Detection of Streptococcus Equi From Environmental Samples

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DETECTION OF *STREPTOCOCUS EQUI* FROM ENVIRONMENTAL SAMPLES

USING AN ELISA

by

Taryn Haller

A Thesis Submitted in Partial Fulfillment
of the Requirement for a Degree with Honors
(Animal and Veterinary Science)

The Honors College

University of Maine

May 2015

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Abstract
Equine Strangles, an upper respiratory disease caused by *Streptococcus equi* subspecies *equi* (*S. equi*) causes significant losses in the horse industry. Surveillance for *S. equi* could be facilitated by quantifying *S. equi* in environmental samples. The objective of this project was to evaluate ELISA in quantifying *S. equi* using two monoclonal antibodies (MAB’s) to the SeM protein (MAB-212 for capture, and biotinylated MAB-211 for detection), with Streptococcal phage lysin releasing SeM from the cell surface. Initial results confirmed a fresh culture of *S. equi* and two *S. equi* lysates stored at -20 C for 2 years as positive, while confirming as negative a stored lysate of *Streptococcus equi* subspecies *zooepidemicus*. A standard curve was created using 12 serial dilutions at 1:10 of *S. equi* and colony counts determined for each of the 12 dilutions. Eight serial dilutions at 1:2 of these 12 dilutions then yielded one dilution for each well in a 96 well plate. Regression of absorbance against colony counts showed that ELISA was successful in quantifying between 78,125 CFU/mL and 15,000 CFU/mL of *S. equi*, with an $R^2$ of 0.93. Water was then seeded with *S. equi*, and 3 swab samples taken, dispersed in PBS, and the 3 suspensions serially diluted 1:10 to provide 4 dilutions (12 total). Colony counts were determined by plating 100 µL of each dilution. All dilutions tested positive for *S. equi*, but there was no correlation between absorbance and colony counts ($R^2 = 0.0028$), possibly due to plate contamination.
Acknowledgements

I would like to thank Dr. Robert Causey for guiding me in my research and helping me navigate some of the difficult aspects of the planning and execution of the assay, as well as financing the project. I would also like to thank Dr. Melissa Ladenheim for helping me with my reading list, and guiding me through the overall thesis procedure. I would like to thank Dr. Rita Seger for helping me with some of the lab equipment, and Dr. Edward Bernard for helping me with the statistical portion of the research. I would also like to thank Nathalie Forster for providing me with a protocol to follow and the antibodies used in the assay. I would also like to thank all of the above mentioned for agreeing to sit on my defense committee. Brenda Kennedy-Wade of the University of Maine Animal Health Lab was also incredibly helpful with the design of the project, and allowed me to use some of her lab supplies, for which I’m very grateful.
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Table 1: Absorbance values (y) and their Corresponding Calculated Concentrations (x) Using the Equation for the Line of Regression (y= 0.00005x – 0.2003), and Colony Counts from the Blood Agar Plates.
Introduction

*Streptococcus equi* subspecies *equi* is the causative agent of the disease Strangles in horses (Sweeney et al., 2005). It forms abscesses in the lymph nodes of the head and neck, and is highly contagious. There are approximately 1000 outbreaks of strangles in the UK each year (Waller 2013). A survey within the US found that infection rates during one outbreak were 17.6% of broodmares, 47.5% of one year old horses, and 37.5% of foals (Sweeney et. al., 1989). An affected horse should be quarantined to avoid infecting other horses, but with the disease being so contagious, quarantine is often not effective to prevent spread of the disease.

The bacteria are shed through mucous secretions and can persist in the environment, being transmitted by people, flies or fomites (Weese et al., 2009). An affected horse may not show clinical signs for six days, and may spread the infection rapidly. Once quarantined, diligent effort must be made to contain the bacteria, but it can be spread by flies: a vector that is impossible to control. After a horse has become infected with strangles, the bacteria migrate to the guttural pouches, and may remain there even after clinical signs have ceased. This can spread the infection to other horses, because the *S. equi* is still persistent in the environment (Webb et al., 2013).

Because the bacteria are typically transmitted in the environment, environmental sampling may be helpful in detecting an infection quickly. Sampling the environment reduces the likelihood of transmission to other horses or contamination of equipment while swabbing the nasal passage of a potentially infected horse. Despite exposure to environmental factors like rain, heat, and sunshine, bacteria may persist on wooden fence posts for up to three days (Weese et al., 2009). Detection of *S. equi* in environmental
samples, such as drinking water or feces, may therefore facilitate surveillance for strangles.

The objectives of this experiment therefore were to develop an assay to detect *S. equi* by targeting the SeM protein, a major virulence factor. The SeM protein is the most commonly studied antigen of *S. equi* because the variance found in the sequence does not affect its ability to be opsonized by the immune system. This also means that the variance should not affect whether the synthesized monoclonal antibodies can detect the different strains (Waller, 2013).

Specific aims were to prepare dilutions of *S. equi* subsp. *equi* in decreasing concentrations, and correlate with the optical density result of an ELISA using linear regression, and then to evaluate the usefulness of the ELISA technique in estimating numbers of viable bacteria in environmental samples such as water.

The hypotheses were that the ELISA will be able to detect *S. equi* at concentrations of 10,000 CFU/mL, that there will be a linear relationship between the concentration of bacteria in the sample and the absorbance values yielded by ELISA, and that the assay can detect the bacteria from environmental samples, such as water.

*Current Diagnostic Methods for Strangles*

Identification of strangles has until recently relied on aerobic cultures from nasal swabs or from nasopharyngeal washes. However, using a plated culture of the bacteria can lead to many false negative results due to intermittent shedding. Taking the rectal temperature daily may be effective in the detection of an infected animal, since horses may show a fever 24 to 48h before the bacteria were able to be cultured from a mucosal
swab, but this is inconvenient in most management settings. For this reason, point-of-care diagnostic tests for strangles would be very valuable in controlling spread of the organism. These tests could be applied to both the live animal and the environment.

The analysis of surface proteins is important in developing point-of-care tests for *S. equi*, and especially for distinguishing *S. equi* from other bacteria. The surface proteins of *S. equi* have been genetically mapped, allowing for comparison to other surface proteins. Most of these proteins are involved in virulence, and in the case of *S. equi*, that is the attachment to the tonsil and resistance to phagocytosis in the lymph nodes. This may help it to persist as a colony in the guttural pouch for such prolonged periods of time (Waller, 2013).

The SeM surface protein appears to be the major virulence factor for the *Streptococcus* species because it is involved in the evasion from phagocytosis, and so it is a good target for diagnostic tests (Sweeney et al., 2005). A diagnostic test to find the presence of SeM, either genetically or antigenically, would appear ideal because experiments have shown that the antibodies to SeM are produced throughout the course of the disease more reliably than any other antibody (Sweeney et al., 2005). The amino terminus is the least variable region, so targeting that sequence shows the most promise.

