DESIGN OF AN IN-HOUSE INSULIN ELISA

BY

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Chapter 1: Introduction

Purpose:

Insulin is a hormone synthesized by beta cells in the pancreas. One of its main functions is to bind to the insulin receptors on a cell which then activates glucose transports in the cell. This in turn allows for glucose to move through the cell wall and be processed by the cell\textsuperscript{1-2}. Type 1 diabetes (T1D), previously known as insulin-dependent diabetes mellitus (IDDM) occurs when the body is unable to produce insulin. Millions of dollars of research goes into finding treatments and cures for this disease. Many approaches involve isolating healthy beta cells in the clusters of cells called islets from the pancreases of animals or human donors. These islets can then be implanted into a recipient and replace the damaged islets in an attempt to reverse or lessen the symptoms of T1D\textsuperscript{3}. In order to quantify the amount of insulin produced by the beta cells, different assays can be used to measure the concentration of insulin. Such assays include ELISAs and RIA. Both of these assays are used in various labs for measuring hormones including insulin. For a long time our lab has specifically been using RIAs to quantify the concentration of insulin in experimental samples. This assay has been ideal because it is sensitive enough to detect picogram concentrations and is relatively inexpensive to run per sample tested. A few drawbacks to the RIA are that the assay takes two days to perform and more importantly, involves the use of radioactive materials. For these reasons, there is significant interest in developing an in-house insulin ELISA that will be faster in generating data than the RIA, be just as sensitive, while being about half of the cost of a commercial ELISA, and involve no radioactive materials.
Statement of Hypothesis or Research Question:

Is it possible to develop an in-house insulin ELISA that will have comparable detection sensitivity as the RIA method with decreased cost compared to standard commercial ELISAs?

Definitions:

ELISA: Enzyme-Linked Immunosorbent Assay
sELISA: Sandwich Enzyme-Linked Immunosorbent Assay
RIA: Radioimmunoassay
Islet: Specialized group of cells in pancreas that make and secrete hormones.
Beta Cells: Found within islets and produce insulin.
Insulin: Hormone made by pancreatic beta cells that helps the body use glucose to regulate metabolism.
Antigen: Any substance that causes the immune system to produce antibodies against it.
Monoclonal Antibody: Antibody that has affinity to one epitope or binding site.
Polyclonal Antibody: Antibody that has affinity to multiple epitopes or binding sites.
Surfactant: Compounds that lower surface tension between two liquids.
OD: Optical Density
TMB: Tetramethylbenzidine
HRP: Horseradish Peroxidase
PBS: Phosphate Buffered Solution
IgG: Immunoglobulin G antibody.

P Ab: Primary Antibody

2 Ab: Secondary Antibody

C Ab: Capture Antibody

D Ab: Detection Antibody

Chapter 2: Literature Review

RIA:

S. Berson and R. Yarlow first described the general concept and reactions of the RIA to measure insulin in 1968. Initially they were interested in tracking insulin once it entered the body through injection. They did this by labeling the insulin with a radioactive isotope. Later on, they discovered that the radiolabeled insulin would not bind as favorably to the detection antibodies as nonlabeled insulin. This led to the development of the RIA. Radioimmunoassays work in a similar manner as the competitive ELISA. In the RIA the target antigen is labeled with a radioactive compound, usually 125-Iodine (125-I), and bound to a known concentration of a specific antibody. An unknown sample of the antigen is then added to the mixture, initiating a competitive reaction. The unlabeled antigen will displace some of the radio-labeled antigen creating a ratio of labeled to unlabeled antigens (Figure 1). The signal given off by the unbound labeled antigen is read using a gamma counter. When the signal increases, there is more unbound radioactive antigen, which means there is more unknown sample bound to the antibodies. Once this reaction is completed, a standard binding curve is created using known concentrations of the unlabeled antigen. This is an extremely simple and sensitive assay that can be relatively inexpensive. For these reasons, it is a very common technique.
for detecting minute concentrations of hormones, such as insulin, in the blood supply or a sample. A major problem with the RIA is that it involves working with radioactive samples. This is never desirable and, if not monitored, can lead to complications in the thyroid, where iodine is stored.

Figure 1: Illustration of RIA steps

ELISA:

The ELISA was first developed in the early 1970s by Peter Perlmann. Later on, Pool et al. developed an ELISA that used beads as tracers to increase the sensitivity and allow for antiserum reactivity measurements to be taken. This was directly related to diabetes research because only serum samples were able to be taken before this
discovery. The ELISA is a common assay used to measure the concentration of a given analyte. Antibodies will bind to proteins and immobilize them to a polystyrene plate. Unbound reagents are easily washed away, which makes the ELISA so easy to perform and optimize to desired specifications. The bound reagents are “tagged” by a detection enzyme which is linked to an antibody. Once “tagged”, a simple reaction with a substrate will cause the enzymes to emit a colored signal. There are many different approaches that can be taken to produce this signal and quantify the analyte concentration of a sample. The direct assay method involves immobilizing the antigen of interest, such as insulin, to the plate and then binding a primary antibody conjugated with the detection enzyme to the antigen (Figure 2a). Then, the substrate can be added, and the concentration of the antigen can be calculated by measuring the optical density (OD) of the signal. The indirect assay method is the same as the direct method except a secondary antibody conjugated with the enzyme is bound to the primary antibody (figure 2b). While this seems more complicated, it is actually a more common method for many reasons. Conjugated secondary antibodies are more widely available. Sensitivity is increased because multiple secondary antibodies can bind to each of the several epitopes on a primary antibody, therefore increasing signal amplification. It is also more versatile because many primary antibodies can be made in one species while using the same secondary antibody for detection. The sandwich method is another ELISA format that binds a capture antibody to the plate first, then the antigen binds to the capture antibody, then a detection antibody is bound to the antigen (Figure 2c). From here, the detection antibody can either be conjugated or another conjugated secondary antibody can be bound additionally. The sandwich ELISA, (sELISA), is the most efficient procedure
because of its high sensitivity, which is due to the multiple antibodies used, and it is robust because the capture antibody binds strongly to the plate. Lastly, a competitive method can be used when the antigen is small and only has one epitope. This method binds a capture antibody to the plate like the sandwich, but then binds both a conjugated secondary antibody and the antigen to the capture antibody at the same time. The antigen and antibody therefore “compete” for binding sites which can indirectly measure the signal, because the more signal there is, the less antigen has bound to the capture antibody (Figure 3). All of the signals that are produced from these various formats are compared to signals emitted from standards with known concentrations.

Figure 2: The direct assay (a), indirect assay (b), and capture or sandwich assay (c) are three different types of ELISAs that use enzymes (E) linked to antibodies to measure the concentration of a specific antigen (Ag).

