REU Program

Mapping MSP1-19 Binding Regions in Band 3 Peptide

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Abstract

The overall purpose for this research is to understand the mechanisms of the Plasmodium falciparum invasion of erythrocytes as band 3 a host receptor in the red blood cells for merozoite surface protein 1 in the malaria parasite. From this knowledge then new vaccines can be created. The objectives are to test the binding strengths of two segments 5ABC and 6A from band 3 peptide with merozoite surface protein 1 found in a malaria parasite. These two segments were found to be host receptors to plasmodium falciparum, which is a deadly strand of malaria. The secondary objectives are to test the binding strengths of two truncated forms of the segment 5ABC, 5 and 6 as well as test two chimeric proteins 5ABC6A and 6A5ABC.

The experiments will use GST-5ABC, GST-5ABC6A and GST-6A5ABC, GST moiety fusion proteins and Trx-MSP1-19, Trx-MSP1-19A, Trx-MSP1-19B, Trx moiety fusion proteins. And the binding measures will use the same method and the analytes concentrations range from 200nM to 1500nM. These samples were placed in the autosampler inside the Biacore, which uses surface plasmon resonance for the interaction between proteins. Surface plasmon resonance detects the interactions due to reflected light between two Medias. When the experiments are finished the data is presented in the form of a sensorgram.

The results of the experiments showed signs of strong binding with GST-5ABC with the three forms of the malaria parasite, Trx-MSP1-19, Trx-MSP1-19A, and Trx-MSP1-19B. GST-6A with the three forms of the merozoite surface protein showed signs of binding. It was not as strong as the first set of experiments. Weak binding was found for the truncated forms of GST-5 and GST-6 with Trx-19. Stronger binding occurred in the experiments of GST-5 and GST-6 with Trx-19A. The two chimeric proteins, GST-5ABC6A and GST-6A5ABC with the Trx-19 showed no signs of binding occurring when GST-5ABC6A or GST-6A5ABC are immobilized on the chip as ligands but binding appears when Trx-MSP1-19A was immobilized on the sensor chip the experiments were repeated and stronger binding seemed to occur. The two segments

5ABC and 6A seemed to bind to the malaria parasite, meaning that the merozoite protein 1 (MSP1-19) has a role in the invasion of the red blood cells.

Introduction

Band 3 peptide, which is located in red blood cells (RBCs), was found to be a host receptor for the invasion of the malaria parasite[1]. Plasmodium falciparum is the most widespread and deadly of the four strands of malaria. The objectives to this experiment are to evaluate the binding strengths of the merozoite surface protein 1 to different types of peptides, for example 5ABC, 6A from the Band 3 peptide. From the study of the binding strengths new vaccines may be designed that will target more of the binding sights on the band 3 peptide.

This experiment is important to the advancement in the understanding of the malaria parasite. It happens that this disease even though it was said to be eradicated in the United States in the 1950's, still exists in third world countries. A million people die from this disease every year; most of the victims are young children. Over 40% of the worlds' population is affected by one of the four strands of malaria[2].

The invasion of p. falciparum occurs when it attaches to one of the binding sites located along the strand of the band 3 peptide. Merozoite surface protein located inside the p. falciparum strand is what attaches the parasite to the RBCs. Once the parasite is inside the cell, it changes the forms of the erythrocytes. Inside the erythrocyte the band 3 peptide has a certain function. That function is to facilitate an electroneutral exchange of the ions Cl^- and HCO_3^- across the plasma membrane[3]. Palsmodium falciparum can induce alterations in the shape of the band 3 peptide. Membrane protein clustering is caused by the conformational changes in the band 3 peptide[3]. Once an erythrocyte is affected by the parasite then knob-like protrusions appear all around the affected RBC. These cells flow through the bloodstream as affected mature erythrocytes, where they can accumulate in the brain causing cerebral malaria[4]. Figure 1 shows how three erythrocytes are affected when p. falciparum invades the red blood cells. In Figure 1a, is a picture of an unaffected erythrocyte, while Figure 1b and 1c depicts the knobs that will form once the erythrocyte becomes affected by the malaria parasite.

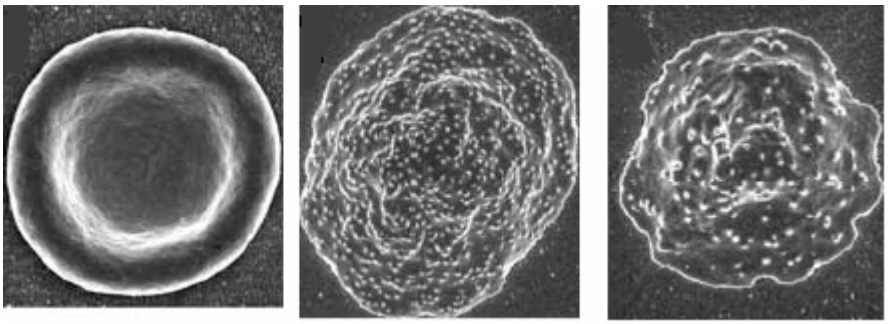

Figure 1[4]. Three pictures of RBCs. (a). A normal unaffected erythrocyte. (b) Knobs form on an affected erythrocyte. (c). Widespread large knobs on an affected erythrocyte.

Band 3 peptide has two main areas that act as host receptors. 5ABC which is a major segment in band 3 bound to MSP1-42[1]. MSP1-19 bound to the RBCs as well as to 5ABC. In this experiment the binding strengths of MSP1-19 are tested to see how much of a role it plays in the invasion of the RBCs.

MSP1-19 is one of the major proteins in p. falciparum. It is part of the MSP1-42, but from this protein MSP1-19 gets a C-terminus domain. This allows the MSP1-19 to carry into the invasion of the RBCs and then later attaches to the band 3 peptide from its C-terminus. The diagram for MSP1 is depicted in Figure 2. Each component is shown. MSP1-19 is the focus of the experiment. The 19 in MSP1-19 is the molecular weight in kDA. It is the last portion of the protein and it splits in two truncated sections MSP1-19A and MSP1-19B. All the portions of MSP1-19 will be studied in the binding of the band 3 peptides.

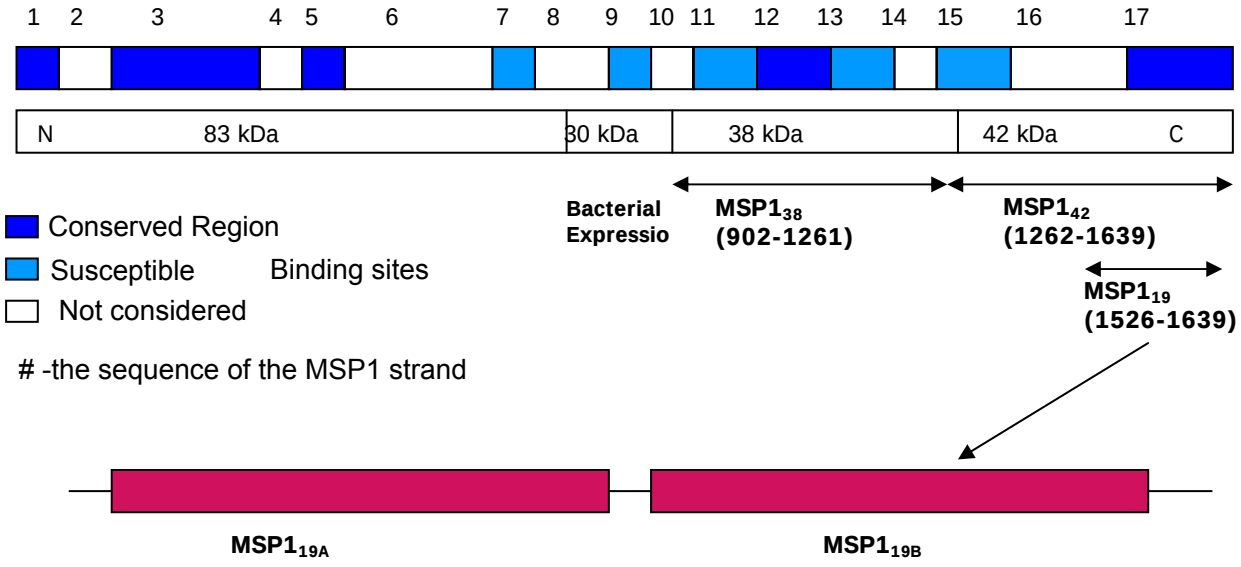

Figure 2. Diagram of the merozoite surface protein 1. It shows the other portions of this protein. The numbers in the parentheses are the number of amino acids for that portion of the MSP1 strand. The main focus is the MSP1-19 and its subsection. It splits into the truncations of MSP1-19A and MSP1-19B.

Band 3 peptide is found in the cell membrane of the RBCs. Another name for band 3 is AE1. AE1 is the human erythrocyte anion exchanger 1[3, 5]. The peptide is named band 3 due to the physical appearance. The focus of the experiments is on the segments 5, 5ABC, 6, 6A, 5ABC6A, 6A5ABC. 5ABC and 6A are the main segments that are host receptors to the invasion of the malaria parasite. In Figure 3[5], 5ABC and 6A are labeled in red. Their location is near the end of the peptide. Experiments were previously done on the other segments of the band 3 peptide.

A previous study was accomplished on mapping of malaria parasite inhibitory antibody located in p. falciparum merozoite surface protein 1_{19}. The protein was mapped using TROSY NMR, which means transverse relaxation optimized spectroscopy using nuclear magnetic resonance. NMR has a method for mapping proteins known as chemical shift perturbation. The study involved the interactions with MSP1-19 with three monoclonal antibodies. NMR epitode mapping showed close relationships of binding of MSP1-19 with two of the inhibitory antibodies known as mABs 12.8 and 12.10. This study is useful for evaluating the anti-MSP1-19 immune response in populations found in the malaria epidemic areas and it's useful in the vaccine studies[6].

In the current project, the binding strengths of certain band 3 peptides and merozoite surface protein 1 will be studied. The interactions between these two components will be tested in the Biacore optical sensor 1000. Biosensors are suited for the studies of molecular interaction in real time. BIA is biospecific interaction analysis. The system uses real time BIA based on the biosensor technology[7]. Surface plasmon resonance is how the bindings are tested. Analyze the results using the Biaevaluation software and see if any patterns appear between the peptides. The end result will be the ability to map MSP1-19 and the binding sites of the band 3 peptide.

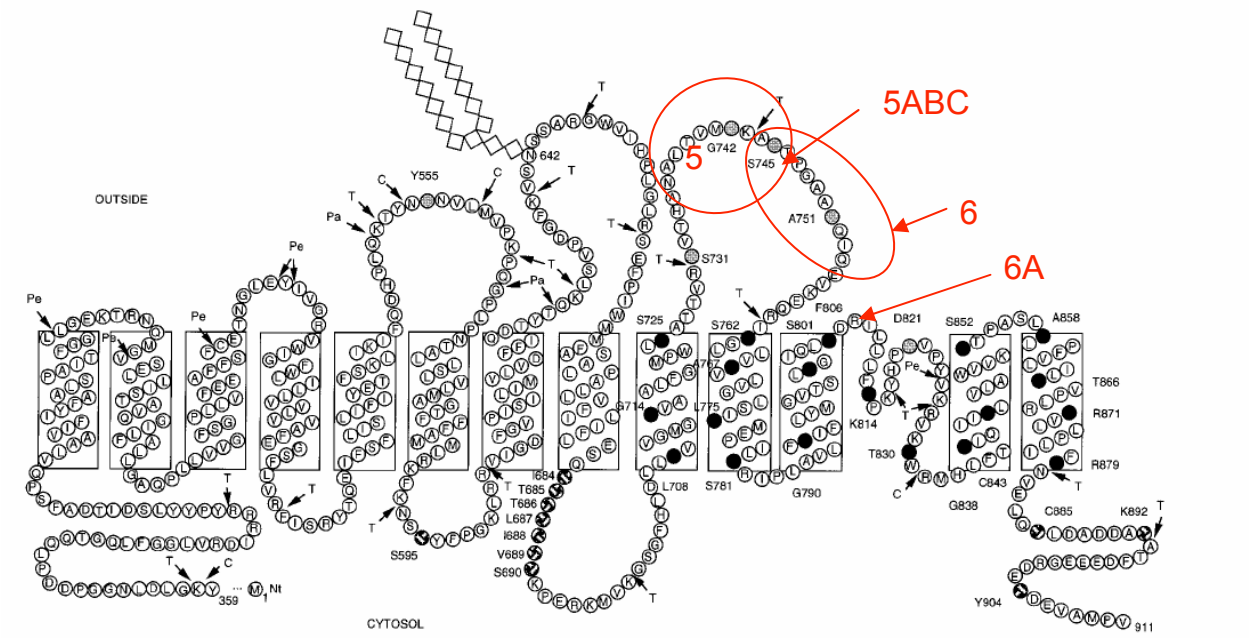

Figure 3. Diagram of Band 3 peptide. The red circles and arrows show the segments of the peptide that are host receptors for the malaria parasite. The tests are usually run on the segments outside peptides. The peptides on the inside are hydrophobic and the peptides on the outside are hydrophilic. The individual circles in the peptide are the amino acids.

Theory

The Biacore uses surface plasmon resonance (SPR) to perform protein interaction analyses. SPR can detect changes in the refractive index, which occurs at the surface of the sensor chip. Mass accumulates on the chip and causes the index to change. The analyte binding to the immobilized ligand on the surface of the chip is an example of mass accumulation.

Surface plasmon resonance is an optical phenomenon[8]. It occurs when light hits a layer of an excited metal plate. For the Biacore the metal plate is a carboxy methyl dextran (CM5) sensor chip, which consists of a layer of glass that is covered by a thin layer of gold film. The chip is derivatized with a flexible hydrophilic polymer to facilitate the attachments of specific ligands to its surface[7]. The light beam is polarized, so when it strikes the sensor all the light is reflected. Reflected light is what the Biacore detects.

