ = 0.98 with edges removed), and sensitivity levels (37.5 pg/mL) appear to approach the requisite levels necessary for usage in a differential isoform assay. Further work is necessary to optimize the assay sensitivity levels and determine if sensitivity suitable for discriminatory testing of isoforms is possible, and if edge effect issues in the assay can be reduced.

# *4 Conclusion*

The data presented here is highly suggestive that a high-sensitivity assay of growth hormone isoforms is achievable using electrochemical techniques. Apart from the accomplishment of assay metrics such as sensitivity and linearity, there are additional advantages offered by the electrochemical growth hormone assay over the existing methodology. Compared to the existing immunoassay system, the electrochemical method developed in this project allows for a low volume, requires 25% less incubation time, can be completely automated, and takes places in a convenient 96-well plate configuration.

| **Factor** | **Electrochemical Method** | **Existing Immunoassay** |
|---|---|---|
| Sample Volume | 25uL | 50uL |
| Sample Medium | Whole Blood | Serum |
| Sample and Detector Incubation Times | 1 hour | 4 hour |
| Testing Format | 96-well plate | Test tubes |
| Automated | Yes | No |

**Table 3. Comparison between the proof of concept electrochemical method developed in this work and the existing chemiluminescent ELISA system.**

While it has been demonstrated that high-sensitivity detection of a protein target is achievable using a streptavidin and biotinylated-antibody based capture layer in blood, there are a number of improvements that seem necessary for the advancement of the methodology towards athletic testing:

1. Whether the 20 kilodalton antibodies provided by Wu and Bidlingmaier et al.[10] are capable of assaying at high sensitivity <5 pg/mL for usage as a biomarker for the isoform ratio test. Based on the known fact that the 20 kilodalton isoform of growth hormone is downregulated following injection of the 22 kilodalton recombinant form, further optimization is necessary to determine whether the sensitivity levels of the 20 kilodalton assay can be improved beyond the 37.5 pg/mL achieved in this study. This will be critical in order to ensure that the downregulated 20 kilodalton can be detected with the electrochemical method.
2. Optimization of protocol to ensure that electrochemical method has stability and functionality. The20 kilodalton protein results for the 96-electrode experiment seem to suggest further optimization is necessary in order to ensure assay stability and prevent edge effects from adversely lowering assay linearity.
3. Further specificity studies to verify that the method used is able to effectively distinguish between the 20 kilodalton and 22 kilodalton growth hormone isoforms. Initial tests conducted on specificity demonstrated that the antibodies do not have elevated signal in the presence of alternative isoforms (Figure 28), but further studies studying specificity of the 5801/5802 and 1c72/5c4 growth hormone antibodies when the 22 kilodalton and 20 kilodalton isoforms of growth hormone are mixed together at different ratios will be important for making sure that the test will function well on collected samples from patients.
4. Performing studies on a population of doped and non-doped blood samples: It will be necessary to conduct studies to determine the sensitivity and specificity of the 20 kilodalton

to 22 kilodalton isoform test in detecting doping by conducting studies on a population that uses growth hormone compared with a population that does not use growth hormone.

With the completion of these additional objectives, the development of a practical isoform whole blood electrochemical assay would be very likely.

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# *Chapter 4 – Low Volume and Reduced Invasiveness Growth Hormone Tests with Saliva and Dried Blood Spots*

## *1 Introduction*

Growth hormone[1] is a prohibited substance in competitive athletics according to the World Anti-Doping Agency, and athletes have agreed to submit to testing procedures for competition in professional sports. Enforcement of compliance with the prohibitions is facilitated by the means of randomized drug testing protocol, where, upon advance notification, the athletes meet with a testing official and an intravenous blood collection protocol is performed as delineated by the World Anti-Doping Agency. This intravenous blood draw sample is then processed by having the serum extracted from the sample, and having the serum sample shipped overnight at controlled temperatures to a World Anti-Doping Agency facility test site, which is a laboratory that has been rigorously certified for measurement of analytes in human specimens.

Other means of collecting samples for growth hormone have been considered and evaluated for effectiveness as a method for growth hormone doping[2], such as testing for the levels of insulin growth factor-1[3] (a hormone that is upregulated for a longer period of time when growth hormone is present) or the testing of athlete urine[4] for growth hormone. From these studies, the apparent deficiencies of the existing growth hormone method can be noted: An alternative method of growth hormone testing that reduces the invasiveness of growth hormone tests and overcomes the limitations of testing for a drug with a high clearance rate would be a

helpful improvement to the existing norm of testing via intravenous blood draw and a chemiluminscent ELISA method of assaying the serum samples.

Being that the overarching goal of this dissertation was to explore a rapid high-throughput electrochemical platform for improved growth hormone testing, studies were made to determine whether a less invasive and cost-effective method of growth hormone sample collection was achievable. The ideal method of growth hormone testing would require a low sample volume, avoid the invasiveness of an intravenous blood draw, be easily integrated into a clinical testing workflow, and potentially be adaptable for more frequent sampling tests. For the accomplishment of these goals, studies of the ability to quantify human growth hormone along two fronts of investigation were made in two directions:

A. **Dried blood spot reconstitution:** The usage of the dried blood spot approach for sample collection would be beneficial because dried blood spots could theoretically be transported in a smaller footprint compared to serum samples, and the usage of dried blood spots could also prevent the need to have extremely rigorous temperature control procedures in order to preserve sample integrity, as occurs in serum based samples (see Figure 39). Furthermore, a spot of blood on a filter paper typically requires less than 100uL of whole blood samples without preprocessing steps, and thus a single finger prick and spotting blood on filter paper would be a less invasive and simpler method for the testing of athlete samples compared to an intravenous blood draw into a blood collection vial, which would require at least 3-4 mL for testing. If a dried blood assay could be successfully developed and validated, it could also potentially allow for a greater amount frequency of sample collection of athletes, and greater sampling frequency could overcome the limitations of infrequent sampling of a compound that possesses a high clearance rate.

Initial explorations explored by Langkamp et al[5] demonstrated that using dried blood spots and an ELISA based system, it was possible to recover human growth hormone at a high efficiency. The investigations conducted in this study build upon the work of Langkamp et al[5] and tests whether the method can be used for quantification of both isoforms using an electrochemical method. These initial tests would test for the viability of using a reconstituted blood spot for a differential immunoassay application in athletic testing as a proof of concept test.

B. **Growth Hormone Detection using Salivary Methods:** Saliva has emerged in the landscape of scientific research as a possible means of non-invasively detecting diseases such as oral cancer[6] and malaria[7]. If Growth Hormone doping could also be easily detected non-invasively through saliva, this would be a productive improvement to the status quo of Growth Hormone testing. However, attempting salivary detection of Growth Hormone is a technical challenge, since Growth hormone has been characterized to be found in the salivary milieu at an estimated thousand-fold lower concentration[8]. Based on these estimates of Growth Hormone concentration in saliva and the known information about Growth Hormone isoform composition, a quantifiable method of growth hormone isoform testing in saliva would possibly require sensitivity levels in the femtogram range.

Initial electrochemical explorations are examined in this work to determine the viability of detecting growth hormone in saliva using electrochemical methods. Exploratory studies were made of applying an electrochemical immunosensor approach and an electrochemical immunoprecipitation based approach. Additional exploratory work was conducted to determine if saliva could be reconstituted and detected from dried filter paper samples. The completion of these would serve as a proof of concept tests for the viability of testing for growth hormone in saliva.

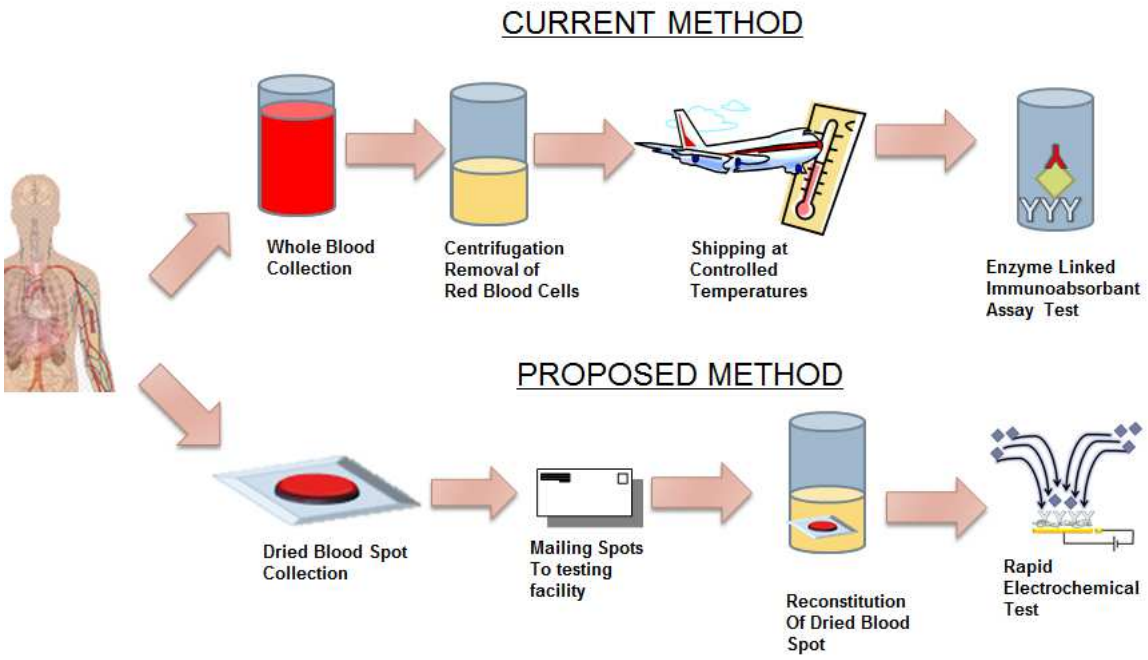


Figure 39. Proposed method of growth hormone collection using dried blood spots compared with current methods of sample collection and growth hormone testing.

