DEVELOPMENT OF A NOVEL IMMUNOASSAY FOR SIMULTANEOUS QUANTIFICATION OF ENDOCRINE PARAMETERS

A Dissertation

presented to

the Faculty of the Graduate School

at the University of Missouri – Columbia

In partial fulfillment

of the requirements for the Degree

Doctor of Philosophy

by

ELIZABETH BENAVIDES

Dr. Duane H. Keisler, Dissertation Supervisor

July 2015
The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

DEVELOPMENT OF A NOVEL IMMUNOASSAY FOR SIMULTANEOUS QUANTIFICATION OF ENDOCRINE PARAMETERS

presented by Elizabeth Benavides,

a candidate for the degree of doctor of philosophy

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Duane H. Keisler

Professor Tim Evans

Professor Jon Green

Professor Kevin D. Wells
To those that have supported me, both intellectually and emotionally throughout this process,

Thank you. I don’t think I can ever truly convey how much your support has meant to me and has kept me going. Thank you for helping me achieve my dream.

Megan, Katy, Katie, Tyler, Mom, Dad and Mark.
ACKNOWLEDGEMENTS

To my committee members, Dr. Evans, Dr. Green and Dr. Wells, thank you for your support and mentorship throughout the duration of my graduate career. Each of you has taught me valuable lessons, whether it be from the bench, in the classroom, or even from the hair salon! Dr. Green and Dr. Wells, your knowledge and experiences helped make this project successful – without your input, I would surely still be in the dark. Thank you for showing me the light. Dr. Evans, you have been a great role model for the classroom. I appreciate the pep talk at the salon, and will take your wise educator words to heart. It is my hope that the passion I currently hold for teaching burns as bright as it does within you!

This project could not have been completed without valuable input from fellow graduate students, especially Benjamin Beaton and Chad O’Gorman. Your assistance and advice helped move this project in the right direction, and it would not have been possible without you! Additionally, I wish to thank Rachel Gerrard and Gabriela Tamassia for their assistance with the experiments. Finally, a special thanks to my undergraduate research assistants, Amanda Schmelzle and Sarah Munzer, for staying dedicated to the lab and being some of the hardest working students I have had the pleasure of working with. I know you will both be very successful in your future endeavors. Thank you so much for helping me through our projects!
Finally, I want to express my sincere gratitude to Dr. Keisler. This project has not been easy, and we both have recognized that. Your patience with me as I worked through the struggles and celebrated the successes does not go unnoticed. Your experience and expertise is what drew me to Mizzou, and I have never once questioned my choice. I thank you for taking me on and sharing your wisdom, both inside and outside the lab with me. You have taught me many lessons that I will carry with me forever. It is my hope to make you proud as I embark out on a new career of my own. Thank you again.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... ii

LIST OF FIGURES .................................................................................................................. vi

LIST OF TABLES ...................................................................................................................... viii

ABSTRACT ............................................................................................................................... ix

Chapter

I. INTRODUCTION .................................................................................................................. 1

II. LITERATURE REVIEW ...................................................................................................... 5
   Early Endocrine Detection Methods .............................................................................. 5
   Bioassays ......................................................................................................................... 5
   Chromatography ............................................................................................................... 7
   Antibody Based Detection Methods ........................................................................... 10
   Antibody Production ...................................................................................................... 10
   Non-label Immunoassays ............................................................................................... 21
   Labeled immunoassays .................................................................................................. 24
   Radioimmunoassay & Enzyme-Linked Immunosorbent Assay .................................. 24
   Immuno-PCR ................................................................................................................... 28
   Early Assay Descriptions ............................................................................................... 28
   Assay Formats and Detection Methods ...................................................................... 30
   Assay Principle ............................................................................................................... 32
   Solid Substrates used in iPCR ....................................................................................... 32
   Summary ......................................................................................................................... 36
   Simultaneous Detection Approaches ........................................................................... 37
   Multiplexing .................................................................................................................... 37
   Illumina BeadXpress ....................................................................................................... 38
LIST OF FIGURES

Figure 1. Schematic representations of a generic antibody molecule..........................13

Figure 2. Antibody and T-cell response to antigen.............................................19

Figure 3. Comparison of conjugation protocols, as measured by the conjugation success assay..........................................................47

Figure 4. Assay configurations investigated on the Luminex xMAP platform..............54

Figure 5. The Luminex bead assay detected LH in ovine and bovine samples in a sandwich assay format..........................................................59

Figure 6. Correlation of LH concentrations in six livestock samples (4 bovine; 2 ovine) measured by RIA and the Luminex bead assay..................................................60

Figure 7. Assay configurations investigated with the iPCR assay.............................72

Figure 8. Immuno-PCR for LH detection was not initially successful......................74

Figure 9. Image of a 3.5% agarose gel with iPCR product.....................................76

Figure 10. Effect of wash frequency on antibody-oligonucleotide detection.............77

Figure 11. The influence of mass of antigen on plate on iPCR...............................78

Figure 12. A biotin detection assay curve..............................................................79
LIST OF FIGURES

Figure 13. Correlation of Ct values obtained from the PCR of detection oligonucleotide diluted in 10 mM Tris and Ct values obtained from the PCR of detection oligonucleotide diluted in 0.01 N NaOH and potassium acetate………………………………………….82

Figure 14. Biotin detection assay carried out in high binding streptavidin plates……….86

Figure 15. Biotin detection assay carried out in standard binding streptavidin plates…..87

Figure 16. Biotin saturation point of standard binding streptavidin plates……………....88

Figure 17. Absence of oligonucleotide release treatment also produces a curve………..89

Figure 18. Schematic of the experiments to determine the influence of shaking steps in the biotin detection assay………………………………………………………………….90

Figure 19. Results of plate shaking effects assay………………………………………..91

Figure 20. Substitution of the biotinylated oligonucleotide in a commercially available ELISA can be a successful detection method…………………………………………………….93

Figure 21. Detection assay for biotin levels in animal sera…………………………...94

Figure 22. Detection assay for biotin recovery in cow serum………………………..95
Table 1. LH values in serum and plasma obtained using the Luminex bead assay and a double antibody disequilibrium RIA…………………………………………………….61
ABSTRACT

Since the advent of the radioimmunoassay in the 1950s, numerous immunologically-based methods have evolved for sample analysis. Although each immunological method possesses unique assets and liabilities, all share limited abilities in range of concentrations detectable and the number of analytes resolvable simultaneously; most procedures are limited to one analyte determined per replicate per sample. The objective of this study was to adapt technology evolving from the genomics “revolution” for multiplexed hormonal analysis in livestock. We sought to complete this objective using the following sequencing technologies and tools: Illumina BeadXpress, Luminex xMAP, and quantitative immuno-PCR. The Illumina BeadXpress and Luminex xMAP both share similar characteristics and each of these proprietary platforms consists of a laser spectrum analyzer and a bead-set. Each bead-set contains microscopic beads and each bead-set has unique identifying signatures. As a test of proof of concept, the surface of a bead set was conjugated to an LH antibody. Using these technologies, we were able to establish an assay for LH on the Luminex platform. We were unable to develop an analyte detection assay on the Illumina platform. The restrictive proprietary constraints of both the Luminex and Illumina platforms however, greatly limited assay flexibility. Therefore, we chose to establish an assay for LH using quantitative immuno-PCR (iPCR). Quantitative immuno-PCR exploits PCR signal amplification in combination with antibody capture and specificity. Briefly, a sandwich immunoassay format is performed with capture antibody immobilized to a PCR plate. A second detection antibody, which is conjugated
to an oligonucleotide, is then added to the solution phase for binding. After a series of washes to remove the unbound oligo-antibody conjugate, the plate contents are subjected to quantitative PCR (qPCR). Detection of LH was achieved, but background binding contributed to significant interference. Subsequently, to simplify the design and demonstrate proof of concept, a biotinylated oligonucleotide was competed with unlabeled biotin in streptavidin coated polystyrene plates and then detected for oligonucleotide via qPCR. The results of this latter experiment demonstrated that the iPCR methodology could detect unlabeled biotin across three orders of magnitude.

In conclusion, background binding contributed to significant interference with quantitative immuno-PCR but one that is believed to be resolvable. Moreover, our observations suggest that immuno-PCR has the potential to improve detection capabilities of current hormonal assays with three or more orders of magnitude in sensitivity, and ultimately provide multiplex capabilities, with the conjugated oligonucleotide serving as both a quantitative detection label and as a barcode for later identification of analyte. The technological leap in capabilities provided by successfully multiplexing can greatly aid in our efforts to understand the complex interaction of endocrine and metabolic signals in the dynamically changing animal and greatly reduce sample throughput investments.
CHAPTER I

INTRODUCTION

The field of endocrinology had rudimentary beginnings based largely on the methods characterized as “ablation – replacement” therapy. For example, early efforts to study the endocrine contributions of a gland, such as the ovary, involved removal of the ovary and observation of the animal’s response to ovariectomy, and/or combine the effect of ovariectomy with replacement of various partitioned homogenates of ovarian extracts. This approach allowed a crude perspective of the function of the ovaries but no information with regards to either the identity of the secretions, the quantity of the secretions, nor the temporal pattern in which they were secreted. In order to move forward with characterizing the secretions from an endocrine gland it was then necessary to categorize the chemical characteristics of the analyte as proteinaceous-like (which would include proteins, peptides, amines) or fat soluble (which would include steroids and lipids). Size estimates of the analyte, along with other chemical characteristics (such as complexity of the analyte) aided in directing the appropriate methodology for quantitation of the analyte. Subsequent advances in assay technology, including the development and use of antibodies in assays, permitted measurement of a specific analyte. This has continued to drive further technological advances in assay technology. The most common analyte detection methods currently available for endocrine and metabolic signals in blood rely predominantly on antibody detection technology. These
methods include the radioimmunoassay (RIA), first developed in the 1950s, and the Enzyme-linked immunosorbent assay (ELISA), first developed in the 1960s. While these methods continue to be in popular use, they are not without their limitations. They can be cost-prohibitive, they may not have the ability to provide the sensitivity and/or specificity necessary for successful measurement, they can be time consuming, they may be heavily regulated (RIAs), and for all practical purposes, these assay methods can only measure a single analyte in a single replicate sample. With the growth in demand for information on more analytes, compounded by limited sample volume in small species, new bio-analytical methodologies are emerging which permit replicate determinations of multiple analytes simultaneously – i.e. multiplexing. Multiplexing is not a new concept, but fairly recently has gained new interest as a way of addressing the challenge of generating more information on multiple analytes in a single sample. In theory, multiplex analysis is similar to current detection methods, however, also incorporated is the unique ability to discern different analytes in a single sample. The ability to measure multiple analytes in a single sample has many advantages including the time and labor costs invested in a multiplexed assay versus a single analyte assay, and the potential to reduce the amount of sample necessary for analysis. From an analytical perspective, the ability to measure multiple analytes simultaneously may also yield a more complete perspective of the interactions that may be occurring between analytes, and may better represent the physiological state of the animal when the sample was taken. Commercial multiplex kits have been developed for human and rodent samples and analytes; primarily cytokines and other immune-related analytes. Few multiplexed analyses are available for detection of analytes in livestock. Therefore, our objective was to determine if tools first developed
for high throughput genotyping, which have the capacity and specificity for making millions of measurements with a high level of precision, could be used for multiplex analysis of livestock hormones. We initially sought to accomplish this using the Illumina BeadXpress reader, which consists of a reader machine with the capability to detect unique barcode signatures embedded in microscopic beads. The surface of the beads were modified for protein attachment. After initial difficulties in setting up the assay, the platform was ultimately abandoned by the manufacturer, making future application of this approach obsolete. We next attempted to establish a multiplex assay using Luminex xMAP technology. The Luminex xMAP platform consists of a specialized reader machine with the capacity to read microspheres with unique spectral signals. However, the proprietary nature of this platform potentially limits assay flexibility (i.e. single proprietary source for assay components). Therefore, we next attempted to develop a hormonal detection assay using a more flexible and adaptable platform: immuno-PCR (iPCR). Immuno-PCR exploits PCR amplification and couples it with the specificity of antibody detection. In the iPCR assay, an antibody may be immobilized onto a support matrix (plate or bead), a second antibody is coupled to an oligonucleotide, either through the streptavidin-biotin interaction, or via covalent coupling, and acts as a detection molecule. In the final step, the completed assay is subjected to qPCR for quantification of the analyte of interest. Using these assay design methodologies (bead based detection and iPCR), the objectives of this study were to: 1) establish a single hormone assay on the BeadXpress and Luminex platform and 2) attempt to recreate the single hormone assay in the iPCR format. The technological leap in capabilities provided by successful multiplexing can be used for understanding the complex interaction of endocrine and
metabolic signals in the dynamically changing animal while greatly reducing sample throughput investments.
Early Endocrine Detection Methods

Bioassays

One of the earliest methods of analyte detection was the bioassay. In a bioassay, the biological response of an organism to a treatment is measured in order to determine the effects of that treatment; whether it be introducing an analyte or removing an organ or tissue. Early bioassays were used to determine the role of a gland or hormone by removing the gland and measuring a physiological response to a replacement therapy (Smith, 1930; Goswami & Williams, 1967; Miller & Bolt, 1987). Today, bioassays are commonly used to assess the effects of a drug or compound on an organism or tissue (Contrera & DeGeorge, 1998). Bioassays are also used as environmental monitors, with their results representing the effect of the environment, or specific environmental contaminants, on the tested organism (Hose, 1985).