The polarized light strikes the gold surface at the interface between the two Medias. The interface exists between a higher refractive index and a lower refractive index. The glass layer of the sensor chip is the high refractive index while the running buffer solution of 10 mM of 4-(2-Hydroxyethyl) piperazine-1- ethanesulfonic with 0.15 M of NaCl (HEPES), and 3 mM of ethylenediaminetetra acetic acid (EDTA) at 0.005 % v/v, otherwise known as HBS, is the low

refractive index. Light that strikes the higher index is partially refracted and partially reflected. In Figure 4[9] it shows the process of the light striking the chip surface. The Biacore will detect the light that is reflected from the chip. From the reflected light off the higher index an evanescent wave is formed[8]. This wave penetrates into the lower index and is absorbed. An intensity dip appears if the light that struck the coated chip is monochromatic and polarized. The intensity dip occurs at a certain incident angle, which is the surface plasmon resonance angle.

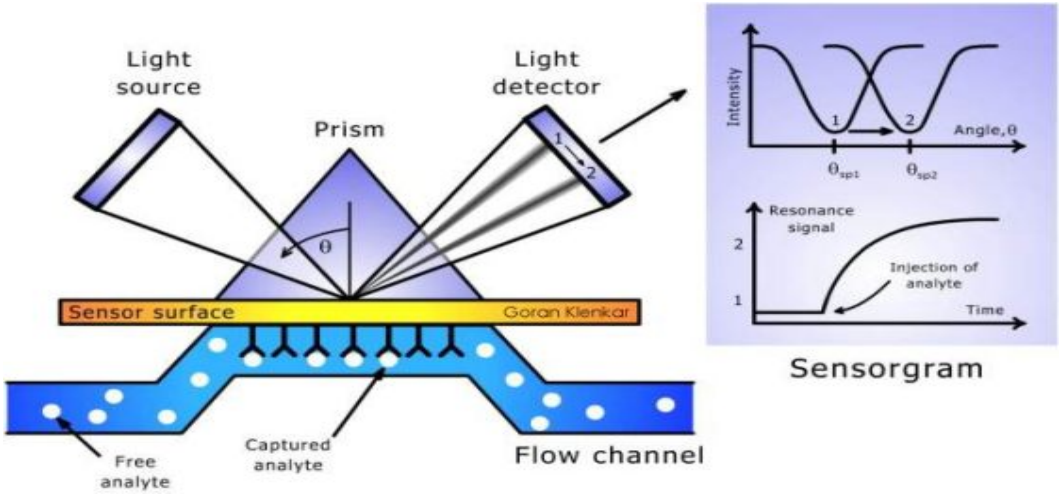

Figure 4. SPR diagram. The light strikes the surface of the chip, the light reflects. An evanescent wave penetrates the low index-the flow channels. The light that is reflected is then detected. Where the light strikes the chip, an intensity dip appears and that is the SPR angle. The intensity dip as it changes the response begins.

SPR occurs with the interaction of the evanescent wave and the reflected light from the interface between the Medias. Plasmons, which are electrons with collective resonant oscillations, are excited through this interaction. From the plasmon resonance the wave becomes amplified. The angle observed is determined by three parameters: the properties of the metal film used, the wavelength of the incident light, and the refractive index on both sides of metal film[8]. While the wavelength and the properties of the metal film are held constant, SPR is used to probe the lower aqueous index. The distance probed depends on how far the wave penetrated into the lower index.

The light source comes from a high-efficient emitting light diode. Its wavelength is near the infrared-red region. In Figure 5a, the light used in the Biacore is shown striking the sensor chip surface and has perfect internal reflection. A wedge shaped beam focuses on the interface of the sensor chip giving the light a fixed range of incident angles. As the refractive index in the solution contacts the surface, the changes in the surface due to binding and dissociation of the

molecules will therefore change the placement of the light causing the displacement of the SPR angle. The change in the incident angle is recorded in real time as a sensorgram.

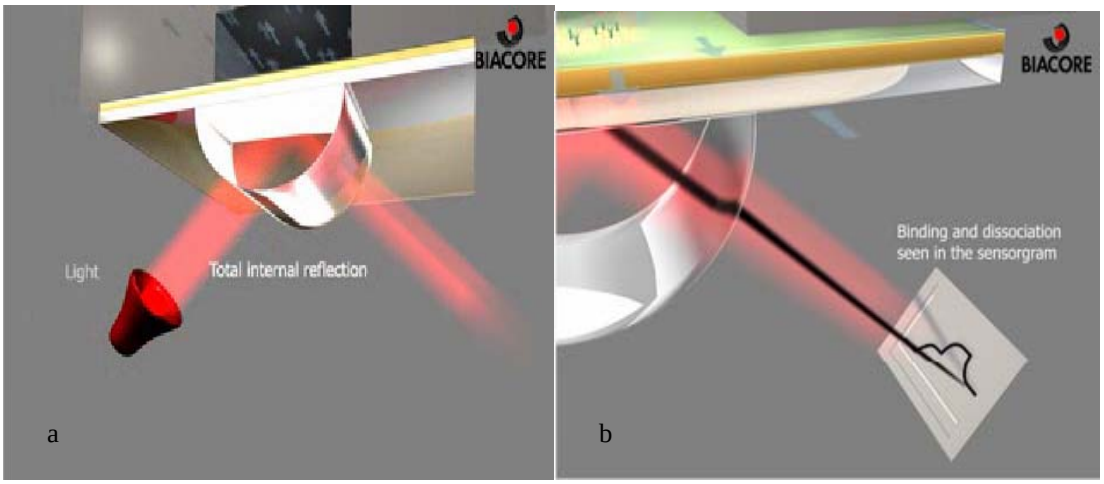

Figure 5[10]. SPR and sensorgrams. (a) The light used in the Biacore and how the light reflects after it strikes the sensor chip. (b) As the sample continues to flow through the channel the association continues causing an increase in the response. As the buffer flows through the channel the dissociation begins.

When the sample flows through the channels in the chip, an increase in the response of the sensorgram is caused due to the molecules in the sample interacting with the immobilized ligand. The response continues to increase as the sample and ligand continue to associate until the interaction reaches equilibrium. In Figure 5b, the buffer is flowing through the channel causing the SPR response to change. The sensorgram finished the association process and it is starting the dissociation process. When the running buffer replaces the sample the response begins to decrease. Dissociation occurs with the decrease of the response. Profiles are created from this process. Kinetic behavior, affinity, and sample concentration can be determined by the profiles.

The profiles can be accessed in the program Biaevaluation. This program allows for the analysis of the sensorgrams, as seen in Figure 6. The cycle goes as follows: the first step involves association, then dissociation, and finally the regeneration, which is the washing of the chip to prepare it for the next sample. For each sample a cycle plot can be accessed, and for an experiment all the cycle plots can be viewed in one window. The analysis begins with curve fitting the experimental data. If the parameters of the fitting (Chi2) is less than two, then the curve fits well to the experimental data[11]. After the curve has been successfully fitted to the data, then the parameters for the experiment appear at the bottom of the plot. Values are found for the

association constant (K_A) and the dissociation constant (K_D). From the values the binding strength can be determined.

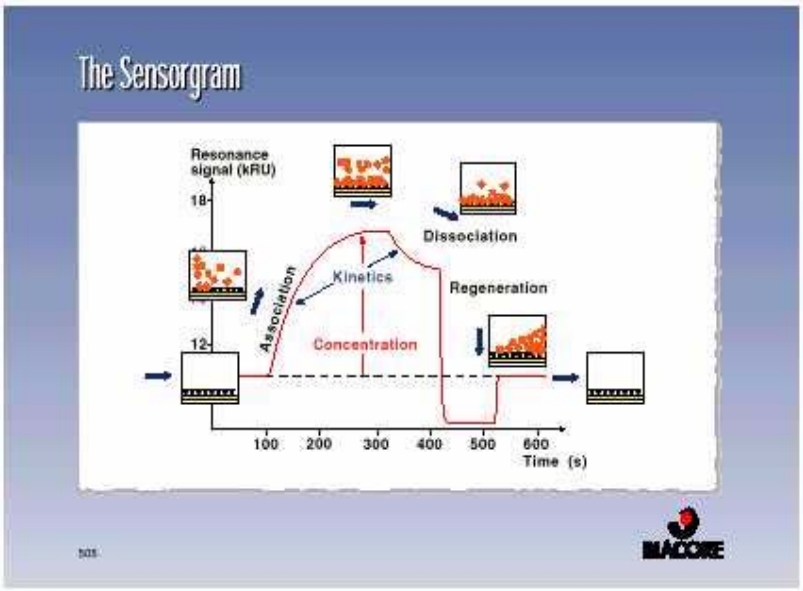

Figure 6[12]. A sensorgram which will be how the data will be presented. The association is the increase in the curve, the dissociation is the dip in the curve, and the regeneration is the process of washing the sensor chip for the next sample.

Principles and Equations

Molecular interactions are characterized by complex formations. If the two interacting species A, B can form a product AB by mixing then the formation will proceed to equilibrium. This is shown in Equation 1[8].

$$A + B \leftrightarrow AB \qquad (1)$$

Where A = 1st reactant

B= 2nd reactant

AB = product

The position of the equilibrium is always dependent on the concentrations of the species A, B, and AB according to the Law of Mass Action. This law can be described by K-the equilibrium constant. Depending on the direction of the reaction then K can be described as K_A for association or K_D for dissociation. In equation 2, it shows that the association constant and the dissociation constant are the inverse of each other.

$$\frac{[AB]}{[A][B]} = K_A \qquad \frac{[A][B]}{[AB]} = K_D \qquad (2)$$

Where A = 1st reactant

B= 2nd reactant

AB = product

K = equilibrium constant

The rate of association and dissociation are important on the microscopic scale. Fluctuations occur in the equilibrium state continuously through the association complexes that are formed and dissociations for other complexes. The equilibrium is dynamic and it reflects the ratios between the probabilities, or rates of formation and dissociation of the complex[8].

Association is the increase in the concentration of the product due to sample flows. In Equation 3 the reaction for association is shown. Rate of association is the increase of the concentration of the product AB over a certain amount of time. Equation 4 will show this expression for the rate constant for association.

$$A + B \rightarrow AB \quad (3)$$

Where A = 1st reactant

B= 2nd reactant

AB = product

$$\frac{d[AB]}{dt} = k_{ass}[A][B] \quad (4)$$

Where A = 1st reactant

B= 2nd reactant

AB = product

dt = change in time

k_{ass}= association rate constant ($M^{-1}s^{-1}$)

Rate constant k_{ass} specifies how fast the concentrations increase for the product AB when the concentration of A is [A] and for B is [B]. For experiments in the Biacore, the values for the association rate constants usually range from 10^3 to 10^7 $M^{-1}s^{-1}$.

Dissociation decreases the concentration of the product AB. In Equation 5 the dissociation reaction is shown. The concentration decrease over a certain time is the rate of dissociation constant. Equation 6 will show the expression for the rate constant for dissociation.

$$AB \rightarrow A + B \quad (5)$$

Where A = 1st reactant

B= 2nd reactant

AB = product

$$\frac{d[AB]}{dt} = -k_{diss}[AB] \quad (6)$$

Where AB = product

dt = change in time

k_{diss}= dissociation rate constant (s^{-1})

Dissociation rate constant indicates the fraction of AB complexes that dissociate per second. The values for k_{diss} usually range from 10^{-5} to 10^{-2} s^{-1}. Depending on what type of biological sample used the half-life could range from a couple of minutes to several hours[8].

The formation or dissociation of the AB complexes as it approaches equilibrium can form an excess either in A, B, or in AB. The excess can be determined as the reaction approaches equilibrium by taking the two sums of the two rate expressions. The expression for the net rate of either formation or dissociation is given in Equation 7.

$$\frac{d[AB]}{dt} = k_{ass}[A][B] - k_{diss}[AB] \quad (7)$$

Where A = 1st reactant

B= 2nd reactant

AB = product

dt = change in time

k_{ass}= association rate constant ($M^{-1}s^{-1}$)

k_{diss}= dissociation rate constant (s^{-1})

At equilibrium the two rate expressions will become zero. The dissociation and association rate constant will be equal to each other. This can be seen in Equation 8.

$$\frac{d[AB]}{dt} = 0 \qquad k_{ass}[A][B] = k_{diss}[AB] \quad (8)$$

Where A = 1st reactant

B= 2nd reactant

AB = product

dt = change in time

k_{ass}= association rate constant ($M^{-1}s^{-1}$)

k_{diss}= dissociation rate constant (s^{-1})

This expression can be rearranged to show that the K, the equilibrium constant, is the ratio of the two rate constants. Equation 9 is the expression for K and the ratio.

$$\frac{k_{ass}}{k_{diss}} = \frac{[AB]}{[A][B]} = K_A \qquad \frac{k_{diss}}{k_{ass}} = \frac{[A][B]}{[AB]} = K_D \tag{9}$$

Where A = 1st reactant

B= 2nd reactant

AB = product

dt = change in time

k_{ass}= association rate constant ($M^{-1}s^{-1}$)

k_{diss}= dissociation rate constant (s^{-1})

The Biacore is monitored in real time, so the complexes that are formed can change in response over time. The concentration of the free analyte-usually the sample, is constant during the flow over the sensor chip. The response curve is the sum of the association rate constant and the dissociation rate constant as seen in Equation 10. As the samples continue to flow complexes are still flowing until the reaction reaches equilibrium.

$$\frac{dR}{dt} = k_{ass} C\left(R_{\max} - R_t\right) - k_{diss} R_t \tag{10}$$

Where dR/dt = rate of formation of surface complexes

k_{ass}= association rate constant ($M^{-1}s^{-1}$)

k_{diss}= dissociation rate constant (s^{-1})

C = concentration of analyte

R_{max}= total number of binding sites

R_{max}-R_t = amount of remaining free binding sites

Method/Methodology

Experiments used with the Biacore test the interactions between two components. Those components can include protein as well as peptides. The Biacore uses surface plasmon resonance to perform protein interaction analysis[11]. For this experiment a peptide from the band 3 peptide will bind to MSP1-19, which is merozoite surface protein- 1. From the Biacore, the bond strengths and the kinetics are analyzed to understand how the two components are affected. Peptides 5ABC, 5, 6, 6A, 5ABC6A, and 6A5ABC from the band 3 peptide were fused to

glutathione S-transferase (GST) to become recombination proteins. The protein used from the malaria strand of plasmodium falciparum is fused with thioredoxin (Trx) and it becomes a recombination protein Trx-MSP1-19 or Trx-19. These combination proteins and peptides are used in the experiments in the Biacore.