Figure 3: Illustration of competitive ELISA. The secondary antibody competes with the antigen for binding space on the primary antibody.
Chapter 3: Competitive Insulin ELISA

Intro: The first method to be examined was the competitive ELISA. The rationale was that our experimental sample concentrations of insulin that were detected using the RIA were extremely small as they were in the range of 50-500 pg/ml. The in-house ELISA would need to be able to detect insulin concentrations in that range in order for it to be useful to our group. An ELISA that measured the concentration of insulin directly, such as “sandwich” or “direct”, would give back a faint signal that would not be distinguishable from any background noise. Also, using an anti-insulin antibody paired with an antibody that would specifically target it would be easier and less expensive than two paired anti-insulin antibodies, which is required for a sandwich ELISA.

Materials and Methods:

Competitive ELISA Insulin Assay Protocol

Materials:

- 96 Well Plate (Fisher Cat# 08-772-53) x1
- PBS (Made in-house) 200 uL/well/wash
- TMB substrate (Immunochemistry Cat# 6276) x100 uL/well
- Stop Solution (Immunochemistry Cat# 6282) x100 uL/well
- 5x Coating Buffer (Immunochemistry Cat# 644) x100 uL/well of 1x solution
  - 8.4 mL diH2O to mix with Coating Buffer to dilute to 1x
- 5% Blocking Buffer (Abcam Cat# ab126587) x200 uL/well
  - 9 mL PBS with 1 mL 10x Blocking Buffer
  - Need additional for dilution with antibodies
Primary antibody (IgG monoclonal bred in mouse) (Abcam Cat# ab6995) 50 ul/well

Secondary antibody (Anti-Mouse IgG (H+L) polyclonal w/ HRP conjugate) (Abcam Cat# ab99603) 50 ul/well

Human Insulin (in-house 1mg/ml frozen samples) 50 ul/well

**Checkerboard Titration**: A common method for optimizing an ELISA is called a checkerboard titration (CBT). The basic principle is to have one variable changing along the rows of the plate, and another variable to change along the columns. This causes 96 different combinations of concentrations between two reagents allowing for quick optimization of an assay (*Figure 4*). If more than two variables need to be optimized, then multiple checkerboard titrations will need to be performed.

**Checkerboard optimization for antibodies**: (Skip step if already optimized)

1. Dilute Primary Antibody (IgG) from column 2 to 11 in coating buffer. Column 12 contains diluent only. Starting concentration should be 10 ug/mL in 100 uL volume total or 1/50 dilution. Dilution doubles at each column. (i.e. 1/50, 1/100, 1/200, etc…)

2. Cover plate and incubate overnight at 4 degree C or 2 hours at room temperature.

3. Wash each well with 200 uL PBS 4x. Flick wells out each time and dab on paper towel to get extra drops out.

4. Block each well with 200 uL/well blocking buffer.

5. Cover plate and incubate for 2 hours at room temperature.

6. Repeat step 3
7. Mix 50 uL of antigen (insulin) and 50 uL of secondary antibody.
   
   a. Dilute antigen from row A to G in Blocking Buffer. Unless specified otherwise dilute starting at 1/1000 and double from there as in part b.
   
   b. Secondary remains constant and can be optimized in an additional checkerboard titration. Dilute at 1/10 in blocking buffer.

8. Repeat step 5 then 3

9. Add 100 uL TMB to each well. Should turn a blue green color and optical density can be read at 370 nm or 620-650 nm.

10. Once colors develop thoroughly, add 100 uL Stop Solution. Wells should turn yellow. OD can be read at 450 nm for 1 hr.

11. Secondary antibody and antigen can be re-optimized to ensure proper efficiency of supplies.

![Figure 4: Illustration of how a possible checkerboard titration looks when completed](image)

Protocol (After optimization with CBT):

1. Prepare PBS, Coating Buffer, and Blocking Buffer as described in materials

2. Dilute primary antibody in Coating Buffer to optimized concentration and add to each well.
3. Cover and incubate overnight at 4 degree C or 2 hours at room temperature.

4. Wash each well with 200 uL PBS 4x. Flick wells out each time and dab on paper towel to get extra drops out.

5. Block each well with 200 uL/well of Blocking Buffer.

6. Cover and incubate for 2 hours at room temperature.

7. Wash each well with 200 uL PBS 4x. Flick wells out each time and dab on paper towel to get extra drops out.

8. Mix 50 uL Antigen (Insulin) with 50 uL secondary antibody at optimized concentration.
   a. Add standards of insulin with secondary antibody to column 1.
   b. Add unknown mixture to the rest of the wells.
      i. A second standard can be added in column 12 if desired.

9. Step 8 then 9.

10. Add 100 uL TMB to each well. Blue Green color will appear.

11. Once color has developed evenly, add 100 uL Stop Solution. Yellow color will appear. OD can be read at 450 nm for 1 hour.

12. Read data in with plate reader.
   a. Note: OD is inversely related to antigen (insulin) concentration.

Notes:

- Both PBS and Blocking Buffer can be used as diluent.
- Can use 50 uL instead of 100 uL to save supplies. Readings may be weaker.
  Divide all volumes in protocol by 2. Keep dilutions the same.
- If whole serum is used as capture reagent, use dilution of 1/100 to start
**Results:** Since there were many variables, the main components being primary antibody, insulin, and detection antibody concentration, multiple checkerboard titrations would need to be performed to complete the optimization.

**Primary Antibody Optimization:** The focus was on the primary antibody and insulin first since changes in those concentrations would have the greatest effect on the optical density. The primary antibody started with a dilution of 1/500 and went to 1/128000. The insulin concentration went from 5.00E-09 to 1.56E-10 g/mL while the secondary antibody was held constant at 1/10 dilution. Once the ELISA was finished, the optical density was read in and is shown in figure 5. In order to optimize efficiency, the least amount of antibody should be used, while not sacrificing signal strength. The 1/8000 and possibly 1/16000 dilutions were optimal for the primary antibody concentrations, since those are the lowest concentrations that still maintain a high OD. There were not many significant trends in figure 6 which illustrated the insulin concentration changes.
**Figure 5:** Optimization of primary antibody concentration against insulin concentration. The circle highlights the concentrations of primary antibody that are most optimal for this assay.

**Figure 6:** Optimization of insulin concentration against primary antibody concentration.
Secondary Antibody Optimization: For the next ELISA, the goal was to optimize the secondary antibody concentrations alongside the insulin concentrations again. The previously optimized primary antibody dilution of 1/8000 was used while ranging the secondary antibody concentration from 1/10 to 1/5120 and keeping the same insulin concentration range as the previous ELISA. There was a predictable trend in decreasing signal with decreasing secondary antibody concentration, but the lack of any trend in the insulin graphs led to some concern with how the insulin and both antibodies were interacting (Figures 7,8). An increase in signal with a decrease in insulin was expected due to the competitive interactions between the secondary antibody and insulin. The secondary antibody was possibly able to bind to many different areas on the primary antibody which allowed for it to bind to antibodies that already had insulin bound to it.