The principle of a bioassay relies on the use of an organism or biochemical reaction in detection of an analyte. There have been a multitude of bioassays that have been described, using a variety of different testing media – from a simple tissue to a whole animal. In fact, the National Center for Biotechnology Information (NCBI) maintains a database of bioassays that have been used in the detection of chemical substances
Bioassays can be used to determine toxicity of environmental contaminants (Farré & Barceló, 2003), the effect of a hormone on a cell or tissue (Tubbs et. al., 2012; Habauzit et. al., 2013), or the physiological effects of a pharmaceutical (Dafale et al., 2015). The basic components of a bioassay include an organism (animal, plant, or microorganism), or components thereof evaluated in the presence of a treatment or compound to be tested. The organism or its components are then subjected to the treatment or compound of interest, and a physiological response is observed and recorded. This response can vary widely depending on the bioassay type, and can include production of analytes in response to the challenge (Parsons et al., 1972), or a measurable physiological response (Dorfman & Dorfman, 1947). An early example of a bioassay for detection of luteinizing hormone (LH) involved administration of mammalian pituitary extracts to frog ovaries. If ovulation or follicular luteinization was detected on the frog ovaries, LH was determined to be present in the sample (Licht et. al., 1976). Moreover, this type of response could be tested with a variety of extracts from different animals and/or tissues, and the potency of the given treatment could be determined based on the nature of the ovarian response. Similarly, in early studies that sought to elucidate the function of the hormone relaxin, the isolated hormone was administered to mice, and the elongation of the interpubic ligaments was measured (Steinetz et al, 1960). In a more modern example, chick embryo hepatocytes and sea urchin embryos have been utilized as indicators of environmental contaminants (Kennedy et al, 1996; Manzo, 2004). In the example of the chick embryo hepatocytes, the objective was to develop a bioassay that identified environmental contaminants that impacted birds. However, as noted by Steinetz et al. as far back as 1960, bioassays are not without their
challenges. Due to the use of animals and tissues, costs can be much higher than other detection methods. Additionally, when measuring a physiological response such as elongation of interpubic ligaments, there can be variation in the method of measurement, which may make comparisons difficult. While it is possible to measure multiple responses to a treatment in a bioassay, it would be difficult to determine the role of the individual treatments if administered simultaneously in a bioassay.

Bioassays provide important information regarding physiological responses to mixtures, and were responsible for some of the early elucidations of the roles of endocrine glands. However, purification of the complex extract mixtures provided more information on the exact hormones or analytes involved in eliciting a response in a bioassay. There are methods available that have the ability to purify complex mixtures based on the chemical composition of these mixtures, and are collectively known as chromatographic methods.

**Chromatography**

Early work in endocrinology required the need to characterize the hormones responsible for the observed physiological response in animals and tissues. Therefore, a number of methods were subsequently developed so as to characterize unique aspects of the analytes using chromatographic techniques. Chromatography has a number of iterations, and is unique in that the type of chromatography used can reveal the structure of a molecule or analyte based on a number of different characteristics of that molecule, including charge, size, solubility, and biological activity. Some of the earliest chromatography assays included specialized paper as the solid phase through which the analytes passed via capillary action. In order to separate analytes based on these features, the solid phase of
the chromatography substrate varies depending on which feature is being exploited to facilitate separation. As an early example, thin layer chromatography is a form of solid support chromatography that uses a solid phase (silica, for example) that is “plated” in a very thin even layer on a glass base. Thin layer chromatography can be used to separate different molecules in a complex mixture, and is used to separate non-volatile compounds. Thin layer chromatography has been used to separate steroidal mixtures from tissue extracts (Mead & Swannack, 1980; Ravindra et. al., 1984). One of the limitations of chromatographic methods is that without further processing, there is not a method of determining analyte identity. Additionally, relying on capillary action to separate mixtures can be time consuming. However, with the advent of high performance liquid chromatography (HPLC), coupled with additional analytical methods, chromatography has become faster and can be used to identify more completely an analyte than with traditional methods. In the HPLC technique, the extracted sample is injected into a solid phase column, and under high pressure (up to 6,000 psi), the sample is separated based on its interaction with the solid phase. High performance liquid chromatography also includes a detection step for sample analysis. The results of HPLC are determined by the time it takes an analyte to exit the solid phase, due to the affinity of analytes to the solid phase. A number of different detection methods can be employed to determine when an analyte leaves the column. These detection methods are based on the structure of each analyte, and their functional behavior. For example, analysis by ultraviolet light absorption is used as a detection method in HPLC; however, not all analytes will absorb UV, which results in the necessity of additional unique detectors. Additional detection methods include fluorescence detection, light scattering detection,
and even ionization methods of detection. Because HPLC separates analytes with differing chemical structures and charges, it has been suggested that using a combination of detection mechanisms will provide the most complete sample analysis. The collected data is then analyzed to determine the composition of the initial sample.

High performance liquid chromatography is often used in pharmaceutical development, and can also be used in environmental monitoring, similar to bioassays (Kaushik et. al. 2012). As early as 1979, HPLC methods have been described for the determination of the carcinogens benzopyrene and other polynuclear aromatic hydrocarbons (Lynch & Weiner, 1979). The versatility in the ability to detect the composition of most any type of analyte makes HPLC a useful detection system. With the right solvent and the right solid phase, any number of analytes may be separated from highly complex mixtures. Furthermore, with the advent of mass spectrometry (MS), HPLC can be used in conjunction with MS to both separate and quantify compounds (Mitulovic & Mechtler, 2006). However, this process results in the destruction of the analyte, limiting further analysis of the analyte.

High performance liquid chromatography is a valuable asset for researchers, especially with the increasing necessity of multiple analyte analysis. The lack of a universal detection mechanism may make comparison between analytes difficult, and introduces sources of variation, depending on the efficiency of each method. High performance liquid chromatography remains a somewhat time-consuming assay approach that may not be useful in scenarios where rapid analyte detection is necessary. Finally, quantification of analytes with HPLC-MS does not maintain analyte structure, which may limit downstream analysis. Regardless, HPLC is a powerful tool for use in analyte detection.
With the use of chromatography comes the ability to separate complex mixtures into their individual components, based on the chemistry and structure of those molecules. However, quantification of molecules subjected to chromatography may be difficult without altering the molecular structure of the analyte of interest. Therefore, methods have been developed that enable the quantification of an analyte of interest without altering its structure. These methods rely on the specificity of antibodies generated by the immune system to bind to the analyte of interest.

**Antibody-based detection methods**

*Antibody production*

Within the context of the immune system, antibodies play an important role in the immune response as major components of humoral immunity. Antibodies produced by plasma cells of the immune system bind to a variety of antigens / pathogens, and assist in their removal from the host through complement activation, opsonization, or neutralization (Schroeder & Cavacini, 2010). One of the important characteristics of antibodies that allows them to target most any pathogen is the ability of antibody populations to greatly diversify antigen recognition using a fixed number of genes (Papavasiliou & Schatz, 2002). There are five different antibody classes, all with similar structure. This structure consists of a constant region, a variable region, and a flexible region. These regions are further classified based on the characteristics of their peptide chains; the heavy and the light chain. Both chains consist of constant and variable regions, with the heavy chain composed of three heavy regions and one variable region, while the light chain consists of one heavy chain and one light chain. The major
differentiating factor between the five antibody classes is the structure of the heavy chains (Davies & Metzger, 1983). Additionally, each of the five antibody classes has a somewhat unique localization throughout the body. For example, IgA is often found at sites of secretions, including tear ducts and milk, while IgG and IgM are predominantly found in serum. Due to the location and abundance of the antibody classes, IgG antibodies are most often utilized in antibody based assays. Within the animal, antibodies are produced by plasma cells after antigen exposure. When generating polyclonal antibodies for use in vitro, the antibody production process is exploited through the introduction of an analyte of interest that may or may not need to be ‘masked’ to appear as a harmful antigen to the immune system of the animal. Regardless, if there is a foreign antigen present or a native analyte that is masked as a foreign antigen, the immune system will respond identically.

The variable region of an antibody is responsible for antigen recognition. However, there is a large variation in the number and types of foreign antigens that invade the body. How can antibodies be specific for such a wide range of antigens (including introduced antigens for assay purposes) when there are only approximately 40 genes in humans that code for the variable region? When antibodies begin to be formed in plasma cells, there are a series of gene rearrangements that occur, known as VDJ recombination. Early experiments using restriction enzyme digest and DNA/RNA hybridization, first revealed that in non-immune cells, the gene regions coding for the constant and variable regions of an antibody were found on separate DNA fragments. In antibody-producing tumor cells, however, the constant and variable regions were found within the same gene fragment, which suggested that a reorganization of the DNA had occurred in the antibody-
producing cells (Hozumi & Tonegawa, 1976). Further investigation of this phenomenon led to the discovery of a unique type of gene recombination that afforded antibodies a diverse population: VDJ or somatic recombination. It is important to understand which antibody structures undergo VDJ recombination and their orientation within the antibody structure. In the antibody configuration, two variable regions exist – the variable light chain and the variable heavy chain (Figure 1). These chains are developed through VDJ recombination.
Figure 1. Schematic representations of a generic antibody molecule. In the top image, the antibody is labeled according to the peptide chains: the constant heavy chains (C_H1, 2 and 3), the constant light chain (C_L), the variable heavy chain (V_H), and the variable light chain (V_L). In the bottom image, the antibody is labeled according to the regions that correspond to the gene segments. The variable, diversity and joining gene segments are found in the variable region, while the constant gene segment is found in the constant region (From Janeway’s Immunobiology, 7th ed. Murphy, Travers & Walport, Eds., 2008).
The genes encoding the chains consist of different segments: multiple variable gene segments, multiple joining gene segments, and multiple constant gene segments. During VDJ recombination of the variable light chain, a single variable gene segment and a single joining segment are combined as an exon. Subsequently, the intron between the variable-joining region and the constant region is spliced post-transcriptionally, forming the mRNA template for the variable light chain. This process is similar in the variable heavy chain, with more gene segments involved. VDJ recombination of the variable heavy chain involves the variable, joining and constant segments, and adds an additional diversity segment. Once again, single variable and joining gene segments and the additional diversity segment are recombined to a single exon, which is then joined to the constant region after intron splicing. One difference with the variable heavy chain is that multiple constant regions are spliced together, whereas only a single constant gene region is used in the variable light chain. How this recombination process results in a diverse population of antibodies is through the number of gene segments that are present on the antibody loci. In the human, approximately 110 gene segments encode for the variable regions on the heavy and light chains (Lederberg, 1959). During the process of gene rearrangement, only one of the segments is used to make a functional antibody. Coupled with the approximately 15 gene segments for the joining region and approximately 25 gene segments for the diversity region, each of the gene rearrangement permutations can result in an antibody population with a unique paratope. The entire process of VDJ recombination is mediated by a group of enzymes known as the VDJ recombinase complex. In addition to VDJ recombination, secondary antibody diversification events occur during antibody development that further increase antibody diversity and
specificity. These events include somatic hypermutation, which is a series of mutations found in the variable region of both the light and heavy chains. These mutations have been associated with altering affinity of the antibody to its target antigen (Papavasiliou & Schatz, 2002). Gene conversion is another secondary diversification event wherein the variable region of the antibody has variable region pseudo genes inserted, which results in increased antibody specificity. Finally, class switching is an event in which the constant region of the heavy chain is replaced by a different heavy chain, which changes the antibody class. In this instance, the paratope is not altered, but the function of the antibody is switched to the new antibody class (Honjo, Kinoshita & Muramatsu, 2002). For example, an IgM antibody that class switches to the IgG antibody class may gain the ability to cross the placenta, as IgG antibodies are the only antibody type that can cross the placenta (Schroeder & Cavacini, 2002; Simister, 2003).

The unique diversification methods employed by the immune system creates antibodies within the five classes that have binding affinity for a wide range of pathogens. The development of a polyclonal antibody pool for use in detection assays exploits antibody production by the immune system to generate antibodies against an analyte that would, under normal circumstances, not be detected by the immune system. In this configuration, antibodies are termed polyclonal due to the fact that antibodies collected are a heterogeneous mixture of antibodies with paratopes that recognize multiple regions of the targeted analyte. This antibody production method is distinct from monoclonal antibody production, which is the production of an antibody pool that recognizes a single paratope, which was first described in 1975 (Kohler & Milstein). Both polyclonal and monoclonal antibodies have applications in a wide range of antibody based assay
Polyclonal antibody production may be viewed as a type of bioassay, in that an animal is injected with a treatment, in this case the analyte that is to be measured, and the animal monitored and evaluated for reaction to the treatment. Specifically, the analyte of interest is injected into the animal, along with an adjuvant. The role of the adjuvant is to assist in the stimulation of an immune response. After this initial injection, blood is drawn from the animal, typically 2 to 3 weeks later (Herbert, 1968). This is analogous to the timing of the immune response when antibody production has been observed at its highest after first exposure to an antigen (Figure 2) (Hanly, Artwohl & Bennett, 1995). Subsequently, additional analyte injections are administered with a second adjuvant that does not stimulate the immune response as aggressively. At this point, the immune system of the animal has been primed for re-introduction of the analyte (Hanly, Artwohl & Bennett, 1995). Antibody response to the analyte is accelerated, and blood may be drawn sooner for antibody isolation. Some considerations regarding selection and use of the target analyte must be made before carrying out polyclonal antibody production. First, the analyte must be sufficiently foreign to the animal being injected. This is due to the fact that the immune system has a number of protective measures to prevent self-recognition by antibodies. Therefore, if the analyte sequence shares similarity to the animal that is to produce antibodies against the analyte, it is unlikely to elicit a robust response of antibody production targeted to the analyte. Second, the size of the analyte plays a major role in the immune response. It has been observed that small molecules (typically less than 10 kDa) generally do not elicit a strong immune response (Hanly, Artwohl &
Bennett, 1995). This however does not prevent antibodies from being made against small molecules such as steroids and amines.
Figure 2. Antibody and T-cell response to infection. During the initial immune response, which is exploited during antibody production, antibody producing cells peak around the second week of infection (From Janeway’s Immunobiology, 7th ed. Murphy, Travers & Walport, Eds., 2008).
When immunizing against small molecules, conjugating them to larger molecules can overcome the size limitation and elicit an immune response. However, polyclonal antibodies will be developed against both the analyte of interest as well as the larger molecule. In these instances, antibody purification methods may need to be considered, typically utilizing some form of chromatography, as discussed previously. If these factors are taken into consideration prior to initiating antibody production, polyclonal antibody production can be successful. Polyclonal antibody production and use remains popular today. With the development of monoclonal antibodies, antibody technology has evolved to include antibodies with greater specificity. Additionally, monoclonal and polyclonal antibodies are often used concomitantly in antibody-based detection methods (Beards et. al., 1984).

