

Predicting a heart attack: Developing a blood test for cardiovascular disease

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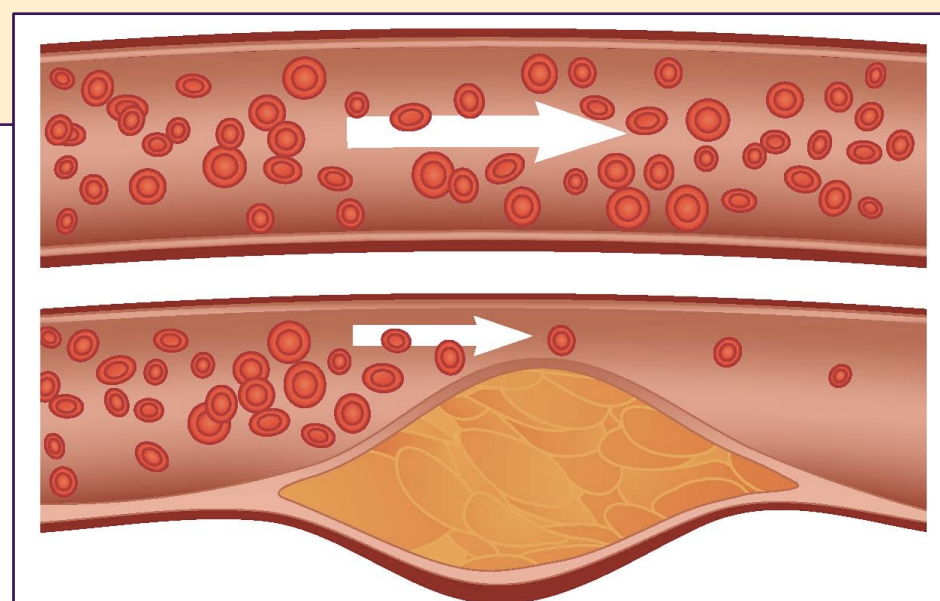


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What if we could develop a non-invasive test to predict a heart attack?

Introduction

- Over 1/3 of Canadian adults will develop heart disease in their lifetime
- There are few non-invasive tests to predict who will have a heart attack
- It is difficult to predict who will have a heart attack by looking at risk factors alone
- RIP3 and MLKL are proteins involved in inflammation, and high levels are found in the vessel wall of coronary arteries with atherosclerotic disease (CAD)
- These two proteins drive necroptosis, a type of pro-inflammatory cell death that results in the release of intracellular content in the necrotic core of atherosclerotic arteries
- Thus, this study hypothesized that high blood serum concentrations of RIP3 and MLKL could serve as markers for necroptosis that would positively correlate with CAD
- The objective was to develop a blood test to identify levels of RIP3 and MLKL using ELISAs in hopes of finding an inflammatory marker to measure and better predict CAD



Methods

Day 1

- 1) Coat plate with 100 μ L capture antibody (Ab) overnight at 4°C

Day 2

- 2) Block with 200 μ L 1% BSA for 1 hour at room temperature
- 3) Add 100 μ L serum, then store at 4°C overnight

Day 3

- 4) Add 100 μ L detection Ab and incubate for 2 hours at room temperature
- 5) Add 100 μ L Streptavidin-HRP and incubate for 30 minutes at room temperature, covered in foil
- 6) Add 100 μ L colour reagent and wait for colour change (~20-30 minutes)
- 7) Stop reaction with 50 μ L stop solution
- 8) Read plate at 450 nm

