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THE DESIGN, DEVELOPMENT, AND OPTIMIZATION OF A LATERAL FLOW ASSAY TO DETECT THE PRESENCE OF MYOGLOBIN

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Abstract

Lateral flow assays (LFA) afford an inexpensive, user-friendly point of care testing alternative that is beneficial in medical diagnoses. The LFA strip comprises an absorbent pad, nitrocellulose membrane, conjugate pad, and a sample pad with laminated backing. Two lines (the control and test line) are placed on the nitrocellulose membrane, and they indicate a positive, negative, or futile test with color changes on both, one or none, respectively. We have successfully created an LFA strip that can detect the presence of myoglobin in a buffer system. This strip can detect the presence of myoglobin at a concentration as low as 2 ng/mL in about 15 minutes. The presence of myoglobin in the blood indicates muscle injury as occurs during heart attack/acute myocardial infarction. The easy, fast, and efficient detection of myoglobin can prove beneficial in diagnosing a heart attack.

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Introduction

Myoglobin is a heme protein found in muscles and serves as the biomarker for detecting heart attacks and muscle injuries. This protein is present in cardiac and skeletal muscles and is released into the bloodstream after heart attacks and muscle injuries.[1] The concentration of myoglobin increases in the blood 2 to 3 hours after the initial symptom of muscle injuries, and it can peak after 8 to 12 hours. The concentration of myoglobin above 900 ng/mL in the blood indicates muscle injuries[2]. The ability to detect myoglobin's presence enables diagnosing diseases like acute myocardial infarction. Several techniques exist to detect this marker, but these techniques are expensive and require high technical expertise and instrumentation, which may not readily be available in some regions of the world. The development of a lateral flow assay (LFA) strip provides quick, inexpensive, and effective detection of myoglobin which can potentially be used to indicate heart attacks and muscle injuries.

Lateral flow assays (LFA) present the opportunity to assess medical emergencies because results are produced quickly, and no expertise is required to read and understand the data. Lateral flow assays are medical tests that are ASSURED (affordable, sensitive, specific, user friendly, rapid, and robust, equipment-free, and deliverable to end-user)[3]. They are also specific to their target antigens/markers and are robust when stored under the proper conditions. A conventional lateral flow assay comprises a sample pad, conjugate pad, nitrocellulose membrane, and an absorbent pad on a laminated plastic backing with the direction of flow from the sample pad to the absorbent pad (Figure 1). The flow direction in a lateral flow assay starts from the sample pad and moves through the conjugate pad, and the nitrocellulose membrane ends finally in the absorbent pad by capillary action. Each of these components has a unique role in the assay presenting various points where optimization of the analysis can occur[4].

The sample pad is the initial receiver of the analyte and serves as a filter to remove all extraneous materials that can potentially influence the assay. Sample pads are usually made of cellulose fiber filters or woven meshes. The conjugate pad, which was glass fiber in our instance, contains an anti-antigen antibody conjugated nanoparticle that binds the antigen (when present). Visualization techniques used in LFAs (Lateral Flow Assays) include gold-nanoparticles based, aptamer-based, carbon nanomaterials based, and magnetic-based[5]. Our studies used the conventional gold nanoparticles in our conjugation with the anti-antigen antibody, giving a characteristic red color in positive assays. The nitrocellulose membrane contains the control and test lines. The test line, which is placed closer to the conjugate pad, includes an antibody that binds to antigen-bound - anti-antigen conjugated gold nanoparticles. The presence of the antigen results in red color at the test line, while the absence of the antigen shows up as no color change. The control line tests the assay's validity. It is made up of an antibody that binds to the anti-antigen antibody conjugated gold nanoparticles irrespective of the presence or absence of the antigen. The control line shows a red color change even in the absence of the antigen. The lack of a red line at the control indicates a non-valid strip, and the assay needs to be repeated with a different LFA strip. The absorbent pad absorbs any extra fluid. A positive result in an assay shows a red color change on both the test line and control line. A negative result shows a red color change on the control line, and an invalid test shows no color change even on the test line (Figure 2).