The first report of monoclonal antibody production in a continuous culture system was in 1975 (Kohler & Milstein). In their experiments, Kohler and Milstein fused ‘permanent cultures’ of mouse myeloma cells with mouse spleen cells that were immunized against sheep red blood cells. It was observed in previous experiments that when two plasma cells were fused, the unique antibodies from each individual cell were produced and secreted by the fused cell. This observation, coupled with the immortal nature of the myeloma cells, resulted in the hypothesis that fusing the myeloma cells with antibody producing plasma cells would result in a fused cell that could produce a single antibody class that recognizes a single epitope and be cultured continuously. This was the first experiment that demonstrated the successful implementation of monoclonal antibody production. Subsequent to this experiment, which earned the authors the Nobel Prize in Physiology and Medicine in 1984, monoclonal antibody production increased. An animal
is still injected with the antigen of interest and an adjuvant, however the plasma cells are harvested from the animal and fused with the permanent cell cultures. Each harvested plasma cell may produce an antibody that recognizes a separate epitope. Therefore, pools of monoclonal antibodies can be established in culture. Furthermore, the fused immortal cell line ensures that the antibodies produced by those cells are renewable. Monoclonal antibodies can be used in the same assay methods as polyclonal antibodies, or the two can be combined for analysis in assays such as the sandwich enzyme-linked immunosorbent assay (ELISA). A monoclonal antibody population can be used to orient antigens on a plate, as the single paratope antibody population will bind to the antigens in the identical location (Lipman et. al., 2005). Many immunoassays use both monoclonal and polyclonal antibodies in their assay designs.

While antibodies are still popular in current immunoassay strategies, analyte detection technologies continue to advance. As the biology of antibodies is further elucidated, and the necessary binding components and sequences identified, new technology has attempted to further refine antibody technology. New antibody-like products such as aptamers and affimers have the advantage of being created in vitro, which eliminates the animal component. Additionally, these small DNA or protein products may have higher binding affinities to their target molecules than antibodies, while remaining non-immunogenic (Jayasenu, 1999; Ni et. al., 2011). While still early in their use, these technologies represent the next generation of analyte detection molecules and may be the catalyst for new detection methods.

The development of antibodies for use in molecular biology represents a method for detecting a specific analyte of interest. This is made possible through the unique antibody
production system in mammalian immune systems. This system, VDJ recombination, results in a wide range of antibodies with different specificities from a limited number of genes. The immune system produces antibodies in response to an insult on the system. By mimicking this process, but without causing disease, the immune system can be exploited to produce antibodies against an analyte of interest. The end result in this process are polyclonal antibodies, a population of antibodies that recognize various epitopes on the analyte of interest. As technology advanced, the ability to produce monoclonal antibodies, an antibody population that recognizes a single epitope was popularized. Both monoclonal and polyclonal antibodies are immensely powerful analyte detection techniques, due to their detection specificity. In addition to their specificity, antibodies can also be conjugated to a wide range of detection molecules which provides visualization and even quantification of the analyte of interest. The use of antibodies in analyte quantification gave rise to the term immunoassay, which combined assay technology with antibodies from the immune system. With the introduction of large scale antibody production, a wide range of both non-labeled and labeled immunoassays were developed. These assays have various levels of detection capabilities, and range and method of detection, all while using antibodies as the common detection unit.

*Non-label immunoassays*

There are a number of assays that use antibodies for detection, both in a non-labeled and labeled context. Non-labeled immunoassays use antibodies for detection, but use secondary detection methods for visualization and quantification, such as gel electrophoresis. For example, two different gel-based detection systems - the radial
immunodiffusion assay and the rocket assay – use gel electrophoresis for antigen quantification. These systems use the antigen-antibody interaction to determine the presence, and in some cases abundance, of the analyte of interest. In the radial immunodiffusion assay, the antibody for the analyte of interest is a component of an agar gel. The antigen or sample is placed into a well in the gel, where it diffuses in a radial pattern. As the sample comes into contact with the antibody, if it contains the analyte of interest, the sample will precipitate out of solution and form insoluble complexes. The diameter of these complexes can be quantified to determine how much antigen is present in the tested sample. The addition of heterogeneous complexes of antigens and antibodies can also determine cross-reactivity of antibodies, and presence of the antigen in a given sample. A somewhat similar assay, the rocket assay or immunoelectrophoresis, also uses a gel to immobilize antibodies, but also utilizes electrophoresis to stimulate movement of the antigen within the gel matrix. The theory behind the rocket assay is similar to that of the radial immunodiffusion assay – as the antigen comes in to contact with an antibody that targets it, insoluble antibody-antigen complexes then form and can be visualized. However, instead of allowing the sample to diffuse into the gel, the rocket assay forces the antigen into the gel matrix via electrophoresis. The result of the forced interaction generates ‘rockets’ of antibody-antigen complexes that remain in specific locations in the gel based on the size and amount of complexes formed. One advantage of a rocket assay over other protein electrophoresis assays, such as SDS-PAGE, is that the rocket assay maintains the structure of the protein, allowing for precipitation of the product after separation (Belfield & Tuite, 1992).
Another unique, non-labeled antibody assay is the reverse hemolytic plaque assay (RHPA). RHPA differs from the previous assay types in that it is more of a bioassay than strictly a detection assay. In RHPA, two cell types are used: the cell of interest, and erythrocytes situated on a plate in a monolayer. The antibody against the analyte of interest is then added to the culture. If the cell of interest secretes the analyte of interest, the antibodies will complex with the antigen, and bind to protein-A on the surface of the erythrocytes. Protein A is a bacterial sourced protein with high affinity for antibody heavy chains. After this part of the assay is complete, complement is added to the assay.

In the immune system, complement is a component of the innate immune system that assists in clearing antigens from an organism. The complement system is activated only when an antibody is bound to its antigen. Once complement is activated on the surface of a cell, the signaling mechanisms produced through complement activation ultimately result in lysis of the cell on which complement was activated. Therefore, in RHPA, the addition of complement to the assay results in lysed erythrocytes only in the presence of an antigen-antibody complex. This is quantified by an area of hemolysis, known as a plaque. The size and rate at which the plaques form can indirectly determine the amount of analyte a particular cell may be producing. The RHPA, similar to the rocket assay, does not alter the function of the secreting cell, meaning that RHPA may be combined with additional cytological assays to characterize a cell type and its secretions (Boockfor & Fidan, 2004).

Antibody based detection methods for analytes have been incredibly useful for quantification of analytes of interest. Additionally, antibody based detection assays have some advantages over other detection methods, as some maintain analyte structure and
function for further analysis, and can be used to characterize specific endocrine cell secretions. Alternatively, other immunoassays that rely on the ability to conjugate molecules to antibodies have also been used for detection and quantification. These assays provide more precise quantification of analyte levels due to the nature of detection. Additionally, depending on the conjugated detection molecule, these immunoassays have the potential to be more specific and sensitive than previously discussed immunoassays.

Labeled immunoassays

The unique feature of labeled immunoassays that differentiate them from non-labeled assays is the incorporation of an element or molecule that can be both detected and quantified. Examples include radioisotopes, enzyme-substrate complexes, fluorophores or even DNA. The nature of this label greatly influences the detection sensitivity of the assay.

Radioimmunoassay & Enzyme-linked immunosorbent assay

In 1959, Yalow and Berson first described the development of a radioimmunoassay (RIA) for the quantification of insulin in a biological sample. Like other antibody-based hormone detection methods, RIA uses analyte-specific antibodies for detection of an analyte of interest, but utilizes radioactive isotopes as the method for quantification. The RIA has been described as a sensitive and specific assay for analyte measurement.

Radioimmunoassay uses radioactive isotopes as the method of quantification. In the most common RIA application, the radioactive isotope 125-iodine is used (Grange et. al., 2014). 125-iodine emits a high energy photon and gamma radiation upon isotope decay,
which can be measured with instruments that have the capability to detect gamma radiation. Because gamma radiation is ionizing, it has the ability to charge particles in which it comes into contact. This change in charge creates an electric field, which is what is measured by the gamma detectors. The more radiation present, the faster the particles are charged. 125-iodine is often used due to its simplistic incorporation onto the amino acid tyrosine (Seevers & Counsell, 1982).

Since the initial description of the RIA, which used a ‘competitive inhibition’ assay format, a number of different assay configurations have been described. In the competitive inhibition configuration, the biological sample that contains the analyte of interest competes for antibody binding with a similar or identical analyte that has been labeled with radiation. After a pre-determined incubation time, (Lenz, Wien & Toth, 1975) a second antibody is subsequently added to the reaction, and is specific for the species in which the detection antibody was built. This second antibody - when binding to the first antibody - adds mass to the first antibody-antigen complex, which results in aggregation of complexes. This subsequently forms a precipitate at the bottom of an assay tube after centrifugation, similar to the insoluble antigen-antibody complexes formed in the rocket or radial immunodiffusion assays. The aqueous component, which contains unbound antigens, is then aspirated or decanted away from the antigen bound precipitated complex, and is measured for gamma radiation via a gamma counter. Since the bound antigens represent a proportional relationship between unlabeled (unknown) antigen and labelled (known) antigen, the amount of measured radiation is inversely proportional to the amount of measured analyte in the sample. Other formats that have been described for radioimmunoassay include a modified competition assay in which the
antibodies are physically immobilized to a solid support surface (Catt & Tregear, 1967). This assay configuration removes the necessity of antibody precipitation and centrifugation, as the bound components are immobilized to the solid surface. Non-bound components are simply removed from the surface and the surface (usually a test tube) is measured for radiation.

Radioimmunoassay has been characterized as a sensitive and versatile assay. It has been reported that a range of 2-3 log units of detection is possible (Dudal et al., 2014). However, there are some limitations with RIA. First, the radioimmunoassay can be somewhat time consuming compared to other analyte detection assays. Many radioimmunoassays typically require 12-24 hours of incubation time for optimal results (Lenz et. al., 1975). The use of radioactivity requires specific training and licensing, and can be a potential health hazard. Finally, radioisotopes have a specific half-life. Therefore, the shelf-life of a radiolabeled antigen may be considerably shorter compared to other enzyme-based methods (Grange et. al., 2014).

The enzyme-linked immunosorbent assay (ELISA) is very similar to the RIA, especially in the assay configurations utilized. However, the major difference is the method of detection. ELISAs utilize colorimetric or fluorescent detection for quantification of analyte concentrations. Due to this detection method, many ELISAs utilize the sandwich assay format (Lequin, 2005). In this configuration, one antibody population is immobilized to a solid support structure, usually a polypropylene plate. The sample is incubated in the plate, allowing the antigen-antibody complexes to form. After washing away non-bound components, a second antibody is added. This second antibody is
specific for different epitopes than the first antibody, and is conjugated to either an enzyme or fluorescent molecule, in the case of fluorescent detection. In colormetric detection, once the detection antibody binds to the immobilized analyte, the substrate of the enzyme is added. Upon substrate cleavage, color is given off that is directly proportional to the amount of analyte in the sample. In fluorescent detection, the fluorescent molecule is directly conjugated to the detection antibody, and no substrate is necessary. The sample only needs to be exposed to the correct wavelength that stimulates fluorophore emission, and can be recorded for quantification purposes.

The current, most popular immunoassays in use today are the RIA and the ELISA. Both assay types share many similarities in assay components, but differ greatly in detection methodology and sensitivity. The RIA utilizes radioisotopes that incorporate into the molecular structure of an analyte without altering its function, and utilizes equipment that has the capabilities to detect and quantify radioactivity in a sample. In the ELISA, the assay principle is similar; however the radiolabeled analyte is replaced by antibodies conjugated to detection mechanisms such as enzymes or fluorophores. The ELISA was originally developed as an alternative to the RIA, which has been reported to be more sensitive than ELISA, although RIAs have a number of regulations relating to human and environmental dangers. Both ELISA and RIA can be carried out in a number of assay configurations, including antibody sandwich and competitive inhibition. In general, ELISAs primarily rely on antibody sandwich assays, while RIAs primarily use the competitive inhibition assay setup. While ELISA and RIA are the most popular immunoassays currently, a new method (immuno-PCR; iPCR) has been described that attempts to improve upon the current sensitivities of RIA and ELISA. Furthermore, iPCR
has built into the assay the potential for measuring multiple analytes simultaneously, which is a limitation of the traditional assays. Multiple analyte measurement has been reported in ELISA based formats, but is limited by the quantity and availability of detection molecules. This new technology, immuno-PCR, may be able to both increase assay sensitivity and improve multiplex capabilities through its detection molecule; i.e. oligonucleotides.

Immuno-PCR

Immuno-PCR (iPCR) is a relatively novel immunoassay format first described in the early 1990s (Sano et. al., 1992). As opposed to traditional labeled detection methods, the iPCR assay utilizes DNA as a detection molecule. The use of DNA for detection has the potential to increase sensitivity and allow for multiplexing of biological samples (Hendrickson et. al., 1995; Adler, Wacker and Niemeyer, 2008). While the iPCR assay is not without its drawbacks, the assay remains a powerful tool for development of sensitive immunoassays and multiplexing.

Early Assay Descriptions

In the first report describing iPCR, Sano and colleagues described the ability to detect attogram quantities of bovine serum albumin (BSA), utilizing DNA as their detection molecule. The sensitivity of the iPCR assay was determined by comparing values obtained from a BSA ELISA to the iPCR results, which found the iPCR assay to be 5-fold more sensitive than the competitive ELISA. This assay method employed basic ELISA technology, but with two important modifications. Instead of using streptavidin as
a carrier for the detection enzyme, a chimeric protein A/streptavidin molecule was used. This chimeric molecule contained the binding motif of protein A, and the streptavidin protein, which strongly binds the molecule biotin. This chimeric protein was used to link the antibody that was bound to the antigen to the DNA for detection of the ELISA complex. The detection DNA was biotinylated and pre-bonded to the chimeric molecule. Using this method, the authors were able to detect as little as 580 molecules of BSA (9.6 x 10^{-22} mol). Shortly after this initial description of iPCR, a separate research group attempted to recreate the assay using commercially available reagents. In 1993, Ruzicka et. al. reported a successful iPCR assay for apolipoprotein E utilizing avidin as the linking molecule between a biotinylated antibody and biotinylated DNA. The assay setup was identical to the first description, except for the use of a biotinylated detection antibody, and avidin as the linking molecule. The authors argued that their assay setup would result in decreased background, as the protein A component of the chimera could potentially bind antibodies that are not occupied by antigens in the sandwich ELISA format. Additionally, the use of commercially available avidin makes the iPCR assay accessible to more researchers. Indeed, subsequent iPCR studies appeared to have moved away from the chimera molecule and toward avidin or streptavidin bridging and subsequently, direct conjugation of oligonucleotides to antibodies (Niemeyer, 2010). With the description of quantitative iPCR in 1999, iPCR advanced another step. Early descriptions of iPCR used indirect quantification methods – gel bands from end-point PCR imaged for pixel density. At the time, this was the only available semi-quantitative method.