# 2 Method

## 2.1 Electrochemical Assay System Used for Testing System

The electrochemical system used to test the effectiveness of a dried blood spot reconstitute and the salivary methods was a Genefluidics 16-sensor gold electrode array that is capable of performing measurement of electrical current over time for protein quantification. The Genefluidics system is able to quantitatively measure the oxidation-reduction rates of a reaction between a peroxidase enzyme and tetramethylbenzidine/hydrogen peroxide solution. In this scheme, the magnitude of the measured electrochemical current measured is proportional to the amount of target protein present[9]. While a variety of different approaches for forming the initial capture layer such as conducting polymer[10] and thiol-probe[11] have been developed and attempted with the Genefluidics platform, the approach taken involves the direct adsorption of

20 μg/mL of capture antibody on the surface, a preliminary assay system that was evaluated to be able to detect growth hormone standards diluted in casein/PBS buffer and whole blood based samples. After wash off of unabsorbed antibody is performed, the sample is incubated on the surface of the electrode for 2 hours and washed off with distilled water. The peroxidase detector antibody is then incubated for 30 minutes before wash off is performed with distilled water. The teteramethylbenzidine/hydrogen mixture (1-Step™ Ultra TMB mixture from Fisher Scientific) and current measurement for 60 seconds is conducted at -200mV, with the average of the last 10 seconds being the final metric for the assay for evaluation of signal.

For this capture layer of the 22 kilodalton isoform of growth hormone, 5802 Antibody from Medix Biochemica in phosphate buffered saline was incubated overnight. For the detector portion of the 22 kilodalton isoform, 5801 Antibody conjugated to a peroxidase enzyme from Medix Biochemica was prepared and diluted in casein/PBS for the detector layer. For the capture layer of the 20 kilodalton isoform of growth hormone, the 1c72 growth hormone used by Wu et al.[12] was used as the initial capture layer. For the detector portion of the 20 kilodalton isoform of growth hormone, the 5c4 antibody used by Wu et al.[12] was conjugated to a peroxidase enzyme in casein/PBS, and was used as the detector antibody in this scheme.

## *2.2 Preparation of Whole Blood Reconstitution Samples*

In the whole blood reconstitute assay, first, whole blood samples needed to be prepared for assay. Two kinds of blood samples were used for the testing of the reconstitution assay for the purpose of experiments:

a. **Sheep blood:** Sheep whole blood mixed with K2 EDTA (Innovative Research) was used as the beginning “human growth hormone free” blood sample negative control, a control which possesses the complex biofluid environment present in human whole blood but has no human growth hormone protein present in it. The sheep blood was

also utilized to create a calibration curve for quantification of human growth hormone in whole blood samples. For the creation of protein standards, the whole blood samples were established by additions of recombinant 22 kilodalton isoform human growth hormone (MyBioSource) or 20 kilodalton isoform growth hormone (Enzo Life Sciences).

b. **Human Blood:** Intravenous blood draws from healthy adult patients were collected in vacutainer K2 EDTA tubes by a trained phlebotomist. Human whole blood samples were evaluated to see if the amount of growth hormone naturally present would be measured in expected physiological range when compared to the standard curves made with sheep whole blood.


Figure 40 Dried blood spot reconstitute. Left figure illustrates the pipetting of 60 **µ**L of whole human blood on the surface of the proteins saver card. Right figure illustrates the hole punching of the dried blood spots on the filter paper into a microcentrifuge tube before shaking at room temperature.

Following the collection and preparation of the whole blood samples, Whatman Protein Saver 903 cards were used as a storage medium for the dried blood spots. This paper spot is a fibre cellulose paper that has been applied to contexts such as neonatal disease screening tests[13] and iron deficiency in adult populations[14]. In this study, 60 µL of total whole blood was pipetted to the surface of the dried filter paper, the pipetting of the samples ideally being within a

designated five circular spots on the protein collector paper. These blood spots then were left to be dried at room temperature for 2 hours before the reconstitution step.

Following the drying of the blood samples at room temperature, a standard 6mm diameter hole punch was used to make punches of the dried blood spot into a microcentrifuge tube. Between punch spots for each different sample of dried blood, a standard cleaning protocol was applied on the hole puncher to prevent cross contamination. The protocol used involved verse immerse dipping the hole punch in a falcon tube of ethanol, following the ethanol immersion with an immersion in water, and then using pressurized air to blow dry the hole punch and remove any additional debris that was not removed during the immersion step.

For this study, ten 6mm whole punches of dried blood per sample were placed in an individual 2.0 mL microcentrifuge tube. 250mL of phosphate buffered saline was added to the hole punch samples in the tube and briefly vortexed in order to agitate the dried blood spots and ensure that they were all properly immersed in the phosphate buffered saline. The vortexed mixture of phosphate buffered saline and the dried blood samples were then placed on a shaker at 250 rpm of shaking for 2 hours at room temperature. Following this incubation on the shaker, the reconstituted tubes were briefly vortexed to make sure the paper was on the bottom of the tube for easier extraction of the liquid.

The reconstituted dried blood spot liquid from the spun down tubes was then pipetted to the surface of the electrode and incubated at room temperature for 2 hours. Following this incubation, the reconstitute solution was washed off with distilled water and a 0.05 mg/mL solution of peroxidase conjugated detector antibody specific for the tested isoform was pipetted to the surface of the working electrode and incubated for 30 minutes at room temperature. The unbound detector antibody was washed off and an electrochemical current readout performed using the standard tetramethylbenzidine/hydrogen peroxide mixture.

## 2.3 Method of Assessing Assay of Saliva Biofluid for Human Growth Hormone

For the assessing of saliva as a biofluid for testing of growth hormone, 10mL of human saliva was collected and spun at 7600xG for 10 minutes at 4 degrees Celsius twice to purify the saliva sample and remove cellular debris. This spin-down sequence is necessary in order to ensure the sample stability for testing and collecting procedures[15].

This cell free saliva had recombinant human growth hormone (myBioSource) added to it at different concentrations, and the samples were assayed using an electrochemical 16-array system with 22kD specific growth hormone as the capture layer. The processed saliva standards were then applied to a Genefluidics 16-array electrode surface and incubated for 2 hours at room temperature. Washoff was performed using distilled water, and then a 0.05 mg/mL solution of peroxidase conjugated detector antibody (Medix Biochemica 5801 Growth Hormone antibody) in casein/PBS was pipetted to the surface of the electrode and incubated at room temperature for 30 minutes. This detector antibody was washed off with distilled water after incubation, and standard current readout with the tetramethylbenzidine/hydrogen peroxide mixture on the surface of the electrode was performed.

## 2.4 Immunoprecipitation Capture Method for Determining Growth Hormone Presence in Saliva

2 mg of Dynabeads Streptavidin T1 beads were transferred to a centrifuge tube and triple washed by magnetizing the beads to the side of the tube, removing supernatant with a pipette, adding 500uL of PBS to the beads, and then vortexing the solution. Following this triple washing, the beads were incubated in 500 µL of a 40 µg/mL solution of biotinylated 22kD growth hormone specific antibody (5802 antibody from Medix Biochemica) with rotating for 30 minutes at room temperature. Following this incubation and rotation procedure, the beads again triple washed with 500 µL of PBS. The beads conjugated with growth hormone antibody were

divided into 150 µL aliquots to each tube, the PBS was removed, and 150uL of cell free saliva sample was added.

The incubation of the salivary samples was performed at room temperature with shaking for 90 minutes. Following the incubation of the salivary samples and removal of saliva sample, the beads were washed with 500uL of PBS. Addition of 5 µg/mL of Peroxidase Conjugated Antibody (Medix Biochemica 5801 Antibody) in casein/PBS and incubation for 30 minutes with rotation was conducted. Following a triple washoff consisting of magnetization, removal of supernatant with a pipette, addition of 500 µL of fresh PBS, and vortexing, 200 µL of tetramethylbenzidine/hydrogen peroxide mixture (1-Step™ Ultra TMB from Fisher Scientific) was added to the solution. At this juncture, the beads which have peroxidase enzyme completing an antibody sandwich will be reacting with the hydrogen peroxide and the tetramethylbenzidine, which is quantifiably measureable by the electrode surface. 60 µL of this bead solution is then pipetted to the Genefluidics 16-array bare gold electrode with a magnet underneath to concentrate the beads on the working electrode, and electrochemical measurement is made of the tetramethylbenzidine, hydrogen peroxide, and peroxidase enzyme completing the bead assay(Figure 41).

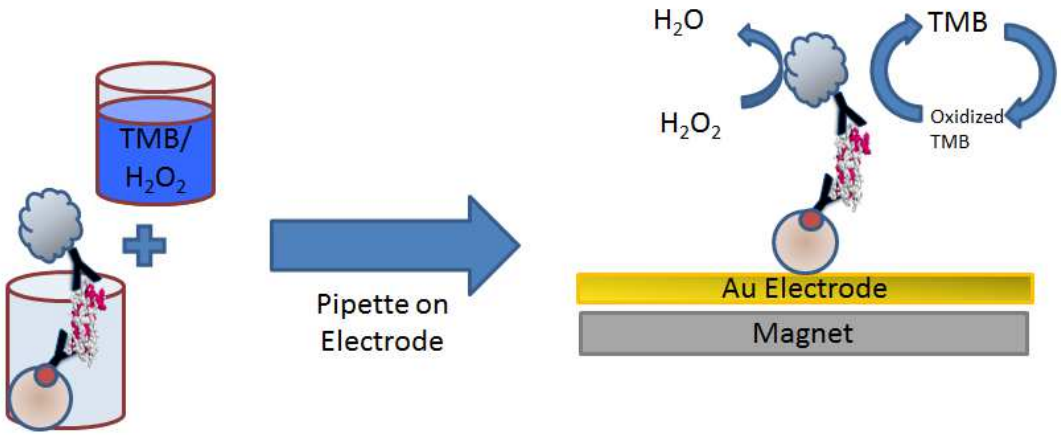


Figure 41. After a complete antibody sandwich is formed by the streptavidin coated magnetic beads and the antibody pair on saliva samples, the sandwich is mixed with tetramethylbenzidine and hydrogen peroxide mixture and

pipetted to the surface of a magnetized electrode. The magnetic sample keeps the beads concentrated on the working electrode while the electrode measures the oxidation and reduction of tetramethylbenzidine.

For this preliminary assessment of growth hormone presence in human salivary sample, the negative control selected was mouse salivary sample, which theoretically has no human growth hormone present in it. The positive control consisted of this mouse saliva spiked with recombinant growth hormone.