Experimental Methods

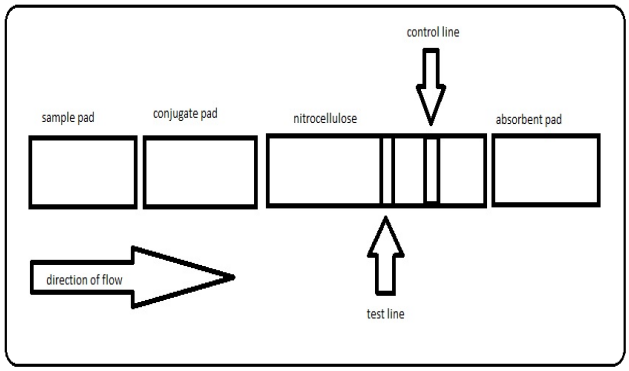

Figure 1: A diagram of a lateral flow assay with all its components except the plastic backing is not shown.

Equipment: Centrifuge, Vortex, Incubator, Pipettes, pH meter
Materials: 15 nm gold nanoparticles (TedPella), Detection Antibody – Mouse monoclonal Myoglobin antibody-10-1361(Fitzgerald), Capture Antibody – Mouse monoclonal Myoglobin antibody-10-1362 (Fitzgerald), control antibody - Goat anti-Mouse IgG1 antibody - 20R-IG003 (Fitzgerald), Bovine serum albumin (BSA) (Sigma Aldrich). Myoglobin (LeeBioscience), sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, potassium carbonate, sucrose, polyethylene glycol (PEG), Polyvinylpyrrolidone (PVP), hydrochloric acid (HCl), tween-20, Phosphate buffered saline (PBS), sodium azide, nitrocellulose membrane Cytiva# 10547129 FF120HP Plus LAM, conjugate pad glass fiber GE# 8133-6621 Standard 14, sample pad CF6 A4 SHEETS 50/PK and sample pads.

Solutions used: storage buffer, nitrocellulose (NC) membrane pretreatment solution, conjugate pad pretreatment solution, sample pad pretreatment solution, antibody dilution solution, 10% BSA, 1% BSA, 0.1 M HCl, 0.2 M K_2CO_3.

1. Storage Buffer (pH 7.8): 0.6360 g Na_2HPO_4, 0.0624 g NaH_2PO_4, 5 g PVP, 1.25 g sucrose, 0.05 g PEG, 0.2 g BSA in 100 mL of Milli-Q water and adjusted to pH 7.8 with a pH probe with 0.1 M HCl and 0.2 M K_2CO_3. The solution was stored at 4 °C until needed.
2. NC Membrane Pretreatment: 1.5 g BSA, 250 uL Tween-20, 5 mL 10x PBS dissolved to 50 mL of Milli-Q. The pH was adjusted to 7.4 with either 0.1 M HCl or 0.2 M K_2CO_3 and stored at 4 °C until needed.
3. Conjugate Pad Pretreatment: 2.5 g sucrose, 0.025 g NaN_3 dissolved to 50 mL with Milli-Q water and stored at room temperature until needed.
4. Antibody dilution solution: 0.1461 g NaCl, 0.025 g NaN_3, 5 mL 10x PBS dissolved to 50 mL with Milli-Q water and stored at room temperature
5. 10% BSA: 1 g BSA dissolved to 10 mL with Milli-Q water and stored 4 °C until needed.
6. 1% BSA: 1 mL 10% BSA dissolved to 10 mL with Milli-Q water and stored at 4 °C until needed.
7. 10% NaCl solution: 2.5 g NaCl dissolved to 25 mL with Milli-Q water and stored at room temperature 4 °C until needed.
8. 0.1 M HCl: 208 uL 12M HCl dissolved to 25 mL with Milli-Q water and stored at room temperature until needed.
9. 0.2 M K_2CO_3: 0.6910 g K_2CO_3 dissolved to 25 mL with Milli-Q water and stored at room temperature until needed.
10. Sample pad pretreatment solution: To make 100 mL of this solution,.0.7627 g sodium borate, 1 g sucrose, 1 g BSA, 0.05 g NaN_3, and 500 uL Tween-20 were stirred with approximate 80 mL of DI water to dissolve and more water was added to the 100 mL mark. The pH of the solution was adjusted to 7.4 and stored at 4 °C

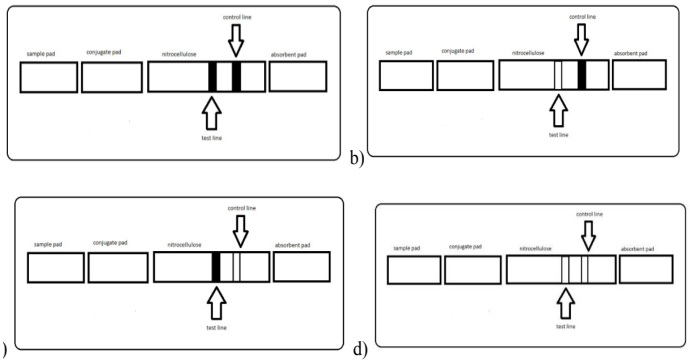

Figure 2: Reading the results of a lateral flow assay: (a) positive results, (b) negative results. When there is an excess of the antigen, results (c) can be seen. After a run, there are also instances when the device is defective, showing results (d).