With the introduction of qPCR, iPCR assays quickly became quantitative in nature. The
first report of iPCR with qPCR for detection was in 2000 (Sims et. al.). In this experiment, a sandwich ELISA was conducted for vascular endothelial growth factor (VEGF). The oligonucleotide was directly conjugated to the detection antibody, and samples were analyzed with qPCR. The results of this assay were compared to a VEGF ELISA, and the sample values were found to be highly correlated. Subsequently, most recent iPCR assays have used qPCR for detection.

**Assay formats and detection methods**

A number of assay formats and detection molecule configurations have been reported for iPCR, ranging from sandwich ELISA format (Mullenix et. al., 2002), antigen competition with antigen immobilized to plate surface, direct conjugation of oligonucleotide to antibody (Adler, Wacker & Niemeyer, 2003), and use of streptavidin as a linker molecule (Saito et. al., 1999). The direct conjugation of oligonucleotide to antibody represents the most direct measurement of analyte in iPCR. Additionally, direct conjugation of antibody and oligonucleotide reduces the number of steps in the assay, as well as reduces handling time, both of which have been associated with decreased performance of iPCR (Ruzicka et. al., 1993; Adler et. al., 2003). The direct conjugation of oligonucleotide and antibody has been used successfully in the sandwich assay format with end-point PCR (Joerger et. al., 1995; Hendrickson et. al., 1995), as well as with qPCR (Sims et. al., 2000; Lind & Kubista, 2005). Various linker chemistries have been used, including the streptavidin bridge. The availability of a number of different linker chemistries allows for a diverse set of potential detection molecules. One of the most popular methods involves non-covalent coupling of the antibody to oligonucleotide via the streptavidin-biotin
interaction. In this format, streptavidin acts as a linker or bridge between a biotinylated antibody and biotinylated oligonucleotide. In the early descriptions of iPCR, the strong bond between streptavidin and biotin was advantageous in order to keep the detection complex anchored to the PCR plate. With the changing availability of iPCR materials, this format may no longer be ideal. Also, as early as 1993, it was suggested that utilizing streptavidin may have additional complications. In their response to the initial description of using avidin as a bridge, Sano and colleagues suggested the use of avidin in the detection complex had the potential to produce detection molecules with 2, 3, or even 4 biotinylated oligonucleotides, which they suggested would decrease assay sensitivity. On the other hand, the commercial availability of streptavidin and both biotinylated antibodies and biotinylated DNA makes the streptavidin bridging detection complex an appealing detection molecule for iPCR.

Another detection complex that has been used extensively in iPCR is the covalently attached oligonucleotide to antibody. In this configuration, the oligonucleotide is conjugated to the antibody via heterobifunctional crosslinkers, often with a spacer arm between antibody and oligonucleotide (Niemeyer, 2010). These conjugated molecules have the advantage of decreasing the number of steps in the iPCR assay, which improves assay sensitivity (Ruzicka et. al., 1993; Adler, Wacker and & Niemeyer, 2008). In a study by Lind and Kubista (2005), three iPCR configurations were tested with two different detection configurations: the streptavidin linker bridge and an oligonucleotide conjugated to antibody via the heterobifunctional crosslinker SSMBC. Increased assay sensitivity was observed in the formats that used the conjugated antibody-oligonucleotide as the detection mechanism. However, all iPCR curves were parallel and all were more
sensitive than the comparable ELISA for prostate specific antigen (PSA). Collectively, the data suggests both streptavidin bridging and covalent conjugation of oligonucleotide and antibody are viable methods of detection in iPCR. While direct conjugation often results in a more sensitive assay, and decreased assay steps, both the streptavidin bridge and direct conjugation are more sensitive than the ELISAs for the tested antigens of interest. While the streptavidin bridge may be less sensitive, the process of conjugating oligonucleotides to antibodies can also be difficult (Barletta, 2006). Regardless, the power of the detection mechanism is what makes iPCR a unique and sensitive assay.

**Assay Principle**

Immuno PCR is a hybrid assay between an ELISA and PCR in most simplistic terms. The specificity of antibody epitope recognition coupled with PCR for detection results in a highly specific, sensitive assay (Barletta, 2006; Niemeyer 2010). Furthermore, a number of different assay designs can be utilized in the iPCR assay, as discussed previously. Many of these designs depend on the analyte being measured, availability of substrate for immobilization, and availability of biological reagents (i.e. antibodies and sample). A range of different assay types on various substrates have been reported for iPCR (Barletta, 2006).

**Solid Substrates used in iPCR**

Early experiments with iPCR conducted separately by Sano et. al. and Ruzicka et. al., utilized the antigen-on-plate method. In this format, the antigen of interest was immobilized on a compatible surface, which was then probed with the detection molecule. The detection molecule was bridged to the DNA via a chimeric protein or
avidin, respectively. Subsequent iPCR studies have also used this approach (Adler, Wacker & Niemeyer, 2003), or have used a sandwich ELISA. One of the variables that makes iPCR both versatile and limiting is the solid substrate on which the assay is performed. Selection of a functional solid substrate is crucial for the success of iPCR. Similar to the assay format, a number of different solid substrates have been reported to be compatible with iPCR, including glass, polycarbonate, and polypropylene, both modified and non-modified surfaces (Saito et al., 1993; Saito et al., 1999; Wang et al., 2008). Additionally, iPCR has also been carried out on functionalized microspheres (Halpein, Jain & Jewett, 2013). For example, Wang and colleagues utilized glass slides to immobilize Epstein Barr virus nuclear antigen (EBNA1), an antigen present in all nasopharyngeal carcinoma cells. The objective of this work was to develop an iPCR assay for EBNA1 in plasma, due to the fact that EBNA1 concentrations are too low to detect in plasma using standard detection assays. The assay, once developed, could be utilized as an early detection assay for nasopharyngeal carcinoma. While the authors reported the successful development of the iPCR assay, it is important to note that prior to immobilizing the antigen, the glass slides were etched with an acidic solution and underwent a unique drying protocol to maintain functionality of the glass. While the success of the assay cannot be dismissed, the extensive modification process makes glass a less than appealing substrate, especially if a rapid and sensitive assay is desired. Another solid support structure used in early developments of the iPCR assays was polycarbonate (Ruzicka et. al., 1993; Hendrickson et. al., 1995), and polypropylene PCR plates (Lind & Kubista, 2005). These substrates are useful because the plastics enable the iPCR to be carried out in a single plate, which improves repeatability of the assay.
Some of the initial descriptions of iPCR were carried out on polypropylene PCR plates (Hendrickson, 1995). Polycarbonate is one of many polymers available for use in molecular biology, and has been observed to be a useable support substrate for biomolecule adhesion (Penney et. al., 1989).

Polypropylene, on the other hand, is an economical alternative to polycarbonate (Smock, 2009). Many of the iPCR methods described have used polypropylene or modified polypropylene plates as the solid support structures in iPCR, with varying levels of success. One potential drawback to the use of polypropylene is that it has been observed to have a low affinity for protein adsorption (Goebel-Stengel, 2011). However, polypropylene may have some capacity to bind antibodies and/or proteins to some degree. Lind & Kubista (2005) carried out one of the more comprehensive analyses of different iPCR assay designs. In all of the experiments, however, the iPCR assay was carried out in polypropylene PCR plates. In one of the ‘assemblages’ tested, streptavidin had been pre-adsorbed to the PCR plate surface. Regardless of the conditions of the plate prior to the iPCR setup, the experiment yielded standard curves for PSA in all assemblages. The only observable difference between assemblages was the result of the detection mechanism: the streptavidin bridge detection mechanism was less sensitive than the assays that used a directly conjugated antibody-oligonucleotide. This demonstrated that both the polypropylene PCR plates and polypropylene PCR plates coated with streptavidin could both be used for development of iPCR assays. It is difficult to determine with certainty which solid structure would be optimal for iPCR in a single plate, as both polycarbonate and polypropylene have both been demonstrated as
functional in an iPCR assay. Regardless, a unique feature of the iPCR assay is the adaptability of the assay to different solid support materials. With the increasing popularity of microspheres and nanoparticles, iPCR can further expand into novel support structure types.

Microparticles have been used in other analyte detection assays, including ELISA-type assays. Microparticles are a unique solid support structure because they can be made in unique batches that can be combined for multiplexed analyte detection (Mullenix et. al., 2002). Additionally, many microspheres can be made ferromagnetic, making it easier to separate them from the liquid component once incubations are complete. Immuno-PCR has been described as working successfully with magnetic microspheres as the solid substrate (Jiang et. al., 2012; Halpern, Jain & Jemett, 2013). In a particularly sophisticated nanoparticle based assay, Nam et. al. (2003) described the use of “bio-barcodes” for protein detection. In this experiment, two sets of nanoparticles were used: a nanoparticle probe and a magnetic microparticle probe. The magnetic microparticle probe had a monoclonal antibody immobilized to its surface. This probe was then mixed with sample to bind the target antigen. The detection nanoparticle was then added. This nanoparticle had a second, polyclonal antibody against the target antigen immobilized to its surface, as well as a number of oligonucleotides for detection purposes. The assay was then carried out similar to a sandwich ELISA. The ferromagnetic property of the microparticles allowed the researchers to separate the microparticles from the washed component with a magnet. What was especially interesting in this assay was that the use of nanoparticles allowed for PCR amplification and detection as well as PCR-less detection, via silver amplification. Whether or not this is a useful and easily adaptable
method for iPCR remains to be seen. Furthermore, the detection limit for PSA in this assay was 3 aM. This sensitivity demonstrates that iPCR could be used as a sensitive tool for protein detection, especially for proteins that may serve as important biomarkers, but have low circulating concentrations in easily accessible biological samples. One area of iPCR that has been proposed, but not investigated, is the ability to utilize iPCR in multiplexed analyte detection.

**Summary**

There are a number of methodologies available for analysis of molecules as they exist in a biological matrix. Many use antibody-based detection, while others use structural determinants for analysis. Immuno-PCR is a relatively new technology available for protein detection. Immuno-PCR has been reported to have increased sensitivity over a number of ELISAs (Sano et. al., 1993; Lind & Kubista, 2005). Success (or failure) of the iPCR assay depends on various factors, including the detection mechanism used, the solid support structure chosen, and the number of steps involved in carrying out the assay. Regardless of these variables, the sensitivity and versatility of iPCR makes it a lucrative target for assay development. The use of DNA as a detection agent also enables movement toward a multiplexed assay that can be easier to analyze than colormetric or fluorometric detection methods. Each assay detection type has their own unique advantages and drawbacks, but many of the discussed assays share a common limitation – these assays generally do not have the ability to determine multiple, specific analytes simultaneously. However, there have been recent advances with assay technology that have begun to investigate simultaneous analyte detection in a single sample. The general
term given to this process is known as multiplexing, and there are at least two methodologies commercially available for this purpose.

**Simultaneous Detection Approaches**

*Multiplexing*

Multiplexed analysis, as first described in 1990 by Fulwyler & McHugh, refers to the measurement of multiple analytes in a biological sample simultaneously. Multiplexing has existed as a method of detection utilizing PCR and microarray since the late 80s and early 90s (Chamberlain et. al., 1988; Schena et. al., 1995). The assays achieved multiplexing utilizing different methods. For example, in multiplex PCR, the detection of multiple products is achieved through the use of unique DNA probes conjugated to different fluorophores. On the other hand, microarrays achieved multiplexed detection by immobilizing multiple, known DNA sequences to specific regions on a glass slip. If the complimentary sequence is present in a sample, it will bind to the plate and the addition of fluorophores can determine its expression relative to a control sample. The advent of multiplexing has challenged the current methodology used for measuring hormones and other molecules in a sample. Multiplexing allows for the ability to use less sample and decrease hands-on time for measurement. The earliest multiplexed assays described were used for detection of various cytokines (Fulton et. al., 1997). In one of the first published uses of multiplex detection, Carson and Vignali (1999) measured 15 different cytokines simultaneously in a multiplex format. The principle of a successful multiplex assay is based on the sandwich ELISA format. Instead of using a plate to immobilize capture antibodies, microscopic beads acted as the solid substrate. Depending on the application,
these beads were uniquely labeled in order to distinguish one bead set from another (Carson & Vignali, 1999; Quinn et al., 2010), which is the technology that makes multiplexed detection possible.

**Illumina BeadXpress**

The Illumina BeadXpress platform was initially described as a novel method for SNP genotyping (Lin et al., 2009). The Illumina BeadXpress platform was first used for multiplex analysis of proteins in 2010, as reported in a white paper published by Illumina (Quinn et al., 2010). In this paper, the principles of the BeadXpress platform were described: glass beads of a diameter of 28 x 240 microns were encoded with a unique barcode. The surface of these beads were functionalized with carboxyl groups, which allowed molecules like antibodies to covalently attach to the surface of the bead via EDC-crosslinked amine residues. Once an antibody was immobilized to the bead surface, the beads acted as a miniaturized assay substrate, with numerous beads being added to a well for analyte detection. Because of the unique barcode etching, the reader machine was equipped with a CCD camera to read the barcode. In this format, multiplexing occurred when the following criteria were met: 1. Immobilization of an antibody for the analyte of interest via covalent coupling mediated by a heterobifunctional crosslinker and 2. The presence of different bead sets with unique barcodes so that the machine could distinguish the spectral signature on the surface of each bead. In addition to the CCD camera, the reader machine was also equipped to measure fluorescence at two wavelengths. The reader machine independently read the bead barcode and the fluorescence on the surface of the bead. Therefore, various bead populations could be
introduced into a biological sample, which is equivalent to multiple ELISAs being carried out in a single reaction well.

With the ability to analyze multiple analytes simultaneously, and using an inert material like glass to immobilize antibodies, the BeadXpress platform had the potential to expand multiplexing further than before. The barcode etching would have potentially increased the number of multiplexed analytes far outside the current limitations. However, support for the machine was abruptly ended by Illumina in 2011, and the reagents are no longer being produced (personal communication).

*Luminex xMAP*

The Luminex method for analyte detection was first described by Fulton and others in 1997. The Luminex technology consists of a flow cytometer measurement machine and polystyrene bead sets. Similar to the BeadXpress platform, the surface of the beads are modified with free carboxyl groups that are activated with the addition of EDC and Sulfo-NHS. Upon the addition of an antibody or protein, the free amine groups on the antibody or protein will covalently bond with the carboxyl groups on the bead surface, resulting in an antibody or protein coated bead surface. Additionally, the beads are internally labeled with proprietary fluorescent dyes, which creates unique bead sets for multiplexed analysis. When first described, the multiplex platform had the capability to detect 64 unique antigens (Fulton et al., 1997). Measurement of the beads in the Luminex platform is achieved through the use of two different lasers – one that excites the fluorescence within the beads, and one that excites the fluorescent detection molecule on the bead surface. The results are then partitioned by bead identification and median
fluorescence intensity (MFI), which corresponds to the surface, or assay fluorescence. Like the sandwich ELISAs, the MFI is directly proportional to the amount of antigen in the sample.