![2nd Ab dilution at different Insulin concentrations](image)

*Figure 7: Optimization of secondary antibody concentration against insulin concentration.*
The third ELISA was setup similarly to the previous ELISA, but with multiple wells of insulin concentrations at 5, 0.5, and 0 ng/mL. The goal was to create a standard deviation of the signals in order to test the sensitivity of the assay. Apart from two outliers in the 5 ng/ml insulin graph, figures 9 and 10 show a fairly high sensitivity. The trends in the graph were still not indirect which is what was expected for a competitive ELISA. Therefore, a new secondary antibody was used that would bind specifically to the variable section of the antibody where the insulin is also binding so that it is true competition.
The fourth ELISA optimized the new secondary antibody. It was a rat monoclonal anti-mouse IgG1 H&L antibody with an HRP conjugate (Abcam Cat# ab99603).
secondary antibody was intended to specifically target the variable region in the primary antibody. The primary antibody concentration was held constant at 1/8000 dilution like the previous ELISAs. The insulin concentrations consisted of multiple wells at 5, 0.625, and 0 ng/mL while the new secondary antibody concentration ranged from 1/2000 to 1/16000 dilution. The product manual suggested a dilution of 1/4000-1/8000 so a step above and below that would be an effective starting point for optimization. This ELISA successfully had a competitive binding environment, because as the insulin concentration increased, the signal decreased (figure 11). A trend that occurred was that as the secondary antibody concentration decreases, the change in signal is not as large between insulin concentrations. This would indicate that sensitivity is not constant and is dependent on the concentrations of the antibodies and insulin. The next step was to reoptimize the insulin and primary antibody concentration range to the new secondary antibody.

![Constant P Ab concentration](image)

*Figure 11: Optimization of insulin concentration against new monoclonal secondary antibody with constant primary antibody concentration (1/8000).*
**Primary Antibody Optimization:** The fifth ELISA used a constant secondary antibody concentration of 1/4000 which was optimized from the results of the previous ELISA. The insulin ranged from 5 ng/ml to 78 pg/ml and the primary antibody ranged from 1/500 dilution to 1/256000 dilution. In *figures 12 and 13*, an optimal primary antibody concentration of 1/8000 is clear because every other concentration is either too small, 1/16000 to 1/256000, or too saturated, 1/4000 to 1/500. By switching the axis on the graph, some trends were easier to see. In a perfectly competitive environment, the graphs would slope downward like a U-shaped parabola and would asymptote at an insulin concentration of zero. The trend shown in those figures has more of a “fish hook” appearance too it. This meant that the assay was reliable and sensitive enough up until the smaller concentrations where the signal starts to get weaker again. To test this, the insulin concentration was increased to range from 100 ng/ml to about 98 pg/ml while keeping the primary antibody concentration at 1/8000 dilution and the secondary antibody at 1/4000 dilution for the next ELISA. The assay did not produce any signal after it was completed. The same solutions were used from the previous ELISA for the current one which were about a week old, and this was thought to be the cause for a failed assay. The assay was run again, but with new solutions and still resulted in poor signal emission. This most likely meant that primary antibody had become unuseable. New antibodies were ordered then aliquoted and frozen to preserve them longer.
Figure 12: Optimization of primary antibody concentration against insulin concentration with constant secondary antibody concentration (1/4000). The blue oval highlights the optimal dilution of 1/8000 while the red oval highlights the higher concentrations of primary antibody that were too saturated.

Figure 13: Zoomed in view of the fifth ELISA. “Fish hook” appearance can be seen by the blue line with the red line illustrating the expected trend in a competitive environment.
**Material/Procedure Optimization:** The eighth ELISA was set up exactly the same as the sixth, but with the frozen aliquots. Identical triplicates of the assay were run to get a standard deviation of the trends. *Figure 14* shows the spread of insulin concentrations. Using the frozen aliquots increased the signal by about 2x which helps to prove that the previous antibodies weren’t fresh. There was a slight positive trend in the signals which actually indicated that there wasn’t a competitive binding between the antibodies. The signal needed to be closer to 1.0 so that the assay was not too concentrated which can mean the signals are unreliable. The next step was to lower the primary antibody concentration which would possibly create a competitive trend by limiting the binding spots that were available to the insulin and secondary antibody.

![Figure 14: Triplicate analysis with constant primary antibody (1/8000) and secondary antibody concentrations (1/4000).](image)