Methods

Conjugation of gold nanoparticles with an antibody: 1000 μL of 15 nm AuNP was pipetted into an Eppendorf tube, and 6 μL of detection antibody was added and vortexed to mix the solution. The solution was allowed to sit for 30 minutes, then 100 uL of 10% BSA was added. The mixture was centrifuged at 3220 xG (6000 RPM) for 1 hour, and the supernatant was removed. The pellets were resuspended in 100 uL of 1% BSA. The mixture was centrifuged at 3220 xG for 1 hour, and the supernatant was removed again. The pellets were resuspended in 1000 μL of 1% BSA. The mixture was centrifuged at 3220 xG for 1 hour, the supernatant was removed, and the pellets were resuspended in 200 uL of storage buffer and stored at 4 °C until use.

Pretreatment of the conjugate pad: The glass fiber conjugate pad (0.8 cm x 0.5 cm for each strip) was soaked in the conjugate pad pretreatment solution for 1 hour. The pad was dried at 50 °C for 2 hours and stored at room temperature until further use.

Pretreatment of the sample pad: The sample pad, also made of glass fiber (1.7 cm x 3 cm for 6 strips) – each strip has a width of 0.5cm, was soaked in the sample pad treatment solution (pH 7.4) for 1 hour. It was then dried at 50 °C for 2 hours and stored at room temperature until ready to use.

Pretreatment of nitrocellulose membrane: The nitrocellulose membrane (5 mm width for one strip) was soaked in nitrocellulose pretreatment solution for 1 hour. It was then dried at 50 °C for 2 hours and stored at room temperature until ready to use.

Immobilization of conjugated antibody gold nanoparticle on the conjugate pad: The pretreated conjugate pad was soaked in a solution of 12 μL of conjugated antibody and 18 μL of storage buffer (pH = 7.8) and immediately dried at 37 °C until completely dry. The dried immobilized conjugate pad is stored at room temperature until used.

Creating Test and Control lines (protocol for one strip): The capture antibody was diluted to 0.5 mg/mL (0.5 μL of 5g/L captured antibody: 4.5 μL of antibody solution (pH 8)). The control antibody was diluted to 0.1 mg/mL (0.2 μL of 1g/L control antibody: 1.8 μL of antibody solution (pH 8)). The capture and control antibodies were pipetted at 1 μL at the test and control lines on the pretreated nitrocellulose membrane, respectively, and dried for 15 minutes at room temperature. The process was repeated one more time to obtain a total pipetted volume of 2 μL. The nitrocellulose membrane with the control and test lines was dried at 37 °C overnight and stored at room temperature.

Assembly of the strip: The pretreated nitrocellulose membranes with laminated backing were used with dimensions of 6 cm (length) x 0.5 cm per strip (total width of 3 cm for 6 strips). A pretreated conjugate pad containing the immobilized gold-nanoparticle conjugated antibody (dimensions of 0.7 cm x 3 cm for 6 strips) was attached to the backing next to the nitrocellulose membrane

on the test line side. Next, the pretreated sample pad (dimensions of 1.7 cm x 3 cm for 6 strips) was attached to the backing next to the conjugate pad. Then, the absorbent pad with dimensions of 1.7 cm x 3 cm for 6 strips was attached to the backing next to the nitrocellulose membrane on the control line side (Figure 1). All the pads were overlapping by 0.2 cm. Lastly, each fully assembled strip was cut with a width of 0.5 cm. The strips were stored at room temperature until used.

Running the strips: 150 μL of myoglobin in its storage solution or buffer was applied to the sample pad for full strips or conjugate pad for half strips, and color changes were observed after 15 – 20 minutes. The strips were allowed to dry before pictures were taken.

Results and Discussion

The design process of our lateral flow assay involved steps outlined below: a) creating the antibody gold nanoparticle conjugation, b) assembly of the strips with their various components, and c) testing the strips to ensure that it works appropriately.