A polymerase chain reaction test can detect a specific nucleotide sequence of the SeM protein of *S. equi*, and is an effective differential test to identify a horse with the infection. The test can identify the gene encoding the SeM protein, and with the right primers the test can be specific enough to catch only *S. equi* and sensitive enough to see all of the bacteria present. The nucleotide sequences found in the gene for the SeM
protein have some homology in strains of *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*), but primers are available that are specific to the non–variable terminus of the gene.

Polymerase Chain Reaction is also an important method used to reduce false negatives, because it can isolate a gene unique to *S. equi* and detect it in a sample. Like other diagnostic tools it targets the SeM gene, and appears three times more sensitive in detecting clinical samples that were positive for *S. equi* than culture or biochemical tests. Alternatively, researchers have developed PCR tests to other targets, the genes *eqbE* and *SEQ2190*, allowing the PCR to produce fewer false negatives, because at least one copy of either gene will likely be present across all strains (Webb et al., 2013). However, a potential draw back of PCR is that dead bacterial DNA may yield a positive PCR result, indicating that the horse is a shedder or currently infected, when it has instead cleared the infection.

An alternative approach to detecting SeM in a point-of-care test is through direct detection of the SeM antigen by Enzyme Linked Immunosorbent Assay (ELISA). Monoclonal antibodies may be targeted to the amino terminus of the protein, because it is less variable than the carboxyl terminus. However, this amino terminus is enveloped in the membrane of the bacteria, requiring that it be digested with phage lysozyme. The phage lysozyme digests the peptidoglycan of the cell wall, releasing the protein so that it can be detected by the antibodies. This is an essential part of any SeM targeted antigenic assay, because antibodies would not be able to detect the bacteria itself-only the exposed protein.
Above all, an antigenic test for *S. equi* must distinguish it from other bacteria in a sample. The closely related *S. zooepidemicus* appears similar to *S. equi* when grown on culture because both are β-hemolytic bacteria such that the agar turns from red to clear where the colony of either organism is growing. However, there is a recurring difficulty for differentiating *S. equi* from *S. zooepidemicus*, in finding a target molecule that is sufficiently unique. Certainly, the SeM proteins on *Streptococcus* bacteria are involved in the virulence, and lie on the outside wall of the bacteria, making them ideal markers for tests. However if there is homology, then an antibody produced by the body could cross-react with *S. zooepidemicus*. In terms of the immune response this is a positive outcome, but in the detection of the disease it could lead to false positive test results.

Monoclonal antibodies to SeM can cross-react with some *S. zooepidemicus* strains, and efforts to improve specificity may decrease sensitivity because failing to detect homologous epitopes will prevent detection of some SeM variants. An ELISA test based on the amino terminus of the SeM protein shows most promise, as the test using both of the two epitopes in that region identified 83/89 positive samples and 138/139 negative samples (Robinson et al., 2013). However, the closely related *S. zooepidemicus* shares a homologous sequence of nucleotides near the carboxyl-terminus of the protein.

The antibodies specific to the SeM protein fragment can opsonize other species of the bacteria, even though there is not a significant amount of homology in the nucleotide sequences. Therefore there are other molecular factors involved in the antibody-protein interaction than just the nucleotide sequence (Timoney et al., 1997). This could pose an obvious problem for the specificity of an assay against *S. equi*. 
Although the SeM protein is a major virulence factor for the *Streptococcus* species because it is involved in the evasion from phagocytosis, not all *S. equi* strains have SeM, or they have a protein with a highly varied sequence. This brings up a sensitivity issue for assays based on the detection of SeM. If the assay cannot detect the strain, it would result in a false negative result, and would not be useful as a diagnostic tool. The amino terminus is the least variable region, so an antibody complementary to that sequence will be the most useful.

An alternative to an assay based on the SeM protein has been proposed which is based on the SzPSe protein. Although it has a homolog in *S. zooepidemicus*, it is less variable and more conserved than the SeM protein (Harrington et al., 2002). However, the occurrences of cross-reactive opsonization of *S. zooepidemicus* and lower opsonization of *S. equi* are a drawback of this type of assay.

Another issue raised by detection of *S. equi* using an ELISA based on the SeM protein (or any other antigen) is that due to the nature of the disease progression, when there are no clinical signs but infection has occurred, as in the first 24 hours after initial contact, the levels of SeM would not be detectable (Waller and Jolley, 2007). This would lead to false negatives, continued spread of the disease and the inability to treat an animal in an appropriate time frame.

*Lessons Learned from Vaccination*

A vaccine targeting the SeM protein sequence has been developed. This is relevant to this research because it has shown that the SeM protein can confer immunity after infection. However, the vaccine used only provides immunity against the particular
strain that was targeted (Galan and Timoney 1988). This research shows the importance of developing an assay to catch all of the strains of *S. equi*, to lower the false negative results.

The strangles vaccines in development anticipate bacterial entry into the tonsil, and then clonal expansion of a given *S. equi* strain in the guttural pouch. Once the bacteria have colonized the guttural pouch, the horse has the potential to become a lifetime shedder of the bacteria. A horse that is shedding the bacteria can be difficult to identify, because clinical signs are not likely to be present anymore. However there may be a change in the SeM during infection, altering the sensitivity and specificity of the assay. This has been hypothesized as a cause for why strangles vaccines may sometimes be ineffective, but also indicates that the diagnostic assay may not yield a positive result when the sample comes from a shedder versus a recently infected horse. Thus the diagnostic testing of the horses will have to be different than for those infected for the first time (Timoney, 2007).

**Materials and Methods**

The ELISA protocol had to initially be tested, to be sure that all of the reagents worked in the detecting of the SeM protein. Once it was known that they worked, a standard curve could be made to correlate the optical density given by the spectrophotometer to known dilutions of *S. equi*. The final step was to test how well the ELISA worked in a realistic setting, so water samples were tested and the results compared to the actual numbers of CFUs in the sample.
Testing the reagents

The first step was to make a Phosphate Buffered Saline solution (PBS). The PBS was made from 8 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate and 2.17 g of sodium phosphate heptahydrate to 900 mL of deionized water. Then the pH was adjusted to 7.6 using 6M HCL and 5M NaOH, and then more water was added to make it 1 L of solution. This was the coating buffer, used as a base to make the wash buffer and the blocking buffer. The blocking buffer was made by adding 1 g of Nonfat Dried Milk to 100 mL of coating buffer. The wash buffer was made by adding 0.05 mL of Tween 20 to 1000 mL of coating buffer. Once the buffers were made, the detector antibody MAB211P, made by Maine Biotechnology Services (Portland, ME), was biotinylated. To do so, 10 mg of biotin was dissolved in 250 µL of Dimethyl Sulfoxide, and then 50 µL aliquots were made. Then 12 vials of MAB211P were combined in a microfuge tube with five µL of biotin. It was incubated for an hour and then dialyzed against 0.15M Phosphate Buffered Saline using a two mL Slide-A-Lyzer Mini dialysis device for two hours. Then the PBS was changed and dialyzed for another two hours. An assay that uses a HABA/avidin complex was used to determine that the biotinylation was successful. In the presence of biotin, the biotin molecule will replace the HABA in the complex, so that the HABA can be measured with an absorbance reading at 500 nm. To run this assay, 180 µL of HABA/avidin was added to five wells and shaken for five minutes, after which the absorbance was read. Then 20 µL of the biotinylated MAB211P was added to each well, shaken for another five minutes, and the absorbance read at 500 nm. Each of the five wells had a different sample of MAB211P added, because 20 µL samples were recovered once every hour during dialysis, and
immediately after the PBS solution was changed. Then the molar ratio of biotin to antibody was calculated using a computer program called R. A successful biotinylation resulted in five to eight biotin molecules per antibody molecule.