The ninth ELISA lowered the primary antibody concentration from 1/8000 to 1/100,000 dilution. This ended up producing no signal and was most likely due to the primary antibody concentration being too diluted. Different well plates, flat bottom and round
bottom, were also compared. Rounds bottoms had been used in the previous tests because they are better for mixing of solutions\(^\text{13}\). The flat bottoms are better for binding antibodies to the plates though\(^\text{13}\). Since there was minimal signal, it was difficult to determine which plate was better. The tenth ELISA set up a CBT of primary antibody from 1/16000 to 1/256000 and secondary antibody from 1/4000 to 1/64000. The insulin concentration was held constant at 1.0 ng/ml. By starting at a primary antibody concentration that is one dilution step below the previously optimized concentration of 1/8000, the trends were compared from this ELISA to the eighth ELISA shown above. Again, this ELISA produced no signal. The reasons for this were most likely due to an error in how the procedure was executed, or that there was an issue with the primary antibody. This ELISA caused some concern with whether the competitive environment was going to be effective enough to use it with our experimental insulin samples. The 11\(^{\text{th}}\) ELISA was set up the same as the ninth in order to compare the different plate bottoms again and also to see if the assay produced any signal since the previous two hadn’t. Unfortunately, the assay gave no signal again. This possibly meant that the antibody concentrations were too dilute. For the next ELISA, the setup was the same as the tenth, which used a higher antibody concentration, to see if the signal could be increased. The antibody stock from the previous day was used to also determine how long the antibodies would last in solution. Since such a small amount of antibodies are used for each assay, being able to reuse samples from a previous ELISA can save a lot of materials from being wasted. What was noticed was that after about 24 hours, signal is reduced to about 1/10\(^{\text{th}}\) the signal at the same concentration pairings from previous ELISAs. This meant that all of the antibody solutions would need to be prepared fresh.
each day. Since the past three ELISAs had not given any signal, there were concerns that the antibodies were no longer working. I performed a sodium azide and PEG precipitate assay to test the quality of an antibody by creating a precipitate of that specific antibody. The results indicated that the antibody was functioning correctly, which meant that the samples were too dilute and that the next assay would use concentrations similar to previous ELISAs that produced signals. The 13th ELISA was set up similar to the 10th ELISA with the primary antibody ranging from 1/8000 dilution to 1/128000 and the secondary antibody ranging from 1/4000 dilution to 1/32000. This was then split into quadrants on the 96 well plate with each containing a different insulin concentration of 1000 pg/ml, 500 pg/ml, 100 pg/ml, and 50 pg/ml. This was a quick way to optimize three different concentrations since it was technically four different ELISAs on one plate. From the results, it was apparent that 1/8000 primary antibody dilution was optimal, since the next dilution’s signals were only slightly larger than the noise of the assay. Knowing this, the data comparing the insulin concentration with the secondary antibody concentrations was plotted (Figure 15). As the graph indicates, there is a direct correlation between insulin concentration and OD, which is not what should be happening in a competitive ELISA.
The last attempt to create a competitive assay was to incubate the insulin overnight in order for the insulin to have enough time to fully bind to the primary antibody and compete with the secondary antibody\textsuperscript{15}. From the previous ELISA, the optimized concentrations of 1/8000 dilution for the primary antibody and 1/4000 dilution for the secondary antibody were used. The insulin ranged from 10 ng/ml to 19.5 pg/ml and created three rows for each overnight and 2 hour incubation. The overnight incubation occurred in a 4 °C refrigerator while the 2 hour was at room temperature on a plate shaker as these were previously found to be optimal conditions\textsuperscript{13}. The results shown in figure 16 show that incubating overnight greatly increases the signal density. There was still no true competitive binding occurring though, which was when the decision was made to switch to the sandwich ELISA.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure15.png}
\caption{Optimization of secondary antibody against insulin concentration.}
\end{figure}
Conclusion: The main advantages of a competitive ELISA are twofold. Because the signal is inversely related to the antigen concentration, a smaller range of signals can be measured than most other ELISAs. Since only one antibody has to bind to the antigen, the competitive ELISA is typically more cost effective than other ELISAs. While an inexpensive ELISA that can detect small levels of insulin the initial goal, there were many problems with how the antigen and antibodies interacted. The insulin and secondary antibody were binding at the same time and this led to many different results that never depicted an actual competitive environment. There should have been an inverse relationship between signal and insulin concentration, but what was most commonly seen was a logarithmic curve with a positive correlation between signal and insulin concentration. The next step was to move to the more robust assay which was the sandwich ELISA.
<table>
<thead>
<tr>
<th>ELISA List</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse IgG (H+L): Donkey Polyclonal to mouse</td>
<td>Optimize P Ab and Insulin. Figures 5 and 6</td>
</tr>
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<td>2</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse IgG (H+L): Donkey Polyclonal to mouse</td>
<td>Optimize Insulin and 2 Ab. Figures 7 and 8</td>
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<tr>
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<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse IgG (H+L): Donkey Polyclonal to mouse</td>
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<tr>
<td>4</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Optimize 2 Ab. Figure 11</td>
</tr>
<tr>
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<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Optimize P Ab and Insulin.</td>
</tr>
<tr>
<td>6</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Optimize Insulin</td>
</tr>
<tr>
<td>7</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Same as 6 but with fresh reagents. Figures 12 and 13</td>
</tr>
<tr>
<td>8</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Same as 6 but using aliquots. Figure 14</td>
</tr>
<tr>
<td>9</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Same as 6 but lower P Ab. Testing flat vs round bottom plates.</td>
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<td>10</td>
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<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Optimize P Ab and 2 Ab.</td>
</tr>
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<td>11</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Same as 9</td>
</tr>
<tr>
<td>12</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Same as 10. Testing using day old antibodies</td>
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<tr>
<td>PEG Precipitation</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td></td>
<td>Tested effectiveness of antibodies with sodium azide and PEG precipitate assay.</td>
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<tr>
<td>13</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Same as 10 but with 4 different sets at different insulin concentrations. Figure 15</td>
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<tr>
<td>14</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Have constant P Ab and 2 Ab. Testing overnight P Ab incubation. Figure 16</td>
</tr>
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</table>

Figure 17: Table of all competitive ELISAs.
Chapter 4: Sandwich ELISA

Introduction: As explained earlier, the sandwich ELISA measures the concentration of a specific antigen by binding two antibodies to it like a sandwich (Figure 18). The initial plan was to find an antibody that could be paired with either of the antibodies that were already being used in order to use my reagents efficiently. Using two antibody pairs specifically designed for sandwich ELISAs was found to be much more reliable and allowed for better sensitivity because they were designed to bind to different epitopes on insulin. The two different antibodies were both anti-insulin targeted. One was conjugated with HRP as the detection antibody and the other as the capture antibody. Just like the competitive ELISA, each reagent had to be optimized with a CBT.

Figure 18: Illustration of the steps involved in the sandwich ELISA\textsuperscript{16}. 
Materials and Methods:

Insulin Sandwich ELISA Protocol

Materials:

- 96 Well Plate (Fisher Cat# 08-772-53) x1 Plate
- PBS (Made in-house) 200 uL/well/wash
- TMB substrate (Immunochemistry Cat# 6276) 100 uL/well
- Stop Solution (Immunochemistry Cat# 6282) 100 uL/well
- 5x Coating Buffer (Immunochemistry Cat# 644) 100 uL/well
- 5% Blocking Buffer (Abcam Cat# ab126587) 200 uL/well
- Capture antibody (Anti-Insulin antibody [3A6] (Abcam Cat# ab1965) 100 uL/well
- Detection antibody (Anti-Insulin antibody [8E2] (HRP) (Abcam Cat# ab24455) 100 uL/well
- Human Insulin (in-house 1 mg/mL frozen samples) 100 uL/well

Protocol:

1. Prepare PBS, Coating Buffer, and Blocking Buffer as described in materials
2. Dilute capture antibody in Coating Buffer to optimized concentration and add to each well.
3. Cover and incubate overnight at 4°C or 2 hours at room temperature.
4. Wash each well with PBS 3x. Flick wells out each time and dab on paper towel to get extra drops out.
5. Block remaining binding sites with Blocking Buffer.
6. Repeat steps 3+4.
7. Dilute samples and add to each well along with known standards.
8. Repeat steps 3+4.
9. Dilute detection antibody and add to each well.
10. Repeat steps 3+4.
11. Add TMB substrate to each well. Blue Green color will appear.
12. Once color has developed evenly, add Stop Solution. Yellow color will appear. OD can be read at 450 nm for 1 hour.
13. Read data in with plate reader.
14. Plot curve with concentrations on x axis (log) and OD on y axis (linear)

Notes:

- Both PBS and Blocking Buffer can be used as diluent.
- Can use 50 uL instead of 100 uL to save supplies. Readings may be weaker.
  Divide all volumes in protocol by 2. Keep dilutions the same.

Results:

**Detection Antibody Optimization:** The first sandwich ELISA (sELISA) was designed to optimize the detection antibody and insulin concentrations. The capture antibody concentration was set at 5 ug/mL, which is a common starting concentration among ELISAs. The insulin concentration ranged from 100 to 12.5 pg/mL which is the around the concentration of our actual experimental samples. The detection antibody concentration ranged from 10 to 1.25 ug/mL, because this was a large enough range around 5 ug/mL for a first test. The results indicate no significant trend with regards to the signal concentration, and the signal was so low that any trend in detection antibody
concentration is not significant either (Figure 19). This was most likely due to the insulin not being incubated overnight. Since the insulin is binding alone without the presence of another antibody to compete for binding spaces, an overnight incubation would be even more beneficial.