# 3 Results

## 3.1 Reconstitution of Growth Hormone using Dried Blood Spots

### Results

Assay was performed comparing an electrochemical quantitation experiment where dried blood spot reconstitute of whole blood growth hormone standards with an electrochemical quantitation experiment where the whole blood growth hormone standards were directly incubated on the surface of the electrode chip. After quantitation on the growth hormone in whole blood standards and the reconstitute was performed, it was suggestive that it is possible to completely reconstitute the dried growth hormone on the filter paper. This conclusion is suggested by Figure 42, where concentrations ranging from 50 ng/mL to 5 pg/mL of growth hormone in whole blood standards were compared to the quantification of growth hormone using a reconstituted dried blood spot based method. In every standard that was prepared and tested in this comparison study, there was no statistically significant difference between the dried blood spot method and the whole blood assay method using the direct antibody based adsorption technique. Testing for the correlation between the average signals of the whole blood assay and the dried blood assay shows that the correlation between the dried blood spot and the whole blood based electrochemical assay is nearly perfectly correlated ($R^2$= 0.99).

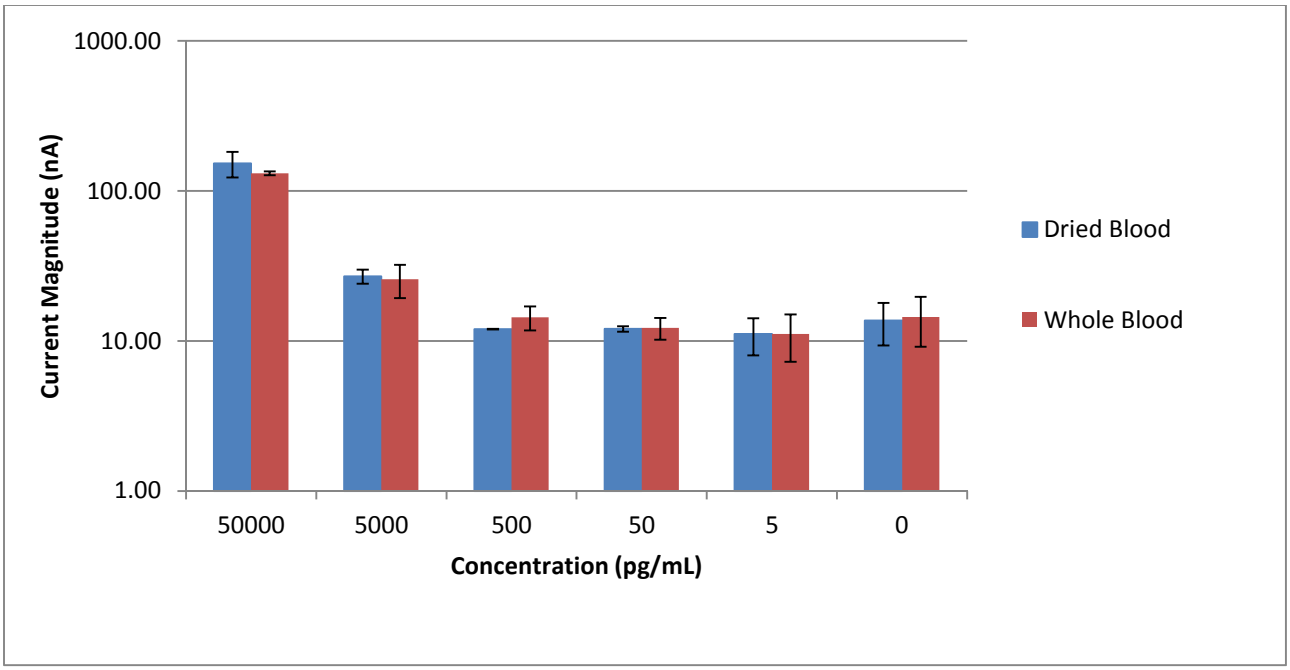


Figure 42. Comparison of whole blood spiked with 22kD recombinant growth hormone quantified through an electrochemical method compared with dried blood spot based reconstituted growth hormone. The results seem to indicate that there is no statistically significant difference between the dried blood spot reconstitutes signal and the results yielded from using growth hormone spiked in whole blood samples. In the scope of this section of the work, the adsorbed antibody based assay was able to yield a sensitivity of 5 ng/mL for assay sensitivity

Further quantitative tests were run on the dried blood spot approach, comparing growth hormone measurement levels in reconstituted human blood spots with reconstituted growth hormone standards in sheep blood. As is indicated by Figure 43, the results of the study seem to perform as predicted. The baseline measurements in reconstituted human samples are elevated compared to the reconstituted whole sheep blood, which is according to expectations that sheep blood does not have human growth hormone as a constituent element. The standards that consisted of sheep blood spiked with recombinant 22 kilodalton growth hormone (at concentrations of 50 ng/mL, 5 ng/mL, 500 pg/mL, 50 pg/mL, and 0 pg/mL) possess a linear relationship ($R^2$ = 1.0) between the measured current of the reconstitute and the amount of growth hormone spiked into the dried blood spot. When the current measurements of the reconstituted human blood spots were compared to the calibration curve of sheep whole blood with growth hormone standards doped, the results seem to suggest that the measured human

growth hormone is between 500 pg to 5 ng/mL, which accords to the known amount of growth hormone present in the human body. Because of these results and the signal measurement of the growth hormone in blood being in a physiologically realistic range when compared to the standard curve, there appear to be grounds to believe that the assay can accurately perform quantitation on human specimens.

A reconstituted whole blood assay using sheep whole blood samples spiked with the 20 kilodalton isoform of growth hormone was also tested, the quantitation of the growth hormone reconstitute being performed with the antibodies 1c72 and 5c4, which were specific to growth hormone[16]. These preliminary tests (see Figure 44) of the 20 kilodalton growth hormone spiked in sheep whole blood and reconstituted from dried blood spots showed that the concentration of 20kD growth hormone spiked in the whole blood correlates well with the measured electrochemical signal( $R^2$ = 0.99). However the baseline measurement of the reconstituted human blood sample is lower than the baseline measurement of sheep blood without any 20kD growth hormone spiked in. This may possibly be attributed to the fact that the existing growth hormone assay may not have the sensitivity of detecting the physiologically 20 kilodalton growth hormone in the blood, a fact suggested by the fact that the assay growth hormone calibration curve in sheep blood only had a sensitivity of 5 ng/mL.

While these results are highly suggestive of the viability of reconstituting dried blood spots and making quantitative measurements of the growth hormone using a dried blood spot approach, it must be noted that further tests and optimizations are necessary in order to make full determination of whether a quantifiable high sensitivity assay of growth hormone is achievable in the dried blood spot based method. Tests on a more sensitive electrochemical assay and additional experiments on human blood samples would be useful in determining whether the reconstituted blood method is a functional assay that can practically replace the existing serum based test for human growth hormone.

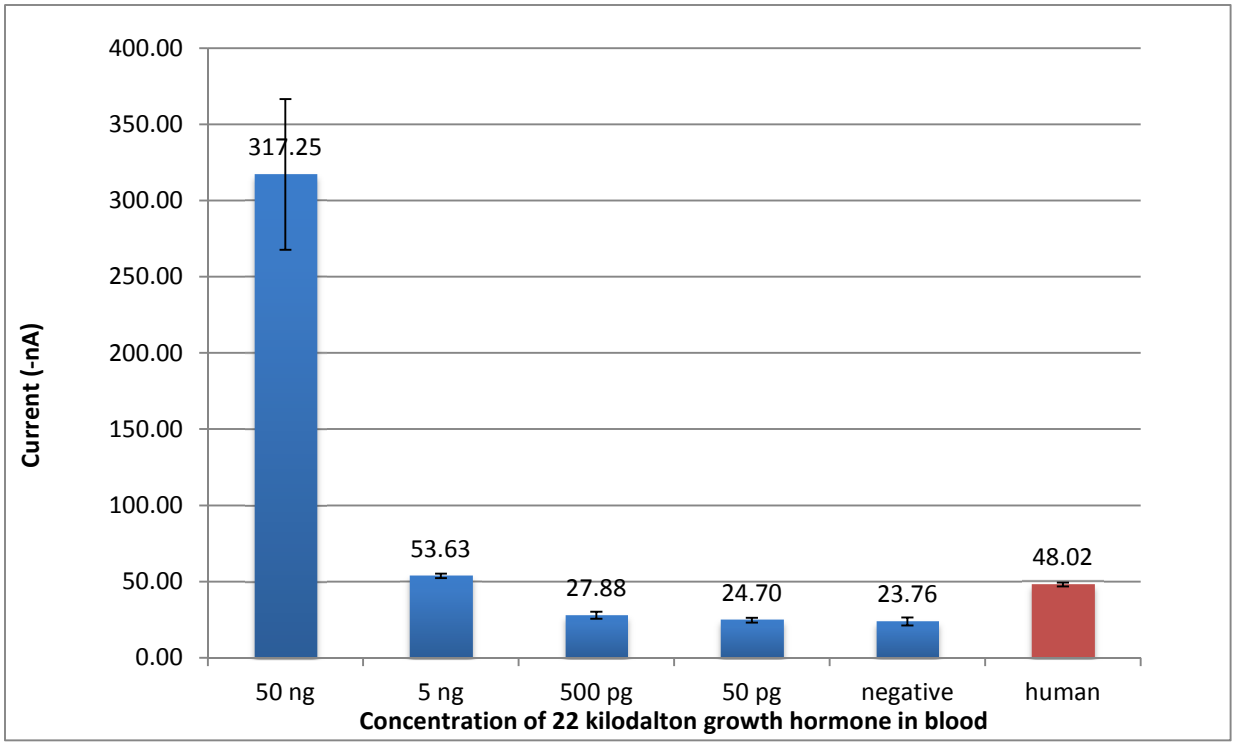


Figure 43. Growth Hormone Assay of 22kD isoform using a reconstituted dried blood spot method. Measurement is of growth hormone using a directly adsorbed antibody strategy (which appears to possess a sensitivity in 5 ng/mL range). The bar indicated by red is the measurement of a healthy adult male blood sample reconstituted and tested in parallel with growth hormone samples prepared in sheep blood.