**Immuno-PCR and Multiplexing**

Little has been explored about the multiplexing capabilities of iPCR, although it has been mentioned in theory (Barletta, 2006; Adler et. al., 2008; Hansen et. al., 2014). Two early experiments explored the concept of multiplex iPCR with three different antigens (Hendrickson et. al., 1995; Joerger et. al., 1995). These iPCR assays utilized gel electrophoresis for detection of three different analytes: human choriogonadotropic hormone (hCG); human thyroid stimulating hormone (hTSH) and β-galactosidase (β-gal). In order to achieve multiplexed detection with this setup, the authors conjugated oligonucleotides of varying lengths to the different antibodies for each antigen measured. As an example, the hCG antibody was conjugated to a 99-base pair oligonucleotide, while hTSH was conjugated to an 85-base pair oligonucleotide. Therefore, due to the different sizes of the oligonucleotides, the samples would separate on the agarose gel according to size, which was confirmed with a molecular weight marker. Additionally, the amount of oligonucleotide in each sample was quantified based on “pixel intensity per unit area”, which represented quantification of the hormones in each sample. This was one of the few experiments demonstrating a successful multiplexed iPCR assay. However, the addition of real time detection to iPCR has eliminated the need for agarose gel electrophoresis for quantification of iPCR products. Additionally, with the advent and advancement of DNA sequencing technology, there is a new possibility for multiplexed iPCR detection. As theorized by Hansen et. al. in 2014, sequencing technologies would
allow for quantification of iPCR products using synthesized oligonucleotides with as little as a single base difference between two probes. Size difference in probes may no longer be a deciding factor or limitation of multiplex iPCR. Additionally, with the increasing demand for knowledge and data, multiplex PCR could fill a gap in endocrine detection; the lack of a reliable multiplex detection system. The utility in iPCR is that it uses fairly common reagents and machinery, and does not rely on proprietary assay technology.

**Future of iPCR and multiplexing**

Although first described over 20 years ago, iPCR has been adapted slowly. It has been suggested that the initial investment, both time and cost, as well as the lack of a standardized method have been barriers to adoption of the technology (Barletta, 2006). This does not change the fact that a wide variety of assay types have been described, from detection of viral antigens (Hashimoto et. al., 2012) to hormones (Sims et. al., 2000). Therefore, it is probable that the next evolution with iPCR would be to investigate multiplexing capabilities, which have been described previously, but with no subsequent follow-ups (Hendrickson et al., 1995). Multiplexed iPCR would be useful in both scientific and clinical settings. For example, it has been established that in cattle, measurement of five hormones from a single sample at slaughter can predict the carcass quality designation assigned by a USDA grader with 95% accuracy (Brandt et. al., 2007). This result leads to additional questions about the capability of endocrine markers to predict any specific phenotype. Having the ability to detect multiple hormones or biomarkers simultaneously may have the ability to generate predictive assays of economically important phenotypes, as well as human or animal disease states. However,
these assays cannot be developed until a more standardized methodology of iPCR assay development can be established. Therefore, the objective of this research was to develop a multiplexed assay for measurement of endocrine analytes, and investigate the use of both commercially available multiplexing platforms and iPCR.
CHAPTER III
DEVELOPMENT OF A MULTIPLEX IMMUNOASSAY ON THE ILLUMINA
BEADXPRESS PLATFORM

MATERIALS AND METHODS

Antibody and Fluorescence Reagents

Monoclonal antibody against progesterone was graciously provided by Dr. Jan Roser. Goat anti-mouse IgG conjugated to the fluorophore Cy3 was purchased from Millipore (Billerica, MA). The monoclonal progesterone antibody was conjugated to the surface of the BeadXpress beads.

BeadXpress Beads: Activation & Coupling

The BeadXpress platform used for this study reads a unique set of silica beads, 28 μm x 240 μm in size. The inside of the beads were laser etched with a unique barcode for each ‘bead set’, while the surface of the beads were modified with carboxyl groups for the attachment of amine groups to the bead surface. In order to prepare the carboxyl groups on the bead surface for conjugation to amines, an activation step was first performed.

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was the crosslinker used to couple the carboxyl groups on the bead surface to primary amine groups found on proteins, including antibodies (Pierce, Rockford, IL). N-
hydroxysulfosuccinimide (Sulfo-NHS) was used as an intermediate stabilizer (Pierce, Rockford, IL). The conjugation process was a protocol modified from the manufacturer (Quinn et al., 2010). Briefly: Silica carboxyl-coated beads (approximately 25,000 beads/conjugation tube) were washed with 0.1 M 2-(N-morpholino)ethane sulfonic acid (MES), pH 6.0. After washing, the carboxyl residues on the bead surface were activated with EDC and Sulfo-NHS in 0.1 M NaH$_2$PO$_4$, pH 6.2. The reaction was mixed and incubated for 20 minutes at room temperature, with intermittent vortexing. After another set of washes with 0.1 M MES and centrifugation to force the beads to the bottom of the tube, the progesterone antibody was added at a concentration of 100 µg/ml in 0.1 M MES buffer. This mixture was incubated at room temperature with rotational mixing for 2 hours. After another set of washes with PABET (0.15 M NaCl; 0.01 M PO$_4$; 0.01% sodium azide; 0.1% gelatin; 0.01 M EDTA; 0.05% Tween 20), then two additional washes with PBS-0.05% Tween 20 (PBST), the beads were stored in KelcoGel (0.05 g/100 mL 5mM NaCl, CP Kelco, Atlanta, GA) at 4°C until assay use.

BeadXpress Platform

The BeadXpress platform consisted of a ‘reader machine’ with unique components: a CCD camera for reading the bead barcode, and two lasers with emission wavelengths of 532 nm and 635 nm to excite the fluorophores on the bead surface. Additionally, the machine had a ridged plate that oriented the rod-shaped beads in a monolayer to prevent the beads from being obstructed when read by the machine. Together, these components first identified the barcode etched within each bead, then determined a fluorescence emission value for each individual bead. Fluorescence was recorded as relative
fluorescence units (RFU) and corresponded to the mean fluorescence of beads measured in the sample.

BeadXpress Conjugation Success Assay Protocol

Beads coupled to monoclonal progesterone antibody in KelcoGel were added to microplate wells (Corning, Corning, NY) in 50 µl aliquots. Coupled beads were then incubated with Cy-3 labeled goat anti-mouse IgG at varying concentrations (500, 50, and 5 ng). After mixing the slurry with a pipette, the fluorescent antibody was incubated with the beads for 60 minutes at room temperature while shaking. The beads were then washed with PBST 3 times, and after the final wash, 100 µl of PBST was added to keep the beads in solution. The beads were then analyzed by the BeadXpress machine, using the 532 nm wavelength for bead surface fluorescence detection.

RESULTS

Initially, there was a great deal of difficulty of conjugating the antibodies to the beads. The initial protocol provided by the company was found to be ineffective in conjugating antibodies to the bead surface (Figure 3, 1 step conjugation). Therefore, we attempted a modified conjugation protocol, similar to alternative existing protocols. The modified protocol provided the greatest conjugation success, as determined by the conjugation success assay protocol (Figure 3, 2 step conjugation). This step reduced assay time by confirming conjugation success before utilizing the beads in downstream assays.
Figure 3. Comparison of conjugation protocols, as measured by the conjugation success assay. Beads subjected to either a single step or two-step conjugation protocol with monoclonal progesterone antibody were incubated with increasing amounts of goat anti-mouse IgG conjugated to Cy3. The bead fluorescence was then measured in the BeadXpress Reader. The 2 step conjugation protocol resulted in higher RFUs compared to the 1 step conjugation, suggesting more monoclonal progesterone antibody was successfully conjugated to the bead surface.
Unfortunately, due to the difficulties in establishing a robust conjugation protocol, the objectives were not successful. However, an assay was developed to determine antibody conjugation success prior to downstream use. Outside of this assay, no other assays for livestock analytes were successfully developed. Prior to this experiment, there was little to no information regarding ways to confirm success of antibody/antigen conjugation prior to downstream use. However, having a quantifiable indicator of conjugation success allowed for greater downstream assay efficiency, as it eliminated the variable of questionable conjugation success. With the high cost of beads and assay reagents, measuring conjugation success prior to using the beads in the assay was cost-effective and minimized unnecessary reagent waste.

KelcoGel was utilized to attempt to disperse beads evenly into the microplate wells. The BeadXpress manufacturer’s protocol suggested the use of filter plates to evenly disperse beads into microplate wells. The introduction of KelcoGel aimed to improve upon the existing assay design by eliminating the use of filter plates for bead dispersement. KelcoGel is a commercially produced gellan gum. 0.05% KelcoGel created an ideal suspension medium for the beads. The medium maintained a liquid consistency while suspending the beads evenly throughout, after vortexing. This allowed for fluid bead handling and consistent distribution of beads into the microplate wells.

The advantage of bead-based assay platforms like the BeadXpress platform is that they have the built-in capability of multiplexing, through the use of multiple labeled bead sets.
Additionally, the BeadXpress platform had the ability to generate a fluorescence profile for each individual bead, which acted as a sample replicate. Therefore, each well in a bead assay had the potential for hundreds of replicates in a single well, corresponding to a single sample. This afforded the use of less sample and improved sampling accuracy. However, there were also a few drawbacks to the platform which hindered its optimum performance. In order to take advantage of improved accuracy with multiple beads in a single well across an assay, bead numbers must be consistent throughout all wells. This became an issue during washing, in which beads were often lost to aspiration. Without a way to prevent the beads from being aspirated, it was difficult to take advantage of the improved accuracy. It has been suggested that the use of a filter plate for the bead assay may be necessary, however that was not necessarily cost-effective and counter to the objectives of the study.

An important consideration when evaluating the use of the BeadXpress platform is that the manufacturer no longer provides support for the machine. As they were the sole manufacturer of the bead sets, without a bead supply, the BeadXpress platform has become obsolete.
CHAPTER IV

DEVELOPMENT OF A MULTIPLEX IMMUNOASSAY ON THE LUMINEX xMAP PLATFORM

MATERIALS AND METHODS

Luminex Microspheres and Assay Reagents

Unlike the Illumina platform, the Luminex platform utilized a detection method based on flow cytometry. The Luminex microspheres were 5.6 \( \mu \)m in size, and made of polystyrene. The polystyrene beads were dyed internally with red and infrared fluorophores at varying ratios in order to give each bead set an individual spectral signature that was decoded by the machine. Similar to the Illumina beads, the Luminex bead surfaces were modified with carboxyl groups that were activated via EDC and Sulfo-NHS in order to facilitate conjugation of antibody to the bead surface. For the Luminex assay, bovine luteinizing hormone (bLH) was chosen as the assay to develop. Monoclonal anti-bovine LH antibody was graciously provided by Dr. Jan Roser. Bovine LH protein (AFP 11743-B) was provided by the National Hormone and Peptide Program (Dr. A. F. Parlow). The detection antibody, a polyclonal, anti-ovine LH was provided by Dr. Tom Adams (TEA oLH #35). Although specific for ovine LH, the homology between bovine and ovine LH\( \beta \) subunit is 99% (UniProt KB, 3.2e\(^{-102}\)). Additionally, the polyclonal antibody has been used to detect both bovine and ovine LH in RIA procedures (Bastidas et. al., 1990; McShane et. al., 1993). The polyclonal antibody was biotinylated with a commercial biotinylation kit (Pierce, Rockford, IL), according to manufacturer’s
instructions. Streptavidin-phycoerythrin was obtained from Sigma-Aldrich (St. Louis, MO).

**Luminex Microspheres: Activation & Coupling**

Similar to the previously described Illumina beads, the surface of the polystyrene microspheres were modified with carboxyl groups for the attachment of primary amines. Therefore, before proteins were coupled to the bead surface, the bead surface was first activated. The protocols used for both microsphere activation and coupling to protein came directly from the manufacturer (Luminex Corp., Austin, TX). Briefly, after a washing step to effectively remove storage buffer, microspheres were activated with EDC and Sulfo-NHS in 0.1 M NaH₂PO₄, pH 6.2. After two washes with MES buffer (0.1 M, pH 6.0), antibody or protein was added at the desired mass (LH antibody & protein: 5 μg/conjugation) in 0.1 M MES buffer, and the solution was allowed to incubate for 2 hours at room temperature. After incubation, beads were washed two additional times with PBS-TBN (PBS, 0.1% BSA, 0.02% Tween-20, 0.05% Azide, pH 7.4). Before storage in PBS-TBN, microsphere suspension concentration was determined with a hemocytometer, in order to divide microspheres evenly within the sample plate. Microspheres were stored in PBS-TBN at 4°C until assay use.

**Luminex IS 100/200 platform**

The Luminex IS 100/200 platform was similar to a flow cytometer. The beads, after being run in the detection assay with samples, were drawn into the machine via fluidics. Within the machine were two lasers (532 nm and 635 nm) with detection filters. The 635 nm laser was responsible for exciting and detecting the internal fluorescence of the
microsphere, while the 532 nm laser was responsible for excitation and detection of the fluorescence on the bead surface. The beads were mapped by median fluorescence intensity and bead fluorescence. This mapping scheme provided information on the bead set present in each sample, as well as the fluorescence from the bead sets.