![Graph showing optimization of insulin concentration against capture antibody concentration.](image)

**Figure 19:** Optimization of insulin concentration against capture antibody concentration.

The second sELISA was set up identical to the first, but with two groups; an overnight and a regular. Surprisingly, there wasn’t a significant difference between the two groups (Figure 20). Having such a low signal again indicated that there was a limiting factor amongst the three variables. The low insulin concentrations were most likely causing the low signals.
The third sELISA had the same capture and detection antibody concentrations, but greatly increased the insulin range to 10-1.25 ng/mL in an effort to determine the cause of the low signal. The signal was inadvertently overdeveloped for 40 minutes instead of the standard 10 due to an occurrence in the lab. Because of this, the signal was too saturated and unuseable. The fourth sELISA was used to optimize the capture antibody and insulin concentrations. The insulin range was reduced from the previous sELISA by a factor of 10 because the concentrations were not physiologically practical as it relates to our research samples. Figure 21 illustrates the trends from this sELISA. The signal was fairly weak and had mostly a linear trend with regards to the change in insulin concentration. What is expected is a Michaelis-Menten-type trend upward with a signal around 1.0 similar to figure 22.
In order to increase the signal density, the insulin was incubated overnight again. This time, the detection antibody was kept constant at 1 ug/mL, while varying the capture antibody and insulin concentrations. By varying the capture antibody concentration, hopefully a wider range of signals could be obtained, because it was the first step and
therefore the most independent variable. This time only 50 uL of reagents were used for each step to see if there was any difference in signal. By cutting down on reagents by 50%, a lot of supplies can be saved which reduces the cost of the ELISA. Only so many antibodies can bind to the plate and the rest is washed away, so this sELISA was determining if there was a significant drop in signal from previous sELISAs. Tween-20, which is a surfactant, was also added to the wash solution. Some of the inconsistencies of the data have been because the detection antibody was indirectly binding to the well plate instead of the insulin. Adding a surfactant is known to inhibit indirect binding. As shown in *figure 23*, there are very significant positive trends for both the capture antibody and insulin concentration. This was the first indication that the sandwich ELISA was working properly. While the signal was still low for the smaller insulin concentrations, the 10 ug/mL capture antibody concentration has the greatest slope and will be used as the standard concentration for future sELISAs. The next step will be to vary the detection antibody, which will hopefully increase the signal strength. Overall, this sELISA was the most significant so far. By adding the Tween-20, a consistent signal was achieved and there was not a major loss in signal when only using 50 uL of samples per well.
Because the Tween-20 inhibited the non-specific binding, the competitive ELISA was performed again with the surfactant in the wash solution to determine if that would be able to fix the problems that were occurring with the signal trends. The previous optimized concentrations of 1/8000 dilution were used for the primary antibody, 1/4000 dilution for the secondary antibody, and ranged the insulin from 1 ng/mL to 62.5 pg/mL. The insulin was incubated overnight with the secondary antibody and found that there was no difference in signal from previous ELISAs. The sandwich ELISA was therefore much more consistent and reliable than the competitive. For the next sELISA, the capture antibody concentration was kept constant at 10 ug/mL and the detection antibody was ranged from 5 ug/mL to 1.25 ug/mL in order to obtain a signal of about 1.0. The previous sELISA showed that a concentration of 1 ug/mL couldn’t achieve that at the insulin concentrations being used.

Figure 23: Optimization of insulin concentration against capture antibody concentration with constant detection antibody concentration (1 ug/mL).
Detection Antibody Optimization: All of the steps were the same as the previous sELISA which became the new standard protocol for this assay. This included incubating overnight and adding Tween-20 to the wash solution. In figure 24, the strength of the signal was greatly increased, but there was no positive trend like in the last sELISA. This could be attributed to using reagents from the previous tests, which were a day old. For the next sELISA, fresh reagents were made to try and prevent this problem.

![Figure 24: sELISA graph with Tween-20 added to wash solution.](image)

The capture antibody was kept constant at 10 ug/mL and the detection antibody constant at 2 ug/mL. This detection antibody concentration was determined from the previous sELISA as it was extrapolated to fit around 1.0 OD. The insulin ranged from 1.0 ng/mL to 15.6 pg/mL. Two versions of the TMB substrate, regular and supersensitive, (Immunochemistry Cat# 6275) were also compared. This is the compound that binds with the HRP and emits a blue color, and to see if there was a noticeable difference in the signals between the two. As shown in figure 25, there is a slight positive trend that covers a range from about 0.45 to 0.6 OD for both groups, but they are almost identical. For cost
effective reasons, regular TMB would be used from now on. An apparent issue with these results is that the “negative control” group that had no insulin concentration still had a signal of about 0.45. This should be around 0.1 which is an acceptable noise level for these assays\textsuperscript{17}. This meant that there was still non-specific binding occurring and that the detection antibody was either binding to the plate or to the capture antibody. This means that there is still an issue with the Tween-20 or the blocking step. This would be the purpose for my next sELISA.

![Comparing TMB Substrates](image)

**Figure 25: Comparison of two different TMB substrates.**

In order to replicate the promising results from *figure 25*, fresh wash solution was made with Tween-20. This seemed to be the most noticeable difference between the two assays that could explain why there wasn’t as strong of a trend in the most recent one. The ninth sELISA was set up exactly the same as the seventh to try and get a better signal and have something to compare to. *Figure 26* shows a significant improvement in the positive trends of the different concentrations. As the detection antibody concentration decreased, the signal decreased, but the slope of each line increased. The detection antibody
concentration of 2 ug/mL would allow for the highest signal with the greatest slope in the trend line. Compared to the previous two sELISAs, the signal trends were much greater which prove that the Tween-20 wash solution has a relatively short shelf life. Since the assay was fairly consistent, the same tests would be run but with multiple wells so that an average and standard deviation of the data points could be created. The insulin concentration range was also decreased to test the sensitivity of the assay.

**Figure 26:** Optimizing detection antibody against insulin concentration with Tween-20 in the wash solution.

**Computational Modeling:** For the tenth sELISA, three identical rows were set up with 10 ug/mL capture antibody, 2 ug/mL detection antibody, and insulin concentrations ranging from 400 pg/mL to 25 pg/mL. Due to an increase in TMB substrate exposure time, the signal emitted was much higher than expected (*Figure 27*). Even though the insulin concentration was smaller, the signal was higher which illustrates the importance of the TMB step. The signal range was also much greater, which was a great indication of the assay’s sensitivity. The standard deviations were also
in an acceptable range for how large the differences were between points. A linear modeling program made with the computer software Matlab was also used. This program used the data points from the graph and created an equation that best fit the binding kinetics of the assay\textsuperscript{19}. The general model used a Michaelis-Menten equation to determine the trendline (\textit{Figures 28 and 29}). The trendline fit with an $R^2$ of 0.9938 which proves that the assay was working how it was intended. Because this sELISA was so successful, the next step was to now test against frozen perifusion samples as unknowns.