For the assay, each well of a 96-well plate was coated with 50 µL of monoclonal capture antibody MAB212P, made by Maine Biotechnology Services (Portland, ME) and incubated overnight, covered and in the refrigerator. To add the reagents throughout the assay a multichannel pipette was used. Then the plate was washed using an automated plate washer, and each well was coated with 50 µL Nonfat Dried Milk in PBS solution, which acted as a blocking buffer. This was incubated for one hour at room temperature and with rotation, and then the wells were washed again using the automated plate washer. Then 50 µL of PBS was added to each well, followed 50 µL of the samples into the wells that were assigned to them. The plate has eight rows and 12 columns, and so the sample went into the first column in each row. Then serial dilutions were made across the rows, by successively taking 50 µL from the previous well and moving it to the next well and mixing it eight times using a pipette. This way, it was known that all of the samples had been diluted significantly and it would be possible to visualize whether the color change was less dramatic with a more dilute solution. Each sample had been digested using five µL of Phage lysin C, and had incubated at 37 degrees Celsius for 30 minutes. The samples incubated in the wells for 30 minutes at 37 degrees Celsius, and then the wells were washed again. Next 50 µL of the detector antibody MAB211P conjugated to biotin was added to each well, the plate incubated for an hour then was washed. The detector antibody and biotin had been diluted to a concentration of 0.5 µg/mL by adding 50µL of the solution to ten mL of PBS. Then 50 µL of Streptavidin Horseradish
Peroxidase, diluted in wash buffer at a concentration of one μg/mL was added to each well to bind to the antibodies. This incubated in the wells for 30 minutes at 37 degrees Celsius and the wells were washed four times. Then 50 μL of TMB (3,3’,5,5’-tetramethylbenzidine) was added to each well, acting as a substrate for the enzyme. As the reaction proceeds the solution in the well turned blue. The reaction was allowed to run for 10 minutes, and then it was stopped by adding 50 μL of 0.6M hydrochloric acid to each well. The results were read at 450nm absorbance using a spectrophotometer.

*Initial assay validation*

Once the MAB211P was biotinylated successfully, the Enzyme Linked Immunosorbent Assay was run using dilutions of samples that were known to be positive (fresh culture of *S. equi* and two *S. equi* lysates stored at -20 C for 2 years as positive), samples that were known to be negative (lysate of *Streptococcus equi* subspecies *zooepidemicus*), and compared to the results of using buffer in place of a sample, and dilutions of bacteria grown on blood agar plates.

*Creating a standard curve*

First a lawn of *S. equi* was grown on three blood agar plates. Then the bacteria was suspended in PBS solution by harvesting the bacteria from the three plates using a cotton swab, and twirling the swab in a micro-centrifuge tube filled with 1 mL of PBS, which released the bacteria into solution. One to ten serial dilutions into PBS were made by adding 900 μL of PBS to 11 tubes and serially transferring 100 μL of bacterial solution across the tubes, and then 10 μL of each dilution was plated onto a separate blood agar plate, and incubated overnight. Then the colonies were counted on each plate.
to calculate the concentration of bacteria that was in each diluted solution. Then 5 µL of Phage lysin C was added to each tube that had one mL of the sample, and the tube was incubated for 30 minutes at 37 degrees Celsius. The samples were treated with phage lysin to release the M protein from the rest of the bacteria. Since there were twelve separate concentrations, they were added across the top row of the plate, and then were serially diluted down their respective columns. Then the ELISA assay was run according to the same protocol as before.

*Statistical Analysis*

To create the standard curve, the absorbance readings were plotted against the concentration of the sample in that well. This data can be found in Appendix 1. The scatter plot of these values can be found in Figure 1. There were some wells in the first two columns whose absorbance was so high that the spectrophotometer results only read overflow, with no numerical value, so they were excluded them from the graph. The horizontal axis of the graph is formatted in a logarithmic form, to better visualize the first part of the data, which was originally too close together to make any analysis. The point where the slope of the standard curve flattens indicates that the assay can no longer detect changes in concentration, because although the concentration is decreasing the absorbance reading is the same. The points on the graph before the curve flattens show the linear relationship of the optical density and the concentration of bacteria, so those points were plotted on a second graph, shown in Figure 2. This data can be found in Appendix 1.
**Testing Water Samples**

The next step was to determine how accurate the standard curve is when used with environmental samples. This step is based on the infectious dose of the bacteria, which is 10,000 CFU, and that the typical horse drinks about 10 gallons of water per day. Working with a 10 gallon bucket of infectious water is not safe, the concentration of the infectious dose was calculated to be 0.264 CFU/mL. This was multiplied by 10 mL, so to make the proper dilution three isolated CFUs were added to 10 mL of tap water.

The ELISA relies on the detection of specific bacteria, so it was important to know that a contaminant was not being used instead of the *S. equi*. To do so, samples that were supposedly *S. equi* were streaked for isolation on blood agar plates. Once they grew and exhibited the typical β-hemolysis of the blood, an isolated colony was gram stained to be sure that it was a gram positive streptococci. The final differential test was the fermentation of specific sugars. *S. equi* ssp. *Equi* does not ferment lactose, mannitol, trehalose or sorbitol, while other subspecies do ferment one or some of those particular sugars. To determine whether the sugar was utilized, colonies were swabbed and added to a solution of each sugar. They incubated overnight at 37 degrees Celsius, and fermentation was indicated by a change in color from red to yellow. None of the sugar solutions changed color, so it was determined that the isolated colonies were in fact *S. equi*.

From the 10 mL of tap water that had been inoculated with three isolated colonies, the bacteria was collected using a cotton swab and transferred to one mL of PBS. This was intended to mimic the realistic collection of bacteria from a sample.
intended to be analyzed. The bacteria needed to be transferred to the PBS because the phage lysin is only functional at the specific pH of the PBS. From the one mL sample, serial dilutions were made, by pipetting 100 µL of the original solution into 900 µL of PBS. Three serial dilutions were made from the original in this way. The process of swabbing the water sample and performing three serial dilutions was then repeated twice more to ensure that the bacteria had been captured from the water sample. This resulted in 12 samples that could then be tested.

