Using Fluorescent Proteins as Biosensors for *Escherichia coli*

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Abstract

During this ASURE research lab, we use biosensors to detect if disulfide bonds are being formed in the periplasm of *Escherichia coli*. Although red fluorescent proteins have already been proven to have high levels of fluorescence, this study focuses on optimizing mKeima as a biosensor by increasing fluorescence intensity and improving sensitivity in the bacterial periplasm where disulfide bonds are formed.

Introduction

Biosensors are devices that produce signals that can indicate the occurrence of a reaction or the presence of organisms in a biological system. Biosensors were created with the ability to detect biological substances in living organisms to allow for further research into molecular physiology (Wang, M. et al. 2023). The signals that biosensors give off are relative to the concentration of the analyte in question in the system, and they can be used to detect diseases or mutations in cells. As such, they can be utilized to prevent diseases from spreading throughout the body and to create and develop new medications (Bhalla, N. et al. 2016).

Fluorescent proteins are proteins that can emit light when they are in an excited state. They can be used as biosensors that are genetically encoded to produce a signal that an experiment is targeting. Fluorescent proteins can be genetically engineered to respond to target molecules in a system that a researcher is trying to detect (Wang, M. et al. 2023).

Background

Leland Clark created the first successful biosensor, which was used for detecting oxygen, in 1956. Further research in similar fields led to the first commercially available biosensor, and since then, the development of new biosensors has grown exponentially (Bhalla, N. et al. 2016). The research in this lab focuses specifically on disulfide biosensors. In the periplasm of *Escherichia coli*, disulfide bonds can form in the presence of cysteines in a given protein, and this is catalyzed by enzymes. These bonds stabilize the structure and can increase the pathogenesis of *E. coli* (Dyotima et al. 2024). The first redox biosensor was the ß-Galactosidase (LacZ) sensor, which can only be active when the formation of disulfide bonds is inhibited. When the enzyme that catalyzes the bonds is not present, the disulfide bonds do not form and the bacteria can turn blue in the presence of the chromogenic indicator XGal. Since this is a redox reaction, the LacZ can be either oxidized or reduced. In the reduced state, the LacZ is active, making the disulfide bonds break and resulting in a blue color. In its oxidized state, the disulfide bonds form and the LacZ becomes inactive and cannot produce the blue color. Even though this was a useful biosensor, there are limitations in its visual readout because the fact that it turns blue is not enough to quantify the measurement in the visible spectrum. The purpose of this research is to use a fluorescent protein instead of LacZ and then create mutations in the fluorescent protein that will cause an increased output of fluorescence, making it a better

biosensor. This method can be quantifiable by measuring the fluorescence with a spectrophotometer.

Preliminary Studies

The first fluorescent protein to be discovered was found in a jellyfish with a bioluminescent protein known as aequorin. Since this green fluorescent protein was identified, many other colors of fluorescent proteins have been discovered or generated. The fluorescent protein I will be working with this semester is mKeima, which is a red fluorescent protein variant from the stony coral Montipora (https://www.fpbase.org).

Fluorescent proteins have features that play a key role in their fluorescence. These proteins have ß-barrel structures that occur in the cytoplasm and periplasm, and they can fluoresce in the periplasm if they are folded in the proper structure. I hypothesize that when disulfides are present, the ß-barrels cannot form the arrangement necessary for the structure to be formed in the conformation that allows fluorescence. Thus, no fluorescence will be observed when the protein is disulfide bonded or oxidized, while the protein will fluoresce in a reduced state. This will be tested in the bacterial periplasm since no disulfide bonds form in *E. coli*'s cytoplasm.

Cysteine is an amino acid that is found in protein chains and forms disulfide bonds. The mKeima fluorescent protein has four cysteines in its sequence, which is necessary for this research because cysteine residues have to be in the protein's sequence in order for the disulfide bonds to form. The inhibition of disulfide bond formation can decrease *E. coli*'s ability to cause disease in the body, so the detection of these bonds in the early stages of pathogenesis can prevent *E. coli* from becoming virulent and help researchers find novel treatments to cure bacterial infection. Over the course of our research, the fluorescent protein mKeima will be used to synthesize an improved biosensor.