![Graph](image_url)

\textit{Figure 27: Sandwich ELISA with fully optimized antibody concentrations and polynomial trend curve overlayed with a best fit model from Microsoft Excel.}
Figure 28: Sandwich ELISA with fully optimized antibody concentrations and Matlab program generated Michaelis Menten curve overlayed.

General model:
\[ cf(x) = D + (A-D)/(1+(x/C)^B)^E \]

Coefficients (with 95% confidence bounds):
\[
\begin{align*}
A &= 0.3959 \quad (-0.5975, 1.389) \\
B &= 1.63 \quad (-31.57, 34.83) \\
C &= 70.46 \quad (-4849, 4990) \\
D &= 20.61 \quad (-2.448e+04, 2.452e+04) \\
E &= 0.01953 \quad (-25.37, 25.41)
\end{align*}
\]

Figure 29: Michaelis-Menten equation used in Matlab code.

**Assay Viability Testing/Background Noise Optimization:** The samples that were used for testing the quality of the sELISA were generated from a islet viability assay known as a perifusion. Similar to the protocol used by C. Ricordi et al., a few hundred islets are suspended on a filter and connected to a pump which delivers a perfusate, usually Krebbs-Ringer solution, through the filter over three hours with low, high, then low glucose concentrations of the perfusate. The solution is collected every ten
minutes and provides a dynamic profile of the characteristics of glucose-mediated insulin release. The perifusion (explain) samples that were used had been taken at different time intervals with varying levels of glucose being perfused through the sample. This allowed for multiple time points to be selected which contain varying levels of insulin. One sample was chosen from the early time points which had low glucose, four from the high glucose section, and another from the later time points which had low glucose again. All of the antibody concentrations were the same as the previous tests and the standards ranged from 400 pg/mL to 25 pg/mL. Each well was doubled so that there would be a standard deviation and be able to compare signals. The standard curve is shown in figure 30, which confirms that the assay worked properly. The signal given off by the unknowns (Figure 31) was very weak and cannot be differentiated from background noise. This means that the insulin concentration was less than 25 pg/mL, or the sample had lost most of its viability while it was stored. The method in which these islets were isolated is known as selective osmotic shock. This is an experimental approach designed by Atwater et al. that attempts to preserve the native extracellular matrix surrounding the islets in order to improve their viability in culture. Instead of digesting the pancreas with an enzyme, rapid changes in glucose concentration is used to cause non-islet cells to lyse under the high osmotic flux. Due to the experimental nature, the viability of the islets is not guaranteed and may also be a cause for the lack of signal. For the next sELISA, the same setup was used, but with a focus on reducing the background noise so that the sensitivity of the assay would higher.
In an attempt to reduce background noise, all fresh reagents were used instead of just making a fresh wash solution. Samples from a different perifusion were used to see if a better signal could be achieved. As shown in figure 32, the standard curve was actually better than the previous sELISA, and the OD of the unknown samples was still very low (Figure 33). The samples actually had a lower signal than the negative controls that contained no insulin. This meant that the background noise is being raised due to some non-specific binding. There are four common causes of this problem: contamination of reagents/samples, detection antibody is detecting coating antibody, insufficient washing of plates, and too much antibody used leading to non-specific binding\textsuperscript{22}. The contamination was unlikely as fresh solutions were made for this sELISA. The detection
antibody binding with the capture antibody is also unlikely because the two antibodies were paired together by the company that they were ordered from. The insufficient washing of plates is possible because the plates can’t be washed too much and the step is extremely important. Having too much of either antibody is unlikely because the concentrations were optimized over the previous sELISAs, and decreasing the concentrations would decrease the signal.

![Trial 2: Fresh Materials](image)

**Figure 32:** Standard curve used in second trial of perfusion samples with fresh materials.

<table>
<thead>
<tr>
<th>Time</th>
<th>OD</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>G10</td>
<td>0.2506</td>
<td>0.0014</td>
</tr>
<tr>
<td>G24</td>
<td>0.23715</td>
<td>0.01215</td>
</tr>
<tr>
<td>G30</td>
<td>0.2494</td>
<td>0.003</td>
</tr>
<tr>
<td>G40</td>
<td>0.2245</td>
<td>0.0048</td>
</tr>
<tr>
<td>G46</td>
<td>0.2065</td>
<td>0.0018</td>
</tr>
<tr>
<td>G65</td>
<td>0.20985</td>
<td>0.00685</td>
</tr>
</tbody>
</table>

**Figure 33:** Table of perfusion time, signal strength (OD), and standard deviation (SD).

For the next sELISA, the blocking step time was increased from 1 hour to 2 hours in an attempt to decrease the non-specific binding. The amount of washes was also increased from 4 to 6 times for each washing step to also reduce the background noise. As shown in
The standard curve used in third trial of perfusion samples with 2 hour blocking step. There was an improvement in the signal, but it remained much lower than the negative control. This indicates that the length of the blocking phase and amount of washes wasn’t directly correlated to the high background noise. The next option to decrease the background signal was to look at the concentrations of the antibodies. Even though they were optimized, reducing the background noise was just as important as having a strong signal.

![Standard Curve](image)

**Figure 34: Standard curve used in third trial of perfusion samples with 2 hour blocking step.**

<table>
<thead>
<tr>
<th>Time</th>
<th>OD</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.17925</td>
<td>0.00135</td>
</tr>
<tr>
<td>24</td>
<td>0.17455</td>
<td>0.00145</td>
</tr>
<tr>
<td>30</td>
<td>0.15965</td>
<td>0.01125</td>
</tr>
<tr>
<td>40</td>
<td>0.16235</td>
<td>0.00795</td>
</tr>
<tr>
<td>48</td>
<td>0.16915</td>
<td>0.00145</td>
</tr>
<tr>
<td>65</td>
<td>0.1847</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

**Figure 35: Table of perfusion time, signal strength (OD), and standard deviation (SD).**

The next sELISA was designed to test the different variables in the blocking step as this was most likely where the problems were occurring. The detection antibody concentration, blocking buffer type, and the addition of Tween-20 were all areas that
could be affecting the signal. Even though the detection antibody was added in a different step, lowering the concentration would rule out if it was a problem with the blocking step or another step. Four different versions of negative controls were set up to determine which variables were affecting the signal the most. Doubles of each group, including standards, were made that went from 400 pg/mL to 12.5 pg/mL. The detection antibody was lowered to 1 ug/mL from the optimized 2 ug/mL. As shown in figure 36, the standard curve was as expected and the trend from the negative controls was somewhat expected too. Without Tween-20 or a blocking buffer at all, the background noise was higher and with a lower detection antibody, the background noise was much lower. As stated earlier, even though the background noise was much lower due to the low detection antibody, the overall signal strength when using 1 ug/mL of detection antibody is quite low (Figure 23). From here, the next way to lower the background noise would be to change the blocking buffer type.