The first sample that was made for the experiment was a mixture of GST-5ABC with sample buffer HBS- this is the first layer analyte, which would attach to the anti-GST immobilized on the sensor chip. GST-5ABC dilution sample was a mixture of 587 µl of running buffer HBS and 13 µl of the recombination protein GST-5ABC. For the rest of the experiments: GST-6A, GST-5, GST-6, GST-5ABC6A, and GST-6A5ABC the mixture of buffer to GST- is different, but the total amount of solution is 600 nM. Those values can be found in Table 1.

Table 1. Sample dilutions made from the recombination proteins. A portion of the sample comes from the pure recombination protein and the rest comes from the running buffer.

Recombination Protein (µg/µL)	Molecular Weight (D)	GST- peptide (µL)	Buffer HBS (µL)
GST-5ABC (0.8)	31413.0	13	587
GST-6A (0.71)	29400.0	12	588
GST-5(0.54)	29800.0	17	583
GST-6(0.60)	30600.0	15	585
GST-5ABC6A(0.4)	34799.0	26	574
GST-6A5ABC(0 .4)	34800.0	26	574

Trx-19 would then also be mixed with a certain amount of the buffer HBS to make 5000 nM solution. To make Trx-19 5 µl sample solution took 235 µl of HBS, the running buffer and 65 µl of the recombination protein Trx-19 sample. MSP1-19 has two components 19A and 19B. The values for the other two recombination proteins of MSP1 can be found in Table 2.

Table 2. 5 µl of sample dilution of the recombination proteins Trx-19, Trx-19A, Trx-19B.

MSP1-19 (µg/µL)	Molecular Weight	Trx-19 protein	Buffer HBS (µL)
Trx-19 (0.68)	29398.0	65	235
Trx-19A (0.79)	23300.0	37	213
Trx-19B (0.59)	24300.0	51	199

The two samples need to be placed in the centrifuge. 5000 nM Trx-19 sample solution will be diluted into the six concentrations: 1500 nM, 1200 nM, 1000 nM, 800 nM, 600 nM, 200 nM. Each sample consisted of a mixture of the HBS buffer with the Trx-19 mixture which can be seen in Table 3.

Table 3. Sample dilution mixtures

Dilution Sample (nM)	Trx-19, -19A, -19B (µL)	Buffer HBS (µL)
1500	60	140
1200	48	152
1000	40	160
800	32	168
600	24	176
200	8	192

Table 3 is used for all experiments of GST-5ABC, GST-6A, GST-5, GST-6, GST-5ABC6A, and GST-6A5ABC as the first layer analyte. For these experiments Trx19A would be used to immobilize on the chip. GST-5ABC6A or GST-6A5ABC was used as analytes whose six concentrations for experiments were: 300 nM, 250 nM, 200 nM, 150 nM, 100 nM, and 50 nM. The sample dilution for either type of experiment does not change. All the samples for every experiment should be centrifuged to remove all the bubbles.

These samples are then placed into one of the thermo racks in the Biacore. The Biacore can be seen in Figure 7a, the autosampler is on the top part of the Biacore, the sensor chip is placed inside the black box, and the buffer is at the bottom of the shelf in the machine. The running buffer solution HBS flows through one of the four channels on the sensor chip, this occurs for cleaning purposes. Anti-GST, the ligand, was immobilized on the sensor chip prior to the initial run of the sample buffer. The purpose of immobilizing a ligand on the surface of the chip is to control the manner of how the proteins bind, without it then the binding becomes uncontrollable and can contribute to loss of biological activity[7]. The sensor chip runs about 72 hours before it needs to be replaced, so once the chip is immobilized then it can run continuously until it reaches the 72 hour limit.

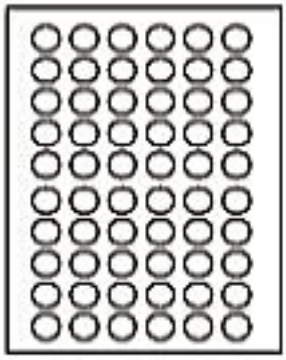

Figure 7. (a)[13]. A picture of the Biacore 1000. (b)[14]. the rack of an autosampler that is found inside a Biacore

A delivery system that consists of a needle connected to an arm takes the sample from the vial to the connector block. The samples are placed in a rack of the autosampler as seen in Figure 7b, each sample has a specified spot that it is placed. From that block the sample runs through one of the flow channels found inside the IFC, which is Integrated µFLuidic Cartridge, to bind onto the ligand attached to the sensor chip. For one sample the process is as follows. The recombination protein of the peptides from the band 3 peptide and GST will flow through the channel where it will bind with the immobilized ligand.

Next the needle will take the diluted sample of the 5 µl sample solution of recombination protein Trx-MSP1-19 and the sample buffer HBS. This sample runs through the same channel as the peptide and GST. There the MSP1-19, or the truncated forms of the protein MSP1-19A or MSP1-19B, will bind to the peptide that is left from the initial binding of GST to ant-GST. During the binding of MSP1-19 to the peptide is when the association begins. As the sample solution with MSP1-19 continues to flow through the chip, the association stops and the dissociation begins afterwards.

Finally, the regeneration occurs. This consists of two fluids-one that will break apart the bonds between the peptide and protein. Regeneration solution is the first solution; for this set of experiments it is 10 mM NaOH and 100 mM NaCl. The second part of the regeneration process includes a run of the sample buffer to wash away the previous samples. Regeneration can be performed 50 times before it affects the ligands ability to bind to the analyte-this is the recombination protein of GST fused with peptides from band 3 peptide.

In a process the running buffer is pumped through as autosampler pump to the autosampler where the samples are placed. The running buffer is sent through a second pump to

the connector block and it flows through the channel before flowing over the sensor chip. Samples and buffer are injected into the connector block from a delivery system that consists of a large needle. This can be in a schematic diagram of the delivery system in Figure 8[8].

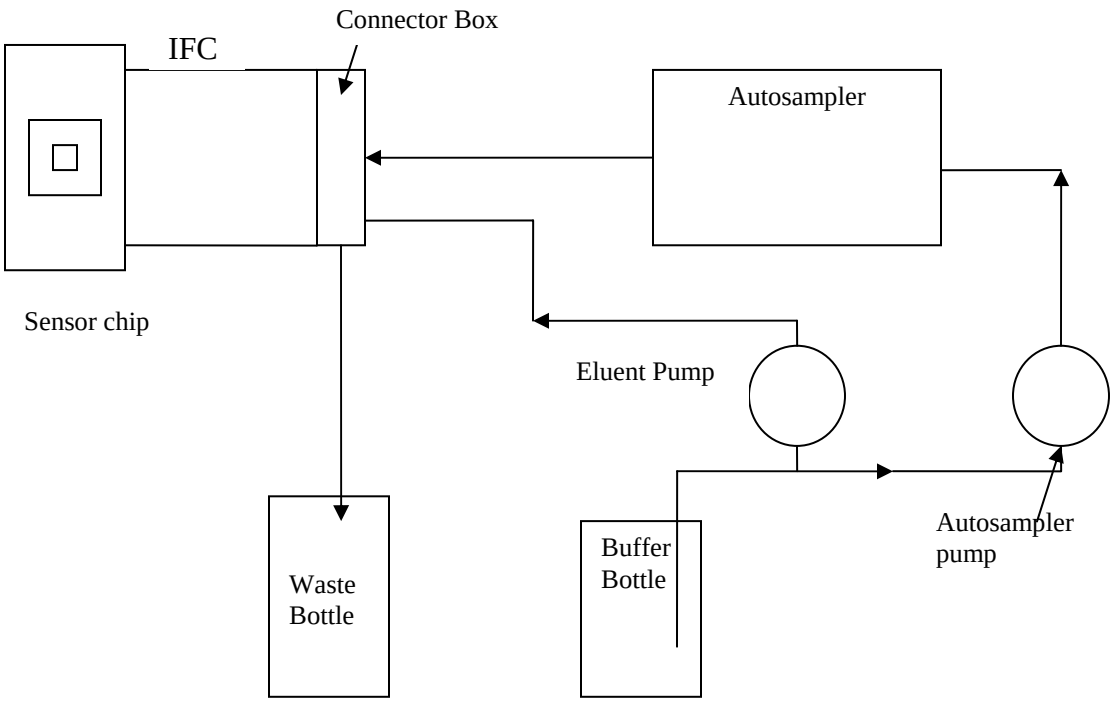

Figure 8. Schematic diagram of the delivery system in the Biacore. Buffer can enter two areas of the process. Samples and buffer can go through the channels once it is inside the connecter block.

Methods are required to run experiments in the Biacore. They are programs that tell the automated liquid delivery system when and how to extract the samples for testing. Method Definition Language (MDL) is the programming language used to create the blocks inside each method[8]. As with any program there are certain aspects that are needed to insure that the program is working. Methods require a Main block that defines the orders of the operations needed to complete the experiment. This block can not be used more than once in a given program.

In the main block one of the first operations listed usually is to set a channel in the sensor chip as the flow cell. This means that the cell listed is where the liquids will bind to the chip and they will flow only through the specified channel or cell. Once the flow channel is set the next operation is to set a certain flow rate for all of the samples to follow. The next operations usually are the Approg solve blocks, where the actual mixing of samples occurs, or a loop block to

insure all the samples repeats the same pattern. In Figure 9, a main block from one of the processes used, GST-6 with Trx-19 shows the operations needed to run the experiment. It starts by listing the racks used in the experiment followed by setting flow channels as well as flow rates.

The Define Aprog blocks are where the operations are written to perform the analysis. In a method many aprog blocks can exist for different processes. This type of block can include mixing and injecting of samples. New flow rates can be set for individual components. Commands are written in these blocks, for example an aprog block needed for regeneration. A command may be inject a fluid from a certain position in the autosampler to the connective block.

```
MAIN
  RACK        1 thermo_c
  RACK        2 thermo_a
  FLOWCELL    2
!  APROG       immob
  APROG       analyse1
  LOOP cycle STEP
    APROG       analyze %sample %position
%volume
    APROG       regeneration
    APROG       analyse1
  ENDLOOP
  APROG       analyse2
  APROG       regeneration
  APPEND      continue
END
```

Figure 9. Sample of a Main block from a method for the experiment GST-6-Trx-19

Aprog blocks can be used for several analyses that are needed during a particular experiment. For many Aprog blocks to exist in a given method the blocks should be placed in order and each block must be complete meaning that when the aprog block is finished End should be written. The method will be able to comprehend all the Define Aprog blocks and accesses them in the order of placement.

```
DEFINE APROG regeneration
    FLOW        5
    INJECT      R2F3 35       ! 100
mM NaCl + 10 mM NaOH buffer
    INJECT      R2F4 50       ! HBS
buffer
END
```

Figure 10. the Define Aprog block for the experiment GST-6-Trx-19. Aprog block is for writing commands for the analysis of an experiment.

Other blocks can exist in a method depending on what operations are needed during the experiment. A loop block is used when the samples of a given experiment follow a certain pattern. Instead of writing several Define Aprog blocks for each of the samples, one loop block can be written and both accomplish the same goal in the program. Loop blocks can be written inside a loop block, the only problem with creating a double loop block is ending the loops. With two loops they should be stopped in this order, the loop inside the initial loop should end first and then the initial loop can be stopped.

The complete method for the experiment GST-6-Trx-19 can be found in Appendix A. Methods should be saved for the individual experiments, so 3 methods can be found for GST-5ABC with Trx-19, Trx-19A, and Trx-19B, as well as GST-6A with Trx-19, Trx-19A, and Trx-19B, ect. Before beginning the program in the Biacore there is an option Pre-run in the method menu. Pre-run will run the program without starting the Biacore, so any mistake within the method can be found before the actual experiment begins.

Experimental Plan

The experiments are: GST-5ABC with Trx-19, Trx-19A, Trx-19B; GST-6A with Trx-19, Trx-19A, Trx-19B; GST-5ABC6A with Trx-19; and GST-6A5ABC with Trx-19. Other experiments for this project consist of using truncated peptides of 5ABC from band 3 peptide: GST-5 with Trx-19 and GST-6 with Trx-19. Because when GST5ABC6A and GST6A5ABC were immobilized on the chip and Trx19 as analyte, there is no binding happen. So another kind of experiment was tested, which is using Trx-19A as the ligand that was immobilized on the sensor chip. GST-5ABC6A and GST-6A5ABC were used as analytes with the different ligand of Trx-19A.

For each of the experiments, 6 diluted samples of Trx-MSP1-19, Trx-MSP1-19A, and Trx-MSP1-19B, depending on the experiments were used. All six samples are the same no matter what form of the merozoite surface protein used. The six samples are 1500 nM, 1200 nM, 1000 nM, 800 nM, 600 nM, and 200 nM concentrations of the Trx-MSP1-19 solution. These samples will be tested with all segments in the 5ABC peptide and 6A peptide of the band 3 peptide found in the membrane of a red blood cell (RBC).