***Note:** the plate was washed with 200 μ L TBST-0.1% 4x between each step

Results

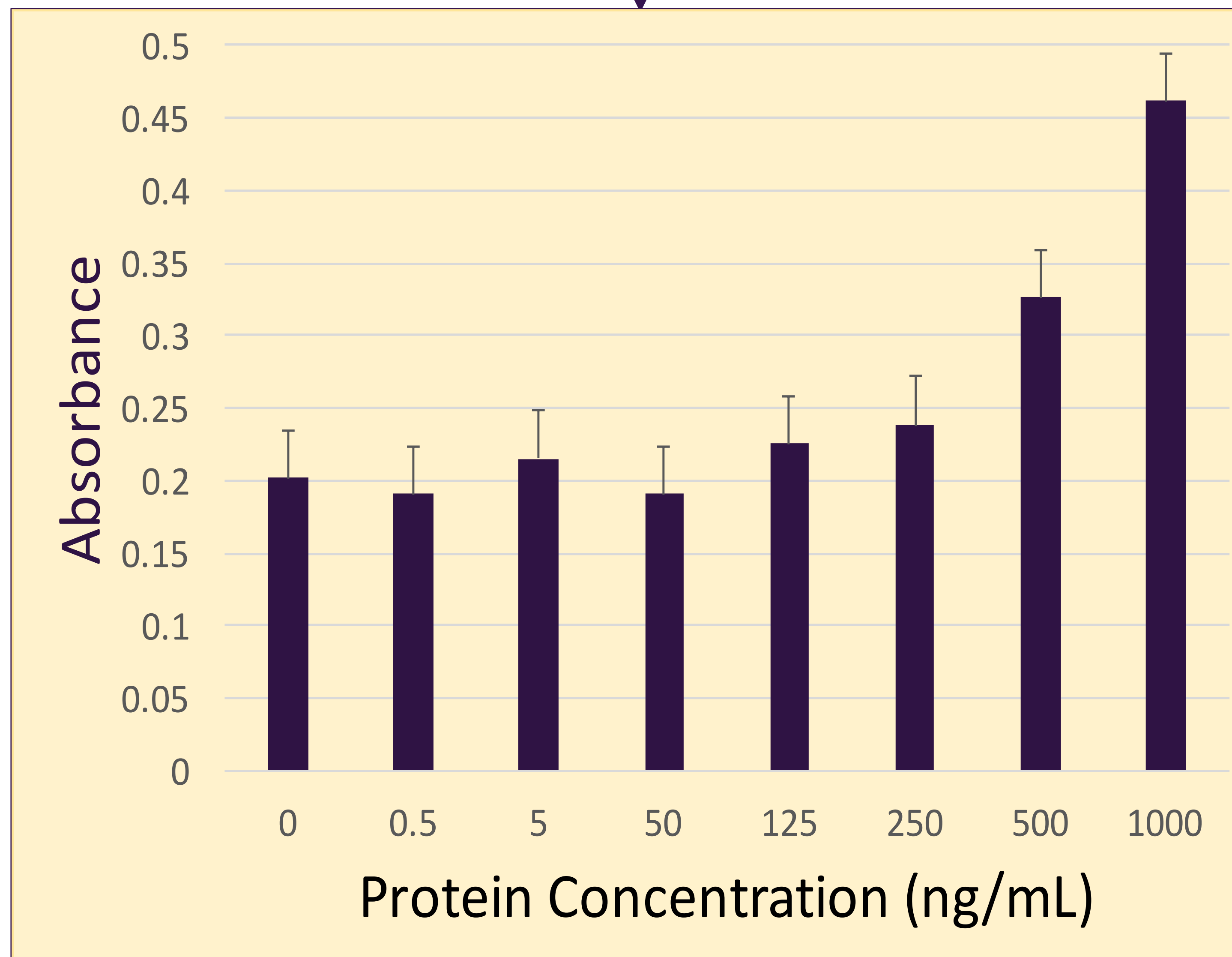
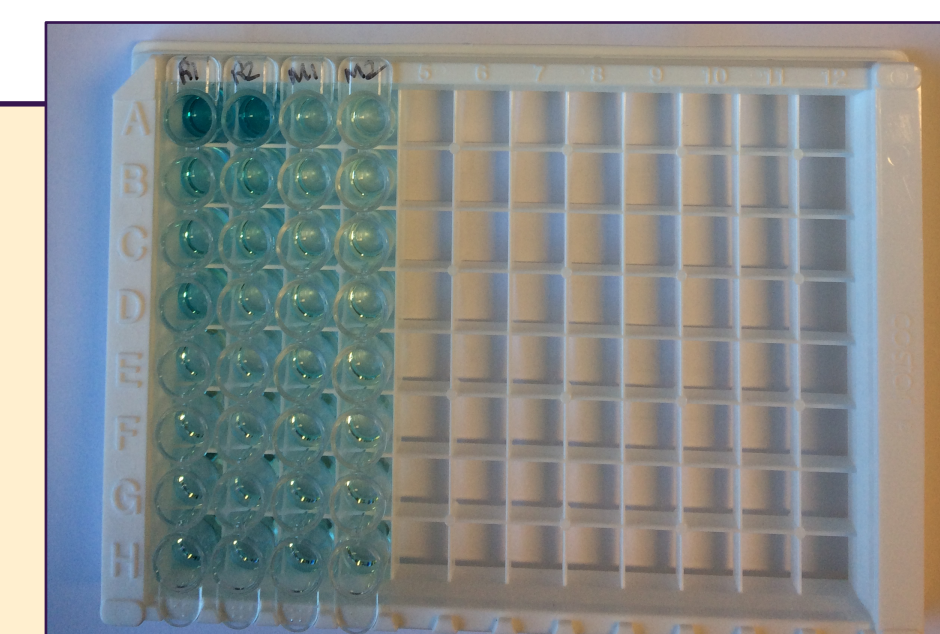