**Luminex Assay Protocol**

The assays were carried out similarly to the suggested protocols provided by Luminex. The following assay designs were attempted: Antibody sandwich, antibody competition and antigen on microsphere (Figure 4). For the antibody sandwich, the protocol roughly followed the Luminex guidelines, with a few minor changes. Filter plates were not utilized. Instead, U-bottom 96-well plates were used (Corning, Corning, NY). After conjugation of monoclonal LH antibodies to the microsphere surface, microspheres were diluted to a concentration of 100 microspheres/μl, based on calculations from hemocytometer counts. The following equation was used to determine microsphere dilution: Total microspheres = microsphere count (1 corner of a 4 x 4 section of hemocytometer) x (1 x 10⁴) x 10 (dilution factor) x 1 (resuspension volume in ml). Fifty μl of the microsphere dilution was added to assay wells. Fifty μl of standards, samples, or assay buffer (PBS-TBN) were added to the appropriate wells. The solution of microspheres and assay components was mixed via repeated pipetting of the solution into and out of the wells. Once mixing was completed, the plate was covered and incubated for 1 hr at room temperature on a plate shaker. After incubation, a wash step was performed (2 washes with PBS-TBN), and 50 μl of a 4 μg/ml solution of biotinylated, polyclonal second antibody was added. The plate was again incubated for 1 hr at room
temperature on a plate shaker. After another round of washes, 50 µl of a 4 µg/ml solution of streptavidin-phycoerythrin was added, and the plate incubated for an additional 30 min at room temperature on a plate shaker. After a final wash, the microspheres were resuspended in 50 µl of PBS-TBN, and then analyzed by the Luminex IS 100/200 (Figure 4A). The antibody competition assay was carried out in a similar manner, however, no second antibody was added, and a fixed mass (1 ng/well) of biotinylated LH was added, to compete for antibody binding on the microsphere surface (Figure 4B). Finally, for the antigen on microsphere assay, bovine LH was conjugated to the bead surface instead of monoclonal antibody, and the LH on the microspheres competed with sample LH for antibody binding. The biotinylated, polyclonal antibody was used in this configuration, and only those antibodies that bound to the LH on the microsphere would be detected by the Luminex IS 100/200 (Figure 4C).
Figure 4. Assay configurations investigated on the Luminex xMAP platform. (A) represents a standard sandwich assay, using a monoclonal antibody on the bead surface, and a polyclonal-biotinylated secondary antibody as the detection antibody. The beads were then incubated with streptavidin-phycoerythrin, a molecule that binds biotin and emits a fluorescence signal that can be measured by the Luminex IS 100/200. (B) represents a competition style assay, in which sample LH competes for binding to the capture antibody on the bead with biotinylated LH. The detection antibody binds both, however only those LH molecules that were captured on the bead were detected. (C)
represents a competition assay, however in this scenario, the sample LH competed for antibody binding with the LH immobilized to the bead surface. Both B and C have the added advantage of only using a single antibody in the configuration. However, it was determined that configuration A resulted in the most robust standard curve.
**Animal Samples & Comparison RIA**

In order to compare Luminex bead results to RIA, animal samples were collected and LH was measured in both the bead assay and a double antibody RIA (Fogwell et. al., 1977). Dairy cows (n=4) were administered a single intramuscular injection of GnRH (2 ml of a 50mcg/ml solution, Cystorelin, Merial LTD, Duluth, GA). Three hours later, blood was collected via jugular veinipuncture, serum was harvested, and stored at -20°C until analysis. Similarly, sheep (n=2) were administered a single intramuscular injection of GnRH (50 µg). Two hours later, blood was collected via jugular veinipuncture, and plasma was harvested and stored at -20°C until analysis.

**RESULTS**

Unlike the Illumina assay, bead conjugation was accomplished quickly and reliably utilizing the Luminex assay protocol. Therefore, there was little necessity for the conjugation success assay, and various assay formats could be rapidly tested. The following assay formats were tested: antibody on bead surface, with antigen competition between sample and biotinylated antigen, antibody on bead surface, with antigen capture in a sandwich ELISA format (detection antibody biotinylated), and antigen on bead surface, with biotinylated antibody competing for binding between antigen in sample and antigen on surface. Of all the assay types tested, the only format that resulted in a dynamic standard curve was the sandwich ELISA format, with a monoclonal antibody on the bead surface, and a polyclonal, biotinylated second antibody acting as the detection antibody (Figure 5). All assays were detected with streptavidin-labeled phycoerythrin,
which has a maximum excitation wavelength of 565 nm and a maximum emission wavelength of 578 nm, which were all within operational range of the Luminex IS 100/200 system. Once the optimum assay design was determined, the assay was tested with bovine and ovine serum, as well as ovine plasma. The assay results were compared to the results obtained from an LH RIA, utilizing the same monoclonal antibody (Figure 6). While the absolute values of the samples differ between the cattle serum samples measured by RIA and Luminex, the correlation between the assays is high (Figure 6, $r^2=0.99$), suggesting that both assays measured a similar amount of LH in the samples.

For example, in both the RIA and Luminex platform, Cow 2352 had the highest level of LH, while Cow 2718 had the lowest level of LH (Table 1). On the other hand, it is important to note that for the sheep samples, both plasma and serum, the values obtained in the RIA are comparable to the values obtained in the Luminex platform.
Figure 5. The Luminex bead assay detected LH in ovine and bovine samples in a sandwich assay format. (A) Serum and plasma from GnRH treated ewes were incubated with Luminex beads in a sandwich assay format. The Luminex beads, conjugated to a monoclonal LH antibody and probed with a polyclonal LH antibody successfully detected LH levels in ovine serum and plasma. (B) Serum samples from GnRH treated dairy cows were incubated with the Luminex beads in an identical manner as the ovine samples. The Luminex beads successfully detected LH levels in bovine serum samples.
Figure 6. Correlation of LH concentrations in 6 livestock samples (4 bovine; 2 ovine) measured by RIA and the Luminex bead assay developed for LH (y=1.1031x – 2.1545 ng/ml, correlation coefficient = 0.99).
Table 1. LH values in serum and plasma samples obtained using the Luminex bead assay and a double-antibody disequilibrium RIA.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Concentration obtained from beads (ng/ml)</th>
<th>Concentration obtained from RIA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy cow 946</td>
<td>4.8 ± 0.9</td>
<td>6.9 ± 0.9</td>
</tr>
<tr>
<td>Dairy cow 2352</td>
<td>9.4 ± 1.9</td>
<td>11.5 ± 1.6</td>
</tr>
<tr>
<td>Dairy cow 2513</td>
<td>1.0 ± 0.8</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>Dairy cow 2718</td>
<td>0.15 ± 0.07</td>
<td>0.9 ± 0.06</td>
</tr>
<tr>
<td>Ewe 111025 serum</td>
<td>17.3 ± 2.8</td>
<td>17.1 ± 0.3</td>
</tr>
<tr>
<td>Ewe 111025 plasma</td>
<td>17.3 ± 2.3</td>
<td>17.2 ± 1.1</td>
</tr>
</tbody>
</table>
DISCUSSION

The Luminex xMAP technology has been widely used for investigative and diagnostic purposes (Faul et al., 2014). Currently, pre-conjugated bead sets are available that test for a wide range of analytes, including cytokines (Palus et al., 2015), human disease biomarkers (Liu et. al., 2005), as well as hormones (Motsinger-Reif et. al., 2013). In addition to pre-made kits, Luminex also offers users the ability to develop their own assays for their specific needs. However, Luminex only provides the machinery and the microspheres. Therefore, the antibodies and antigens must be supplied by the end user. The ultimate success of these user-designed assays then rests on the specificity of the antibodies for their target antigen (Darwish, 2006). Additionally, a sandwich assay format, which is how many pre-fabricated kits are designed, requires two antigen specific antibodies, which may be problematic for investigations in livestock. Due to the limited availability of livestock-specific antibodies, a variety of assay designs were investigated in order to determine the best assay design for measurement of livestock analytes. The assay designs were based on other assay types that use similar methodologies (Fogwell et. al., 1977; Lequin, 2005). After numerous attempts, it was determined that for the analyte being investigated (LH), the sandwich assay was the most successful. The sandwich assay was dynamic over 3 orders of magnitude, with a range of 0.005 ng – 5 ng. Reported ranges of Luminex assays are variable, and depend heavily on analyte (Dudal et. al., 2014). In order to compare the Luminex assay to a conventional assay using the same antibodies, an RIA was run in parallel and animal samples were compared. The animal samples were taken from female cattle and sheep, after
administration of GnRH. Gonadotropin releasing hormone injections will result in a surge of LH release from the pituitary gland, and can cause a sustained rise in serum LH values 3-5 h post-injection (Thatcher et. al., 1993). These samples were used in order to ensure detectable levels of LH in the collected serum and plasma. As a negative control, commercially available fetal bovine serum (FBS) was also run in the Luminex assay. Commercial FBS has been demonstrated to have low to moderate levels of LH (Honn et. al., 1975). When compared to the RIA, the absolute values of the cattle serum samples differ from one another. However, it is important to note that the overall pattern of LH levels in the different cattle is comparable between both assays. Furthermore, comparing the RIA LH values to the LH values obtained in the bead assay suggests a strong correlation between both assays. This demonstrates that both assays measure similar levels of LH in the same samples. Additionally, it been suggested that in order to compare two different assays, if the same sample values fall within 30% of one another, the assays can be considered comparable (Dudal et. al., 2014). While only 2 of the 4 cattle samples fall within this range, the remaining samples are likely out of range due to the low LH values obtained from both assays. Regardless of these absolute values, the correlation between assays is an important indicator of the success of the LH bead assay. With the potential to multiplex hormonal analysis, the Luminex assay provides a slight edge over the RIA, with the potential ability to measure multiple analytes in a single sample, overall decreased hands-on work time, and increased sample throughput (Leng et. al., 2008). On the other hand, both a serum and plasma sample obtained from sheep resulted in almost identical LH values from the Luminex assay and the RIA. There could be a number of potential explanations for the detection differences observed in serum and
plasma, including antibody specificity for bovine and ovine samples, or differing matrix effects in cattle and sheep serum (Rosenberg-Hasson et. al., 2014). However, the particular antibodies used in the Luminex assay have been previously validated for measurement of ovine LH (Adams et. al., 1975). It has been suspected that bead based assays have the potential to be affected by serum matrix effects at varying degrees (Leng et. al., 2008; Dudal et. al., 2014). While the differences were not specifically investigated in the present experiments, it is plausible that the different proteins present in the serum of cattle and plasma of sheep may influence the Luminex assay in ways that are not observed in the RIA.

Regardless of the matrix effects, the Luminex assay appears to be a feasible assay for measuring bovine and ovine LH in serum or plasma samples.

An additional objective of this experiment was to establish a routine methodology for setting up a new analyte for detection on the Luminex platform. Once the methodology was established, a second analyte was attempted on the Luminex platform (bovine GH, bGH). However, the lack of an available monoclonal/polyclonal antibody set made the development of this second analyte extremely difficult. Therefore, it was determined that in order to advance multiplexing in a way useful to livestock investigations, it would be important to consider an alternative assay type that utilizes less antibodies in the assay design. Therefore, the Luminex platform was abandoned in order to attempt a third assay type for hormonal analysis. This third assay provided an additional advantage aside from utilizing a different antibody configuration: the ability to measure analyte levels without reliance on a proprietary technology, which may be considered a drawback with existing platforms. Utilizing a machine more readily available in modern molecular biology labs
may prevent a similar outcome as the Illumina platform – abrupt withdrawal of machine support. Based on these considerations, an experiment was designed to test the hypothesis that a multiplex assay could be developed based on an assay first described by Sano et. al., in 1994: immuno-polymerase chain reaction (iPCR).
CHAPTER V

DEVELOPMENT OF A NOVEL IMMUNOASSAY FOR SIMULTANEOUS DETECTION OF ENDOCRINE ANALYTES

MATERIALS AND METHODS

Assay Reagents

For the iPCR assay, two major assay designs were tested: assays similar to an ELISA utilizing LH reagents, and a biotin detection assay. The ELISA-like assays were tested using reagents for LH detection. Monoclonal LH antibody was provided by Dr. Jan Roser, while polyclonal LH antibody was provided by Dr. Tom Adams (TEA #35). LH standard (AFP 11743-B) was obtained from the National Hormone and Peptide Program (Dr. A. F. Parlow). Biotinylation kit was from Pierce (Rockford, IL) and used according to manufacturer’s instructions. Biotinylated oligonucleotides were manufactured by IDT (Coralville, IA). The oligonucleotide was a 71-mer, previously used in a successful iPCR experiment (Sims et. al., 2000). The sequence was as follows: 5’ –

CCAACCTCCTGTCCACCAACTCTTTCTGATGATCTGCGGCTTTATGTTGGTTCTCCTGGACTGGAA – 3’, with the biotin molecule attached at the 5’ end.

Forward and reverse primers were also manufactured by IDT. In addition to the 5’ biotinylated oligonucleotide, the same oligonucleotide was also synthesized with an amine group at the 5’ end, for direct conjugation of oligonucleotide to antibody. A commercially available conjugation kit was purchased from Solulink (Antibody-
oligonucleotide All-in-One Conjugation Kit, San Diego, CA), and used according to manufacturer’s instructions. SYBR Green PCR Master Mix was obtained from Life Technologies (Applied Biosystems SYBR Green PCR Master Mix, Grand Island, NY). A commercially available human LH ELISA kit was purchased from Enzo Life Sciences (Farmingdale, NY). For the biotin detection assay, biotin was obtained from Sigma (St. Louis, MO), and streptavidin-coated polystyrene plates (both standard and high capacity) were obtained from Pierce (Rockford, IL). According to the manufacturer, the standard binding plates had the capacity to bind ~10 pmol biotin/well, while the high binding plates had the capacity to bind ~125 pmol biotin/well.