Research Methodology

In order to generate and improve upon our fluorescent protein, we have to clone the biosensor with mutations that can increase the intensity of the fluorescence. To clone the biosensor, we began by performing a polymerase chain reaction (PCR) that was meant to amplify the number of copies of template DNA. To verify that the PCR generated the intended product, we used agarose gel electrophoresis. In this step, there were other unwanted products that were detected with the electrophoresis. We had to use PCR purification to remove the unwanted products (salts and enzymes) from the DNA sample. This purification process involves DpnI-digestion, which removes the DNA template from the sample, and column purification, which removes any other impurities. Right after the purification, we verified that we did not lose the DNA while removing the impurities by measuring the concentration of DNA with a nanodrop. Then, we ligated the mKeima protein fragments into the plasmid, which joins the pieces of DNA together. The product of ligation is used for bacterial transformation. We synthetically transformed the product through electroporation, in which we shocked it to create pores in the cell membrane so that the bacteria can be placed inside the cell. We had to add LB media to the solution and place it in an incubator so that the cells could recover after the pulse and the membrane could seal around the bacteria. Finally, we used miniprep for plasmid preparation to

separate the plasmid DNA from all the other elements of the bacterial cell. In our next steps, we will verify that our biosensor is working as predicted by comparing our wildtype and mutant to a control that will fluoresce in any condition. In our independent research, we proceeded to measure the fluorescence of our biosensor and then used directed evolution to improve its fluorescence under reduced conditions.

After ligating the insert DNA into the biosensor plasmid and transforming it into electrocompetent cells, we performed PCR purification and sequenced the DNA to verify that it matched the sequence of our fluorescent protein. We analyzed the sequence data using SnapGene. After the successful transformation of our bacterial product, we prepared our first fluorescence assay by growing overnight cultures of the three strains: the control, the wildtype (oxidized, with disulfide bonds), and the mutant (reduced, without disulfide bonds). We measured the OD600 of each culture with the spectrophotometer to standardize cell density and determine the volume that would be transferred to fresh media before adding CUMA, or the inducer. This protocol was meant to induce fluorescence in the mutant strain, which was in the reduced state. We optimized the fluorescence assay by repeating it four times with different media and CUMA concentrations. We also performed another round of random mutagenesis with PCR followed by gel electrophoresis to achieve more mutations with higher fluorescence than the first round. After performing a ligation reaction, we transformed the resulting plasmid into HK317 (a strain of *E. coli*) electrocompetent cells with electroporation. In the next transformation that we performed, we used DH10ß cells and heat shock for the ligation instead of HK317 and electroporation.

After our final round of mutagenesis, we tested our mutants by growing overnight cultures in a 96-well plate. In each well, we had individual colonies from the transformation, the original wildtype, mutant, and control from our first transformation, and a blank with just media. After an incubation period, we calculated the volume of each culture we would need to add for the inducer to be effective at the ideal concentration that we found from the fluorescence assays. The DH10ß cells that we used for this transformation had the Dsb gene instead of being a Dsb mutant like HK317. To mimic the reduced environment of the HK317 strain, we modified the DH10ß cells by inactivating the protein with an inhibitor of the disulfide bonds. This would allow us to test for fluorescence in the reduced environment without the formation of disulfide bonds. We then compared the values of fluorescence with and without the inhibitor in two different 96-well plates, both with the same colonies in the corresponding wells. We scanned for fluorescence and absorbance for both plates with the Gen5 program, and for the fluorescence we used the excitation and emission we found during the bootcamp. Finally, we analyzed the data to identify the colonies with the highest normalized fluorescence in the plate with the inhibitor and compared them to the wells with the same colonies in the plate without the inhibitor to determine if our mutagenesis was successful at increasing the fluorescence capacity of the protein.

Results

In the final steps of the bootcamp, we performed our first transformation and successfully grew hundreds of colonies on agar plates containing antibiotic. Significantly more colonies grew on

the reaction plate with ligated DNA than the control plate without ligated DNA. This means our ligation reaction was successful and the plasmid DNA entered the cells and passed on antibiotic resistance. When we sequenced the DNA in SnapGene, the chromatogram appeared to show a population mixture because there were many mutations throughout the sequence and several positions with more than one peak (Figure 1.). This was likely due to accidentally picking up more than one colony during plasmid preparation, resulting in a mixed culture that included multiple variants of the plasmid. Because my clone had deviations from the known sequence of mKeima, we used Olivia's transformation product, which had fewer mutations near the plasmid backbone and the primer binding sites.