With the reproduced experiments, the setup is different because the Trx-19A recombination protein is already the ligand. The six diluted samples are made from the running buffer and the sample of GST-5ABC6A or GST-6A5ABC. Also the concentrations are different

for the diluted samples. The concentrations start at 300 nM, 250 nM, 200 nM, 150 nM, 100 nM, and 50 nM. This setup will be tested on other peptides to see the comparison between the two ligands.

Comparison will be made of three segments of GST-5ABC, and GST-5 plus GST-6 with the three forms of merozoite surface protein 1-19. The two truncated segments GST-5ABC6A and GST-6A5ABC will also be compared with the two truncated forms of MSP1-19. The samples for this experiment were prepared differently. Only three samples were needed along with MSP1-19 and its truncated forms. From the comparison plots binding strengths can be distinguished between the three peptides.

Results and Discussion

Three experimental runs were performed using the recombination protein of GST and 5ABC from the Band 3 peptide with the recombination protein of MSP1-19 and the truncated forms of this protein. The recombination protein for the merozoite surface protein 1: Trx-19, Trx-19A, and Trx-19B. The purpose of using all three types of the protein from the malaria parasite is to observe how important MSP1-19 is to the invasion of the red blood cells (RBCs). Each of the three experiments used the same six diluted samples of the recombination proteins Trx-19, Trx-19A, and Trx-19B. A plot of the response of the complexes vs. the time can be obtained for the experiments. The response is in units (RU), which means response units. This is the unit conversion for the RU shown in Equation 11.

$$1000RU = \frac{1ng}{mm^2} \tag{11}$$

The first set of experiments use GST-5ABC with Trx-19 shows signs that strong binding has occurred. In Figure 11, the sensorgram shows 5 of the 6 samples. The samples used in the analysis for is Trx-19 with the concentrations of 1500, 1200, 1000, 600, and 200 nM. The sample of Trx-19 at 800 nM did not show good results of binding as it flowed through the channels of the sensor chip, so the data was left out of the analysis. Strong binding has occurred and that can be seen around the curve of the plot as well as the base lines. The red circles on the sensorgram point where the binding has occurred.

At the beginning the base line is low that is when the sample is first flowing through the channels. As the curve slowly increases is the time when the sample is binding to the immobilized ligand then as the curve comes to an end the reaction has reached equilibrium and then dissociation begins where the last of the red circles are located. The two baselines are different from each other the last base line is higher then the first, so binding has occurred.

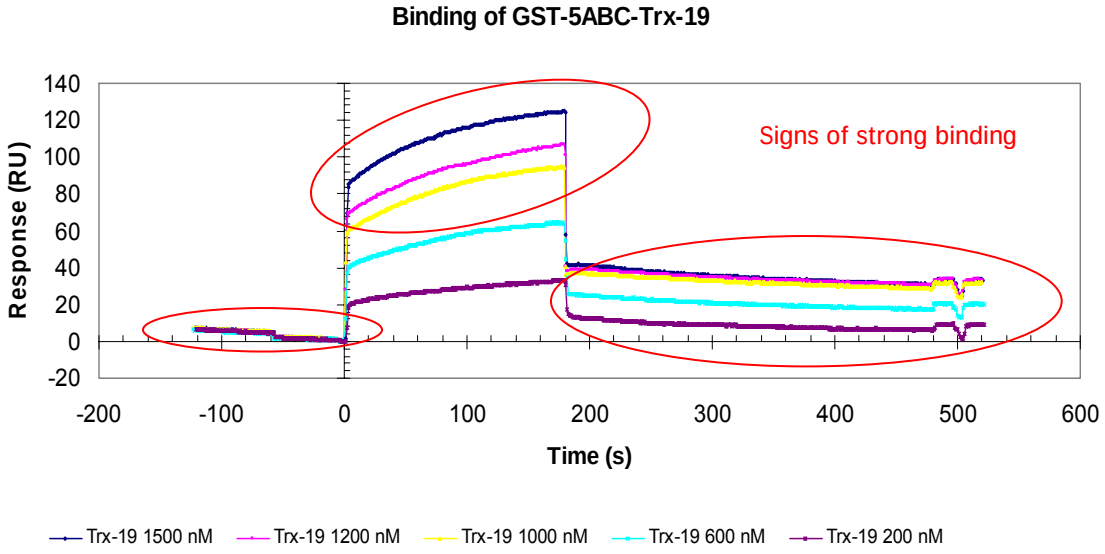

Figure 11. The sensorgram for the experiment GST-5ABC and Trx-19.

The second experiment with GST-5ABC and Trx-19A also showed the same results. The binding that occurred was stronger than the first experiment. The curve showed a slower increase in the curve meaning that more of the analyte bound to the ligand. The differences in the baselines are more defined. The results for this experiment can be seen in Appendix B, Figure 1.

The last experiment using GST-5ABC and Trx-19B also had binding occur, but it was not as strong as Trx-19 or Trx-19A. The curve is steep so the analyte did not bind to the ligand as well as the other two experiments. Many of the binding sites were left open. Even though the binging is weaker than the last two the base line is defined better than the first experiment. This plot can be seen in Appendix B, Figure 2.

The second set of experiments involves a different segment from the band 3 peptide, 6A. It is also a recombination protein GST-6A. Two segments from band 3, where said to be host

receptors for the malaria parasite, 6A is the second host receptor. This experiment also involves the same recombination proteins of the merozoite surface protein 1.

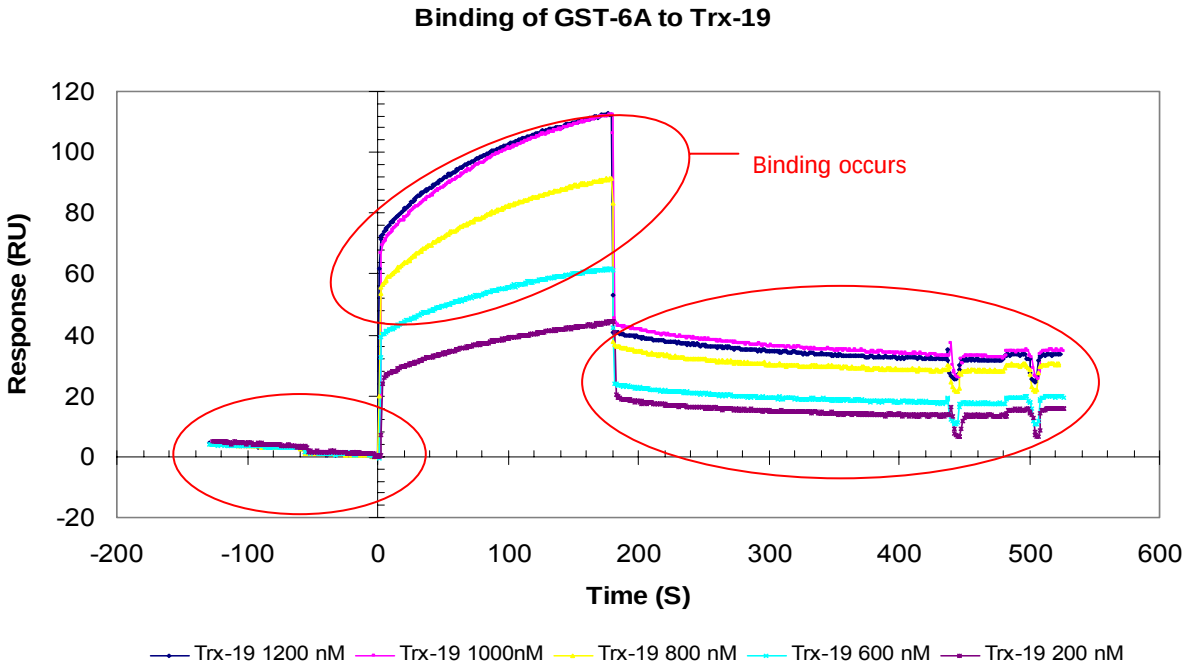

Figure 12. The sensorgram for the experiment GST-6A with Trx-19.

The first experiment involves GST-6A with Trx-19 and it also showed signs of binding. The binding seemed to be as strong for GST-6A as it was for GST-5ABC. Figure 12 shows 5 of the 6 concentrations. Trx-19 concentration of 1500 nM was the first sample that ran through the channel; its response was higher than the others. It was removed from the analysis, so it couldn't affect the other 5 concentrations. With the rest of the samples the binding is visible, there is a difference between the first and second baseline. The curve shows the analyte binding slowly to the ligand as the curve increases.

The other two experiments involved GST-6A with Trx-19A and Trx-19B. Patterns can be seen between the two sets of experiments. Like the GST-5ABC, the GST-6A with Trx-19A had stronger binding because of the sloping curve and the baseline. The curve was more defined and the time it took for the curve to form was longer. This plot can be seen in Appendix B, Figure 3. GST-6A with Trx-19B also showed the occurrence of binding. A pattern was formed with this experiment as well. GST-5ABC showed weaker signs of binding with Trx-19B than with Trx-19. It is the same for GST-6A, the Trx-19B did not bind as well as the Trx-19. The base line for this experiment is more defined in the differences between the beginning of the

sample flow compared to the ending where the analyte bound to the ligand and the dissociation began. The sensorgram is Figure 4 in Appendix B.

Another set of experiments was using chimeric band 3 peptides. This was also concentration dependent experiments. A chimeric peptide is a combination of peptides. The recombination proteins used is GST with the two recombination peptides from band 3, GST-6A5ABC, and GST-5ABC6A. The chimeric peptides are the two segments that are host receptors in the band 3 peptide. Only one experiment was run with these peptides and the recombination protein Trx-19.

GST-6A5ABC with Trx-19 showed no occurrence of binding. Out of the six samples only three were good enough to be analyzed. The other three samples didn't have the analyte bind to the ligand. With the three good samples it did not show any signs binding. The baseline goes below zero and the curve shows no sign of binding. The curve has a steep increase and then the curve barely increased and then there was a steep decrease. In Figure 13 the red circles show where the supposed binding should occur. The second experiment that used the chimeric recombination protein GST-5ABC6A with Trx-19 and it also did not show signs of binding. More of the sample dilution concentrations were able to be used. The baseline was also near zero. The curve looked the same as the previous experiment. This plot can be seen in Figure 5, Appendix B.

The next set of experiments that involved the two chimeric recombination proteins of GST-5ABC6A and GST-6A5ABC had a different ligand immobilized on the sensor chip. When anti-GST was used on the sensor chip, the two experiments showed no signs of binding. The experiments were reproduced using a different ligand. Trx-19A was immobilized on the sensor chip, using this as a ligand took out an extra step in the method of the experiments. The sensorgrams, however did show signs of binding, the curves showed slow increases as the sample bonded with the ligand. GST-6A5ABC with Trx-19A showed signs of strong binding. The sensorgram can be seen in Figure 6 of Appendix B. In Figure 7 of Appendix B showed the binding of GST-5ABC6A with Trx-19A. The baseline showed a tremendous increase at the end of the experiment then at the beginning.

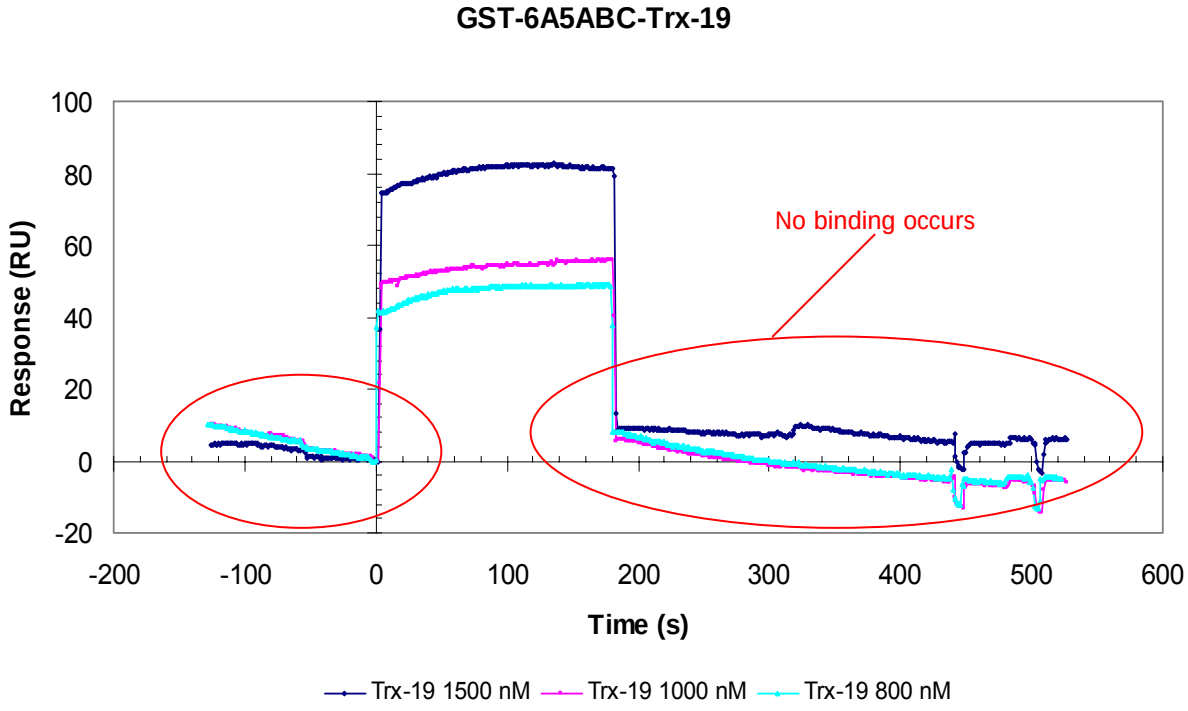

Figure 13. The sensorgram for the experiment of GST-6A5ABC and Trx-19. No occurrence of binding found.