Our initial steps involved the conjugation of the gold nanoparticle antibody. As part of the research process, we tested different conjugation protocols by changing the amount of the antibody in the conjugation protocol. We also tried different pHs from 7.5 to 8.5 and tested protocols using several buffer systems. For example, we used PBS (pH = 7.8) and sodium phosphate buffer (pH = 8.5) as an initial resuspension solution before adding the detection antibody. Both systems failed, and we concluded that resuspending the AuNP in these buffer systems caused them to aggregate. We, therefore, removed an initial resuspension of gold nanoparticles from our protocol. We also experimented with 2 sizes of gold nanoparticles (15 nm and 40 nm), choosing 15 nm for our final optimized protocol, which we reported under methods. The 40 nm gold nanoparticles frequently resulted in aggregation in all the methods tested. When aggregation occurred, the gold nanoparticle solution would turn black, and we discarded the protocol (Figure 3). With each run, we measured the peak wavelengths and compared that to our original wavelength of the gold nanoparticle solution to ascertain if our conjugation was successful (Figure 3).

Once we had a successful conjugation protocol, we focused on actual testing by assembling a strip. As we were designing from first principles, we used half strips in most of our assays and optimizations protocols. Half strips do not include sample pads to increase the speed of testing. All tests were done in triplicates, and we split the control and test line in our initial testing and tried dif-

AuNP size	Buffer used during conjugation	Peak wavelength absorbed (nm)	Color of conjugated solution
15 nm	Not conjugated	520	
	PBS pH 7.8	636	
	Sodium phosphate pH 8.5	422	
	No added buffer pH 5.3	528	
40 nm	Not conjugated	527	
	PBS pH 7.8	637	
	Sodium phosphate pH 8.5	422	
	No added buffer pH 5.3	637	

Figure 3: Comparing different conjugation protocols and their products.

We tried to establish that the antibodies in our control and test lines were working as they should. We used a half strip with no pretreatment on the NC membrane and pretreatment on the conjugate pad. The antibody concentration on the control and test lines was 1mg/mL. We did not experience any flow issues in both instances, and both the control and test lines worked as expected except for a slight false positive test on our test line in the absence of the antibody (Figure 4). To establish if the concentration of the antibodies on the control and test lines affected the assay's sensitivity; we tested two different concentrations aside from our original 1mg/mL (5mg/ml and 0.5 mg/mL). We used similar conditions to test the 1 mg/mL test lines. We found that the more concentrated lines gave sharper lines, but aside from that, all the concentrations gave similar data (Figure 5). We decided to use 0.5 mg/mL in optimization assays to save the antibody and make the project more affordable on our undergraduate lab research budget.

Our attention switched to fixing the false positives occurring on test lines with no myoglobin in the test solution. One proven way of removing false positives is through the pretreatment of the nitrocellulose membrane. However, initial testing with 3% BSA in our nitrocellulose pretreatment solution following a process by Zhou et al.[6] resulted in slow flow issues on our strips (data not shown). We decided to delete the pretreatment, which introduced false positives (enhanced red color on the test line in the absence

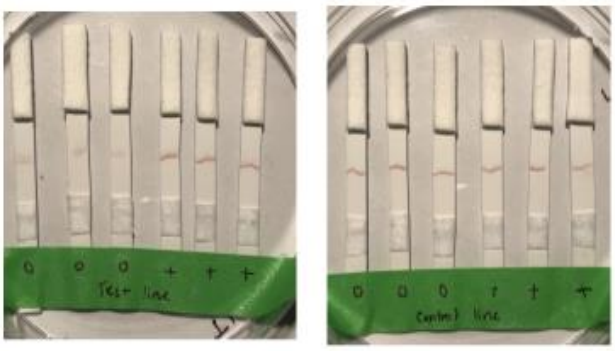

Figure 4. Testing the validity of our selected antibodies and conjugations using a half strip. In both instances, the half strips worked as expected except for a slight positive test on the test line, which became a goal in our optimization processes. There was no pretreatment on the nitrocellulose pad, the conjugate pad was pretreated. The concentration of antibody on both the control and test line were 1g/L. Reader should note that in all images there is a shadow where the absorbent pad meets the nitrocellulose pad.