Figure 1. Standard Curve for RIP3 Recombinant Protein. Graphical analysis of the beginnings of a standard curve for RIP3 protein concentration in the RIP3 ELISA assay. The standard curve begins to form at around 250 ng/mL. Each bar represents the average duplicate absorbance of each concentration value. Error represents one standard deviation of the results.

- Five rounds of optimization were performed for the MLKL and RIP3 proteins
- One consistent problem was high background, caused by random binding
- For the second optimization, Ab concentrations were increased to increase detection, to no effect
- To reduce background in the third optimization, the concentration of the blocking agent, BSA, was increased to 10%, and 1% skim milk was used as well, to no effect. Ab concentrations were returned to their original values
- For the fourth optimization, a new capture Ab was used (same brand, but newer bottle) and fresh aliquots of recombinant protein were used to reduce background and increase detection
- The fourth optimization provided promising values for the protein standard curve. In the fifth optimization, the same protocol was followed with higher protein concentrations in an attempt to increase the amount of protein on the curve
- The fifth optimization produced the beginnings of a standard RIP3 protein concentration curve, displayed in **Figure 1**. Lower concentration values, between 0-125 ng/mL, demonstrated similar absorbance values. A steady increase in absorbance values began to develop with concentrations of 250 ng/mL and up



Recombinant Protein (ng/mL)		Samples	Recombinant Protein (ng/mL)		Samples	
0.238	0.222	0.232	0.496	0.427	0.299	+ Serum (1:3)
0.206	0.201	0.209	0.311	0.341	0.295	
0.194	0.189	0.165	0.229	0.248	0.191	+ Serum (1:10)
0.206	0.197	0.163	0.231	0.220	0.185	
0.205	0.196	0.202	0.191	0.190	0.201	Buffy Coat (1:1000)
0.186	0.185	0.195	0.195	0.236	0.211	
0.215	0.195	0.175	0.191	0.191	0.200	Lysates (1:2000)
0.235	0.231	0.178	0.206	0.198	0.206	

Figure 2. Results of RIP3 ELISA Optimization #5. The effectiveness of both the old and new hRIP3 capture antibodies (represented in the left three and right three columns, respectively) was compared. A serial dilution of RIP3 recombinant protein was performed to build a standard curve for protein detection.