The last set of experiments was truncated forms of the segment of 5ABC from the band 3 peptide. Peptide 5 and 6 are a part of the segment of 5ABC as can be seen in Figure 3. This experiment is done to see if these segments of 5ABC are the host receptors instead of the actual segment 5ABC of the band 3 peptide. The recombination protein of GST-5 and GST-6 with Trx-19 and Trx-19A were the experiments performed.

GST-5 with Trx-19 showed signs of weak binding occurring. Weak binding means that the anaylte only bonded with certain ligands and many of the free binding sites were not used. In Figure 14 the baselines are hard to distinguish whether binding occurs. The second baseline is above the beginning line. The curve shows some binding but it is not a smooth and slow increase as the components bind. The binding that occurs happens quickly before the dissociation process begins.

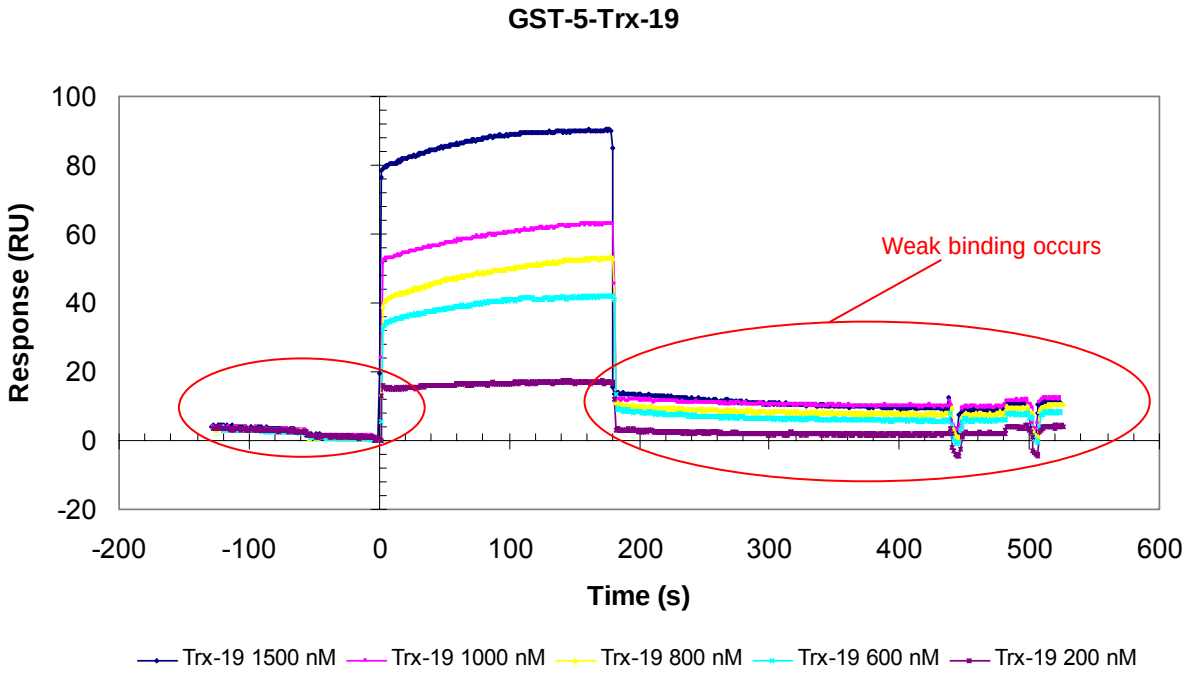

Figure 14. Sensorgram of the experiment of truncated GST-5 with Trx-19. A case of weak binding.

GST-5 with Trx-19A showed a different pattern of binding then the previous experiment of GST-5 with Trx-19. Trx-19A was the ligand immobilized on the sensor chip. In the sensorgram for GST-5 with Trx-19A, stronger binding was seen. During association for each of the concentrations the curve in Figure 8 in Appendix B, slowly increased as the two components bonded together. This is similar to the results of the initial experiments of GST-5ABC and GST-6A with Trx-19A.

The second experiment with GST-6 and Trx-19 shows the same type of weak binding. The baseline is barely higher than when the sample first flowed through the channels. This is another pattern occurring. The sensorgram for this experiment can be seen as Figure 9 in Appendix B. GST-6 with Trx-19A showed binding patterns that followed the experiment of GST-5 and Trx-19A. Stronger binding can be seen in the way the curve slowly increases as the association continues and the baseline at the end of the experiment was higher than the beginning. The recombination protein Trx-19A had better binding properties than the full Trx-MSP1-19 strand. In Figure 10 of Appendix B shows the sensorgram for the experiment of GST-6 and Trx-19A.

Patterns have occurred between the segments of 5ABC and 6A. The strengths of binding have been really close to each other. From the kinetic values given from each plot the pattern can be seen there. The kinetic values include the constants for the association and dissociation terms as well as the curve fitting values. The association terms are how well the analyte bond to the ligand and dissociation is how quickly the bond was broken. The association constant is the reciprocal of the dissociation constant as seen in Equation 12.

$$K_A = \frac{1}{K_D} \qquad K_D = \frac{1}{K_A} \qquad (12)$$

All the experiments with GST-5ABC have association constants in the same range. GST-5ABC with Trx-19, Trx-19A, and Trx-19B have values for the association constant 6.45E+06 1/M, 1.31E+07 1/M, and 8.52E+06 1/M respectively. The values for the dissociation constant are as follows Trx-19, Trx-19A, and Trx-19B are 1.55E-07 M, 7.64E-08 M, 1.17E-07 M respectively.

Table 4. Parameters for the experiments.

Bindings Kinetic Constants	KA (1/M)	KD (M)	σ^2 Chi2 value
GST-5ABC-Trx-19	6.45E+06	1.55E-07	1.67
GST-5ABC-Trx-19A	1.31E+07	7.64E-08	6.07
GST-5ABC-Trx-19B	8.52E+06	1.17E-07	1.77
GST-6A-Trx-19	8.01E+06	1.25E-07	9.34
GST-6A-Trx-19A	4.08E+06	2.45E-07	2.17
GST-6A-Trx-19B	1.24E+07	8.07E-08	7.81
GST-5ABC6A-Trx-19	1.86E+04	5.39E-05	12.0
GST-6A-5ABC-Trx-19	0.828	1.21	12.3
GST-6A5ABC-Trx-19A	9.49E+07	1.05E-08	2.88
GST-5-Trx-19	4.62E+06	2.17E-07	0.93
GST-5-Trx-19A	1.41E+07	7.09E-08	1.18
GST-6-Trx-19	3.97E+15	2.52E-16	0.968
GST-6-Trx-19A	1.14E+07	8.75E-08	0.812

The values for the constant for GST-6A are similar to the constants that were found from the experiments involving GST-5ABC. These values can be found in Table 4. Table 4 has all the association constants and dissociation constants for all the experiments performed. There is also a Chi2 value, this is a fitting parameter used when curve fitting the data. If this value is

lower than 2, then the curve fits well to experimental data. Since the values for the two experiments are similar then that means the merozoite surface protein 1 (MSP1-19) has an important role for the p. falcipaurm invasion of the red blood cells. The segments of MSP1-19, 19A and 19B contribute to the overall binding of the protein to the host receptor.

The experiments with GST-5ABC6A and GST-6A5ABC did not seem to have any binding occur. The values for the association constant and the dissociation constant are lower than for the other experiments. GST-5ABC6A with Trx-19 had a value for the association constant- $1.86E^{+04}$ 1/M and the value for the dissociation constant- $5.39E^{-05}$. These values are lower than the previous experiments. For GST-6A5ABC the values for both constants are very low, so there is no sign of binding. These values can be seen in Table 4. Since only one experiment was done for each protein, the values may be wrong.

Experiments were reproduced for GST- 5ABC6A and GST-6A5ABC where Trx-19A was the immobilized ligand. The results for the kinetic values were close to the values of the previous experiments of GST-5ABC and GST-6A, which can be seen in table 4.

GST-5 with Trx-19 shows signs of weak binding. The values for the association constant and dissociation constant were similar to the values for GST-5ABC and for GST-6A. With GST-6 the two values were very high, for an experiment that showed weak binding. Since one experiment was done on this combination something may have gone wrong to produce the values that were given during the analysis. Values for GST-6 can be found in Table 4. The experiments have to be reproduced. The two experiments of GST-5 and GST-6 with Trx-19A had similar values as most of the experiments. Trx-19A seems more important to the binding of the proteins then the whole form of the merozoite surface protein.

Comparisons then were made between the segment 5ABC of band 3 peptide and the two truncated forms 5 and 6. This involved using the three merozoite surface proteins Trx-19, Trx-19A, and Trx-19B. The first comparison was GST-5, GST-6, and GST-5ABC with Trx-19. Binding strengths are as followed: GST-5ABC had higher response for the binding results with Trx-19 than the other two segments. In Figure 15 the sensorgram shows the three peptides and the binding that occurs with the one meorozoite surface protein. With the other two comparison plots involve the three peptides with Trx-19A and Trx-19B were seen binding together. All the experiments showed how GST-5ABC had a higher response, followed by GST-6 and lastly GST-5. The comparison sensorgrams can be seen in Figure 11 of Appendix B. This sensorgram uses

Trx-19A, which shows stronger binding compared to the whole form of Trx-19. Figure 12 in Appendix B shows the strong binding of the three recombination proteins with Trx-19B. Again this truncated form of MSP1-19 also shows signs of better binding then the original experiment with Trx-19. The purpose of the comparison plots is to see which forms of the merozoite surface protein 1 as well as what segments of bind 3 peptide interacts the best together.

Comparison plots were also used for the two recombination proteins of GST-5ABC6A and GST-6A5ABC with the two forms of the truncated forms of the merozoite protein, Trx-19A and Trx-19B. The first comparison of the two peptides with Trx-19A showed no signs of binding occurring. This can be seen in the sensorgram in Figure 13 of Appendix B. The same results were found for the second comparison of the two peptides with Trx-19B, as in Figure 14 of Appendix B. These peptides seem not to bind with Trx-19, 19A, and 19B while the ligand immobilized on the chip is anti- GST. The experiments done with the same peptides and Trx-19 as the ligand had results of binding.

The kinetic values for the comparison experiments seem to follow the same values as previous experiments. There is a case of a few experiments were no binding occurred due to the type of protein used. It is like table 4 which expressed the constants for both association and dissociation. The values for the comparison plots can be seen in Table 5 in Appendix B. The association constants and the dissociation constant are of similar magnitudes.

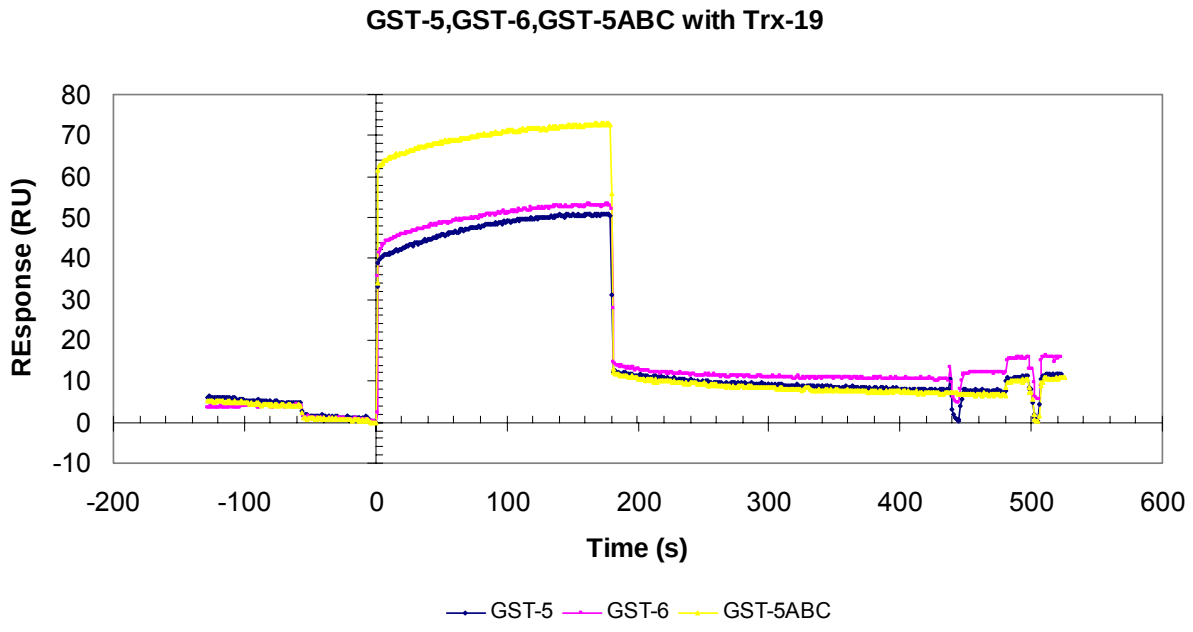

Figure 15. Comparison plot of GST-5, GST-6, and GST-5ABC with Trx-19. Weak binding is still occurs, but there is higher response with the GST-5ABC segment.