Figure 5. Testing different antibody concentrations on control and test line to establish the effect of more concentrated antibody lines versus dilute antibody lines. Half strips were used. In these half strips, the test and control lines were separated on different strips. 0 represents the absence of myoglobin, and 1 represents the presence of myoglobin There was no pretreatment of the nitrocellulose membrane but the conjugate pad was pretreated. One set of strips used antibody concentration of 5 g/L (conc.) on both test line and control line whilst the other set was 0.5g/L (dilute).

of myoglobin). We hypothesized that we could reduce how much BSA was in our pretreatment solution and, in the process, tested 3 different concentrations of BSA (1.5%, 0.75%, and 0.375%) to find which one was optimal. We did not increase the BSA concentration past 1.5% because we wanted to avoid flow issues. The optimal concentration was found to be 1.5%, so to confirm, we ran the experiment in triplicate. There was no red color change on our half strip in our myoglobin-absent sample (Figure 6).

With the success of these initial tests, the next step in our optimization process was to include both the test line and control line on one strip, as occurs in the conventional lateral flow assay. In triplicates, we tested the presence of the control line and test line on the same half strip in the absence and presence of myoglobin. Assay conditions included pretreatment on the nitrocellulose pad with 1.5% BSA nitrocellulose pretreatment solution and conjugate pad. Our test and control lines had 0.5 mg/mL of antibody concentration. In the absence of myoglobin, there was no color change on our test line but a red color change on our control line, as expected. In the presence of myoglobin, however, we had a color change on our test line (as expected) but no color change on our control line (not expected) (Figure 7). We speculated that all the anti-myoglobin antibodies-conjugated gold nanoparticles were binding to the test line with no excess left over to bind to the control line. As we advanced, we thought of 3 different routes to tackle this issue - reducing the amount of myoglobin in the test, reducing the antibody concentration on the test line, or increasing the amount of immobilized anti-myoglobin antibody conjugated gold nanoparticles on the conjugate pad.

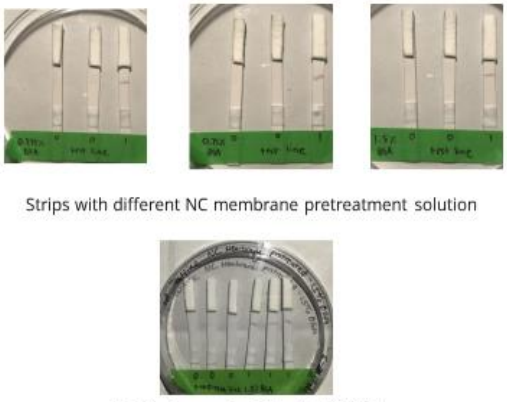

Figure 6. Eliminating False Positive results by using different concentrations of BSA (1.5%, 0.75%, and 0.375%) in the nitrocellulose pretreatment solution. Half strips were used in these assays. In these half strips, the test and control lines were separated on different strips. 0 represents the absence of myoglobin, and 1 represents the presence of myoglobin. The optimal concentration was found to be 1.5% BSA. The nitrocellulose membrane was pretreated with different concentrations of BSA and the conjugate pad was also pretreated. The test line and control line had antibody concentration of 0.5 g/L.

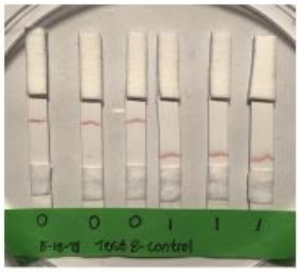

Figure 7. Testing the effect of having both the control line and the test line on the same strip. 0 represents the absence of myoglobin, and 1 represents the presence of myoglobin. In the absence of myoglobin, the strip works as expected, but there is no red color on the control line in the presence of myoglobin. The nitrocellulose membrane was pretreated with pretreatment solution containing 1.5% BSA. The conjugate pad was also pretreated. The concentration of antibody on the control and test line were both 0.5 g/L.