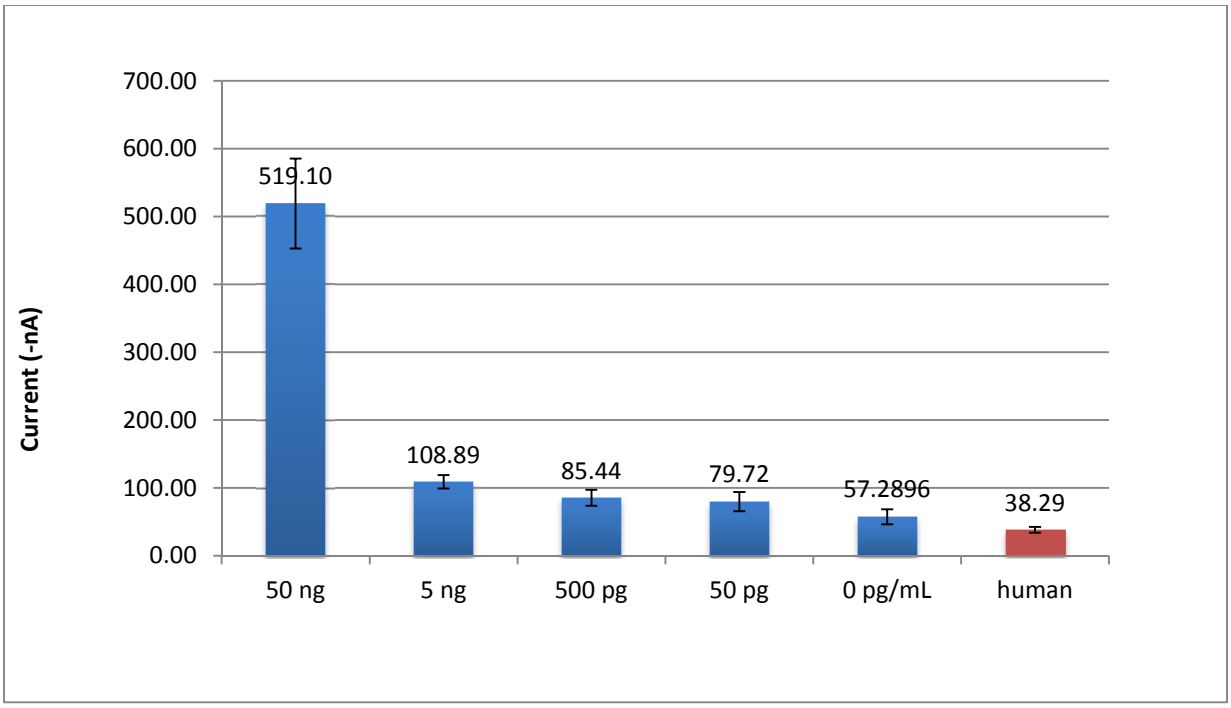


Figure 44 Growth Hormone Assay of 20kD isoform spiked into whole blood samples using a reconstituted dried blood spot method. Measurement is of growth hormone using a directly adsorbed antibody strategy (which appears to possess a sensitivity in 5 ng/mL range). The bar indicated by red is the measurement of a healthy adult male blood sample dried blood spot reconstitute with no growth hormone spiked in and tested in parallel with growth hormone sample prepared in sheep blood (indicated by the bars colored in blue).

## *3.2 Saliva Based Detection System for Growth Hormone Results: Elimination of Matrix Effects*

Initial investigation into the usage of processed saliva supernatant as a medium for testing of growth hormone seemed to be in the negative on an electrochemical immunosensor setup due to the fact that a calibration curve with spiked growth hormone was not recoverable when tested on the electrochemical sensor. In order to examine this effect more, dilutions were made of the processed saliva supernatant in order to examine more thoroughly the effect of the salivary matrix on the ability to recover human growth hormone in electrochemical assay (see Figure 45). As the results indicate, in the 1:1 supernatant sample, no discernable trend is observable for the 500 pg/mL standard of growth hormone, while in dilution ratios greater than 1:2 a 500 pg/mL measured current is statistically significant from salivary samples without growth hormone spiked in. When the average signals of the results of these experimental

results are performed, it appears that the 1:3 and 1:4 dilutions of salivary samples have a higher average signal with the 1:1 and 1:2 dilutions of growth hormone.

Based on the results of the salivary experiment performed, it appears that in order for an assay for saliva to be implementable on growth hormone, it would be necessary to dilute to at least 1:2 saliva to buffer in order to overcome matrix effects. It should be specifically reiterated that the spiking in of growth hormone in the salivary samples was done after the dilution protocols were enacted, so the amounts spiked are theoretically higher than would be observed in actual salivary samples of human growth hormone. The electrochemical immunosensor system tested in this project does not appear to be sensitive to the levels required for salivary samples, but the experimental work performed here is proof of concept to show that diluting salivary samples can help overcome matrix effects present in salivary samples and allow for the quantitation of growth hormone in saliva.

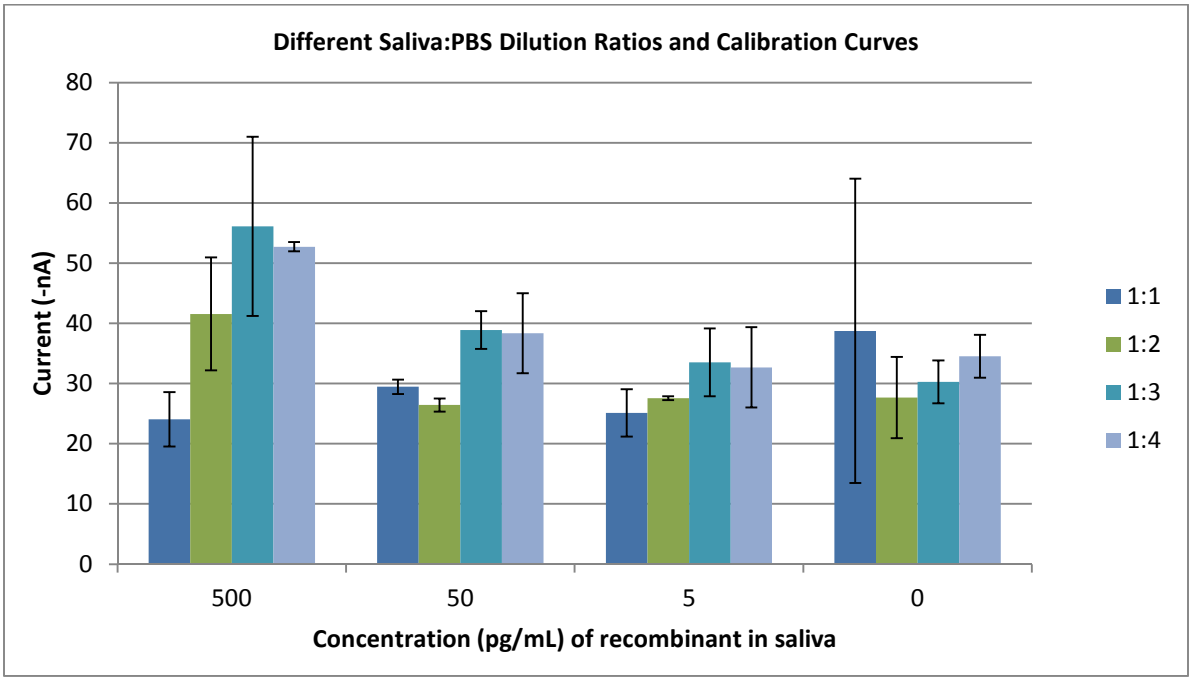


Figure 45. Performance of growth hormone standards after dilution of saliva in PBS and spiking in growth hormone in different concentrations. Results indicate that until the 1:2 dilution ratio, there is a significant effect of matrix effects that renders growth hormone spiked into saliva unrecoverable in electrochemical assay.

## 3.3 Determining of Growth Hormone Presence in Salivary Samples

Following the exposure of the 22kD growth hormone specific antibody beads to the milieu of cell-free saliva by mixing for 90 minutes, quantitation was made using the magnetic beads and electrochemistry on the three samples: positive control of recombinant growth hormone mixed in saliva, the negative control of mouse saliva without addition of growth hormone, and a human salivary sample. The quantitation of the results using electrochemistry demonstrated that there was a statistical significance between the different controls and the human growth hormone sample. Figure 46 shows that the bead based magnetic nanoparticle approach was able to cause significant elevation of measured current in a mouse saliva sample spiked with 500 pg/mL of 22 kilodalton recombinant growth hormone compared to the mouse saliva that had no 22 kilodalton recombinant growth hormone spiked in. This shows that the bead based system is able to successfully capture growth hormone present in undiluted saliva samples.

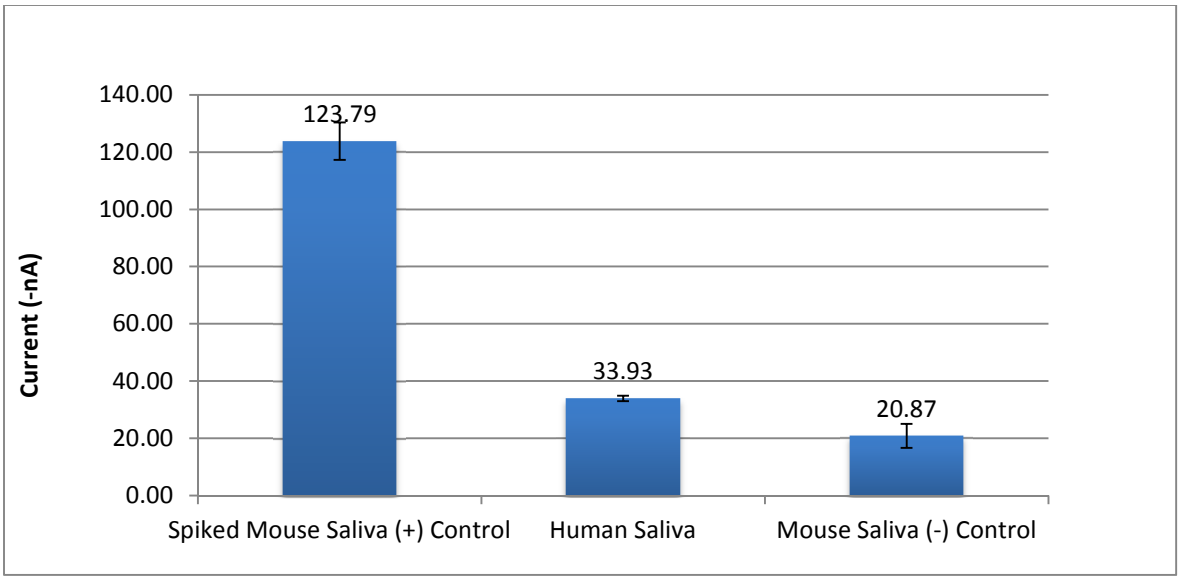


Figure 46. Comparison between electrochemical measurement of growth hormone bead-based electrochemical assay in saliva. These results corroborate predictions that the human salivary sample would have a measurement in

the spectrum between a positive control which consisted of mouse saliva spiked with 500 pg/mL of growth hormone, and a negative control of mouse saliva without addition of recombinant growth hormone, which has no growth hormone present.