Conclusions

Successful runs were performed for the experiments involving GST-5ABC with all truncations of the merozoite surface protein 1 and for all the experiments run with the recombination protein GST-6A and GST-5. The experiments involving GST-5ABC6A, GST-6A5ABC and GST-6 with Trx-19 were all unsuccessful. The purpose to the project was to test the binding strengths of segments of the band 3 peptide with the merozoite surface protein 1. GST-5ABC and GST-6A were found to be the segments of band 3 peptide that seemed to have the strongest bonds to the malaria parasite. MSP1-19 seemed to have weak bonds to the truncated recombination proteins of GST-5 and GST-6. Strong binding seemed to occur for GST-5 and GST-6 when Trx-19A was used. For some reason the chimeric proteins GST-5ABC6A and GST-6A5ABC didn't seem to bind to the parasite. During the experiment most of the sample ran through the chip without binding to the ligand. When the experiment was reproduced using Trx-19A as the immobilized ligand then the sensorgrams showed an increase in the binding properties. The two experiments followed the binding pattern seen in the experiments of GST-5ABC with Trx-19, Trx-19A, and Trx-19B.

Segments 5ABC and 6A from band 3 peptide from the results seem to be the host receptors for p. falciparum. The experiments resulted in strong bonds between the strand of malaria and the two segments. Combined regions of 5ABC and 6A are also receptors to the invasion of the malaria parasite. The experiments where Trx-19A was the ligand immobilized on the sensor chip resulted in strong binding between the analyte and Trx-MSP1-19A. This also proves that MSP1-19 has a role in the parasite invasion of the red blood cells.

Future developments would be finishing the experiments where only Trx-19 was tested. GST-5ABC6A, GST-6A5ABC, GST-5, GST-6 would have to be experimented with two forms of MSP1-19, Trx-19 and Trx-19B. Reproduce the experiment using GST-5ABC6A with Trx-19A were that particular protein is immobilized on the chip. One problem with the pure protein could be that the concentration was too low, so when the time to make the diluted sample of GST-5ABC6A with the running buffer there was barely any protein for binding with the ligand. Also reproduce the experiment GST-6 with Trx-19 to see if the kinetic values will differ from the original analysis. Finally see if any of the early experiments using the other peptides such as GST-5ABC or GST-6A to compare the results of having the immobilized ligand as anti-GST to Trx-19 as a ligand.

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3. Professor Athar H. Chishti and Lixiao Zeng

4. Members in Prof. Chishtis' lab and AMReL lab

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8. *The Biacore System Manual.* Version September 1991. Pharmacia Biosensor AB, Sweden. 1991

9. SPR image: www.ifm.liu.se/ applphys/sensor/spr.html—spr

10. www.biacore.com

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12. What are the basics? **http://memo.cgu.edu.tw/inscorelab/corelab/document/BIAexpDesign.pdf**

13. image of biacore- giga.sct.ub.es:800/ ImatgesHQ/biacore.jpg

14. picture of the rack in autosampler www.biacore.co.jp/ 2_5_2.shtml

Appendix A- The Method

```
!               KINETICS EXPERIMENT
!
! Association and dissociation of an analyte to and from an
! immobilized ligand surface at varying concentrations are
! monitored in real-time scale.
!
! RACK1= Thermo_C
!    2= Thermo_A
! FLOW CELL= 3
!
! Position r2d4:  Anti-GST Ligand (400nM)
!         r2a1:  Analyte--GST-6 (500nM)
!         r2b1-b6:  Analyte--Trx19 (or Trx)

DEFINE APROG immob
   FLOW       5
! Mix NHS and EDC
   TRANSFER   R2D1 R2D3 50
   TRANSFER   R2D2 R2D3 50
   MIX        R2D3 150

! Active sensor chip
   INJECT     R2D3 35

! Inject 35 ul ligiand
   INJECT     R2D4 35

! Deactivate excess linking groups
   INJECT     R2D5 35

END

DEFINE APROG regeneration
   FLOW       5
   INJECT     R2F3 35      ! 100 mM NaCl + 10 mM NaOH buffer
   INJECT     R2F4 50      ! HBS buffer
END

DEFINE APROG analyse1
   FLOW       50
 * KINJECT    R2A1 150 300    ! GSTGST-5 Conc =400nM Dissociation 5 min
END

DEFINE APROG analyze
```

```
  PARAM %sample %position %volume
  KEYWORD sample %sample
    FLOW       50
 * KINJECT    %position %volume 300    ! Dissociation 5 min
END

DEFINE LOOP cycle
 LPARAM %sample %position %volume
        Sample1  R2B1       150              !Trx19 Conc =1500nM
        Sample2  R2B2       150              !Trx19 Conc =1200nM
        Sample3  R2B3       150              !Trx19 Conc =1000nM
        Sample3  R2B4       150              !Trx19 Conc =800nM
        Sample3  R2B5       150              !Trx19 Conc =600nM
END

DEFINE APROG analyse2
    FLOW       50
 *  KINJECT    R2B6 150 300    ! Trx19A Conc =200nM  Dissociation 5 min
END

MAIN
  RACK       1 thermo_c
  RACK       2 thermo_a
  FLOWCELL   2
!  APROG      immob
  APROG      analyse1
  LOOP cycle STEP
    APROG      analyze %sample %position %volume
    APROG      regeneration
    APROG      analyse1
  ENDLOOP
  APROG      analyse2
  APROG      regeneration
  APPEND     continue
END
```

Appendix B-Sensorgrams of the Experiments & Tables

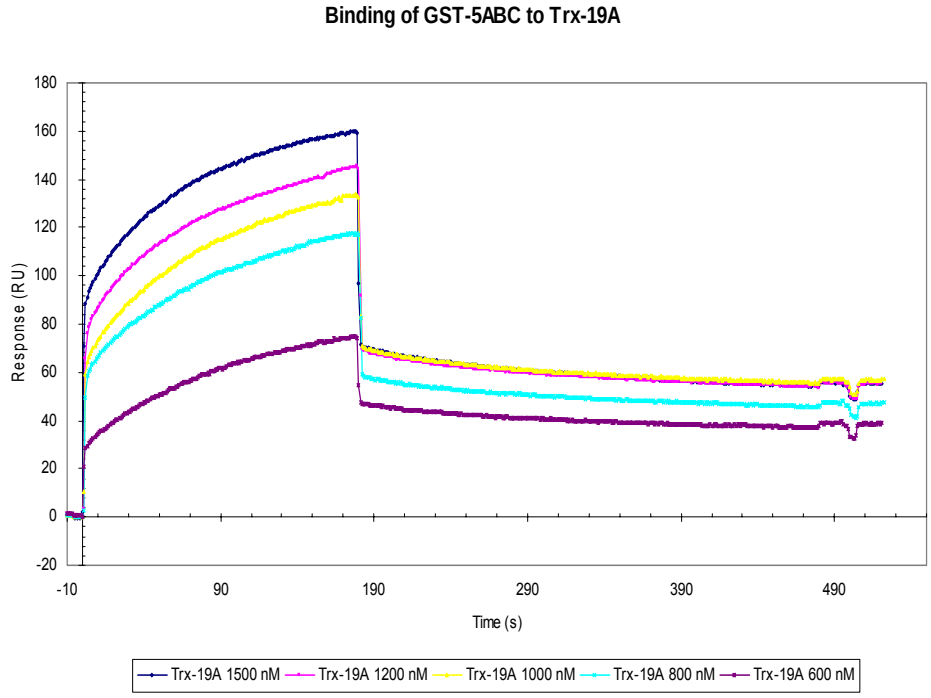

Figure 1. Sensorgram of GST-5ABC with Trx-19A. Binding has occurred, this is can be seen from the increasing curve.

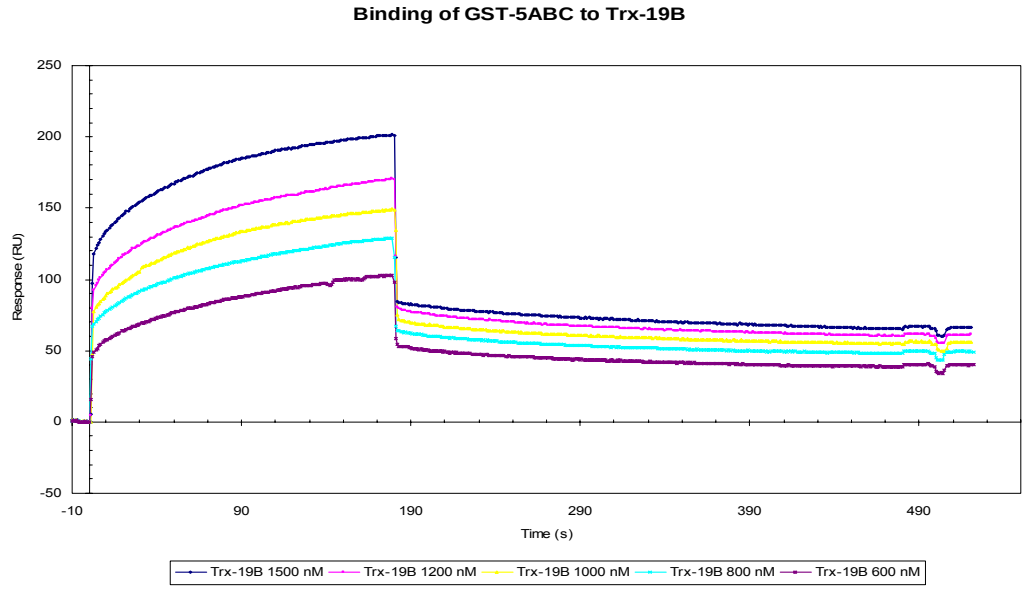

Figure 2. Sensorgram of GST-5ABC with Trx-19B. Binding has occurred due to the increase of the curve.

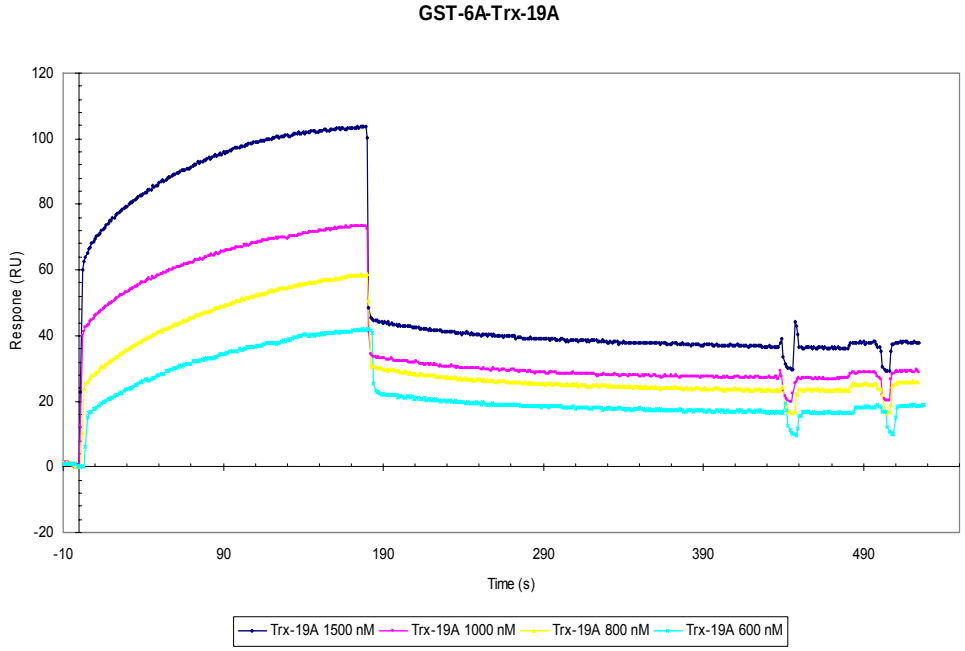

Figure 3. Sensorgram of GST-6A with Trx-19A.

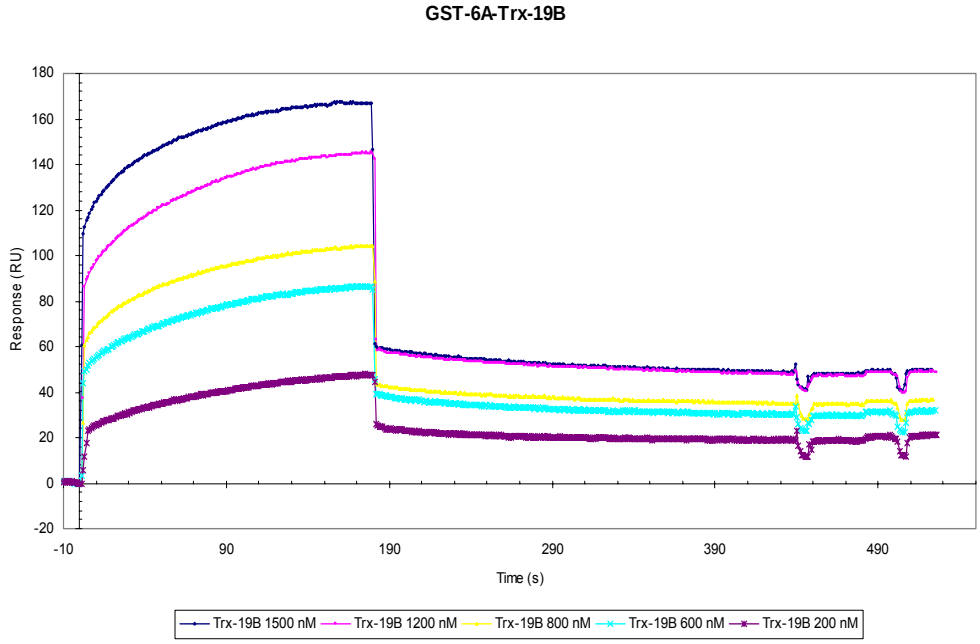

Figure 4. Sensorgram of GST-6A with Trx-19B

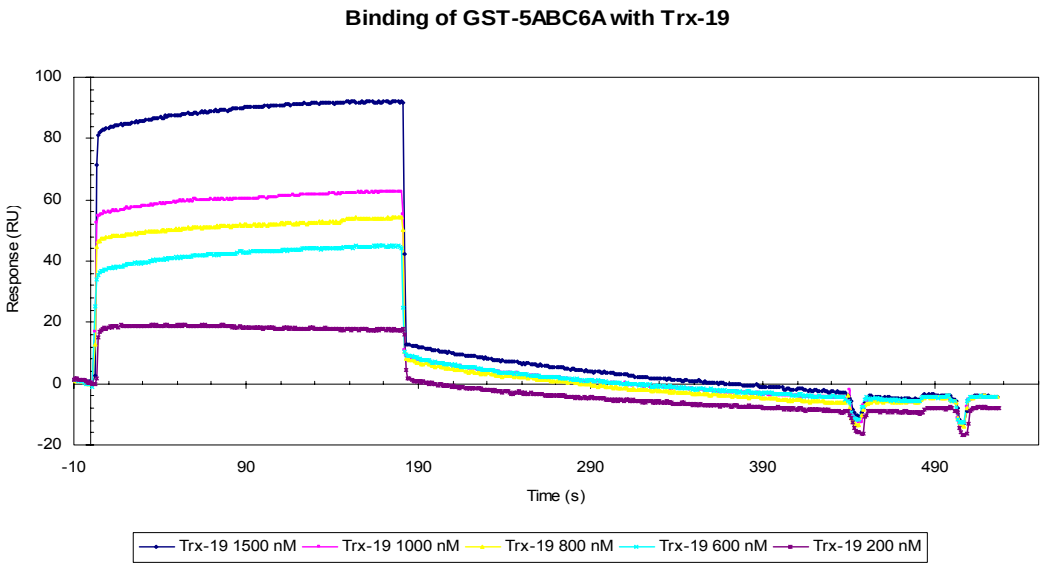

Figure 5. The sensorgram for GST-5ABC6A with Trx-19. There are no signs of binding.