Fluorescence Assays: Wildtype vs Mutant Biosensor

In our first fluorescence assay, we expected the control to produce the highest fluorescence with increasing concentrations of CUMA because of the ability to fluoresce independently of redox sensing. We also expected the wildtype biosensor to show little to no fluorescence and the mutant biosensor to have slightly higher fluorescence than the wildtype. However, the wildtype biosensor had the highest fluorescence and the fluorescence stayed relatively constant across the CUMA concentrations (Figure 2.).

To address these unexpected results, we repeated the assay with a new 96-well plate and fresh overnight cultures. In this second trial, we increased the starting concentration of CUMA to 1000 μM to broaden the range of fluorescence and compared two types of media (LB and minimal media) to determine if the fluorescence of the LB itself interferes with the fluorescence readings. Again, the results did not align with our expectations. In LB, the mutant biosensor was higher in fluorescence than the wildtype biosensor but still less than the wildtype control (Figure 3). In minimal media, the wildtype control had the highest fluorescence, and the wildtype biosensor again produced more fluorescence than the mutant biosensor (Figure 4). These results suggested that minimal media may be better for the assay than LB and that we still had not reached the ideal concentration of the inducer.

For our third assay, we used only minimal media and raised the starting concentration of CUMA to 2000 μM. The level of fluorescence remained consistent through most of the serial dilution but began to decline at the highest concentrations (Figure 5.). This suggests that we had surpassed the optimal concentration of the inducer, possibly inactivating the protein. Our fourth and final fluorescence assay showed consistent results despite contradicting our original hypothesis. The wildtype biosensor still produced the highest fluorescence (Figure 6.), so we concluded that mKeima may inherently fluoresce under oxidizing conditions and its fluorescence is not limited to the reduced state. It is also possible that mKeima is capable of proper folding and fluorescence even in the presence of disulfide bonds, allowing it to remain functional in the oxidized state. On the other hand, it may misfold or become less stable in the reduced state, which would explain the lower fluorescence expressed by the mutant biosensor.

Directed Evolution: Mutants with Improved Fluorescence

In order to improve our biosensor's performance, enhance the fluorescence in the reduced state, and improve its sensitivity to redox conditions, we performed random mutagenesis using

PCR to introduce mutations into the mKeima sequence. Gel electrophoresis confirmed successful amplification. After ligating the mutated inserts into the plasmid backbone, we initially conducted a transformation into HK317 electrocompetent cells with electroporation. However, this trial produced false positive colonies, and the transformation was complicated by a slimy pellet that made it difficult to remove the supernatant. Due to these challenges, we repeated the ligation and performed a transformation into DH10ß chemically competent cells with heat shock. For this trial, we successfully grew hundreds of colonies, allowing us to screen for improved fluorescence in individual mutants.

To screen for improved fluorescence, we prepared overnight cultures in each well of a 96-well plate. The first three wells contained the original wildtype, mutant, and control strains, and each remaining well was inoculated with an individual colony from our transformation. After an incubation period, we added a disulfide bond inhibitor to one plate to create a reduced environment. We expected high fluorescence in the presence of the inhibitor and low fluorescence without it. We scanned both plates for fluorescence and absorbance to compare the normalized fluorescence of each colony in reduced versus oxidized conditions. We analyzed the data and identified three colonies that displayed significantly higher fluorescence with the inhibitor than without, indicating improved sensitivity to the reduced state (Figure 7.). These levels of fluorescence were also higher than the original wildtype and mutant biosensors, indicating that our directed evolution successfully enhanced the fluorescence of mKeima in the reduced state.