![Background Noise Optimization](image)

**Figure 36: Optimization of background noise compared to standard curve in sandwich ELISA.**
Currently, a General Blocking Buffer (Immunochemistry Catalog# 632) was being used to block the plates. The goal was to test the original blocking buffer against two different kinds that were targeted towards sandwich ELISAs. SynBlock ELISA Blocking Buffer (Immunochemistry Catalog# 641) is a synthetic blocking formulation designed to eliminate nonspecific binding of enzyme-labeled conjugates and is targeted towards sELISAs. Monster Block ELISA Blocking Buffer (Immunochemistry Catalog# 6295) is a heterogenous mixture of nonmammalian blocking agents that is useful for testing mammalian serum samples and reducing a high backround noise. Testing both a protein based and non-protein based buffer alongside the original buffer would determine the most efficient blocking type for the sELISA. The next assay was set up similar to the previous sELISA, but the standards went from 200 pg/mL to 12.5 pg/mL and were blocked with the general blocking buffer. Each standard and buffer was doubled to allow for a standard deviation. The standard curve was not as good as some of the previous sELISAs since the lowest signals were around 0.6 (Figure 37). This could have been due to various reasons, but the signal of the negative controls that were comparing the different blocking buffers was still clear. The original blocking buffer provided a significantly lower background noise than the two new buffers.
With this last assay, it was confirmed that using the original blocking buffer, adding Tween-20 to the wash solution, making the reagents fresh, and using the optimized antibody concentrations was the best way to increase the signal to noise ratio. The next step was to compare the in-house sELISA to a commercial kit that is used by our lab to measure insulin concentrations.

**Commercial ELISA Comparison:** A Mercodia Insulin ELISA was used to compare the signal strengths and sensitivity of my assay. The documented range of this ELISA is 3 - 200 mU/L\(^{23}\). A unit (U) of human insulin was calculated by means of amino acid analysis and states that 1 mole corresponds to 166.8 x 10\(^6\) U or that 1 mU/L = 6.00 pmol/L\(^{24}\). This means that the range of the commercial ELISA is 104.5 – 6970 pg/mL. As stated earlier, the range of the samples we test in our lab are between 25 and 200 pg/mL.

First, the commercial sELISA was compared to the in-house sELISA with a static incubation of islets. These islets were isolated in the lab and cultured for 3 days. Then, about 10-15 islets were incubated at a low glucose concentration of 3 mM for an hour,
then at a high glucose concentration of 25 mM for an hour. The islets process the glucose and release insulin which can then be detected in the solution by the ELISAs. Both assays produced low signals from the samples which meant that the insulin concentration was not detectable due to low concentration or the islets weren’t viable after culture and unable to produce insulin. The commercial standard curve was still analyzed (*Figure 38*) and showed a linear trend that had a very low background signal. The in-house sELISA used both the normal standards and the commercial standards along with the samples from the static incubation (*Figure 39*). The in-house sELISA had a much steeper standard curve which shows it was more sensitive than the commercial ELISA at the lower ranges from around 20-200 pg/mL.

![Commercial Standard Curve](image)

*Figure 38: Commercial ELISA standard curve.*
From the data shown above, the in-house sELISA has proven to be more effective at detecting lower insulin concentrations than the commercial kits that had been used in the past. Since the commercial ELISA is meant for higher insulin concentrations, using plasma samples instead of perifusion or static culture samples is expected to get a detectable signal. This is why our lab has used RIAs instead of commercial ELISAs for measuring our insulin samples in the past, but because of time and, most importantly, radiation concerns there has been a need to switch to the ELISA as a means to detect human insulin concentrations. For the next sELISA, culture media that the islets were incubated in was used. This is because there are more islets per mL of liquid compared to static incubation samples and they are incubating for a longer period of time. After culturing the islets for 4 days, the media was collected from 3 different culture plates along with a conical tube that had leftover islets from a different assay that was performed earlier. The islets had been culturing in that particular media for 48 hours. Therefore, this media should be the most concentrated with insulin, if the islets were...
viable. The sELISA produced a fairly strong standard curve (Figure 40) with only some concern in the lower concentration ranges where the negative control is actually higher than the first standard. The signal that the media showed (Figure 42) was very strong so the lack of sensitivity at the smaller ranges wasn’t as much of a concern to me. The signal was then input into a similar matlab code that generated the standard curve (Figure 41), but inverses the equation to then calculate the concentration that best fits with the standard curve\textsuperscript{19}.

![Figure 40: Standard curve graph using Matlab. Axis units are pg/mL of insulin for the x axis and OD for the y axis.](image)

General model:

\[
\text{cf}(x) = D + (A-D)/(1+(x/C)^B)^E
\]

Coefficients (with 95% confidence bounds):

- A = 0.2157 (-0.447, 0.8784)
- B = 1.603 (-35.82, 39.03)
- C = 172.3 (-2.945e+04, 2.98e+04)
- D = 2.709 (-1062, 1060)
- E = 0.2051 (-134.5, 134.9)

![Figure 41: Michaelis-Menten equation generated by Matlab code.](image)
<table>
<thead>
<tr>
<th>Culture Plate</th>
<th>OD</th>
<th>Concentration (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.1605</td>
<td>689.1895</td>
</tr>
<tr>
<td>1</td>
<td>1.1581</td>
<td>685.6</td>
</tr>
<tr>
<td>2</td>
<td>0.8307</td>
<td>341.1671</td>
</tr>
<tr>
<td>2</td>
<td>0.8195</td>
<td>332.9892</td>
</tr>
<tr>
<td>3</td>
<td>1.1923</td>
<td>738.8411</td>
</tr>
<tr>
<td>3</td>
<td>1.1131</td>
<td>622.0557</td>
</tr>
<tr>
<td>Conical Tube</td>
<td>1.0816</td>
<td>581.4763</td>
</tr>
</tbody>
</table>

Figure 42: Table of signal given off by media samples. Insulin concentration is calculated using matlab.

The signal produced by the media was well within the range of my sELISA, but the commercial kit would not be able to confidently detect these signals since it is on the very low end of the described range.