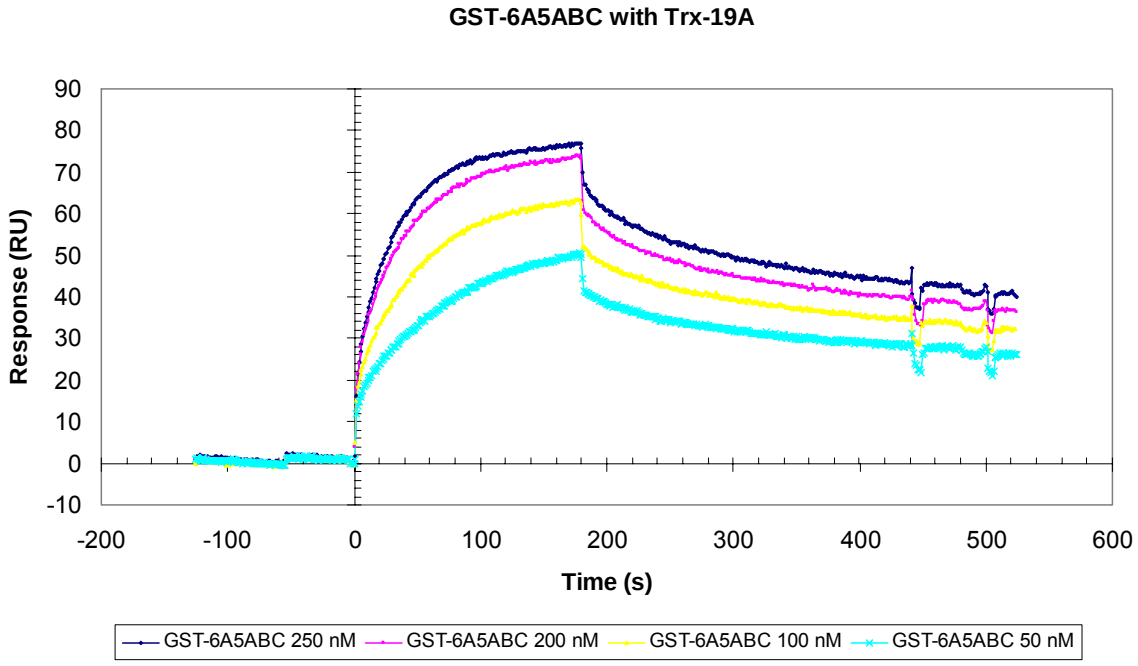

Figure 6. GST-6A5ABC with Trx-19A. This sensorgram shows signs of binding. The difference in this experiment is that the ligand immobilized on the sensor chip is Trx-19A.

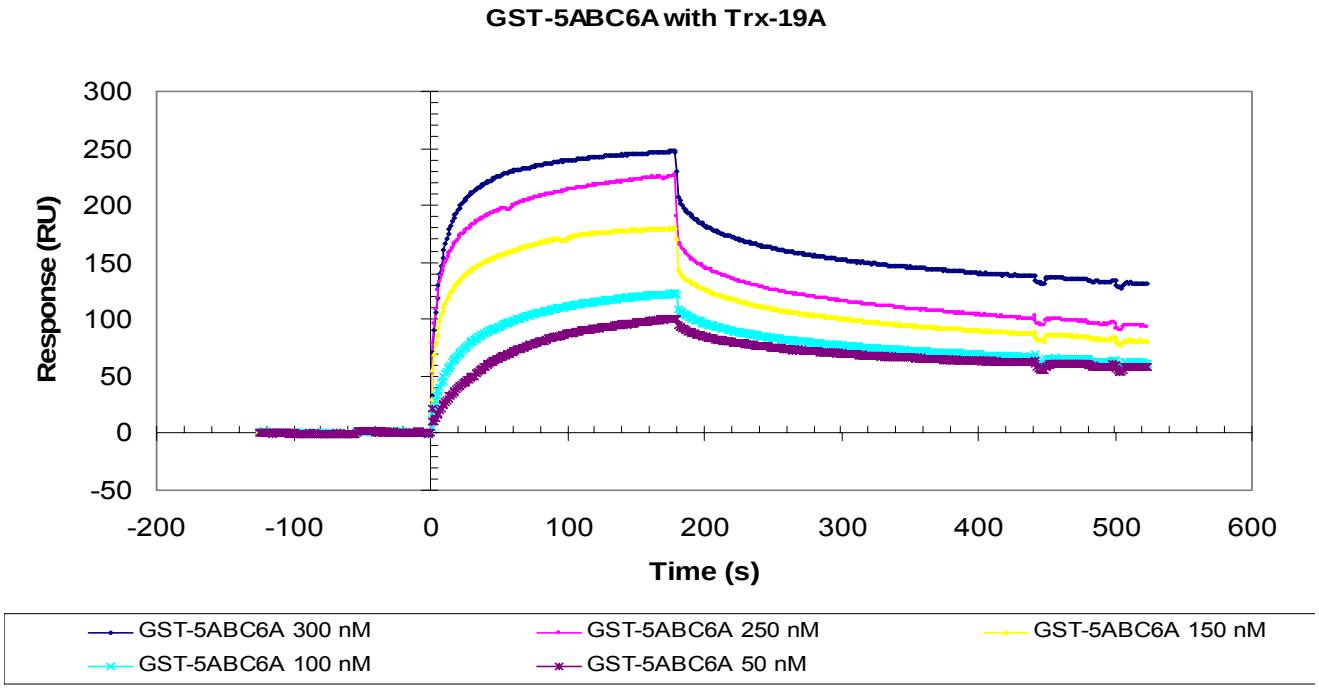

Figure 7. GST-5ABC6A with Trx-19A also shows signs of binding. The immobilized ligand for this experiment was also Trx-19A.

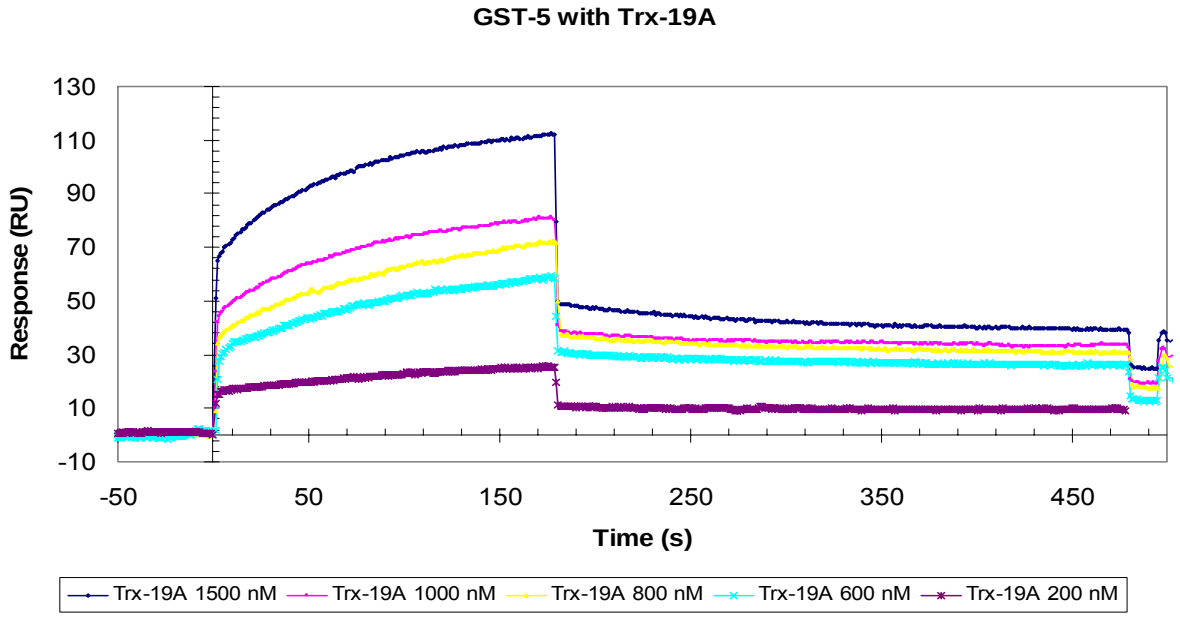

Figure 8. GST-5 with Trx-19A. This experiment follows the strong binding patterns of the GST-5ABCs and GST-6A with the three recombination proteins.

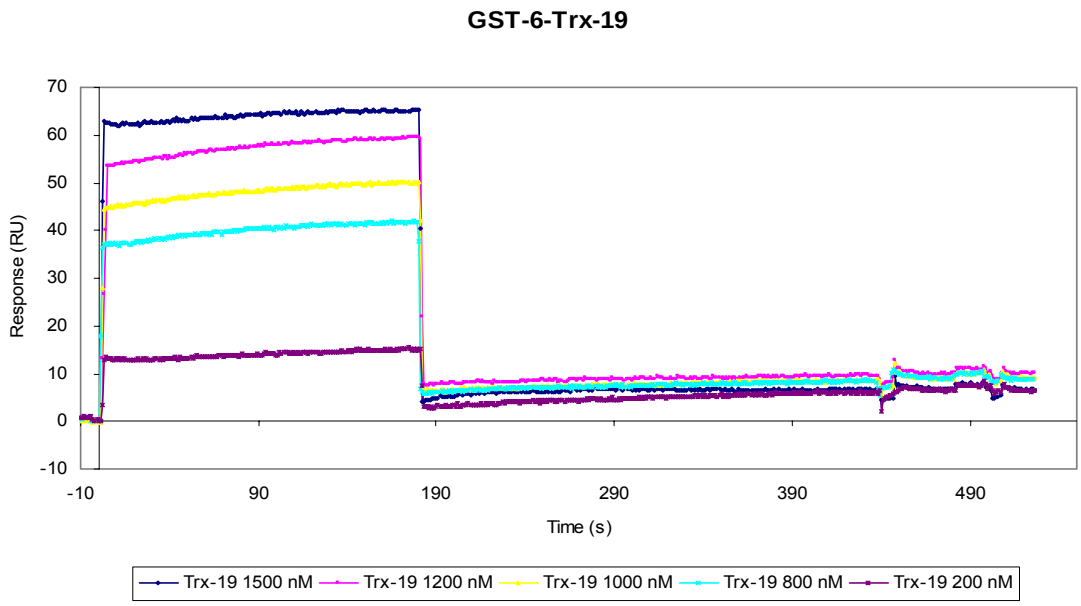

Figure 9. The sensorgram for GST-6 with Trx-19. Weak binding has occurred.

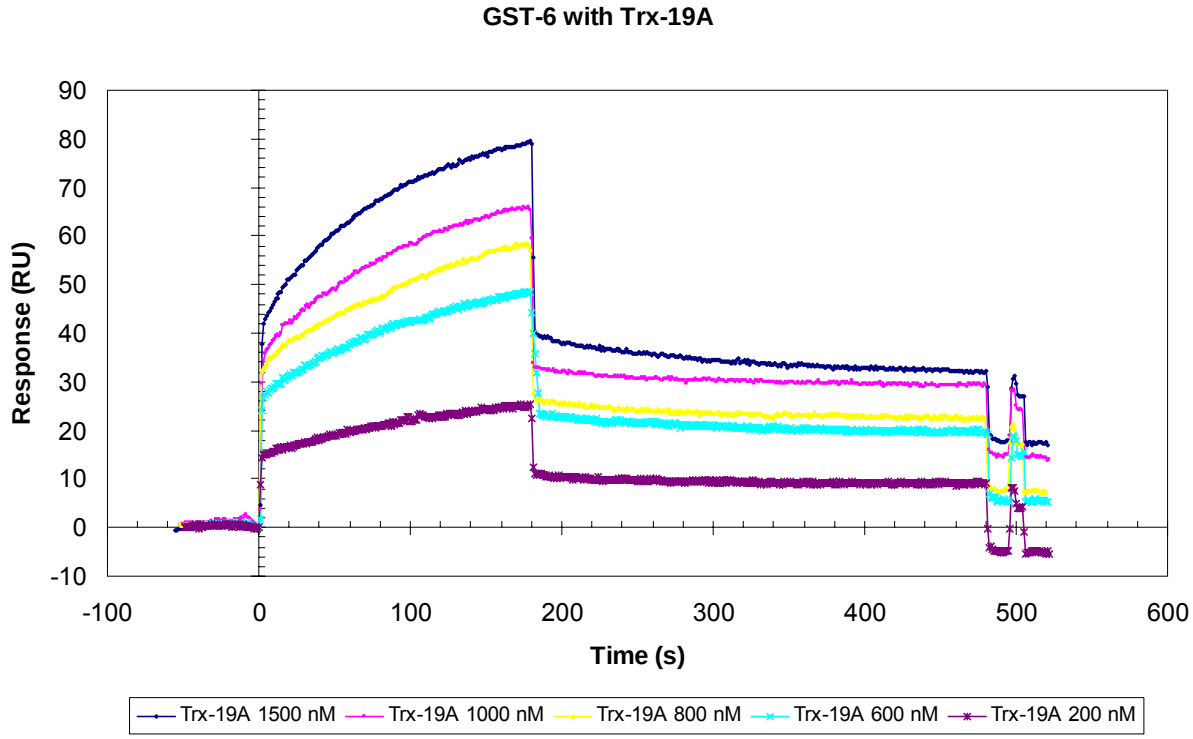

Figure 10. The experiment of GST-6 with Trx-19A. Unlike the previous experiment with GST-6 and Trx-19 this shows a better and stronger signs of binding.