The results of Figure 46 corroborate our predictions that the human salivary sample fell in the spectrum between the negative control of mouse saliva (which has no human growth hormone present in it) and the positive control of mouse saliva (which was spiked with 500 pg/mL of recombinant growth hormone). This preliminary assay is suggestive that human growth hormone is present and can be extracted using a bead based assay, and could be extended by performing the bead assay with more standards of growth hormone spiked into mouse saliva in order to thoroughly ascertain the performance of a bead based assay method for extracting growth hormone in cell-free saliva samples.

# *4 Conclusion*

Alternative matrixes would be highly beneficial to the advancement and improvement of growth hormone testing because they would be useful in reducing the invasiveness of existing methods and reduce the sample requirements. The methods and results that are here presented are useful studies inasmuch as they demonstrate that it is possible to recover growth hormone from alternative matrixes of detection such as dried blood spots and salivary samples. The work in dried blood spot assay is highly suggestive that quantitation and recovery of growth hormone isoforms in dried blood spots is efficient in the electrochemical context (100% recovery between the dried blood spot and whole blood assay of growth hormone), and agrees with the existing literature of growth hormone dried blood spot assays.

In order to reach the endpoint of developing an improved growth hormone sensor system that reduces the cost and inconvenience of testing for growth hormone, the dried blood spot reconstitute method explored in this section must be combined with a highly sensitive assay system. In the studies made in this work, the sensitivity levels of the assay systems are

still in the 10 ng/mL to 500 pg/mL range, while the ideal limit of detection for a quantitative isoform assay must be in the low picogram range for the 22kD and 20kD isoforms of growth hormone (50 pg/mL to 5 pg/mL) in blood.

The work regarding the salivary studies for a saliva based growth hormone, which would be the least invasive of all methods of sample collection, leads to two conclusions:

A. **Magnetic Bead Approaches can successfully capture salivary growth hormone:** Preliminary studies of a magnetic microparticle based approach for extracting growth hormone from saliva (and overcoming the difficulty of the low concentration of growth hormone) are suggestive that this strategy of growth hormone extraction is possible. The data collected was only a preliminary proof of concept to determine the presence of salivary growth hormone using a single sample of human saliva, one positive control of growth hormone spiked in mouse saliva, and one negative control of mouse saliva.

   Further development of the magnetic bead method would necessitate tests on a "standard curve" of growth hormone spiked into a growth hormone free saliva in order to comprehensively assess key quantitative properties such as limit of detection and linearity. That is, multiple samples of mouse saliva would need to be spiked with recombinant human growth hormone and run with the growth hormone capturing magnetic beads and the resulting readout quantified. These tests would require measurement of growth hormone standards down to the femtogram level in order to prove 20kD and 22kD proteins were quantifiable in the saliva. It would also prove useful in validation of the bead based approach to perform tests on additional individuals. This would be prudent in establishing a broader understanding of the variability levels of growth hormone in human salivary samples.

B. **Electrochemical immunosensor approach shows matrix effects in saliva:** Based on the results demonstrated in this work, it appears that the quantitation of growth hormone on an electrode based biosensor approach (as opposed to the bead approach) possesses matrix effects. The results indicate that a 1:2 or greater dilution of salivary samples is necessary in order to be able to have a discernable relationship between electrical current measurement and growth hormone. Because of these results, and the reported values of growth hormone in salivary samples, it seems that a great degree of biosensor optimization is necessary in order to reach the sensitivity levels made necessary by the low natural abundance of salivary growth hormone in saliva and the effects of diluting saliva.

Being that the dried blood spots approach worked in a highly effective manner and that the electrochemical limit of detection of growth hormone isoforms is currently out of range for growth hormone, it seems that dried blood spots is the most technically feasible non-serum sampling approach that is the most likely to be able to be put into practice soon. But the data presented in this work has not entirely disqualified the salivary quantification of growth hormone approach.

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# *Chapter 5 –Low-Voltage Electric Field Study Using High-Throughput Electrode Array*

## *1 Introduction*

Previous studies[1, 2, 3,] in electrochemistry have suggested that the application of low voltage electric fields may be able to enhance the performance of protein biosensors by facilitating the binding between proteins and antibodies in an antibody sandwich (such as is used for the detection of human growth hormone). Enhancements to assay sensitivity and speed seemed to be the primary enhancements that resulted from the application of these low-voltage electric fields. This low voltage electric field method seems to operate at voltages different from other immunoassay enhancing methods such electrophoresis[4] (which applies 100-300 Volts direct current) to attract target molecules based on their net charge or electrothermal[5] flow (which applies greater than 100 kHz alternating current waveforms at a peak-to-peak potential of 8-20 voltages) to generate thermal gradients that stir a solution.

In this portion of my dissertation, I wish to make initial investigations into these low-voltage electric fields and determining whether they affect the capture of growth hormone from whole blood. The present methods for examining the method[1, 2, 3] seem to be based on simply evaluating one low-voltage waveform at a time (see Figure 47A), and with this approach it is difficult to thoroughly evaluate the effect of the low-voltage method on sensor performance. The method taken in this work is to investigate by rapidly screening for the optimal parameters for enhancing the detection of growth hormone by using a high-throughput electrochemical sensor

array in combination with parallelized application of different low-voltage electric waveforms (see Figure 47B). An electrochemical potentiostat has been previously developed for rapid high-throughput parallel readout of electrochemical current on a 96-electrode array, but this system was limited in its ability to control the potential applied to the individual electrodes. As a result, a controlling system was developed to generate the waveform functions necessary for this systematic study. This method allows for electric-field screening studies at a rate unrivaled by existing methods. Ultimately, the implementation of this method will create a unique tool that assist in elucidating the effect of the low-voltage electric field and determine whether binding between a protein and antibody is promoted using these low-voltage effects.

# 2 Method

## 2.1 Implementation of Control Layer Developed for Electric Field Study

In this study an Ivium Compactstat potentiostat device was used. This potentiostatic device can run a large variety of electrochemical techniques including cyclic voltammetry (where the applied voltages are varied for an electrode and the associated current response is measured) and chronoamperometry (where measurement of current over time is made). Additionally, the Compactstat can be integrated with add-on modules that allow for multiple channels to be read in parallel. These methods that are typically used in a potentiostat were capable of performing readout and uniform application of a single waveform to multiple electrodes (see Figure 47A), but the ability to manipulate and test multiple different waveforms simultaneously on different electrodes was limited.

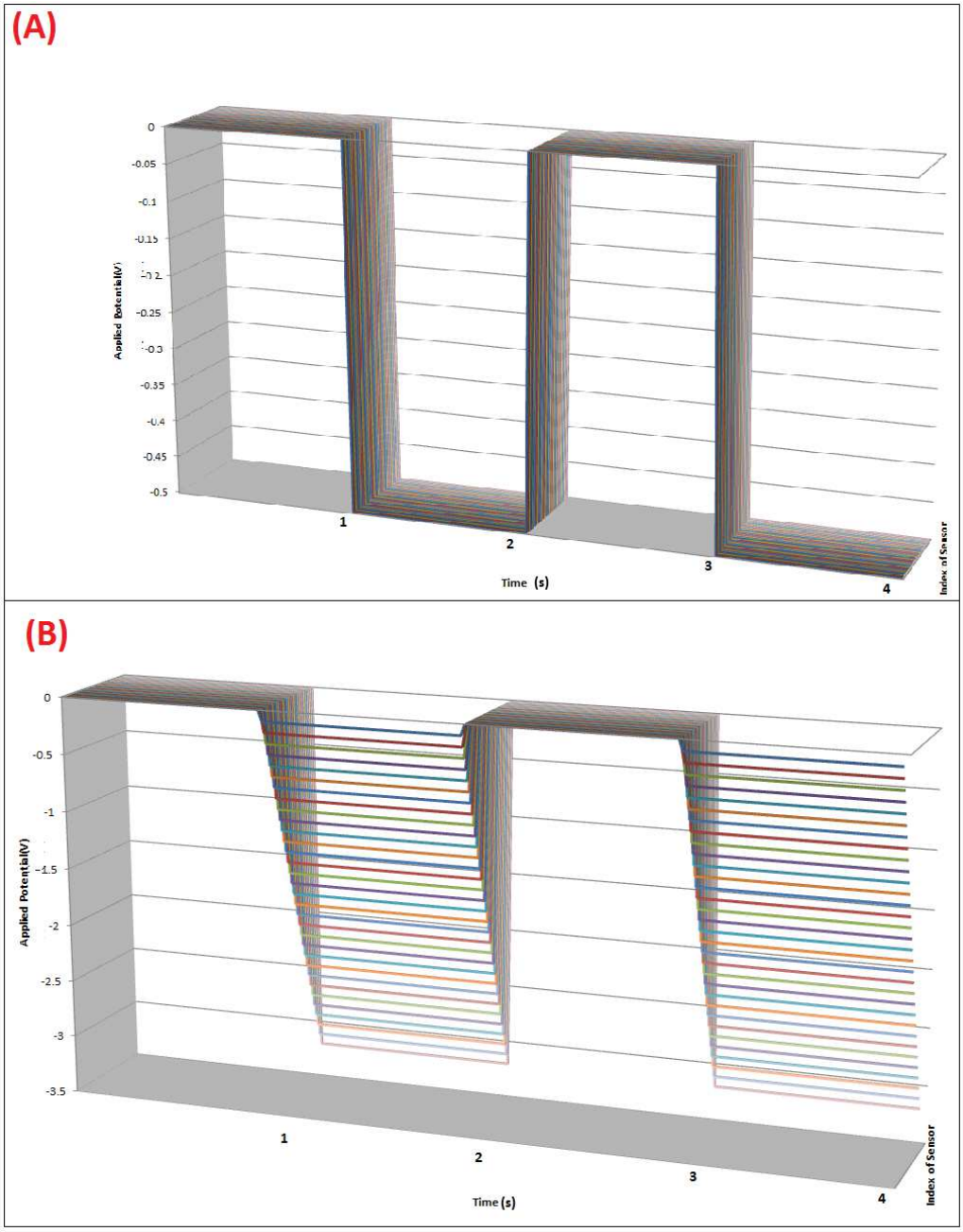


**Figure 47.** (A) Example waveform function that could be applied to the 96-electrode array using potentiostat (B) Following implementation of the control layer, the potentiostat is able to be controlled and waveform functions at different potentials can be run in parallel.