Before the 12 samples were digested in phage lysin, 100 µL was taken from each and pipetted onto a corresponding blood agar plate, then spread for colony isolation using a sterile loop for each plate. This step was intended to be a check against the ELISA results, because the colonies could be counted and compared to what the test indicated.

Then 50 µL of each of the 12 samples were run through the same ELISA protocol as described previously, and their absorbance was measured using the spectrophotometer. The blood agar plates incubated overnight at 37 degrees Celsius, and the colonies were counted based on the hemolysis that they showed.

Results and Discussion

Initial Assay Validation

Results of the initial assay are shown in Figure 1 and 2. The assay correctly identified *S. equi* positive samples. The decrease in signal strength of three dilutions of *S. equi* indicated a quantitative relationship. Additionally, the assay did not recognize samples of *S. zoöepidemicus* or reagent buffer free of *Streptococci*. 
Standard Curve

Graphical plot of all the data from 96 points used in standard curve estimation is shown in Figure 3. The data from the linear part of the graph shows that there is correlation between the absorbance value and the concentration of bacteria, with the slope of the line of regression as 0.00005 (Figure 4). The $R^2$ value is 0.9258, indicating that the correlation shown by the line of regression is strong. The scatter plot of the data confirms the hypothesis: an increase in the concentration of SeM will result in a linear increase in the absorbance value that is read. The other hypothesis, that the test could detect the SeM protein and result in a color change was also correct. The assay could accurately detect a range of concentrations from 78,125 CFU/mL to 15,000 CFU/mL. This is not accurate enough to match the infectious dose of the bacteria, but more tests can be done to refine these results. The ELISA plates before and after addition of the stop solution are shown in Figures 5 and 6.

Environmental Sample Testing

The second part of the project, which involved a realistic water bucket sampling, did not yield a significant relationship. The output value of the concentration was calculated when absorbance values were entered into the regression model given by the standard curve. This data is shown in Table 1. The colonies on the plates were more than a factor of 10 different from what the absorbance values indicated. This may be due to several factors. First, a colony forming unit is not necessarily one bacterium, especially in
the case of a Streptococcus, which tends to be grouped as a string of bacteria. Therefore it was impossible to get an accurate count of bacteria just by doing colony counts. Also, since each CFU that was formed can have different numbers of bacteria, the colonies used to inoculate the water sample had a significant level of variation. Another source of inaccuracy is that since tap water was used as the initial sample, there were contaminants on the plates that clouded the S. equi colonies on top of the agar. This forced the colony count to rely on the hemolysis of the blood that showed through to the outside of the plate (Figure 7). Anecdotally, there is usually more than one colony per cleared circle on the plate, so by only counting the circles of hemolysis, the actual number of colonies would be underestimated. All of the plates that were grown using a sample which had not been diluted had growth that was too confluent to count (Figure 8), so the colony count was estimated to be 300 CFUs, which is the upper limit of what is countable, and then it was multiplied by 10 to account for the fact that the plates had one tenth of the original PBS solution, as was done for all of the plate counts. The regression coefficient obtained when predicted colony counts from absorbance data were compared with actual colony counts (Figure 9) did not indicate a strong relationship (R² = 0.0028).

**Conclusion**

In conclusion, the ELISA assay was sensitive enough to detect a concentration of at least 15,000 CFU/mL, which is slightly higher than the 10,000 CFU/mL stated in the hypothesis, but within the order of magnitude expected of the test. The relationship had a strong regression coefficient (R² = 0.93). Overall the data would tend to support the hypothesis, but further testing is necessary. While positive results were obtained in water
samples containing *S. equi*, contamination and confluence of colonies made it impossible to confirm a linear relationship between absorbance and colony counts.
Tables and Figures

Figure 2. Same as Figure 1, following addition of stop solution (0.6 N HCl).

![Figure 2](image)

Figure 3. Graphical representation of absorbance readings (450 nm) following ELISA of 96 different concentrations of *S. equi*.

![Figure 3](image)
Figure 4. Regression of absorbance values derived from ELISA of *S. equi* at concentrations in the linear region of the standard curve shown in Figure 3.

![Linear Relationship of the Standard Curve](image)

\[ y = 5 \times 10^{-5}x - 0.2003 \]
\[ R^2 = 0.92581 \]

Figure 5. ELISA plate from standard curve calculation after the addition of tetramethylbenzidine, but prior to addition of 0.6 N HCl. Row A1 – A12: serial 1:10 dilutions of *S. equi* lysate. Rows B – H, serial 1:2 fold dilutions of columns A1 – A12.
Figure 6. Same as Figure 5, following addition of stop solution (0.6 N HCl).

Figure 7. β-hemolysis and colony counting method (black dots). Darker growth in zigzag pattern behind hemolytic colonies shows contaminant.
Figure 8. Confluent hemolysis that prevented the colonies from being counted accurately.

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<table>
<thead>
<tr>
<th>Plate Number</th>
<th>ABS (y)</th>
<th>CFU/mL (x)</th>
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<td>3000</td>
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<tr>
<td>C3</td>
<td>0.397</td>
<td>11946</td>
<td>30</td>
</tr>
</tbody>
</table>
References


Author’s Bio

Taryn Haller was born in Ellicott City, Maryland on June 17th, 1993. She lived most of her life in Groton, CT and graduated with an International Baccalaureate diploma from Robert E. Fitch Senior High School in 2011. Taryn graduated from the University of Maine with a Bachelor’s Degree in Animal Science, and was involved with the Catholic Student Association, the Pre-Veterinary Club, and the National Society of Collegiate Scholars on campus. After graduation, Taryn will attend the University of California, Davis School of Veterinary Medicine to pursue a Doctorate of Veterinary Medicine and pursue a career as a veterinary surgeon.
## Appendix 1: Data

Table A 1: Absorbance Values and Concentrations of Bacteria Used in the Standard Curve.