Conclusion

Redox-sensitive biosensors like mKeima are valuable tools in molecular biology because they allow researchers to distinguish between oxidized and reduced cellular states. The goal of this research is to design a redox fluorescent biosensor using the fluorescent protein mKeima. Although we expected the mutant biosensor to express higher fluorescence in the reduced state and the wildtype to express very little fluorescence, our results suggest that mKeima maintains fluorescence even in the oxidized state. To improve the biosensor's responsiveness, we used random mutagenesis and directed evolution. When screening for fluorescence, we identified three mutant colonies that had higher fluorescence in the reduced state and higher fluorescence than the original wildtype and mutant strains. This is likely because the mutations introduced to these colonies improved redox sensitivity and minimized protein misfolding.

To continue developing our understanding of fluorescent proteins as biosensors for redox environments, we should sequence these mutants with the highest fluorescence to identify specific mutations that may have led to enhanced fluorescence. This could determine how the structure and chemical composition of mKeima influences the behavior as a redox biosensor. This improved sensitivity would allow researchers to screen for new drugs that can target redox-regulated processes and identify when bacteria are less virulent. Further optimization of the biosensor will make this a valuable tool both in research and clinical practice.

References

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Appendix

Figure 1.

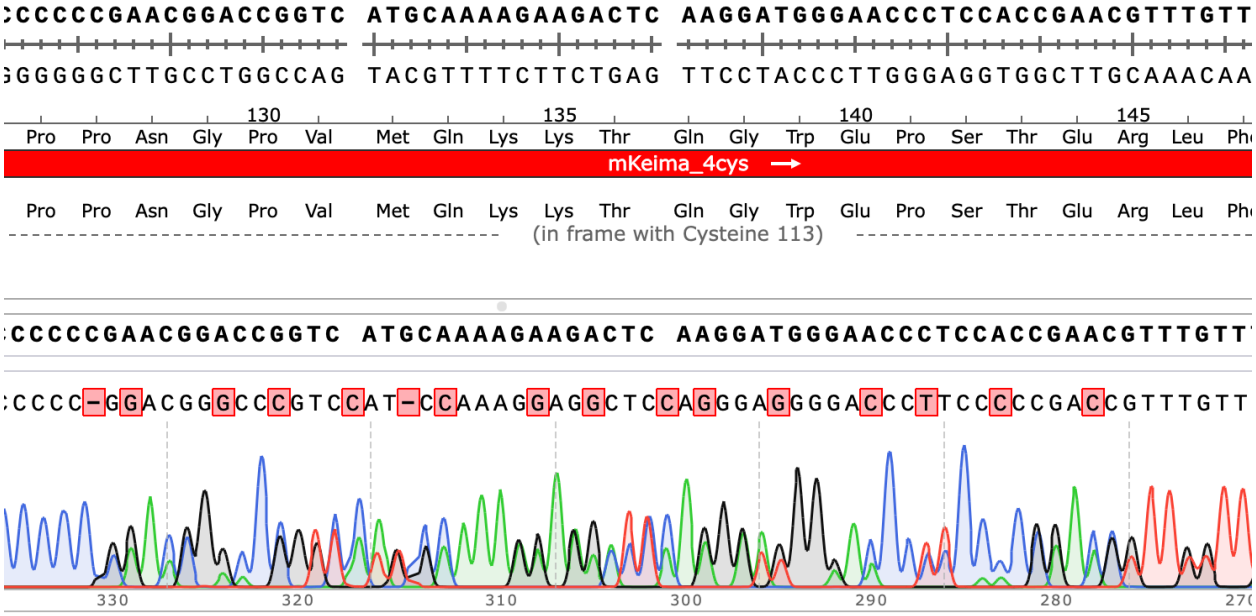

Figure 2.