Assay Procedures

A number of ELISA-like assays were tested with iPCR. The objective was to identify a solid support structure that would be compatible with the PCR step in the protocol. Previous experiments have used polypropylene plates modified for protein attachment, as polypropylene generally is not a suitable surface for protein binding (Lind & Kubista, 2005; Niemeyer, Adler & Wacker, 2007; Goebel-Stingel et. al., 2011). The following solid supports were investigated: polypropylene PCR plates (Bioexpress, Kaysville, UT) modified for protein attachment via plasma ion treatment and glutaraldehyde treatment; magnetic microspheres coated with streptavidin (NEB, Ipswich, MA); and streptavidin-coated polystyrene plates (Fisher, Rockford, IL). For each of these experiments, the general assay design was similar: the monoclonal capture antibody was adsorbed onto the surfaces, either through passive adsorption or binding with streptavidin. Once the capture antibody was immobilized, LH standard was added at various masses, from 500 ng to 0.005 ng of LH/well. From here, the methods slightly diverge, depending on the detection
method tested. Initially, a streptavidin bridge was employed, similar to the observations of Ruzicka et al. In this configuration, the detection antibody was conjugated with biotin, and the oligonucleotide was modified with a biotin molecule on the 5’ end. These components were then added simultaneously to the assay and allowed to self-assemble. In the second method, the detection antibody was covalently conjugated to a 5’ amine modified oligonucleotide using the commercially available conjugation kit. This detection molecule was then incubated with the assay for 1 hr before PCR analysis. In all tested assays, wash steps with TETBS (1x Tris-NaCl, 5 mM EDTA & 0.05% Tween 20) were performed between each incubation step, and included a final wash before subjecting the surfaces to PCR. For the polypropylene PCR plates and the magnetic beads, after the final incubation, the plate or beads were immediately subjected to PCR. The PCR master mix was added on top of the assay reagents that should have been immobilized to the bottom of the plate or on the bead surface. Quantitative PCR was carried out with an ABI Prism 7500 System (Life Technologies, Grand Island, NY). The reaction was cycled under the following conditions: an initial incubation at 95°C for 10 min to activate the polymerase, and 30 cycles of a 95°C incubation for 15 sec as a denaturing step, and a 60°C incubation for 1 min as an annealing and extension step. After completion of PCR, select samples were subjected to agarose gel electrophoresis in order to confirm the PCR products were the correct size. Due to the small product size, PCR products were run on a 3.5% agarose gel. Twenty µl of PCR product was loaded into wells, and the gel was run at 140V for 1 hr. For the biotin detection assay, streptavidin-coated polystyrene plates, both high and standard binding were washed three times with assay buffer (TETBS). Biotin standard, from 500 ng to 0.005 ng and a fixed
number of biotinylated oligonucleotides (3.67 x 10^{10}) were added simultaneously to the streptavidin-coated plates. In this assay configuration, the biotin and biotinylated oligonucleotide competed for binding to streptavidin on the plate surface. This assay was incubated at room temperature under shaking conditions for 1 hr. After another series of washes (4x with TETBS for 30 sec and 3x with TETBS for 4 minutes, with shaking), 0.01 N of NaOH was added to all wells, and incubated for 15 minutes with shaking. In this step, the streptavidin was denatured in the presence of NaOH, without altering the detection DNA. With the denaturation of streptavidin, the bound oligonucleotides should be freed from the plate surface, allowing for detection via qPCR. Once the incubation was finished, potassium acetate (3M) was added to wells to neutralize the NaOH. From this solution, a 1 µl aliquot was amplified in the qPCR assay, under the same conditions as previously described.

*Polypropylene Plate Modification*

In an attempt to modify the polypropylene PCR plate surface for favorable protein binding conditions, two methods were attempted: plasma ion treatment, and glutaraldehyde treatment. For the plasma ion treatment, polypropylene plates were subjected to plasma ions in a vacuum chamber for 5 minutes. The plasma ion treatment etched the surface of the polypropylene, and this etching adds a type of static charge to the surface, increasing the capacity of the polypropylene to bind molecules such as proteins (Liston et. al., 1994). In the second method, glutaraldehyde treatment of the polypropylene surface, followed by immediate addition of the antibody to the polypropylene plate facilitates crosslinking between the plate and the antibody.
RESULTS

Schematic representations of the ELISA-like assays can be found in Figure 7. Representative curves from the ELISA-like assays are shown in Figure 8. For the different types of solid support structures tested, none of the assays were dynamic in their standard curves. The assays demonstrated minimal displacement of the oligonucleotide based on the PCR results. However, PCR product from the assays was the expected size (Figure 9). Due to the number of steps involved in these assays, any of the steps could have potentially contributed to the lack of displacement in the assay. The conjugation of the oligonucleotide to antibody; the bridging assay between the streptavidin and biotinylated antibody and oligonucleotide; the modification of the polypropylene plates; and the loss of magnetic beads during washing steps could all be contributing factors to the failure of the assay. As a specific example, the oligonucleotide conjugation of the antibody, which was carried out according to kit instructions, resulted in much lower yields than the kit suggested. Additionally, in the case of the magnetic bead assay, during washes, many beads were aspirated out of the plate before analysis, complicating analysis, due to the varying number of beads within each individual well. Similar observations were reported with other bead based assays. Due to the number of steps that could have influenced the assay negatively, the assay was ‘stripped-down’ to its basic components in order to determine if a standard curve could be developed using only the most basic assay components, which in this case were biotin and streptavidin. This was
the basis for the biotin detection assay. The simplicity of the biotin detection assay afforded the opportunity to evaluate some of variables that may have contributed to the failure of the ELISA-like assays. These variables included washing conditions (Figure 10), density of molecules on the plate surface (Figure 11), as well as effectiveness of biotin conjugated oligonucleotide.
Figure 7. Assay configurations investigated with the iPCR assay. (A) Various assay designs were tested on polypropylene PCR plates, due to their compatibility with the thermal cycler. Because polypropylene does not generally bind proteins well, assays were designed to attempt to manipulate the polypropylene surface to make it amenable to protein binding, including plasma-ion and glutaraldehyde treatment of the polypropylene plates. In 1., a biotin-streptavidin-biotin ‘bridge’ was used as the detection complex. Streptavidin and a biotinylated oligonucleotide were added after the initial incubation of antigen with antibody. Additionally, the polypropylene plates were subjected to plasma ion treatment In 2., a direct detection complex was employed though conjugation of an amine-labeled oligonucleotide to an antibody. Additionally, the plates were treated with
glutaraldehyde to facilitate crosslinking between the antibody and the plate. In 3., non-treated polypropylene plates were used to carry out a sandwich iPCR format, and in 4., the antigen was immobilized to the plate surface and probed with the antibody-oligonucleotide conjugate. (B) Magnetic beads coated with streptavidin were also used as a solid substrate. In this configuration, the assay closely mirrored a sandwich ELISA, with the magnetic beads subjected to PCR. (C) Polystyrene plates, with streptavidin immobilized to their surface were also utilized. In this assay, biotinylated LH antibody was incubated on the plate in order to orient the antibodies on the plate. Subsequently, a sandwich ELISA was carried out, using the direct detection method, in which the oligonucleotide was directly conjugated to the detection antibody.
Figure 8. Immuno-PCR for LH detection was not initially successful. In (A), polypropylene PCR plates were treated with plasma ions. It was assumed that plasma ion treatment of polypropylene plates would etch the surface, making it more amenable for protein binding. Unfortunately, there was very little difference in curve dynamics between plasma treated and non-plasma treated plates, suggesting that the plasma ion treatment did not influence protein binding to the polypropylene plate surface. In (B), an iPCR reaction for the detection of LH was carried out on streptavidin coated magnetic beads. In this configuration, biotinylated LH was incubated with the streptavidin coated magnetic beads at varying concentrations, then probed with an anti-LH antibody conjugated to an oligonucleotide. After 8 washes, the beads were subjected to PCR. The
PCR detected differences in oligonucleotide concentrations; however it was not consistent across the varying biotin-LH concentrations. It was expected that as biotin-LH concentration decreased, the corresponding Ct values would increase, due to decreased presence of antibody-oligonucleotide conjugate on the bead surface. In (C), a streptavidin-coated polystyrene plate was first used to carry out the ELISA portion of the assay. In this setup, unlabeled antigen was co-incubated with a fixed concentration of biotinylated antigen and antibody-oligonucleotide conjugate. After a 1 hr incubation, the plate was washed, heated to 95°C, and a 1 µl aliquot was removed from the plate for PCR. This protocol did not result in a curve for detection of LH. (D) LH protein was adsorbed to an untreated polypropylene PCR plate and probed with an oligonucleotide-conjugated detection antibody. Similar to the other designs, this assay configuration did not result in a curve for LH detection.
Figure 9. Image of a 3.5% agarose gel of PCR products. The detection oligonucleotide was 71 bp in length. 20 µl of PCR product was run on the gel for 1 hr at 140 V, and was at the expected location in the gel.
Figure 10. Effect of wash frequency on the detectability of the antibody-oligonucleotide conjugate. A fixed concentration (green triangle) of antibody-oligonucleotide was added to wells containing streptavidin-coated magnetic particles and biotinylated LH. Each well was subjected to an increasing number of washes, and the well contents were analyzed by PCR (blue diamonds). Additionally, an aliquot of the wash solution was also analyzed by PCR (red squares). It was expected that with each subsequent wash, the Ct of the wells would gradually increase, with more non-bound antibody-oligonucleotide being washed away. Similarly, it was expected that the Ct of the wash would also increase with each wash, as the amount of antibody-oligonucleotide being washed off the plate and beads decreases with each subsequent wash, driving the Ct higher. These results suggest that there is a level of non-specific oligonucleotide that cannot be washed from the plate surface in 12 wash steps.
Figure 11. The influence of mass of antigen on the plate on iPCR results. In this assay configuration, immobilized LH competed for antibody binding with standard LH in solution. The antibody was conjugated to oligonucleotide, and after washing, only those antibodies that bound the plate LH should remain. The plate was then subjected to PCR. In this configuration, the assay was not successful. It is difficult to determine if the assay setup or the amount of LH theoretically bound to the plate surface may be the cause for assay failure.
Figure 12. A biotin detection assay curve. In this assay, biotin and biotinylated oligonucleotide were co-incubated on commercially available polystyrene plates pre-coated with streptavidin for 1 hr. After a series of 7 washes to remove non-specific oligonucleotide binding, 0.01 N NaOH was added to the plates and incubated for 15 mins. The NaOH was then neutralized by the addition of potassium acetate. A 1 µl aliquot was used for qPCR analysis (blue diamond). The red square and green triangle represent the number of oligonucleotides added directly to the PCR and not competed with free biotin, respectively.
As observed in Figure 12, the biotin detection assay was much more successful in generating a dynamic standard curve than the ELISA-like assays. For the biotin detection assay, the components included a commercially available polystyrene plate with streptavidin adsorbed to its surface, a biotin standard, and the biotinylated oligonucleotide, which was commercially produced. This assay setup was a competition assay, with the biotin standard competing for binding of streptavidin on the plate with the biotinylated oligonucleotide. Therefore, the assay required only a single incubation, and a single wash step, the simple combination of which decreased potential sources of variation. However, this assay also introduced a new source of variation not observed previously: due to the necessity of the polystyrene plates, the oligonucleotide had to be freed from the plate surface in order to be measured via PCR. Therefore, we modified the well-known alkaline lysis step of plasmid preparation (Birnboim & Doly, 1979; Birnboim, 1983; Ehrt & Schnappinger, 2003) for use in the biotin detection assay. After the washing step to remove non-bound components, a 0.01 N solution of NaOH was added to the wells. This step targeted the streptavidin molecule, denaturing it, without adversely affecting the oligonucleotide. After a 15 min incubation with NaOH, without shaking (as demonstrated in Figure 20), a solution of potassium acetate (3M) was added to neutralize the NaOH. This NaOH/potassium acetate solution was tested in PCR in order to determine if the solution had any negative influences on PCR (Figure 13). The results indicated that NaOH/potassium acetate did not negatively influence PCR results. Once it was determined that the assay components did not negatively influence PCR results, the assay reagents and steps in the assay were investigated, in order to determine if and
Figure 13. Correlation of Ct values obtained from the PCR of detection oligonucleotide diluted in 10 mM Tris, and Ct values obtained from the PCR of detection oligonucleotide diluted in 0.01 N NaOH and potassium acetate. The NaOH/potassium acetate solution does not appear to interfere with the PCR detection of the oligonucleotide ($y = 0.81x – 0.3683; r^2 = 0.9664$).
how these variables influence the assay results. Initially, the plates that were used with
the assay were classified as high-binding plates, with the capacity to bind ~125 pmol of
biotin/well. These curves, while acceptable, were not very dynamic or sensitive (Figure
14). Therefore, standard capacity binding plates (~10 pmol biotin/well) were employed,
and the standard curves were visually compared. It was determined that the standard
capacity binding plates provided a more dynamic standard curve than the high binding
plates (Figure 15). Additionally, the binding capacity of the standard capacity
streptavidin plates was verified in-house with the biotinylated oligonucleotide that was
used in the biotin experiments (Figure 16). Therefore, for further experiments, the
standard capacity streptavidin plates were employed.