<table>
<thead>
<tr>
<th>CFU/mL</th>
<th>ABS</th>
<th>CFU/mL</th>
<th>ABS</th>
<th>CFU/mL</th>
<th>ABS</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000000</td>
<td>OVRFLW</td>
<td>3125</td>
<td>0.505</td>
<td>3.75</td>
<td>0.961</td>
</tr>
<tr>
<td>2500000</td>
<td>OVRFLW</td>
<td>3000</td>
<td>0.433</td>
<td>2.34375</td>
<td>0.977</td>
</tr>
<tr>
<td>1250000</td>
<td>OVRFLW</td>
<td>1875</td>
<td>0.586</td>
<td>1.875</td>
<td>0.474</td>
</tr>
<tr>
<td>6250000</td>
<td>OVRFLW</td>
<td>1562.5</td>
<td>0.463</td>
<td>1.5</td>
<td>0.712</td>
</tr>
<tr>
<td>5000000</td>
<td>OVRFLW</td>
<td>1500</td>
<td>0.5</td>
<td>1.171875</td>
<td>0.781</td>
</tr>
<tr>
<td>3125000</td>
<td>OVRFLW</td>
<td>1500</td>
<td>1.244</td>
<td>0.9375</td>
<td>0.388</td>
</tr>
<tr>
<td>2500000</td>
<td>OVRFLW</td>
<td>937.5</td>
<td>0.63</td>
<td>0.75</td>
<td>0.446</td>
</tr>
<tr>
<td>1562500</td>
<td>OVRFLW</td>
<td>781.25</td>
<td>0.887</td>
<td>0.46875</td>
<td>0.708</td>
</tr>
<tr>
<td>1250000</td>
<td>OVRFLW</td>
<td>750</td>
<td>0.406</td>
<td>0.375</td>
<td>0.57</td>
</tr>
<tr>
<td>781250</td>
<td>OVRFLW</td>
<td>750</td>
<td>0.456</td>
<td>0.234375</td>
<td>0.937</td>
</tr>
<tr>
<td>625000</td>
<td>OVRFLW</td>
<td>468.75</td>
<td>0.597</td>
<td>0.1875</td>
<td>0.393</td>
</tr>
<tr>
<td>500000</td>
<td>3.17</td>
<td>390.625</td>
<td>0.873</td>
<td>0.15</td>
<td>0.664</td>
</tr>
<tr>
<td>390625</td>
<td>OVRFLW</td>
<td>375</td>
<td>0.528</td>
<td>0.117188</td>
<td>0.601</td>
</tr>
<tr>
<td>312500</td>
<td>OVRFLW</td>
<td>375</td>
<td>0.393</td>
<td>0.09375</td>
<td>0.3</td>
</tr>
<tr>
<td>250000</td>
<td>2.867</td>
<td>234.375</td>
<td>0.488</td>
<td>0.075</td>
<td>0.442</td>
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<tr>
<td>156250</td>
<td>3.819</td>
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<td>0.046875</td>
<td>0.895</td>
</tr>
<tr>
<td>125000</td>
<td>1.999</td>
<td>187.5</td>
<td>0.477</td>
<td>0.0375</td>
<td>0.653</td>
</tr>
<tr>
<td>78125</td>
<td>3.571</td>
<td>150</td>
<td>0.736</td>
<td>0.023438</td>
<td>0.681</td>
</tr>
<tr>
<td>62500</td>
<td>1.04</td>
<td>93.75</td>
<td>0.541</td>
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<tr>
<td>50000</td>
<td>0.693</td>
<td>93.75</td>
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<td>0.015</td>
<td>0.443</td>
</tr>
<tr>
<td>390625</td>
<td>3.237</td>
<td>75</td>
<td>0.602</td>
<td>0.011719</td>
<td>0.816</td>
</tr>
<tr>
<td>31250</td>
<td>1.079</td>
<td>46.875</td>
<td>0.559</td>
<td>0.009375</td>
<td>0.48</td>
</tr>
<tr>
<td>30000</td>
<td>1.394</td>
<td>46.875</td>
<td>0.309</td>
<td>0.0075</td>
<td>0.803</td>
</tr>
<tr>
<td>25000</td>
<td>0.565</td>
<td>37.5</td>
<td>0.405</td>
<td>0.004688</td>
<td>0.728</td>
</tr>
<tr>
<td>15625</td>
<td>1.019</td>
<td>23.4375</td>
<td>0.463</td>
<td>0.00375</td>
<td>0.411</td>
</tr>
<tr>
<td>15000</td>
<td>0.404</td>
<td>23.4375</td>
<td>0.733</td>
<td>0.002344</td>
<td>0.36</td>
</tr>
<tr>
<td>12500</td>
<td>0.424</td>
<td>18.75</td>
<td>0.987</td>
<td>0.001875</td>
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</tr>
<tr>
<td>7812.5</td>
<td>0.479</td>
<td>15</td>
<td>0.427</td>
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<td>0.531</td>
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<tr>
<td>7500</td>
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<td>11.71875</td>
<td>0.655</td>
<td>0.000938</td>
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<tr>
<td>6250</td>
<td>0.646</td>
<td>9.375</td>
<td>0.295</td>
<td>0.000469</td>
<td>0.517</td>
</tr>
<tr>
<td>3906.25</td>
<td>0.776</td>
<td>7.5</td>
<td>0.708</td>
<td>0.000234</td>
<td>0.396</td>
</tr>
<tr>
<td>3750</td>
<td>0.696</td>
<td>4.6875</td>
<td>0.297</td>
<td>0.000117</td>
<td>0.509</td>
</tr>
</tbody>
</table>
Table A 2: Data points in the linear region of standard curve that showed a linear relationship.

<table>
<thead>
<tr>
<th>CFU/mL</th>
<th>Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>78125</td>
<td>3.571</td>
</tr>
<tr>
<td>31250</td>
<td>1.079</td>
</tr>
<tr>
<td>30000</td>
<td>1.394</td>
</tr>
<tr>
<td>25000</td>
<td>0.565</td>
</tr>
<tr>
<td>15625</td>
<td>1.019</td>
</tr>
<tr>
<td>15000</td>
<td>0.404</td>
</tr>
</tbody>
</table>
Appendix 2: Protocol Flow sheets and Reagent Preparation Sheets

Elisa protocol flow sheet

Name:
Date:

I. Hazard Assessment

To protect yourself from any possible hazards associated with this task wear eye protection. You should also wear latex, nitrile, or vinyl gloves and a lab coat with long sleeves. To protect your legs and feet wear closed shoes and long trousers. Do not wear sandals, shorts or a short skirt. Wash your hands before eating and when leaving the laboratory. You should review the MSDS for any chemical used in this procedure. In case of a spill with a toxic chemical remove all contaminated clothing and wash affected areas with copious quantities of water. Check location of the nearest safety shower. Eyes should be washed copiously for 15 minutes.

When preparing 0.6 N Hydrochloric acid solution wear goggles that form a seal and nitrile gloves. Always add the acid to the water, not vice versa.

This protocol is based on the Maine Biotechnology Services ELISA protocol for SeM.