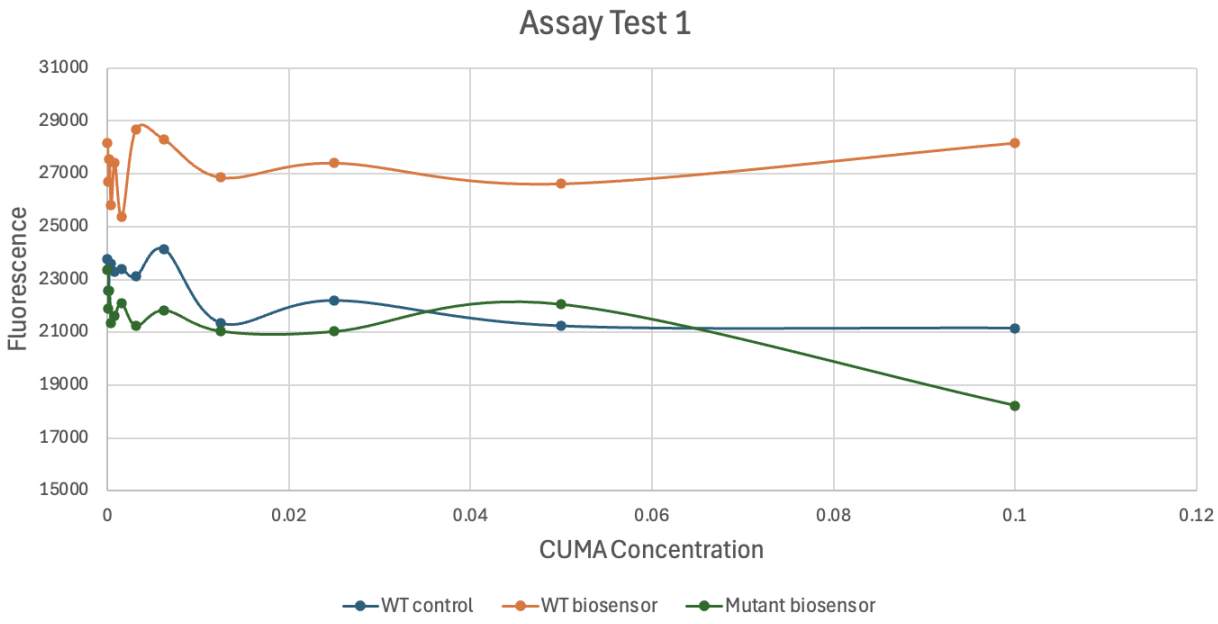

Figure 3.

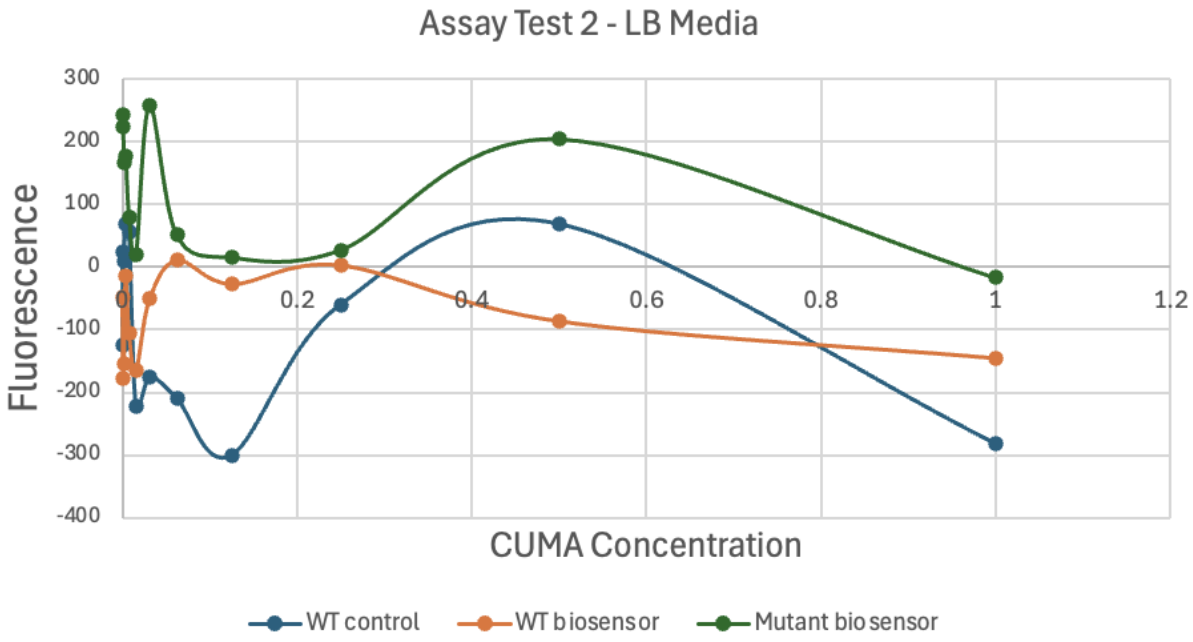

Figure 4.