**Conclusion:** Overall, the sandwich ELISA proved to be the most effective way to measure low concentrations of insulin, and while it was more expensive than the competitive ELISA, the accuracy and consistency that was shown was far better and more reliable.
<table>
<thead>
<tr>
<th>ELISA</th>
<th>Capture Antibody</th>
<th>Detection Antibody</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Optimize Insulin and D Ab. Figure 15</td>
</tr>
<tr>
<td>16</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Same as 15 but testing overnight incubation of C Ab. Figure 16</td>
</tr>
<tr>
<td>17</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Same as 15 but with increased insulin concentration. Figure 17</td>
</tr>
<tr>
<td>18</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Incubating Insulin overnight and added Tween. 50 ul per well. Figure 19</td>
</tr>
<tr>
<td>19</td>
<td>Anti-Insulin: mouse monoclonal</td>
<td>Anti-mouse (H+L): Rat Monoclonal</td>
<td>Incubating Insulin overnight and added Tween for competitive.</td>
</tr>
<tr>
<td>20</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Optimize D Ab and Insulin. Figure 20</td>
</tr>
<tr>
<td>21</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Testing sensitive vs regular TMB. Figure 21</td>
</tr>
<tr>
<td>22</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Same as 20, Fresh reagents with flat well plates. Figure 22</td>
</tr>
<tr>
<td>23</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Same as 22 in triplicates for standard deviation. Figures 23, 24, and 25</td>
</tr>
<tr>
<td>24</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Perifusion test. Figures 26 and 27</td>
</tr>
<tr>
<td>25</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Same as 24, with fresh reagents. Figure 28 and 29</td>
</tr>
<tr>
<td>26</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Same as 24, with 2 hour blocking step. Figures 30 and 31</td>
</tr>
<tr>
<td>27</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Background noise optimization. Figure 32</td>
</tr>
<tr>
<td>28</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>Blocking Buffer Optimization. Figure 33</td>
</tr>
<tr>
<td>29</td>
<td>Commercial</td>
<td>Commercial</td>
<td>Commercial Test with static incubation. Figure 34</td>
</tr>
<tr>
<td>30</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>ELISA to compare commercial in 29. Figure 35</td>
</tr>
<tr>
<td>31</td>
<td>Anti-Insulin</td>
<td>Anti-Insulin HRP</td>
<td>SOS media test. Figures 36, 37, and 38</td>
</tr>
</tbody>
</table>

**Figure 43: Table of all sandwich ELISAs**

**Summary and Conclusion:**

Prior to designing this ELISA, my lab had used RIAs to effectively detect the very low concentrations of insulin present in our experimental samples. This involved using radioactive samples to quantify the concentration. For health and safety reasons, we wanted to avoid using the RIA and switch to a different assay. The ELISA is another popular way to measure concentrations of an antigen, but it is not as sensitive as the RIA and is also much more expensive. For this reason, designing an inexpensive in-house
ELISA that can measure the small concentrations of insulin was the overall goal of this project. This project first started with a competitive ELISA as this is usually the version that can detect the smallest amounts of antigen. For various reasons, the assay was unable to generate a standard curve that reflected the binding activity that occurs in a competitive environment. For this reason the competitive ELISA, would not be reliable or effective. The next step was to create a more robust ELISA which was the sandwich ELISA. After optimization, a standard curve was created that fit within the range that was needed to detect the low insulin concentrations in our experimental. Multiple different samples were tested to compare my in-house assay to a commercial version. From the data shown above, I have demonstrated that my ELISA can successfully detect minute concentrations of insulin, and at about half of the cost of a commercial ELISA [Appendix B][19]. Going forward with this project, more samples produced by our lab will need to be tested to better understand how sensitive this assay is and what strategies can be developed to make our experimental samples detectible by the in-house assay. These would include increasing the number of islets used in functional tests of islets with glucose incubations. Also, looking at different surfactants, such as SDS, instead of just Tween-20 could allow for a greater decrease in background noise. Overall, the in-house ELISA has successfully achieved the goal of the project.

**Acknowledgement**

I would like to express my gratitude to my master thesis advisor, Dr. Emmanuel Opara for all of the help, guidance, and opportunities he has given me this past year. I also would like to thank my coworkers JP McQuilling and Sittadjody Sivanandane for all of their guidance and support in the lab every day. I’d like to thank my Committee
members Dr. Aleksander Skardal and Dr. Nicole Levi-Polychencko for their suggestions and help this year. In addition, I would like to thank anyone who has assisted me in my growing process of becoming a successful researcher at WFIRM, especially Dr. Giuseppe Orlando for his guidance and trust in me. Lastly, I would like to thank my family and loved ones for supporting me along the way and being just as excited as I am about my research.

I would also like to thank the financial support of the Helen Bell Fund. None of this would be possible without your contributions.
References:


Appendix A: Example of a checkerboard titration. Primary antibody is in the top row, then insulin, then secondary antibody concentration.

<table>
<thead>
<tr>
<th>Material</th>
<th>Price</th>
<th>Amount</th>
<th>Unit</th>
<th>Assay</th>
<th>$/unit</th>
<th>Assay per unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture Antibody</td>
<td>376</td>
<td>100</td>
<td>µg</td>
<td>1</td>
<td>180.48</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Detection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody</td>
<td>370</td>
<td>200</td>
<td>µg</td>
<td>1</td>
<td>17.76</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>Well Plate</td>
<td>161.45</td>
<td>50</td>
<td>plates</td>
<td></td>
<td>3.23</td>
<td></td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>64</td>
<td>50</td>
<td>ml</td>
<td>1</td>
<td>0.61</td>
<td>10 x</td>
</tr>
<tr>
<td>TMB Solution</td>
<td>44</td>
<td>100</td>
<td>ml</td>
<td>1</td>
<td>2.11</td>
<td>1 x</td>
</tr>
<tr>
<td>Stop Solution</td>
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<td>PBS +</td>
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<td>miscellaneous</td>
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<td>Coating Buffer</td>
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<td>ml</td>
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<td>Total Assay</td>
<td>210.35</td>
<td></td>
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</tr>
</tbody>
</table>

Wells 96

Appendix B: Cost analysis of in-house ELISA.
Curriculum Vitae:

KEVIN ENCK
Cell: 585-781-0577 Email: kenck@wakehealth.edu

EDUCATION

University at Buffalo Buffalo, New York
Bachelor of Science Degree in Biomedical Engineering

Virginia Tech – Wake Forest University Winston-Salem, North Carolina
2014-Current Masters of Science Degree in Biomedical Engineering

RELEVANT EXPERIENCE

Wake Forest Institute for Regenerative Medicine Fall 2014-Current
-Master’s Thesis Research conducted under Dr. Emanuell Opara
- Thesis: Designing an in-house insulin ELISA.

RELEVANT SKILLS AND CERTIFICATIONS

Computer Language
- Java, C++, MatLab, Visual Basic, BioPac, and LabView
Lab
- ELISA training and optimization. Designing of in-house insulin ELISA for lab use.
- Cell Isolation techniques specifically for human and porcine islet collection.
- Cell culture techniques
- Grant Writing and Protocol design skills

Certification
-IACUC animal care and use certification Fall 2014

Research
-Thromboresistant Stent research: Stent design, Stent creation, FDA/Patenting, etc. Fall 2013-Spring 2014
-In-house ELISA assay development Fall 2014-Current
-Alginate Microbead Encapsulation for Human Islet Transplantation Fall 2014-Current
-In Vivo Analysis of Human Islet Isolation Techniques Fall 2014-Current
-Alginate Microbead Physical Testing for Ovarian Cells Spring 2014-Current
-Dopamine Modified Alginate Encapsulation of Omega-3 Oil Fall 2015-Current

Presentations
-Effect of Alginate Microcapsule Stiffness on Encapsulated Ovarian Cell Viability
Virginia Tech SBES Presentation: Blacksburg, Va Spring 2015
BMES Conference: Tampa, FL Fall 2015
NCTERMS: Winston-Salem, NC Fall 2015