II. Reagents and supplies

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Separate SOP?</th>
<th>Ready</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating buffer (0.15 M PBS, pH 7.6)</td>
<td>1 liter</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Capture Antibody - MAB212 diluted in coating buffer (2 ug/mL)</td>
<td>10 mL</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Wash Buffer (0.15 M PBS, 0.05% tween 20)</td>
<td>1 liter</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Blocking Buffer (1% Non Fat Dried Milk (NFDM) in 0.15 M PBS)</td>
<td>100 ml</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Sample: phagelysin digested sample, variable dilution profiles, 50 ul. per well,</td>
<td>as needed</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Detector Antibody MAB211-biotin diluted in blocking buffer (0.5 ug/mL)</td>
<td>10 mL</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Tracer Streptavidin Horse Radish Peroxidase (HRP) diluted in wash buffer (1:10,000)</td>
<td>10 mL</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Substrate, which is turned blue by HRP, TMBW (undiluted - single step reagent)</td>
<td>10 mL</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Stop solution which causes blue to yellow color change (0.6 N Hydrochloric acid)</td>
<td>10 mL</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

Material

ELISA Plates
Plate washer
Multichannel pipette
Unichannel pipettes
Pipette tips
Reagent tubes
III. Coating Plates
   Label Plates
   Dispense 50 ul of 2 ug/ml MAB212 capture antibody to all wells**
   Cover and refrigerate at 4 C overnight

**Use Multichannel pipette

Comments:

IV. Wash Plates post-coating
   Wash # 1 with automatic plate washer (0.15 M PBS, 0.05% tween 20), fill 300 uL
   Flick then blot dry

Comments:

V. Blocking
   Prepare blocking buffer (1 g NFDM in 100 mL PBS)
   Label expiration date 1 week from date of preparation
   Dispense 300 ul of Blocking Buffer (1% Non Fat Dried Milk in 0.15 M PBS) to all wells**
   Refrigerate any excess blocking buffer
   Incubate plates at room temperature for 1 hour or overnight at 4 C

**Use Multichannel pipette

Comments:
VI. **Wash Plates post blocking**
Wash #1 with automatic plate washer (0.15 M PBS, 0.05% tween 20), fill 300 uL ____.  
Flick then blot dry ____.  
Comments:  

VII. **Arrange of samples by serial dilution 1:2 (or 1:6)**
Prepare phagelysin digested samples/controls in dilution tubes, label and record on summary sheet ____.  
Dispense 50 ul of 0.15 M PBS to all wells ____.  
Add 50 ul (or 10 ul) of samples to rows in column 1* ____.  
Transfer 50 ul (or 10 ul) to column 2** ____.  
Repeat transfer through row 12** ____.  
Discard the last 50 ul (or 10 ul) from row 12 ____.  
Incubate plates with rotation at 37 C for 30 minutes or at room temp for 1 hour ____.  
**During this incubation, prepare a 0.5 ug/ml solution of the detector antibody MAB211P-biotin, in blocking buffer (1% NFDM in 0.15 M PBS; see separate SOP)** ____.  

*Use single channel pipette  
**Use Multichannel pipette, mixing 8 X before each transfer  
Comments:  

VIII. **Wash Plates post sample incubation**
Wash #1 with automatic plate washer (0.15 M PBS, 0.05% tween 20), fill 300 uL ____.  
Flick then blot dry ____.  
Comments:  

IX. **Addition of Detector Antibody MAB211P-Biotin**
Pipet 50 ul well of the detector antibody (MAB211P-biotin) to each well** ____.  
Incubate at 37C for 30 minutes or 1 hour at RT, with rotation if possible. ____.  
**During this incubation, prepare a 1:10,000 dilution of the Streptavidin HRP in wash buffer. See separate SOP** ____.  

30
**Use Multichannel pipette**

Comments:

X. Wash Plates post incubation with MAB211P-Biotin Detector
   Wash # 1 with automatic plate washer (0.15 M PBS, 0.05% tween 20), fill 300 uL ____
   Flick then blot dry ____
   Comments:

XI. Addition of Streptavidin HRP
   Pipet 50 ul of the Streptavidin HRP at 1:10,000 to each well** ____
   Incubate at 37C for 30 minutes or 1 hour at RT, with rotation if possible. ____
   **Use Multichannel pipette**
   Comments:

XII. Wash plates post incubation with Streptavidin HRP
   Wash # 1 with automatic plate washer (0.15 M PBS, 0.05% tween 20), fill 300 uL ____
   Wash # 2 with automatic plate washer (0.15 M PBS, 0.05% tween 20), fill 300 uL ____
   Wash # 3 with automatic plate washer (0.15 M PBS, 0.05% tween 20), fill 300 uL ____
   Wash # 4 with automatic plate washer (0.15 M PBS, 0.05% tween 20), fill 300 uL ____
   Flick then blot dry ____
   Comments:

XIII. Addition of TMBW color reagent
   Add 50 ul of the TMB to each well on plate ** ____
   Start timer for 10 minutes and cover plate** ____
   After 10 minutes, stop the enzyme reaction by adding 50 ul of 0.6N HCL ____
Read the plate at A(450 nm)

**Use Multichannel pipette

Comments:
Reagent Preparation Sheet.

0.15M PBS, pH 7.6, coating buffer

ELISA Coating Buffer

Date prepared:

By:

I. Hazard Assessment

To protect yourself from any possible hazards associated with this task wear eye protection. You should also wear latex, nitrile, or vinyl gloves and a lab coat with long sleeves. To protect your legs and feet wear closed shoes and long trousers. Do not wear sandals, shorts or a short skirt. Wash your hands before eating and when leaving the laboratory. You should review the MSDS for any chemical used in this procedure. In case of a spill with a toxic chemical remove all contaminated clothing and wash affected areas with copious quantities of water. Check location of the nearest safety shower. Eyes should be washed copiously for 15 minutes.

II. Buffer

0.15M PBS, pH 7.6, coating buffer

III. Purpose of buffer

Basis buffer in SeM ELISA protocol for coating plates with MAB212p, also the 1X PBS used in blocking, washing and antibody dilution.

IV. Buffer recipe

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium chloride</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2 g</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na2HPO4.7H2O</td>
<td>2.17 g</td>
</tr>
<tr>
<td>water</td>
<td>900 ml</td>
</tr>
<tr>
<td>Adjust pH to 7.6</td>
<td></td>
</tr>
<tr>
<td>with 6N HCL or 5N NaOH as necessary</td>
<td></td>
</tr>
</tbody>
</table>

Add water to 1 liter

V. Comments
Reagent Preparation Sheet.

Working dilution of MAB212p in coating buffer for coating ELISA plates at a concentration of 2 ug/mL

Date prepared:

By:

I. Hazard Assessment

To protect yourself from any possible hazards associated with this task wear eye protection. You should also wear latex, nitrile, or vinyl gloves and a lab coat with long sleeves. To protect your legs and feet wear closed shoes and long trowsers. Do not wear sandals, shorts or a short skirt. Wash your hands before eating and when leaving the laboratory. You should review the MSDS for any chemical used in this procedure. In case of a spill with a toxic chemical remove all contaminated clothing and wash affected areas with copious quantities of water. Check location of the nearest safety shower. Eyes should be washed copiously for 15 minutes.