Up until this point, we had been immobilizing 3 μL of anti-myoglobin antibody conjugated gold nanoparticles on the conjugate pad with 1 μL of myoglobin per strip. Our next set of tests included increasing the amount of the immobilizing solution to 6 uL and reducing the myoglobin concentration to 0.5 μL per strip. Aside from making these changes, we decided to include in the buffer solution 1% BSA to our running buffer. BSA is a protein blocker and forms non-specific contacts to the nitrocellulose surface and avoids the formation of such contacts by conjugates; this reduces the background and improves the band intensities[7]. With the changes made, we started to see a slight color change on our control line in the presence of myoglobin. Adding 1% BSA to the running buffer made no difference in the color change detected (Figure 8). With this data in hand, we decided to try a varied amount of immobilized conjugated antibodies ranging from 12 μL/strip to 21 μL per strip. The antibody concentration on the test line was also reduced to 0.25 mg/mL. Aside from the 12 μL/strip, with

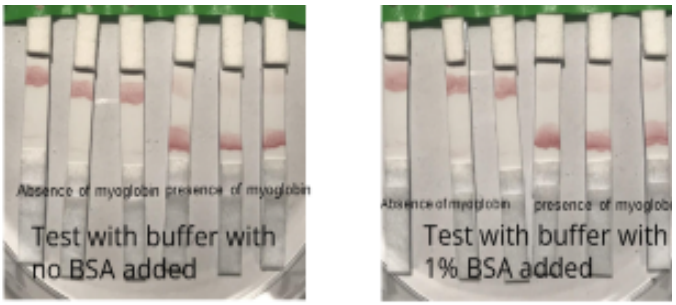

Figure 8. Testing the effect of an increased concentration of immobilized conjugated antibody and a reduced myoglobin concentration in the running buffer. 0 represents the absence of myoglobin, and 1 represents the presence of myoglobin. We did all tests in triplicates, including using 1% BSA in one of our buffer systems (on right). The nitrocellulose membrane was pretreated with pretreatment solution containing 1.5% BSA. The conjugate pad was also pretreated. The concentration of antibody on the control and test line were both 0.5 g/L. The immobilized conjugated antibody used was 6 uL/strip.

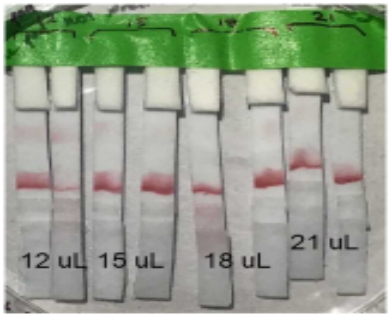

Figure 9. Testing the effect of different amounts of immobilized anti-myoglobin conjugated gold nanoparticles on the conjugate pad. The amount of myoglobin was 0.5 uL/per strip in each run - each run was done in duplicates. The nitrocellulose membrane was pretreated with pretreatment solution containing 1.5% BSA. The conjugate pad was also pretreated. The concentration of antibody on the control line was 0.5 g/L and test line was both 0.25 g/L.The immobilized conjugated antibody used were 12, 15, 18, 21 μL/strip.

slightly improved color, all the others did not provide any positive data sets. 12 uL/strip of immobilized conjugate antibodies with 0.25 mg/mL on the test line and 0.5 mg/mL on our control line became our standard conditions for subsequent runs (Figure 9).

As we had optimized the amount of immobilized conjugated antibodies, we decided to further reduce the antibody concentration at the test line from 0.25 mg/mL to 0.125 mg/mL. We maintained all other optimized conditions. This reduction of the antibody at the test line resulted in a marked improvement of our red color change on the control line in the presence of myoglobin (Figure 10). We finally had a half strip that could detect the presence and absence of myoglobin as expected, so we decided to include the sample pad in our strip.

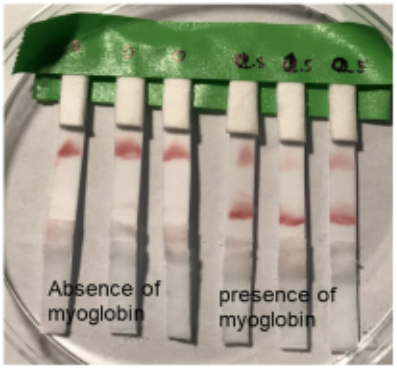

Figure 10. Testing the effect of a test line at an antibody concentration of 0.125 mg/mL in the presence and absence of myoglobin. The nitrocellulose membrane was pretreated with pretreatment solution containing 1.5% BSA. The conjugate pad was also pretreated. The concentration of antibody on the control line was 0.5 g/L and test line was 0.125 g/L. The immobilized conjugated antibody used was 12 μL/strip.