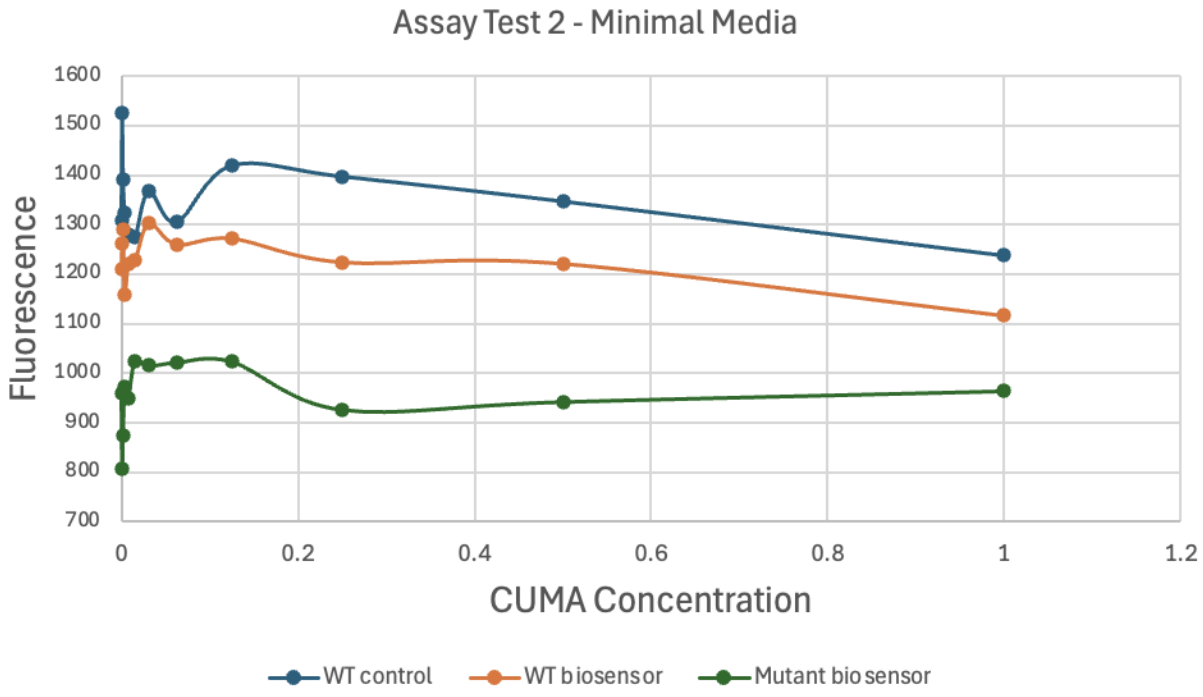

Figure 5.