II. Reagent

MAB212p dilution in coating buffer for SeM ELISA

III. Purpose of reagent

Serves as the capture antibody MAB212p for SeM ELISA. This antibody serves as the coating agent on the ELISA plate to concentrate target SeM and Sem containing streptococci.

IV. Reconstituted vial and storage

5 Vials (1 mL) received at 1.4mg/mL. Aliquotted into 50 ul aliquots and frozen at -20°C.

V. To prepare a working dilution

Number of moles in 10 mL @ 2 ug/mL = Number of moles in x mL @ 1.4 mg/ml (added to 10 mL buffer)
10 mL X 2 ug/mL = x mL X 1.4 mg/mL
10 mL X 2 ug/mL = x mL X 1400 ug/mL
x mL = (10 mL X 2 ug/mL)/1400ug/mL solve for x
x ul = 1000*(10 mL X 2 ug/mL)/1400ug/mL give volume in ul
x ul = (10 mL X 2 ug/mL)/1.4 mg/mL 1000’s cancel out
x = 10*2/1.4 yields volume (in ul) of MAB212p @ 1.4 mg/mL to add to 10 mL to make a 2 ug/mL solution in ul

The R code to generate table below

> y=c(1.2,1.3,1.4,1.5) # a variety of concentrations, including the 1.4 mg/mL used in the example
> x=10*y; x #prints volumes (uL) to add of each concentration

<table>
<thead>
<tr>
<th>Original concentration MAB212p</th>
<th>1.2 mg/mL</th>
<th>1.3 mg/mL</th>
<th>1.4 mg/mL</th>
<th>1.5 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume MAB212p to use</td>
<td>16 ul</td>
<td>15 ul</td>
<td>14 uL</td>
<td>13 ul</td>
</tr>
<tr>
<td>Volume of coating buffer (0.15M PBS, pH 7.6)</td>
<td>10 mL</td>
<td>10 mL</td>
<td>10 mL</td>
<td>10 mL</td>
</tr>
<tr>
<td>Check which volume used</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
</tbody>
</table>

VI. Comments
Reagent Preparation Sheet.

Preparation of Phage Lysin digested sample for SeM ELISA

Date prepared:

By:

I. Hazard Assessment

To protect yourself from any possible hazards associated with this task wear eye protection. You should also wear latex, nitrile, or vinyl gloves and a lab coat with long sleeves. To protect your legs and feet wear closed shoes and long trousers. Do not wear sandals, shorts or a short skirt. Wash your hands before eating and when leaving the laboratory. You should review the MSDS for any chemical used in this procedure. In case of a spill with a toxic chemical remove all contaminated clothing and wash affected areas with copious quantities of water. Check location of the nearest safety shower. Eyes should be washed copiously for 15 minutes.

II. Reagent

Phage lysin digest of sample for SeM ELISA.

III. Purpose of reagent

The Phage Lysin C (PlyC) releases SeM from the wall of Streptococcus equi by digesting the peptidoglycan. This exposes epitopes on the SeM protein which are recognized by MAB212p (capture) and MAB211p (detector) monoclonal antibodies.

IV. Reconstituted vial and storage

Phage lysin was received at 5 mg/ml (i.e. 5 ug/ul) and aliquotted into 50 ul aliquots and frozen at -20 C.

V. To digest sample

The following method is used to digest the sample. The sample is intended to be on a cotton swab. This would be either a clinical swab, a swab placed in compost, a swab swept through the water (or over the plastic surface) of a water bucket, or a swab drawn over a blood plate to harvest streptococcal colonies

Twirl swab in 1 mL of PBS (0.15 M, pH 7.6) ______
Add 5 ul of phage lysin ______
Incubate 30 minutes at 37 C ______
Label, date and store unused digest at - 20 C ______

VI. Comments
Reagent Preparation Sheet.

Preparation of Biotinylated MAB211p, SeM detector antibody in ELISA protocol.

Date prepared:

By:

I. Hazard Assessment

To protect yourself from any possible hazards associated with this task wear eye protection. You should also wear latex, nitrile, or vinyl gloves and a lab coat with long sleeves. To protect your legs and feet wear closed shoes and long trowsers. Do not wear sandals, shorts or a short skirt. Wash your hands before eating and when leaving the laboratory. You should review the MSDS for any chemical used in this procedure. In case of a spill with a toxic chemical remove all contaminated clothing and wash affected areas with copious quantities of water. Check location of the nearest safety shower. Eyes should be washed copiously for 15 minutes.

II. Reagent

Biotin conjugated MAB211p.

III. Purpose of reagent

The monoclonal IgG antibody used in detection of SeM antigen of Streptococcus equi subspecies equi. In ELISA format, it is used in conjunction with MAB212, the latter being a capture antibody to concentrate target on the ELISA plate.

IV. Reconstitution and storage of Biotin

Dissolve vial of biotin (10mg) in 250 ul of DMSO. This gives a final concentration of 40 mg/mL.

Make 50 ul aliquots of biotin at 40 mg/mL (i.e. 5 aliquots at 50 ul) and store at - 20 C

V. Biotinylation procedure

In a 1.5 mL microfuge tube combine 12 (50 ul) vials of MAB211p (tracer antibody)

(12 X 75ug = 900ug of MAB211p and 12 X 50ul = 600 ul total volume)

To this add 5 ul of biotin (5 ul X 40 mg/mL = 0.2 mg)

Mix and incubate at room temperature for an hour

Dialyse this volume (600 ul) against 0.15M PBS using a 2 mL Slide-A-Lyzer MINI Dialysis Device for 2 hours at room temperature, twice with 20 kD Molecular weight cut-off (20K MWCO).

Reconstitute with PBS up to a volume of 900 ul (~1 mg/mL) and make 50 ul aliquots (each at ~1 mg/mL i.e. 50 ug) and freeze aliquots at -20 C.

When ready to conduct ELISA, add 1 aliquot of biotinylated antibody (50 ug) to 10 mL of blocking buffer (0.15 M PBS with 1 % NFDM) to have a final concentration of 0.5 ug/mL of detector antibody in blocking buffer.
VI. Assessment of biotinylation using HABA/AVI D IN BIOTIN replacement assay

The assay provides a colometric assessment of displacement of HABA from its complex with AVI D IN by biotin. The HABA/AVI D IN complex is measured at A500, and its reduction in absorbance can be directly correlated to moles of HABA being replaced by biotin.

Reconstitute HABA/AVI D IN vial (Sigma H 2153) with 10 mL of deionized water. (Use needle and syringe.)