Implementation of this layer was performed through the usage of a dynamic-linked library meant for interfacing with the original hardware and software of the Ivium Potentiostat. The python programming language was used as wrapper to simplify the complex function calls

to the dynamic linked library (and by extension, the potentiostat). These abstractions allowed us to conveniently specify the electrodes, potentials, and waveform functions to be applied to the electrode array and specify offsets so as to modify the set potential on individual electrodes.

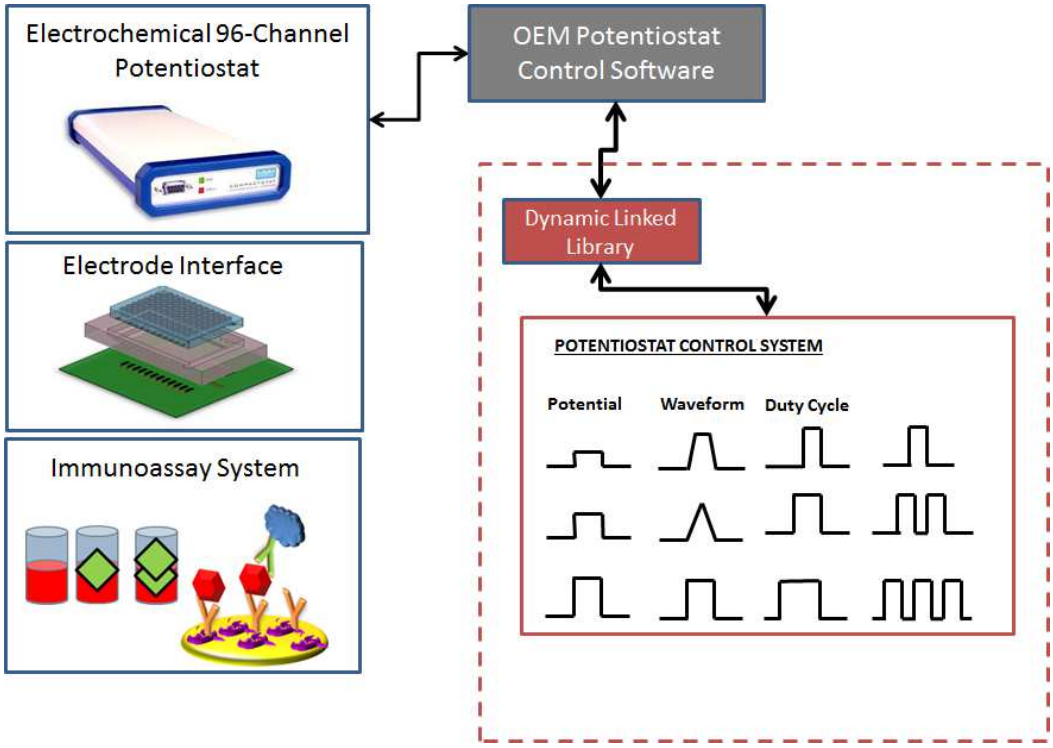


**Figure 48.** Overview of the high-throughput electrochemistry array system. A dynamic linked library was interfaced with a control method for rapid control of the individual waveforms that could be sent to the potentiostat and 96-electrode plate.

## *2.2 Technical Limitations of Approach*

Since the potentiostatic control software consists of three 32-electrode multiplexing modules connected with each other, the offset settings for one 32-electrode unit will be

propagated to the other two 32-electrode array units, meaning that the potentiostat is ultimately capable of applying a maximum of 32 unique potential settings to the 96-electrode system.

Furthermore, because of the technical limitations of the offset method, the manipulation of the electrode potentials is a more discrete process, and usage of the potentiostat to attempt different profiles such as sinusoidal waves at different frequencies would be unachievable. The current method developed based on the hardware only allows for waveforms such as sinusoidal waves of the same frequency, fixed potentials, and ramping potentials at the same rate.

## *2.3 Electrochemical Experimental Method Used with Software Control*

The aforementioned electric-field control method on the Iviumstat Compactstat and multiplexing method was applied to an immunosensor system present on a 96-electrode plate that was interfaced with the potentiostat. This immunosensor system consists of 96-electrodes that had an electropolymerized polypyrrole layer combined with streptavidin protein, with a biotinylated antibody specific for human growth hormone (5802 antibody from Medix Biochemica) incubated on top of this streptavidin polymer layer. This initial streptavidin-polypyrrole layer with growth hormone specific antibody incubated on the electrode surface serves as the initial capture layer for the high-throughput electrode plate.

Following the creation of the capture layer on the electrode plate, the samples and standards for the target analyte are pipetted to the surface of the electrode, and an electric field pulsing protocol is applied to the surface according to the control program devised in this study. The sample and standards that have been pulsed with low-voltage electric fields are then washed off with distilled water, and a 5 µg/mL solution of peroxidase conjugated antibody (5801 Antibody from Medix Biochemica) is pipetted to the surface of the electrode and incubated for 30 minutes. This peroxidase conjugated antibody functions as the detector antibody and completes a complete antibody sandwich.

After the detector antibody has been incubated to complete an antibody sandwich, the unbound detector antibody is rinsed off distilled water, and a tetramethylbenzidine/hydrogen peroxide mixture is pipetted to the electrode surface. The reaction between the peroxidase enzyme and the tetramethylbenzidine/hydrogen peroxide solution will cause a measureable current. This measured current magnitude is proportional to the amount of a target analyte that has been captured by the biotinylated capture antibody and the peroxidase-conjugated detector antibody.

## *2.4 Method of Surveying Parameters*

Our study combined the electrochemical assay system for growth hormone with the potentiostatic control program in order to assess the effect of a low-voltage electric field on the binding between human growth hormone samples in blood and a growth hormone specific capture antibody. The subject of our study was the cyclic square wave profile design that had previously proved successful in the biodetection of nucleic acid[3] and protein-based systems[6].

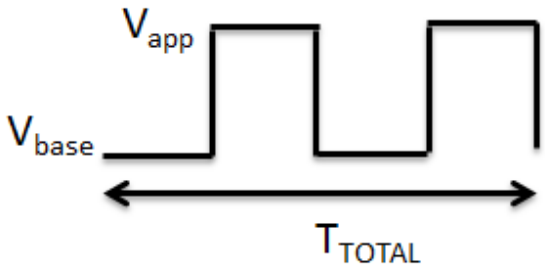


**Figure 49.** Cyclic square wave profile that was evaluated for the study. This study investigated kept the $V_{base}$ at a constant 0 mV, and examined the assay performance when $V_{app}$ and $T_{total}$

The search approach used in this study proceeded in two phases:

A. **Phase One: Surveying Optimal Applied Potentials ($V_{app}$).** Using the offset program that was designed for the electrochemical system, different potentials were applied at

100mV increments from 0 to -700 mV in order to survey which signal was able to optimally maximize the signal to background current measurement.

A standard 96-electrode electrochemical plate is coated with a streptavidin-polymer matrix and a biotinylated antibody specific for growth hormone is prepared. Growth hormone standards are pipetted to the electrodes (50 pg/mL of growth hormone in whole sheep blood for a positive control, and whole sheep blood without growth hormone spiked in for the negative control), and different electrical potentials are applied to the 96-electrodes. The base potential ($V_{base}$) was kept constant +0 mV for 1 second and the applied potentials ($V_{app}$) ranging from 0 to -700mV were applied for 1 second to individual wells. Seven cycles of this square wave were applied, totaling 16 seconds of the applied square wave profile for each well.

Following the application of the cyclic square wave to each well, the blood standards are washed off, a detector antibody is incubated on the surface for 30 minutes, and readout of electrical current is made for each well. In this experimental setup, each potential was tested with six positive controls consisting of 50 pg/mL of growth hormone spiked into whole blood and six negative controls consisting of whole blood without growth hormone spiked in. The optimal candidate is selected based on the potential that had the largest difference between the positive and negative control measurements.

B. **Phase Two: Surveying Optimal Cycle Lengths ($T_{total}$).** The optimal square wave profile from the phase one experiment is applied at different cycle lengths to determine the optimal time of applied signal that best maximizes the difference between the positive and negative control. In this study, 6 replicates of the positive control and 6 replicates of the negative are tested for each cycle length.

This follows a very similar workflow to the phase one: A standard 96-electrode electrochemical plate is coated with a streptavidin-polymer matrix and a biotinylated antibody specific for growth hormone is prepared. Positive and negative controls are then pipetted to the surface of the electrode and the optimal cyclic square wave from phase one is tested at different cycle lengths. Following the application of the cyclic square wave to each well, the blood standards are washed off, a detector antibody is incubated on the surface for 30 minutes, and readout of electrical current is made for each well. The optimal cycle length that maximizes the difference between the positive and negative controls is selected for the third phase.

# *3 Results*

The results of the study indicated observable trends in the performance of the positive and negative controls and the relationship to the applied potential. For the phase one experiment in which 8 cyclic square waves were applied, with each cycle consisting of 0 mV applied for 1 second and different potentials ranging from 0 to -700 mV were applied for 1 second, trends were observed(). The difference between the positive and negative control value begins in the 0 mV range with a difference of approximately 39 nA, while there is a sudden drop in the difference between the positive and negative control samples in the -100 to --300 mV range. After the -300mV range, however, the difference between the positive and negative control values raises to the 60-80 nA range. Based on the results of this experiment, the best candidate potential seemed to be in the -500mV potential setting, and it was used in the next phase of the experiment.