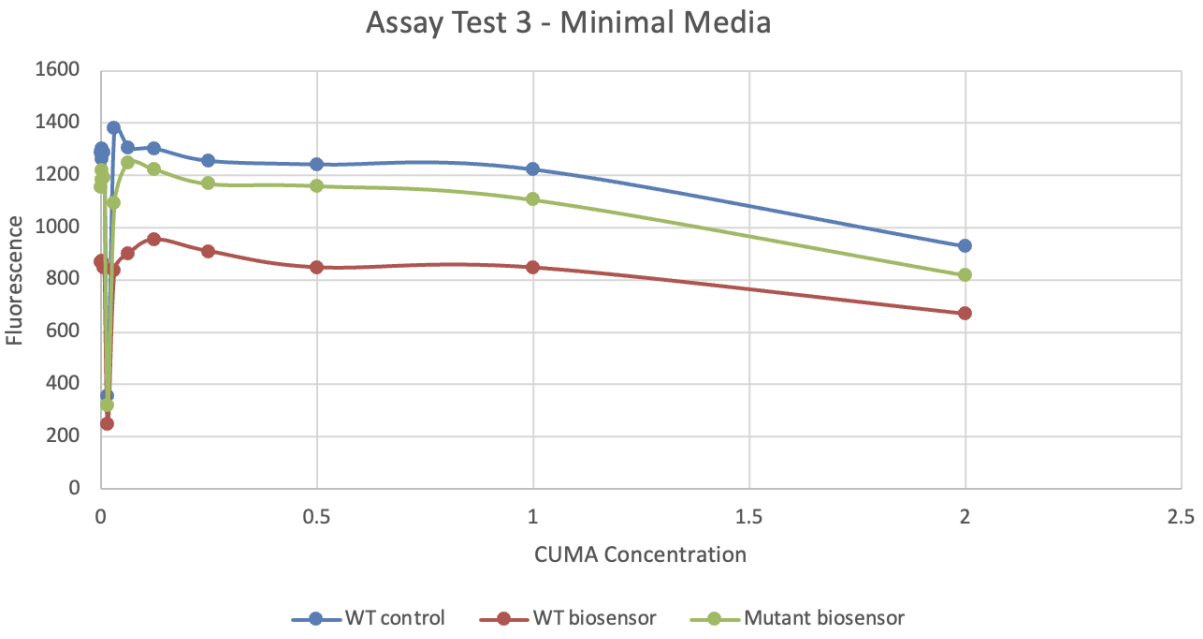

Figure 6.

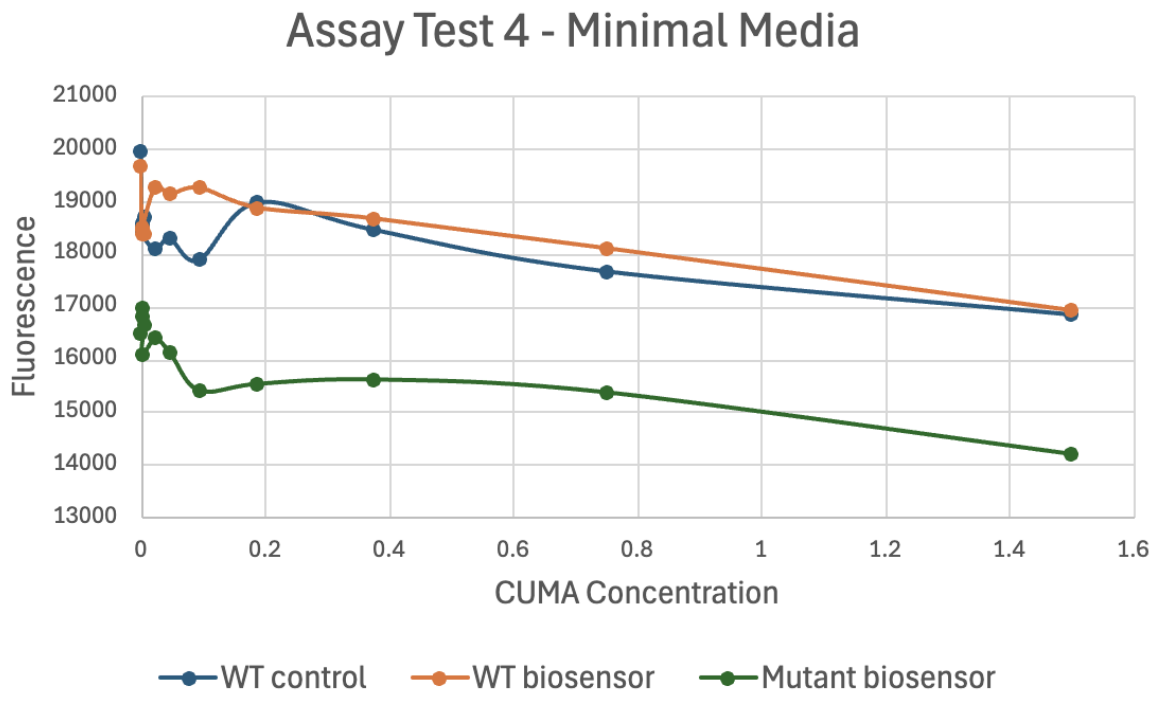

Figure 7.