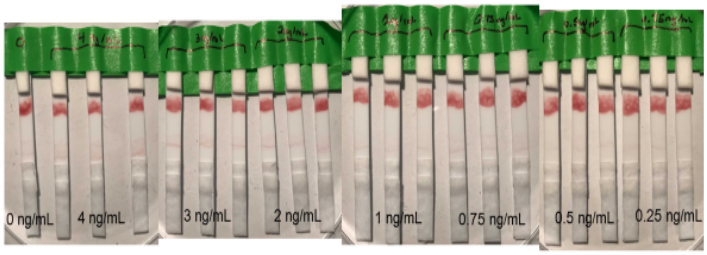

Figure 11: Testing the minimum concentration detected with the device. The strips ensembled can detect up to 1ng/mL of myoglobin. Less than that concentration, there was no difference between the strips with no myoglobin and the ones that had myoglobin.

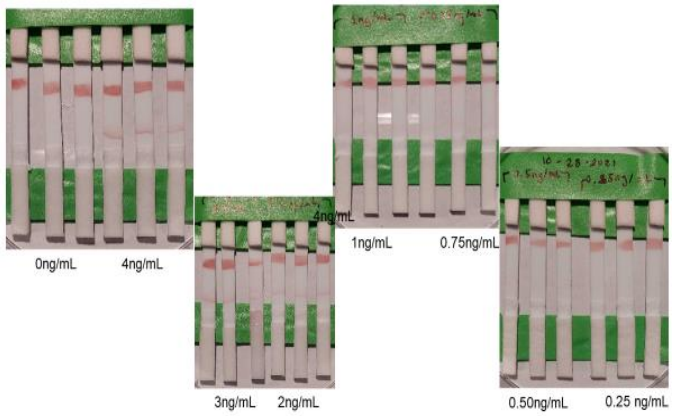

Figure 12: Limits of detection test for a strip. The strip works until 2 ng/mL

With a full strip in hand, we moved on to the final piece of our optimization process. We reduced the concentration of our myoglobin to 500 ng/mL and 5ng/mL. We tried multiple volumes and realized that we needed 9 μL of conjugated antibody on the conjugate pad. We increased the antibody concentration of the test line back to 0.5 mg/mL and decreased the control to 0.1 mg/mL based on the results we observed. Even at the low concentration of 5 ng/mL, our strip still worked as it should (Figure 11). We proceeded to find limits of detection of our strip when it tests buffers spiked with myoglobin. We tested concentrations of 4 ng/mL, 3 ng/mL, 2 ng/mL, 1 ng/mL, 0.75 ng/mL, 0.5 ng/mL, and 0.25 ng/mL. The results indicated a faint color change until 2 ng/mL and no visible color change less than 1ng/mL (Figure 12).

Conclusions

At this point, we have a strip that can detect the presence of myoglobin in a buffer system to a concentration as low as 2 ng/mL. As part of our future work, we hope to try different sample pads to understand which type of sample pad will serve as a better filter for myoglobin in blood or plasma. We plan to use a sample pad that can efficiently filter out blood or plasma constituents to test the strip with blood with or without myoglobin. We also hope to quantify the color change that has occurred with a cell phone spectrometer or ImageJ.

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Table 1: Summary table of all conditions used in this lateral flow assay process.

Figure	Half strip or Full strip	Pretreatment of nitrocellulose	Pretreatment of conjugate pad	Volume of immobilized conjugated antibody on conjugate pad	Concentration of control line	Concentration of test line
4	Half strip – separate test and control lines	No	Yes	6 uL	1 g/L	1g/L
5	Half strip - separate test and control lines	No	Yes	6 uL	5 g/L and 0.5 g/L	5 g/L and 0.5 g/L
6	Half strip - separate test and control lines	Yes (1.5%, 0.75%, 0.375% BSA)	Yes	6 uL	0.5 g/L	0.5 g/L
7	Half strip – control and test lines on same strip	1.5 % BSA	Yes	6 uL	0.5 g/L	0.5 g/L
8	"half" strip – control and test line on same strip	1.5 % BSA	Yes	6 uL	0.5 g/L	0.5 g/L
9	"half" strip – control and test lines on the same strip	1.5 % BSA	Yes	12 uL 15 uL 18 uL 21 uL	0.5 g/L	0.25 g/L
10	"half" strip – control and test lines on the same strip	1.5 % BSA	Yes	12 uL	0.5 g/L	0.125 g/L
11	"half" strip – control and test lines on the same strip	1.5 % BSA	Yes	12 uL	0.5 g/L	0.25 g/L
12	Full strip – control and test lines on the same strip	1.5 % BSA	Yes	9 uL	0.1 g/L	0.5 g/L