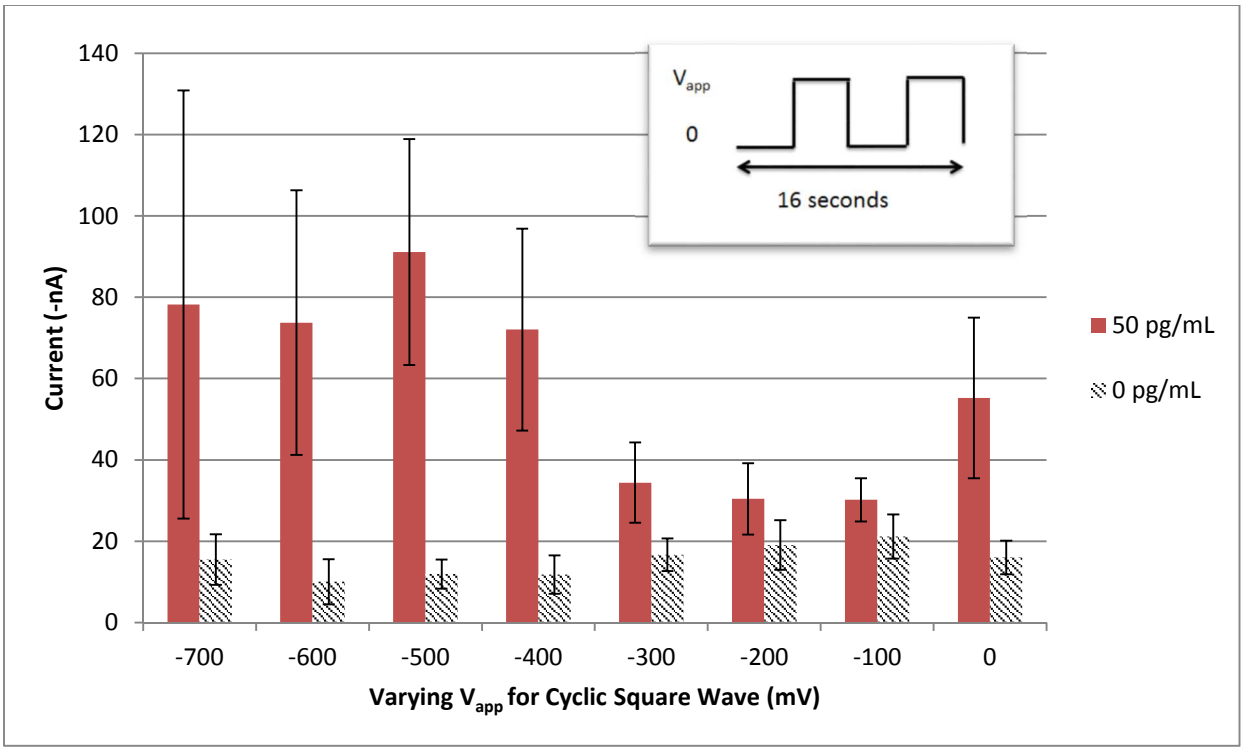


**Figure 50**. Results of comparing a constant 8 cycles (. $T_{total}$ = 16 seconds) of cyclic square wave with a uniform $V_{base}$ =0mV and variations to $V_{app}$ for the sample incubation step (where the capture antibody attempts to bind to the target of 22 kilodalton growth hormone in blood)

Following the selection of the optimal potential, further tests were run using this -500mV for 1 second and 0mV for 1 second cyclic square pattern at different lengths of time to determine the optimal length of electric field application that would be able to maximize the signal between the positive and negative controls. shows the result of investigation using this -500mV and 0mV cyclic square wave. In this experimental result, there seems to be an elevation of the potentials observed in the 1, 2, and 4 cycle settings, but a significant drop-off in the signal after the 4 cycle setting. Based on the result of this experimental work, the 2 cycles of applying -500mV for 1 second and 0 mV for 1 second were decided as the optimal electric field settings for the electric field driven incubation method.

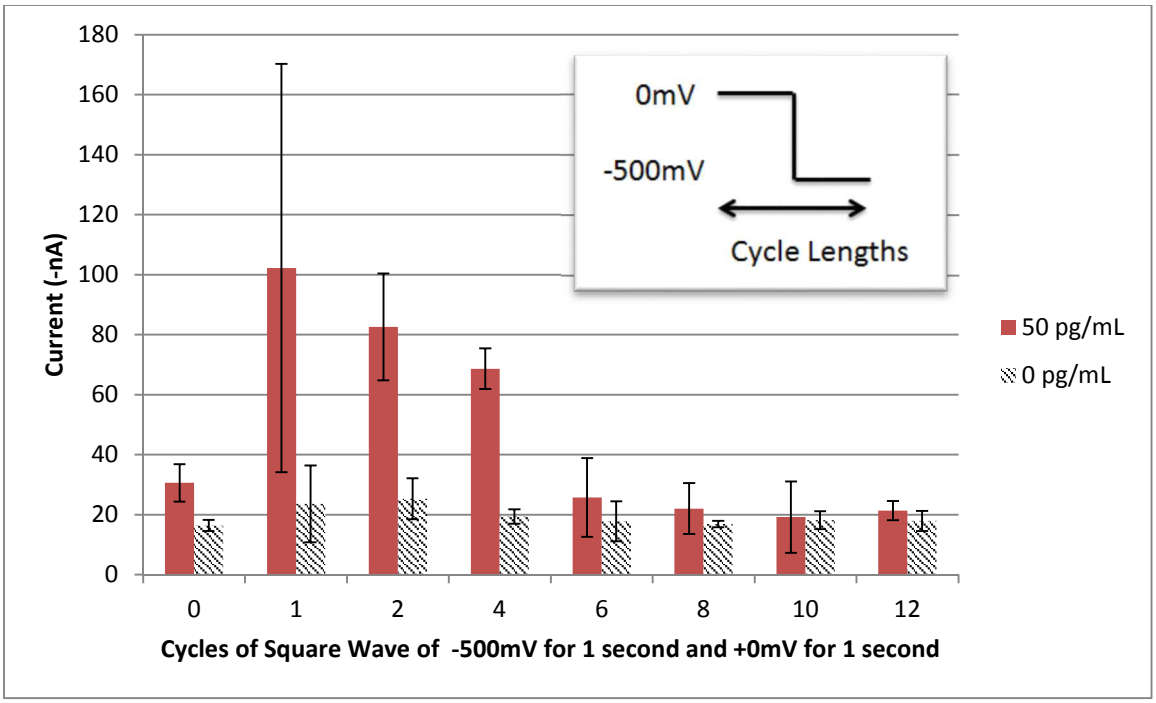


**Figure 51**. Results of comparing a constant $V_{base}$ = 0mV and a constant $V_{app}$ =-500mV on a variable amount of cycles of applied cyclic square waves ($T_{total}$ is variable) for the sample incubation step (where the capture antibody attempts to bind to the target of 22 kilodalton growth hormone)

The mechanism of this low-voltage phenomena is currently unknown, but the results of both phases of this experiment conducted using the control algorithm and the high-throughput electrode array demonstrably show that the potential of the applied voltage and the duration of the applied voltage can both affect the binding of growth hormone protein to the antibody. The results of studying different potentials show that there are regions where immunocomplexing can be suppressed and regions where immunocomplexing is possibly improved. The result of studying different applied cycle lengths seem to show that there are regions where immunocomplexing is elevated, but going behind this region can lead to the suppression of immunocomplexing.

# 4 Conclusion

This study attempts to lay an initial groundwork for the study of low-voltage electric field based phenomena for the enhancement of immunosensor function. A control system was developed that utilizes a dynamic-linked library to interface with a 96-channel multiplexed potentiostat control system, and this control system allows for easy adjustment of the voltages that can be applied to each electrode on the electrochemical array. This control system would allow for rapid and evaluation of different applied waveforms and their effect on the performance of an electrochemical immunosensor system.

A preliminary investigation was conducted by using the control algorithm to test square wave patterns and their effects on immunosensor performance. Results conducted in this method demonstrate that certain potentials degraded the assay performance, and some results () also suggest that the usage of electric fields could potentially enhance performance when a short duration of electric field is applied. Future investigation seems prudent to further elucidate the mechanism by which these low-voltage electric fields affect the immunocomplexing process, and a deeper understanding this phenomena may aid in creating a tool that can be leveraged for enhancing biosensor performance. The control system developed in this work would be a useful tool for accomplishing these investigations for a wide variety of protein targets.

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# *Appendix A: Specific Aims Section from Oral Qualifier*

Human growth hormone is a protein produced in the anterior pituitary gland that systemically circulates through the body for the promotion of cartilage, bone, and muscle growth. It is of interest to competitive athletics to develop effective testing of recombinant growth hormone because it is a prohibited substance. This test requires the development of an assay platform that can effectively detect for the different isoforms of the protein and make a discriminatory determination based on the ratio of isoforms present in human serum. Screening for growth hormone usage is a time-consuming, cost-inefficient, and highly invasive process involving large volume of blood drawn, expensive supply-chains for facilitating transportation to clinical labs, and time-intensive laboratory procedures for hormone testing.

Amperometric electric sensors have been demonstrated in previous investigations to be able to perform detection of biomarkers with high sensitivity and specificity for both DNA and protein based biomarkers. Amperometric biosensors have also proven to be robust enough to be adaptable for detection of protein biomarkers in both serum and saliva biofluids. Investigations into protein-based amperometric detection for interleukein-8 have also yielded results that are suggestive that a pulsed electric-field methodology can be used to accelerate protein-antibody interactions and improve the speed with which samples can be processed for biomarkers.

While literature exists concerning the usage of constant voltages for manipulating biomolecules in bioassay, there appears to be no systematic investigation into the nature of pulsed low-voltage electric-fields as a methodology for the detection of protein. Wei et al. (1) has specified parameters that accelerate the binding of intelerukin-8 as a biomarker using pulsed electric-fields, further determinations appear to be necessary for deducing the precise mechanism of pulsed-electric-field driven antibody-antigen binding and determining whether this method can be applied to additional protein biomarkers.

The scope of the present project attempts to ascertain the capabilities of electrochemical amperometric biosensors on reconstituted dried blood spots for human growth hormone isoform tests. The long-term objective is to develop a general platform and method to utilize pulsed-electric field driven electrochemical assays for multiple biomarker panels in collected body fluids. The central hypothesis that is pursued is that pulsed electric-field driven biosensors will be able to detect recombinant human growth hormone users in reconstituted whole blood samples. The immediate objective is to demonstrate that in spiked whole blood samples that have been reconstituted, it is possible to rapidly detect individual isoforms of human growth hormone with high sensitivity and specificity.