Label vial "exp" and date one month from today, store at 0-4 C

Add 180 ul of HABA/AVI D IN solution to well. Shake 5 min

Record absorbance at 500 nm ______ A500 HA

Record concentration of biotinylated MAB211p mg/mL ______ mg/ml mgmlpro

Add 20 ul of biotinylated MAB 211p to same well. Shake 5 min

Record absorbance at 500 nm ______ A500HAB

(If absorbance < 0.15 dilute sample and repeat assay)

Calculate molar ratio of biotin to protein (target is 5 - 8) moles of biotin per mole of protein

VII. R code Calculations

In the formula below fill in values xxx for mgmlpro, A500HA, and A500HAB. The formula generates the biotin to protein molar ratio and an array with values including DA (difference in absorbance), mmol per mL of biotin (mmolmlbio), mmol per mL of protein (mmolmlpro) and the last value is also the biotin to protein molar ratio (bio2pro).

Formula for microplate

\[
\text{mgmlpro} = xxx; \ A500HA = xxx; \ A500HAB = xxx; \ DA = (A500HA) - A500HAB; \ mmolmlbio = DA(34000*0.5) / mmolmlpro = mgmlpro/150000; \ bio2pro = (mmolmlbio*10)/mmolmlpro; \ bio2pro; \ \text{rbind(mgmlpro,} A500HA, \ A500HAB, \ DA, \ mmolmlpro, \ mmolmlbio, \ bio2pro)#for microplate
\]

In R paste the text after the command prompt ">". For example:

> mgmlpro=0.69; A500HA=0.904; A500HAB=0.771; DA=(A500HA)-A500HAB; mmolmlbio=DA(34000*0.5)/mmolmlpro=mgmlpro/150000; bio2pro=(mmolmlbio*10)/mmolmlpro; bio2pro; rbind(mgmlpro,A500HA,A500HAB,DA,mmolmlpro,mmolmlbio,bio2pro)#for microplate

[1] 17.00767

[,1]

mgmlpro   6.900000e-01
A500HA    9.040000e-01
A500HAB   7.710000e-01

38
DA 1.330000e-01
mmolmlpro 4.600000e-06
mmolmlbio 7.823529e-06
bio2pro 1.700767e+01

Formula for cuvette.

\[ mgmlpro = xxx; \ A500HA = xxx; \ A500HAB = xxx; DA = (0.9*A500HA) - A500HAB; \]
\[ mmolmlbio = DA/(34000*1); mmolmlpro = mgmlpro/150000; bio2pro = \]
\[ (mmolmlbio*10)/mmolmlpro; rbind(mgmlpro,A500HA, A500HAB, DA, \]
\[ mmolmlpro,mmolmlbio,bio2pro)#for cuvette \]

Refer to instructions for HABA/AVIDIN, for how to perform assay in a cuvette system. The
formula is adjusted slightly to adjust for change in absorbance with addition of sample (0.9),
and different pathway of cuvette (1 cm) versus microtitre plate (0.5 cm)

VIII. Comments

Biotinamidocaproate N-Hydroxy-Succinimide ester provides more detailed information
about biotin

(Sigma H 2153) gives detailed information about the HABA/AVIDIN reagent from Sigma
used in this protocol, including instructions on how to reconstitute and store reagent.

HABA/AVIDIN gives detailed explanation and background information on the assay, and
how to perform calculations. But instructions on how to reconstitute the vial are not
applicable to this protocol since we use a different product (Sigma H 2153)

Slide-A-Lyzer detailed explanation and background information for slide-alyzer dilaysis
system

Ordering Information:

www.Sigmaaldrich.com

- Biotin - Biotinamidohexanoic acid N-hydroxysuccinimide ester 10 mg # B2643-
10MG $29.90

- Dimethyl sulfoxide CHROMASOLV Plu # 34869-100ML $66.30

- HABA/Avidin Reagent lyophilized powder # H2153-1VL $39.50

www.piercenet.com

- Slide-A-Lyzer MINI Dialysis Device, 20 Kdalton Molecular Weight Cut-Off (20K
MWCO), 2mL (pack of 25 devices) catalog # 88405 $220.
Reagent Preparation Sheet.

Conjugated-Streptavidin horse radish peroxidase

Date prepared:

By:

I. Hazard Assessment

To protect yourself from any possible hazards associated with this task wear eye protection. You should also wear latex, nitrile, or vinyl gloves and a lab coat with long sleeves. To protect your legs and feet wear closed shoes and long trousers. Do not wear sandals, shorts or a short skirt. Wash your hands before eating and when leaving the laboratory. You should review the MSDS for any chemical used in this procedure. In case of a spill with a toxic chemical remove all contaminated clothing and wash affected areas with copious quantities of water. Check location of the nearest safety shower. Eyes should be washed copiously for 15 minutes.

II. Reagent

Peroxidase-conjugated Streptavidin

III. Purpose of reagent

Streptavidin is a protein derived from *Streptomyces avidinii*. Streptavidin binds biotin (vitamin B7) in one of the strongest non-covalent interactions known.

The HorseRadish Peroxidase enzyme is made visible when it catalyzes oxidation of a substrate by hydrogen peroxide. Substrates are chosen which change color on oxidation, and the presence of the HRP or an HRP-conjugate can then be detected by spectrophotometry. In this case the HRP is conjugated to Avidin, which binds to the biotin on MAB211p, which is the monoclonal antibody binding specific epitopes on SeM.

The substrate used in the SeM ELISA protocol is tetramethylbenzidine (TMB). When oxidized by HRP it turns blue (read absorbance at 650 nm), but following stopping the reaction using 0.6 N HCL, it turns yellow (read absorbance at 450 nm)

IV. Reconstituted vial and storage

Add 1.0 mL dH2O to 1 vial (1.0 mg) freeze dried powder ___ (1 mg/ml)

Centrifuge product if not clear

Store at 2-8 C

Label vial "exp" followed by date 6 weeks in the future ___

V. To prepare a working dilution

Should be done fresh each day:

Dilution

0.1 ug/mL 1 ug/mL 2 ug/mL
Check dilution used

<table>
<thead>
<tr>
<th>Final &quot;Dilution&quot;</th>
<th>1:100,000</th>
<th>1:10,000*</th>
<th>1:5,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase-HRP stock diluted 1:10 (1 mg/mL)</td>
<td>1 uL</td>
<td>10 ul*</td>
<td>20 ul</td>
</tr>
<tr>
<td>Volume of wash buffer (0.15M PBS, 0.05% tween 20)</td>
<td>10 mL</td>
<td>10 mL*</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

*This is the dilution specified in the SeM ELISA Protocol.

VI. Comments

**HRP-Streptavidin** provides more technical details.

Ordering Information:

www.jacksonimmuno.com

Jackson Immunoresearch, Peroxidase-conjugated streptavidin, 1.0 mg as freeze-dried powder. The catalog # is 016-030-084.

**TMB** provides more detailed technical information

Ordering Information:

www.surmodics.com

TMB One Component HRP Microwell Substrate # 1000-01 TMBW 100mL