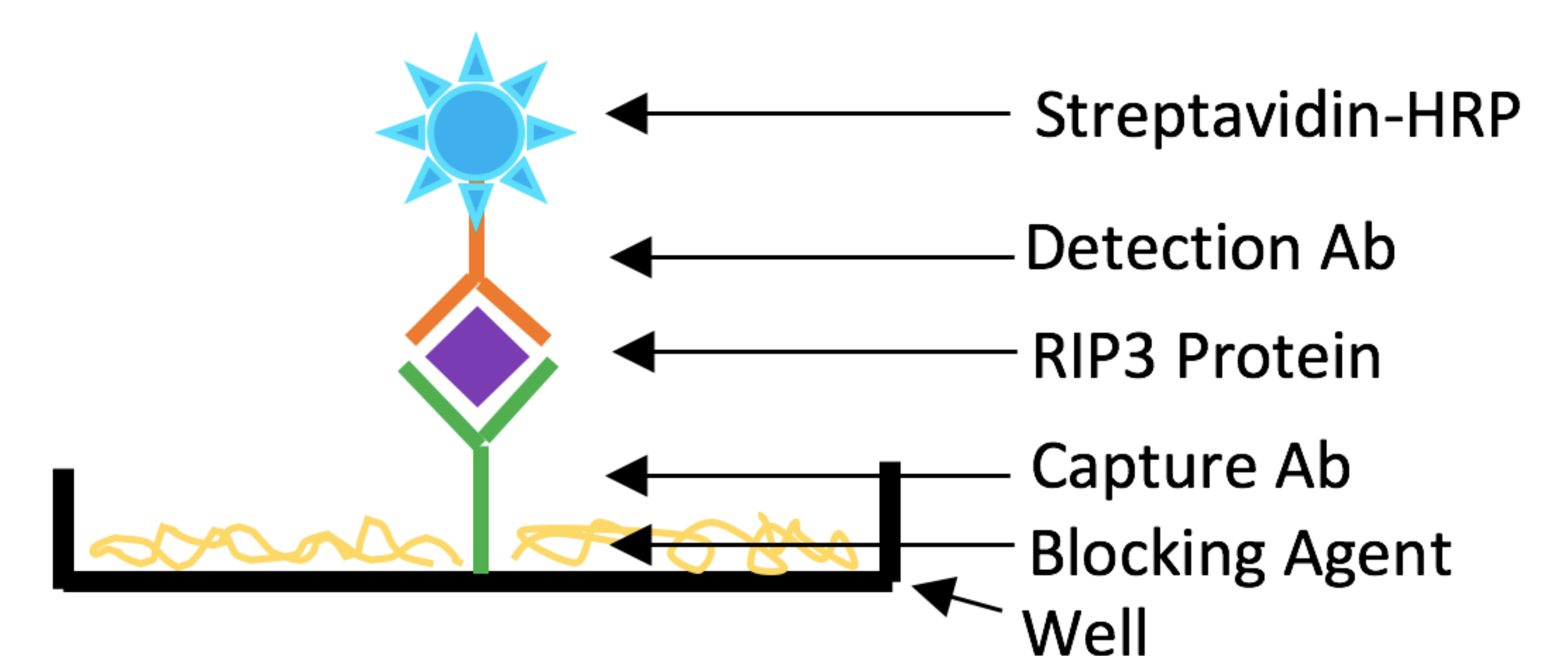


Figure 3. Graphic representation of the antibody "sandwich" formed to capture and detect RIP3 in the ELISA.

Conclusions

- Protein concentrations will continue to be raised to increase the amount of protein on the standard curve
- Better ELISA antibodies need to be developed for the capture and detection of RIP3 and MLKL
- Protocol will be further optimized to reduce background
- Both the RIP3 and MLKL ELISAs must be further optimized before the hypothesized link between RIP3, MLKL and CAD can be adequately supported

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