**Specific Aim 1. Developing a rapid pulsed-electric field driven electrochemical assay sandwich format for human growth hormone.**

Investigations must be made in the course of experimentation to determine whether the electrochemical assay methodology can be adaptable to the detection of human growth hormone isoforms. Experimental result definitively showing the possibility of utilizing pulsed electric-fields for creating a surface coating of IgG capture antibodies, target molecules, and enzyme reporter molecules must be performed. The goal of this step is to explore this step and see whether initial coating of antibody can be improved through electrically manipulating capture antibodies to the biosensor surface. This has potential to reduce assay time and increase assay effectiveness. Inasmuch as pulsed electric-field driven assays for protein targets have not been thorough investigated, electrochemical instrumentation will be utilized to more thorough assess the relationship between antibody-antigen binding and its dependence on parameters such as applied frequency, voltage levels, ramping rates, and pulse duration. The working hypothesis of the delineated study is that pulsed electric-field driven electrochemical assays can perform a highly sensitive and rapid isoform assay.

Milestone 1 – Experimental Result showing that electric-fields can drive capture antibodies to streptavidin surface should be acquired. The goal of this step is to explore this

step and see whether initial coating of antibody can be improved through electrically manipulating capture antibodies to the biosensor surface. This has potential to reduce assay time and increase assay effectiveness.

Milestone 2 – Experimental result showing that electric-fields can drive samples to streptavidin-antibody surface should be acquired. This step, if accomplishable, will have the potential of enhancing assay time, sensitivity, and specificity.

Milestone 3 – Experimental results showing that electric-fields can drive peroxidase reporter molecule to antibody-antigen complex should be acquired. This step, if accomplishable, will be able to reduce assay time and possibly reduce nonspecific binding interactions that can lower assay signal.

**Specific Aim 2. Determination of growth hormone isoforms in reconstituted dried blood spots using electrochemical sensors.**

Experimental work must be intiated to determine whether collection of dried blood spots can be applied to the isoform ratio for distinguishing between users and non-users of recombinant human growth hormone. In other for this to be accomplished, the tests must be performed at the appropriate sensitivities and specificities necessary for adequately distinguishing between users and non-users of recombinant human growth hormone by a ratio of the isoforms. Previous studies have demonstrated success along the lines of detecting growth hormone reconstituted blood spot samples (2), however, a proper isoform ratio test using the dried blood spots is necessary in order to move the method into a clinically practical reality. Clinical samples must then be tested in order to also aid in the development of proper discriminatory ratio levels of growth hormone isoforms. The proposed work is innovative because it attempts to rapid quantification of growth hormone isoform in a low volume and efficient collection format. The outcome of the study will be to create a rapid low-volume method of sample collection and testing for quantifying isoforms for growth hormone usage detection.

Milestone 1. Establishing antibody pairs with optimal sensitivity and dynamic range, and a calibration curve for 22kD and 20kD isoforms of human growth hormone should be acquired. This step is necessary in order to establish whether the candidate antibodies for the capture of the isoforms can perform at the levels needed for testing in dried blood spots.

Milestone 2. Establishing antibody set with adequate specificity with various mixtures of 22kD, 20kD, and pituitaryHGH forms of human growth hormone. This step is absolutely critical in order to develop a working isoform assay method for detecting illegal growth hormone usage.

Milestone 3. Validation of adequate calibration curve using spiked whole blood samples on best candidate antibodies set. This step is necessary to show that the assay is robust and begins to move it into a practical clinical reality as opposed to test systems using buffer. It would be helpful to determine the effect of the whole blood red blood cells and interfering proteins on the sensor performance.

Milestone 4. Validation of adequate calibration curve using spiked dried blood samples. This will validate whether proper reconstitution and elution of the growth hormone off of the collection paper is possible and whether it occurs in a fashion with which data about the original amount of growth hormone in whole blood can be acquired.

Milestone 5. Testing of isoform mixtures in conjunction with dried blood spots. This is absolutely necessary to test the specificity of the assay and developing the isoform assay.

Milestone 6. Collect blood and dried blood spot samples for candidate patient population and determine whether quantification on electrochemical sensor with dried blood spots correlates to serum tests using existing clinical assay.

Milestone 7. Collect blood and dried blood spot samples for candidate patient population (rHGH users and non-users as patient populations), have both methods perform assay on blinded-sample. Perform statistical analysis on patient population to determine the appropriate cutoff values for users and non-users of HGH, and also perform analysis to see whether the

ratio test developed is equivalent or superior to the existing commercial ELISA chemiluminescent method.

# *Appendix B: Current Method Used for Growth Hormone Isoform Assay Compared to the Electrochemical Method*

The gold standard method for the detection of growth hormone isoforms is the differential immunoluminometric assay developed by CMZ-Assay GmBH. Molecularly, this assay works by assaying and quantifying the amount of recombinant form of growth hormone (22 kilodalton molecular weight) and quantifying the total amount of pituitary growth hormone (which would have the 22 kilodalton isoform of growth hormone in addition to an assortment of other growth hormone isoforms) and comparing the ratios of the quantified growth hormone. Practically, these tests utilize a polystyrene test tube based antibody sandwich that allows for the quantification of growth hormone isoforms for 25 patient samples (if duplicates are performed) at a time by leveraging a chemiluminescent reaction. The samples tested are human serum samples. These serum samples are acquired through making an intravenously drawn blood sample and then spinning down samples to remove the red blood cells.

According to the protocol issue by the vendor, the calibration curve for the differential isoform assay is performed by preparing protein standards of growth hormone. The protein standards of growth hormone are prepared by diluting lyophilized growth hormone standards in sheep serum samples. These protein standard solutions (which are both for recombinant growth hormone and pituitary growth hormone) are also assayed by incubating them in the polystyrene tube for 2 hours, washing out the unabound analyte, and then incubating a reporter antibody solution in the polystyrene tube for 2 hours. Following a final washoff, a substrate is added and the solution absorbance is measured for measurement. This assay reports a sensitivity of 0.021 ng/mL for recombinant human growth hormone and 0.023 ng/mL for the pituitary growth hormone. This was calculated by performing a calibration curve with the standards and inferring the sensitivity from calculating the mean of 40 measurements of a growth hormone free sample

and three standard deviations. This immunoassay protocol requires 4 hour of total incubation time (2 hours for sample incubation and 2 hours for the incubation of the reporter antibody).

The vendor protocol states that assay linearity was assessed by running five sample concentrations of 8.6 ng/mL, 4.0 ng/mL, 2.8 ng/mL, 2.2 ng/mL, and 1.2 ng/mL and diluting these five different concentrations at different amounts in sheep serum (reported by the vendor to be 1:2, 1:4, 1:8, and 1:16). The vendor states that extrapolated concentrations corresponded to 100 ± 6.9%. The vendor datasheet states that assay specificity was assessed by spiking five serum samples with haemoglobin, bilirubin, triglyceride, and human serum albumin, and all these tests showed that these additions did not affect the signal. As for variability within the assay, the vendor states that a correctly performed assay with duplicates of each sample and standard will have a coefficient of variation of 10% or less. The performance of this current gold standard method for growth hormone isoform detection is excellent, which should be expected from an assay that is designed for application in sports drug testing facilities. One should note, however, that the vendor states that these values are only orientational in nature, and that individual tests should be conducted at a specific testing site in order to assess assay

performance.

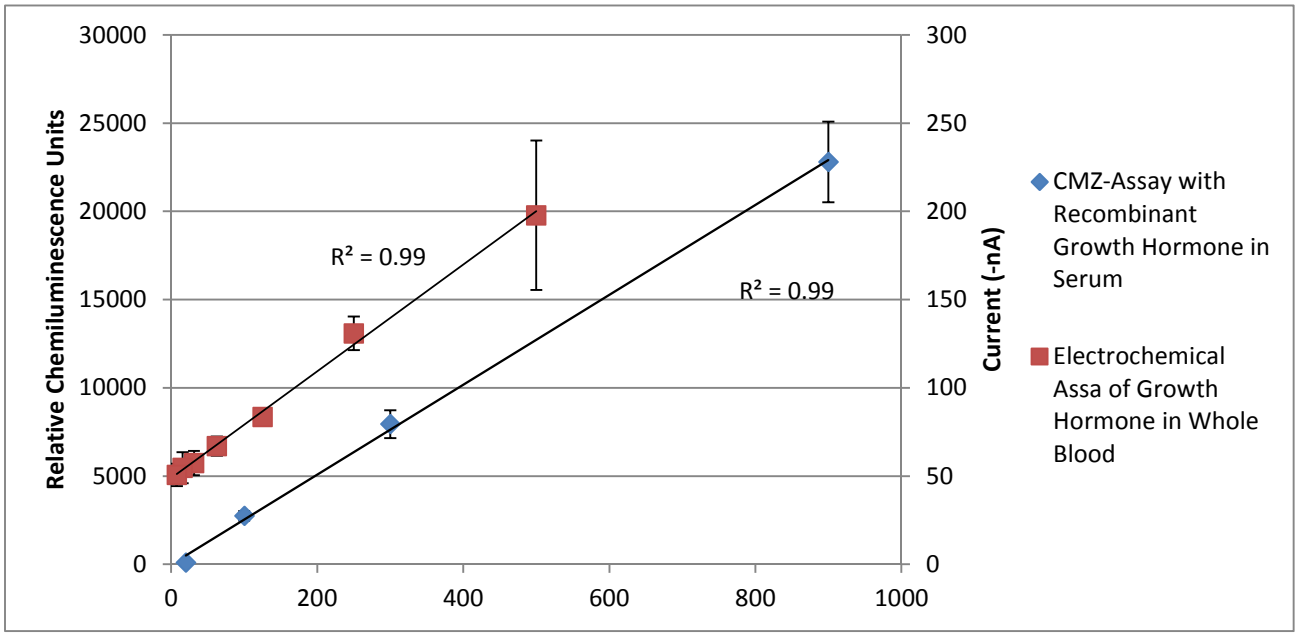


**Figure 52. Comparison test between CMZ-Assay kit using serum samples of recombinant growth hormone and assay of recombinant growth hormone standards in whole blood.**

Comparison of the data provided by the CMZ-Assay kit and the electrochemical method demonstrate that the performance of the current electrochemical assay in whole blood also possesses a high-linearity and picogram level sensitivities. Further study is necessary to determine the critical factors contributing to variability and if the electrochemical array is capable of even higher degrees of sensitivity. The data suggests that electrochemical array for protein detection in whole blood is a viable approach that is comparable to the conventional chemiluminescent approach used in the CMZ-Assay kit.