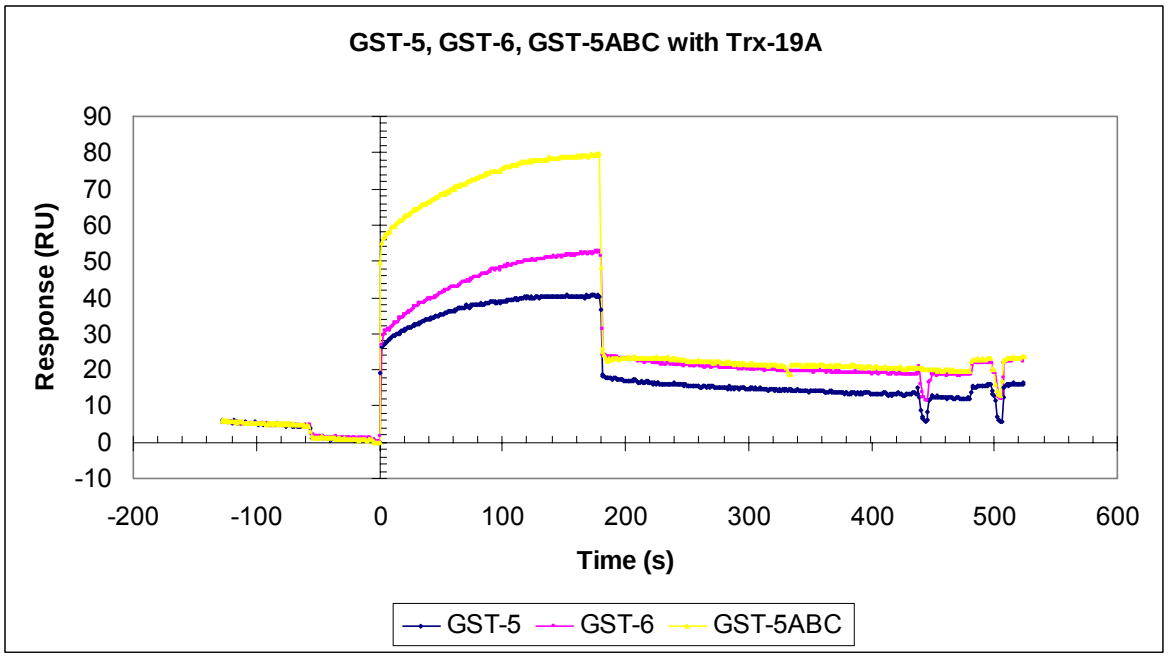

Figure 11. Comparison plot of GST-5, GST-6, and GST-5ABC with Trx-19A. This shows that Trx-19A binds with all three peptides. The difference in binding can be seen in this plot.

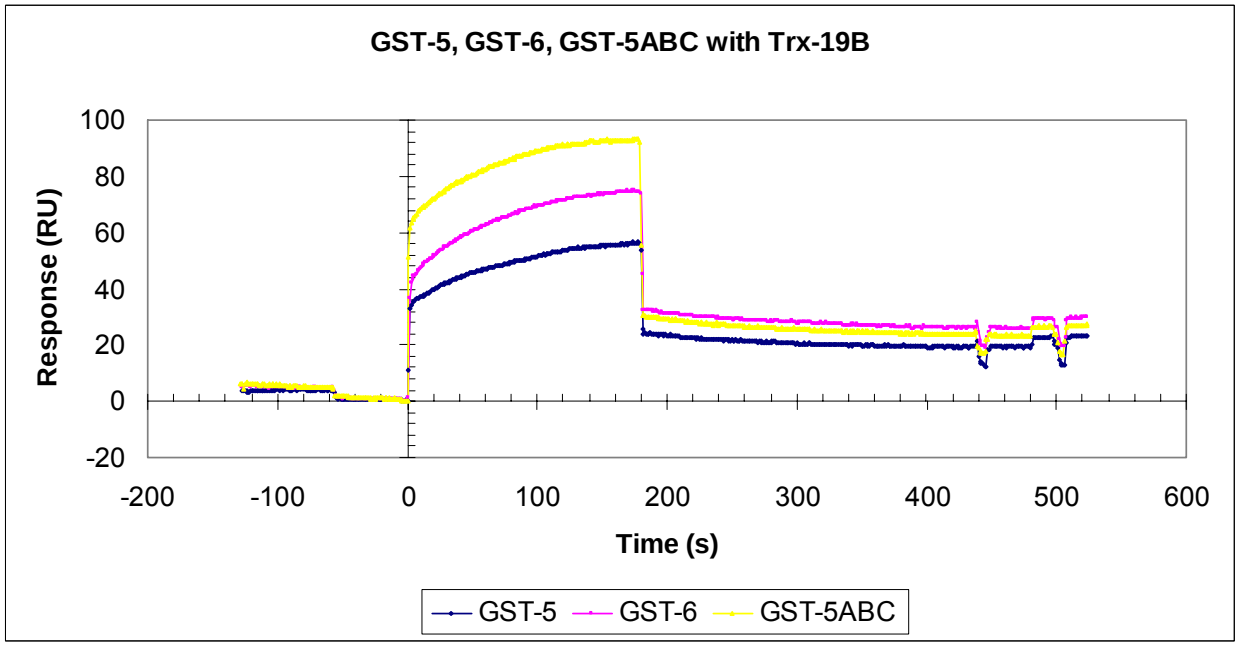

Figure 12. Comparison plot of GST-5, GST-6, and GST-5ABC with Trx-19B. The values of the binding for all three peptides are closer.

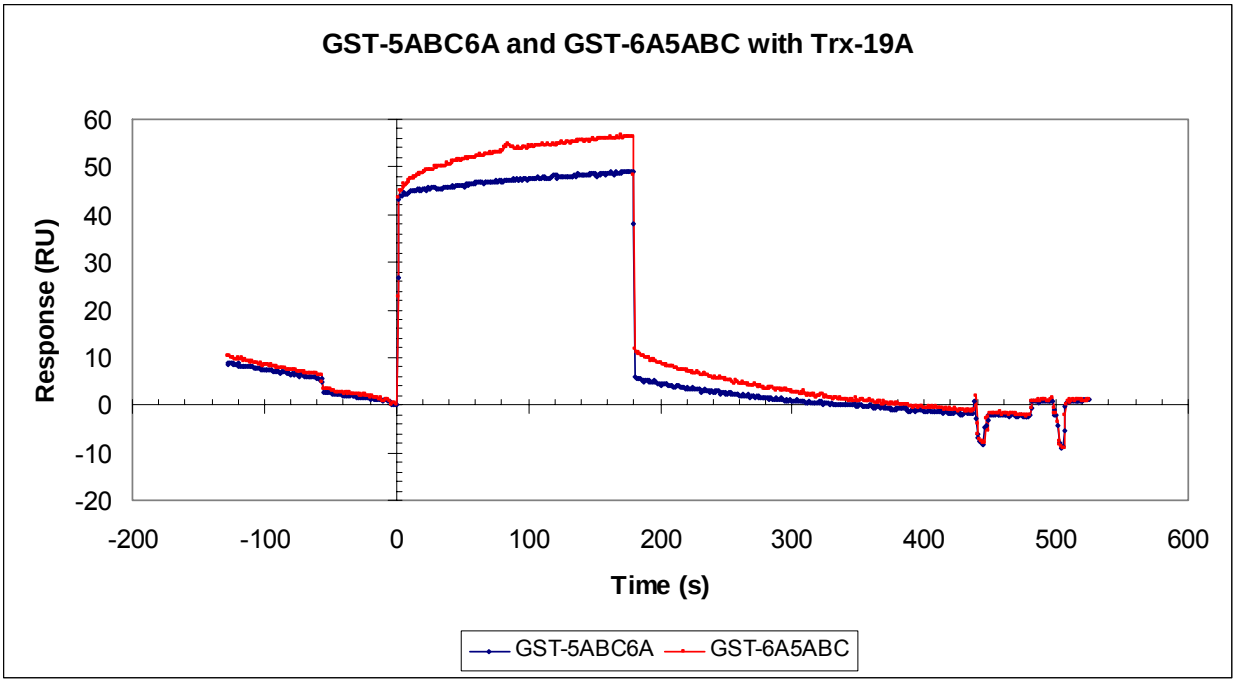

Figure 13. Comparison plot of GST-5ABC6A and GST-6A5ABC with Trx-19A. These two recombination proteins showed weak binding with Trx-19A. The immobilized ligand is anti-GST.

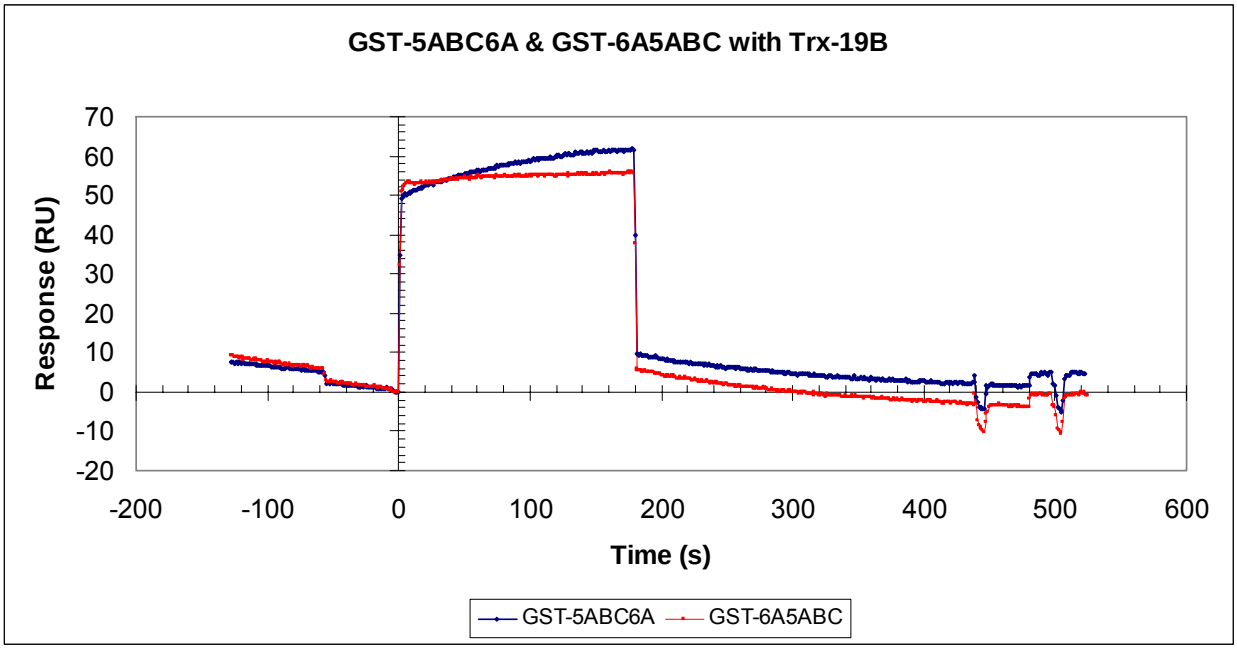

Figure 14. Comparison plot of GST-5ABC6A and GST-6A5ABC with Trx-19B. Weak binding has occurred again.

Table 5. Comparison plots parameters

Binding Kinetic constants	KA (1/M)	KD (M)	σ^2 Chi2 value
GST-5 with Trx-19	1.04E+07	9.58E-08	0.644
GST-5 with Trx-19A	3.16E+07	3.16E-08	0.637
GST-5 with Trx-19B	2.51E+07	3.98E-08	0.681
GST-6 with Trx-19	4.46E+07	2.24E-08	0.14
GST-6 with Trx-19A	2.00E+07	4.99E-08	0.677
GST-6 with Trx-19B	2.62E+07	3.82E-08	0.584
GST-5ABC with Trx-19	1.21E+07	8.24E-08	0.0694
GST-5ABC with Trx-19A	2.81E+07	3.56E-08	0.167
GST-5ABC with Trx-19B	2.16E+07	4.63E-08	0.214
GST-5ABC6A with Trx-19A	1.78E+04	5.61E-05	0.418
GST-5ABC6A with Trx-19B	191	5.25E-03	0.174
GST-6A5ABC with Trx-19A	6.76E+05	1.48E-06	0.255
GST-6A5ABC with Trx-19B	2.48E+06	4.04E-07	1.19