One observation that was a cause for concern was that wells that were subjected to the
assay, but not treated with NaOH/potassium acetate resulted in a dynamic curve as well
(Figure 17). Thus, we attempted to determine if this result could be altered. Due to the
nature of the assay, the washing step was considered to be the step that would most
influence this result. An experiment was designed to test the effect of plate shaking on the
curve in the presence and absence of NaOH/potassium acetate. Four plates total were
tested, and these plates either were or were not shaken at different washing steps, as well
as during the final NaOH incubation step. Figure 18 is a schematic representation of the
experiment. Standard curves both treated and not treated with NaOH were run
concomitantly in the same plate. The tested groups were as follows: Wash step with plate
shaking and NaOH step with plate shaking (shake/shake); wash step with plate shaking
and NaOH step without plate shaking (shake/no shake); wash step without plate shaking
and NaOH step with plate shaking (no shake/shake); wash step without plate shaking and
NaOH step without plate shaking (no shake/no shake). The results of this experiment are summarized in Figure 19 and suggest that plate shaking during the wash but not during the NaOH step was required in order to generate the most dynamic standard curve, and minimize oligonucleotide detection in samples that were not treated with NaOH. Collectively, the data has provided experimental evidence of optimal assay design for the biotin detection assay. The results suggest that after co-incubation of biotin standard at varying concentrations, and a fixed concentration of biotinylated oligonucleotide, washing the plate 4 times with TETBS for 30 sec, and then 3 times with TETBS for 4 minutes while shaking at room temperature, coupled with no shaking of the plate during the 15 min incubation with NaOH results in the most dynamic and sensitive curve produced, while simultaneously minimizing the non-treated curve. With the optimum steps for a successful assay determined, two additional experiments were conducted in parallel, in order to further elucidate whether an oligonucleotide could serve as a novel detection mechanism in an immunoassay format. In the subsequent experiment, the biotinylated oligonucleotide was substituted into a commercially produced ELISA. In this case, the kit reagents would provide for a streptavidin ‘bridge’ that would link a biotinylated antibody to streptavidin, then to the biotinylated oligonucleotide for detection purposes. Essentially, the ELISA was carried out according to kit directions, until the addition of substrate. At this step, the biotinylated oligonucleotide was added and incubated in place of the substrate. For the remainder of the assay, the steps were carried out according to the biotin detection assay. The ELISA wells were washed 7 times according to the wash sequence previously described, then treated with NaOH for 15 mins and neutralized with potassium acetate.
Figure 14. Biotin detection assay carried out in high binding streptavidin plates. Limit of detection in the high binding plates was 10 ng/ml of biotin.
Figure 15. Biotin detection assay carried out in standard binding streptavidin plates. Limit of detection in the standard binding plates was 1 ng/ml of biotin, one order of magnitude higher than the high binding streptavidin plates.
Figure 16. Biotin saturation point of standard binding streptavidin plates. According to the manufacturer, the plates had the capacity to bind approximately 10 pmol biotin/well. 10 pmol biotin is equivalent to $1.5 \times 10^{13}$ biotin molecules. With a single biotin molecule attached per oligonucleotide, the number of oligonucleotides should be equivalent to the number of biotin molecules present. These results demonstrate that the results obtained in-lab are similar to those reported by the manufacturer. The maximum number of oligonucleotides that these plates can bind was $2.35 \times 10^{12}$. 
Figure 17. Absence of oligonucleotide release treatment also produces a curve. Two identical biotin curves were assayed in the polystyrene plate. One was treated with NaOH (blue diamond), while the other was treated with assay buffer (purple x), and both resulted in a similarly dynamic curve. This may be due to non-specific binding, or mechanical dislodging of biotinylated oligonucleotide from the streptavidin on the plate.
Figure 18. Schematic of the experiments to determine the influence of shaking steps in the biotin detection assay. For each plate, treatments were divided between NaOH treatment and no NaOH (- NaOH) treatment. The biotin assay was carried out as described and shaking treatments carried out according to the schematic. The shaking occurred at the washing step (First shake or no shake) and then at the NaOH treatment (Second shake or no shake).
Figure 19. Results of plate shaking assays. Based on these results, the optimum assay configuration was a shaking step during the incubation of the assay components, but to not shake during the NaOH incubation. While this curve appears to be less dynamic than the No Shake/Shake treatment, it is important to note that the no NaOH treatment is too similar to the NaOH treatment to select the No Shake/Shake configuration.
The results of this experiment suggest that it may be possible to substitute a biotinylated oligonucleotide in an ELISA assay for sensitive detection of an analyte (Figure 20). Finally, testing the ability of the assay to measure biotin in a biological sample was investigated. Initially, animal serum samples (bovine, ovine, and porcine pooled sera) were added directly to the assay, at varying volumes. The biotin detection assay was unable to detect biotin levels in the serum samples (Figure 21). The next attempt treated the animal sera as a matrix, in order to determine if the results were due to unknown matrix effects, or due to low levels of endogenous biotin in serum. The same sample pools were spiked with a known amount of biotin (10 ng), and were run similar to the standard curve. The results of this assay suggest that the biotin assay, using an oligonucleotide as a detection molecule, can measure biotin in a biological sample (Figure 22).
Figure 20. Substitution of the biotinylated oligonucleotide in a commercially available ELISA can be a successful detection. (A.) represents the OD values obtained from the colorimetric detection of the assay, to ensure the assay was working as described. In (B.), the substitution of the biotinylated oligonucleotide in place of the TMB Substrate results in a dynamic standard curve.
Figure 21. Detection assay of biotin in animal sera. Sera from 3 different species was competed with the biotin-oligonucleotide in the biotin detection assay. The results of this assay suggest that endogenous biotin levels in these species are not detectible by the current iPCR method.
Figure 22. Detection assay for biotin recovery in cow serum. In order to determine if the low detection of biotin in serum samples was the result of low endogenous biotin levels, and not potential serum matrix effects, cow serum was spiked with 10 ng of biotin and subjected to the biotin detection assay. These results suggest that serum matrix effects are minimal in the biotin detection iPCR assay.
DISCUSSION

While the initial objective of this experiment was to simply adapt the iPCR assay for hormonal analysis, it subsequently became an experiment that aimed to determine the ideal mechanics for the iPCR assay. While there are numerous references to the success of the assay (Liu et. al., 2014; Bonot et. al., 2014), it was a struggle in the current experiments to establish a functional assay without numerous issues. However, there are additional reports of the iPCR being cumbersome and difficult to set up (Barletta, 2006; Hansen et. al., 2014). Regardless of the reports, the results of the present experiment suggest that iPCR could be a feasible assay for analyte detection and multiplexing.

In the initial iPCR descriptions, chimeric detection molecules and end-point PCR were utilized, which differed considerably from the current experiments. Immuno-PCR experiments subsequently described direct conjugation of oligonucleotide to antibody and/or using a streptavidin ‘bridge’ to link a biotinylated antibody to a biotinylated oligonucleotide (Barletta, Edelman & Constantine, 2004), which provided the reference and starting point for the current series of experiments. The availability of commercial kits for conjugating oligonucleotide to antibody, as well as kits for biotinylation of protein were employed in order to quickly establish an iPCR assay with readily available commercial reagents. Initially, once the iPCR assay was adapted, the goal was to utilize iPCR in a multiplex format. To do this, DNA would be used as the ‘barcode’ to differentiate one analyte from another. While there is little evidence of multiplexed iPCR
analysis at present, a study conducted in the early 1990s demonstrated varying DNA length as a method for multiplexing (Hendrickson et. al., 1995). In our proposed method, different antibodies or antigens – depending on assay design – would be conjugated to different oligonucleotides, and detection would be carried out through qPCR. Once the specific products were amplified, they could potentially be sequenced to determine the specific DNA sequences present in the sample, and correlate those sequences with the analyte measured. However, as evidenced by the results, there was some degree of difficulty in adapting the iPCR assay for livestock hormonal analysis. The difficulty in adapting the assay has been suggested previously, and may partially explain why this technique is not currently in widespread use (Barletta, 2006). While it could not be determined exactly where the assay failed, there are a number of steps and variables which may have contributed to the failure of the initial iPCR adaptation. Some of these variables include: the conjugated antibody-oligonucleotide, the lack of availability of specialized PCR plates for antibody adsorption (Niemeyer, Adler & Wacker, 2007) and/or the success of washing steps between assay steps. In the initial attempt of iPCR adaptation, these variables were tested and met with limited success (Figure 8).

Regardless of the outcome, the biotin-streptavidin interaction should be unaffected. Therefore, the pitfalls of the initial iPCR assay were parlayed into a stripped down experiment to determine if a simple iPCR assay was possible to adapt. Using commercially available reagents and plastics, as well as an adapted cell lysis protocol, we were able to establish a very basic iPCR assay for the detection of biotin. In this approach, a polystyrene plate pre-adsorbed with streptavidin was used for the ELISA portion of the assay. In this format, the biotinylated oligonucleotide competed for
streptavidin binding with a biotin standard that was added simultaneously. The competition incubated for 1 hr., as it has been suggested that biotin-streptavidin binding is complete within the range of 30 min – 1 hr (Piran & Riordan, 1990). Once the ELISA assay was completed, the polystyrene plate was treated with 0.01 N NaOH. This NaOH treatment comes from the modified cell lysis protocol – The NaOH denatures the streptavidin molecules without damaging the detection oligonucleotide, which theoretically should release the complexes from the plate. After a short, 15 min. incubation in NaOH, the reaction is then neutralized with the addition of potassium acetate. After the extensive washing protocol, it is logical that the polystyrene plate should contain only the bound assay components. After neutralization of the potassium acetate, a 1 µl aliquot is used for qPCR analysis. While this introduces a new step that may increase variation, it also removes the other sources of variation that may have contributed to the issues in the earlier iPCR experiments. Additionally, the assay volume in the polystyrene plates is useful for sampling repeatability, as the large volume allows for multiple investigations of the assay.

While using biotin as the analyte to be detected may not be advantageous from an analytical perspective, it has advantages from the technical perspective. The small size of biotin (244.3 Da) makes it usable as a molecule for conjugation to an oligonucleotide. Additionally, the strength of the bond between biotin and streptavidin has been described as “the strongest non-covalent biological interaction known” (Holmberg et. al., 2005). Therefore, using this strong and specific bond would be the most efficient method to determine success of the iPCR assay. Additionally, altering the number and type of variables in the assay design (addition of transfer but subtraction of plate and antibody
conjugation) keeps the assay simple and provides a working template that can be utilized when establishing additional assays that require additional variables.

In order to determine if the oligonucleotide was a feasible method of detection in hormone-based assays, we next substituted the biotin detection assay method in a commercially available ELISA. The purpose of this experiment was to determine if the oligonucleotide could be used as a detection molecule in a hormonal detection assay. In this configuration, the biotinylated oligonucleotide was used in place of the TMB substrate in a commercially available ELISA for the measurement of human LH. Colorimetric assays have been described as being less sensitive compared to other assay types (Tang & Hewlett, 2010). While improving sensitivity may not have been a stated objective of the experiment, it has been documented that iPCR may be capable of improving assay sensitivity (Saito et. al., 1999; Barletta, 2006). Regardless of sensitivity data, it is important to note that the substitution assay successfully measured LH from the commercially available kit. Therefore, utilizing an oligonucleotide as a detection molecule in a hormonal detection assay appears to be possible and feasible. It is probable that the iPCR assay can be adapted for measurement of both small molecules and larger proteins, although extensive optimization is necessary before use, which may or may not be advantageous in the future.

The importance of the iPCR assay cannot be understated. The previous iPCR experiments have provided evidence that a DNA sequence can be used as a detection molecule for a hormonal assay. While RIAs and ELISAs are still in widespread use, with the increased demand for complex data, their usefulness may be overshadowed by the necessity of
multiplexing (Olsson et. al., 2005). While bead-based platforms attempt to meet this demand, they still have some limitations. For example, the bead-based assays are limited by the number of bead sets that are available for use – currently 100 sets are available through Luminex, one of the many bead-based platforms in use. An interesting possibility with iPCR is that the detection molecule has the potential for a much greater number of detection molecules ($4^4$ per base per length of oligo). While this may not serve useful in the present, it could prove valuable as multiplexing evolves.
CHAPTER VI

SUMMARY AND IMPLICATIONS

In the current set of experiments, the overarching goal was to develop an assay methodology that could successfully carry out multiplexed detection of multiple analytes in livestock samples. A number of setbacks made this objective difficult to achieve with speed and accuracy. The initial experiments attempted to meet this objective through the use of proprietary instrumentation and reagents. The advantages of these systems are that the details and variables had already been optimized by the companies, and the materials necessary for the assays were readily available from the companies that have developed the technologies. However, during these experiments, it became readily apparent that it may not be in the best interest of researchers to solely rely on a single company for reagents and technical expertise, as evidenced by the decision of Illumina to remove support for the BeadXpress machine. Furthermore, while the Luminex assays have pre-made kits available for many human and rodent applications, livestock applications are limited. Therefore, some investigation into assay development was required in order to develop assays specific for livestock hormonal analysis. While this increased labor, it also provided the advantage of potentially developing various multiplexed assays that could be highly specialized for particular phenotypes or other investigations. However, most successful Luminex assays, including the assay developed in the current experiment, utilized a sandwich assay format. This presents a further complication for livestock hormonal analysis, as quality livestock antibodies, both monoclonal and
polyclonal are commercially limited. While it is indeed possible to generate the antibodies in-house, the time and resources required for each analyte to be measured may outweigh the usefulness of the bead-based multiplex assay, especially if antibody pairs are necessary. While there was no indication of Luminex removing support for their multiplex bead-based assay, it remained a concern that working with proprietary technology may result in losing support of that technology if it ever reached end-of-life. Technology and antibody limitations resulted in the exploration of an alternative assay format. Immuno-PCR provides the ability to multiplex hormonal analysis without the use of proprietary technology. Thermocyclers are widely available in today’s molecular biology laboratories and PCR continues to be a commonly used protocol in molecular biology. However, the current experiment indicated that there are many variables that influence the success of iPCR. While numerous reports exist on successful iPCR assays, many do not directly address these variables. The results suggest that these variables can greatly influence the outcome of the assay. Previous work in iPCR suggests this may be a common occurrence during iPCR development (Lind & Kubista, 2005). However, a very basic iPCR assay for the detection of biotin was developed in the present experiment. This assay was useful as a proof-of-concept assay, and also provided a simplistic method of exploring some of the complex variables in iPCR development. The reported methods should be easily adaptable for hormonal analysis. While not all variables were explored and resolved, enough of the assay components have been investigated to make a successful biotin detection assay. Additional work with re-introducing the immunoassay component of iPCR will be necessary to further explore the contributing variables in the assay. The ability of the iPCR to detect biotin in a biological matrix suggests that sample
variability may be less of an issue compared to the bead based assays. Finally, substituting the detection oligonucleotide in a commercially available ELISA for LH resulted in the successful measurement of the ELISA LH standard via iPCR. The iPCR may be slightly more sensitive than the ELISA for LH detection. This result provided positive evidence that iPCR may be able to measure analytes of interest.

In order to move iPCR and multiplexing forward, more work needs to be done with the iPCR methodology. Investigating other solid substrates, such as polycarbonate and their ability to bind molecules may be necessary to combat non-specific binding that was prevalent in the current experiments. Conjugation of antibody and oligonucleotide is the most direct method in iPCR, and should be considered over streptavidin bridging in future iPCR investigations, as it decreases total hands on time with the assay. Finally, other assay designs should be considered when exploring future iPCR assays. Of particular interest is conjugation of an analyte of interest to an oligonucleotide and using it in a competitive inhibition assay format. This is analogous to an assay that would closely mimic the biotin detection assay and may provide the greatest chance for success.

Regardless of the detection method used, the development of multiplexed hormonal analysis is necessary for a number of reasons. In animal studies, hormonal profiles are often generated in order to examine the relationship between endocrine signaling and phenotype (Möstl & Palme, 2002). Furthermore, a study in 2007 established phenotypic relationships between carcass characteristics of cattle and three hormones associated with growth and fat depositions (Brandt et. al., 2007). The ability to measure multiple hormones or analytes simultaneously could be useful as predictive indicators of livestock
performance. The current method for multiple hormonal analysis requires a lot of time, sample, and labor. Multiplexed analysis lowers all these requirements, which may also lead to generation of additional multiplexes and phenotypes at a more rapid rate than is currently occurring.


Elizabeth Ashley Benavides is originally from McKinney, Texas. She received a B.S. in Agriculture and an M.S. in Animal Science from Texas A&M University – Kingsville, in Kingsville, Texas. Her thesis was entitled “The effect of season and milk source on puberty and endocrine parameters in doe kid goats”. She relocated to Columbia, Missouri in 2010 to pursue her Ph.D. under the direction of Dr. Duane Keisler. She has accepted a position as an Assistant Professor at Texas State University in San Marcos, Texas, where she will be responsible for teaching introductory and advanced animal science courses to undergraduate and graduate students.