Title: Exploring the safety of bioreactive AHLs- an investigative look at current standards and future directions

ASU iGEM 2016

Introduction

Here we address the safety and proper disposal of acyl-homoserine lactones (AHLs). AHLs are a signaling molecule commonly utilized in a bacterial communication method known as quorum sensing (QS), first observed in *Vibrio fischeri* (1). QS allow bacteria to detect the concentration of their species-specific signaling molecule. When in high enough concentration, AHLs can induce a concerted response across a population. In nature, AHLs are produced by a synthase protein. When these AHLs are in high enough concentration, they can then bind to a receiver, which then binds to a specific promoter and initiates transcription of genes relevant to group behaviors, such as biofilm formation, bioluminescence, sporulation, competence and virulence (2). QS is also commonly used synthetic biology tool, as it allows cells to communicate directly across space. AHLs can also be chemically synthesized and used as inducer compounds for cells containing QS receiver proteins. Due to both the use of AHLs in the activation of virulence pathways and their use in synthetic systems, we investigated safe disposal methods for AHLs. As synthetic biology increases in scope and scale, so will the use of these QS pathways. This calls for the need to address possible safety concerns that might arise from the utilization of molecules that are known to induce pathogenesis, disrupt signaling in important environmental systems, and are key in biofilm formation.

Why is this important?

The primary concern over AHL disposal is the possible risk of activation of pathogens and soil and plant microbes. Over 100 species of bacteria are known to possess LuxIR homologues, and many of them utilize QS to regulate virulence and pathogenicity (3). For example, the well studied Las and Rhl QS systems in the opportunistic pathogen *Pseudomonas aeruginosa*, which causes chronic lung infection in cystic fibrosis patients (4). The *P. aeruginosa* QS network utilizes two LuxIR homologues, LasIR and RhlIR. It is suggested that both QS systems play a significant role in all stages of infection, and allow *P. Aeruginosa* to regulate the production of toxins (2). Additionally, both QS systems are commonly used in synthetic biology (6). Not only is QS used by human pathogens, but it has also been shown that QS plays a role in a bleaching disease in coral and is involved with many plant-pathogens (4, 5). In many cases, the QS system activates a concerted pathogenic response when a host weakness is detected (7). While QS is not innately harmful, these few examples suggest QS plays a central role in pathogenic activity, and has the potential for harm if used inappropriately in engineered systems, particularly is AHLs or their synthases are released into the environment.

Some QS systems display crosstalk or the ability to activate other systems. This presents an issue as AHLs from non-pathogenic bacteria may have the potential to active

pathogenic receivers(3). Cross-talk then poses problems for the inclusion of QS networks in therapeutics, as even non-pathogenic QS AHLs may still activate pathogenic receivers, and thus activate virulence factors. Additionally, while many pathogens do not produce AHLs, they still retain QS receivers (1). Many pathogens can 'eavesdrop' on AHLs signals from other species, despite not having a complete QS network. Both the widespread use of QS across many species of pathogenic bacteria, and the possibility of cross-talk and eavesdropping, suggests the need for careful use and proper disposal of the QS activation molecules, AHLs.

Who uses QS networks?

QS is a widely studied and utilized system in synthetic biology, and has the potential to be used for many applications. QS can act as a "bio-wire" in complex biological circuits. It is possible to perform computation, and coordinate cell response using QS (3). As an example, Tabor et al. (2009) created an edge detection system using the LuxI/R system. Bacterial cells placed in the dark produced AHLs, but no signal response. The system would express LacZ, a pigment, only when the cells were exposed to both light and AHLs. This computation is possible because of a unique design, and the capabilities of the LuxI/R system.

The simplicity of QS systems is part of the great popularity of their use. At least 11 teams from the 2015 iGEM competition utilized QS to develop their projects. These systems are used not only at iGEM but across research in academia and industry. Synchronized gene expression controlled by QS across a population increases product synthesis (9, 10, 11). QS also has the potential for therapeutics, coordinating bacterial responses to detect and disrupt cancer cell environments, or targeting other microbes (12). Greater quantities of AHL in laboratories could lead to more people coming into direct contact with AHLs. Expanded use in industrial applications without proper disposal methods could leave large quantities of AHLs in the water, possibly activating dangerous microbes in the environment. Placing AHLs inside a human being could activate latent diseases in patients if possible crosstalk with pathogens is not considered as a design parameter. All of this suggests the necessity of a shift in thought about AHLs, which have previously been considered non-toxic agents that do not require regulation or special disposal protocols. Because of their bioactivity and possible reactivity with environmental microbes, we decided to further explore the state of AHL regulation as it is now.

Where are we now?

We reached out to synthesis companies, professionals in the field, the iGEM safety committee, and chemical providers to evaluate how AHLs are currently regulated. After giving them background on the project, we asked whether they had considered the potential harm of DNA sequences that produce AHLs, and if they have any policies regulating these sequences. We received an interesting variety of responses from each company and individual contacted; however none mentioned any specific considerations for AHLs. We received the following specific information from IDT, one of the largest DNA synthesis companies:

Integrated DNA Technologies (IDT)'s screening process involves asking three questions:

- Could this sequence be harmful to our lab personnel who are making it?
    a. AHLs and AHL proteins do not fall into this category because they are safe by themselves.
- Would inserting these genes into a different species lead to a new highly pathogenic strain?
    b. QS genes do contribute to pathogenicity, but so will any promoter IDT synthesizes.
    c. QS is a common process in both pathogens and nonpathogenic related species.
    d. Because of these reasons, IDT concludes that QS genes, by themselves, cause major concern regarding their use to create super bugs.
- Could an accidental transfer to a different species lead to a highly dangerous pathogen?
    e. Again, AHLs do not fall under this category as they are well studied, and are not likely to result in super pathogens if transferred.

GeneWiz, another DNA synthesis company contacted, had a different screening process from IDT, but also did not include AHLs or QS genes. GeneWiz screens for toxins and agents from a list given to them by the FBI. If the sequence being synthesized is not listed, then they are able to synthesize it. They also suggested that it was the responsibility of the researcher to determine how safe their orders may be.

We also contacted Dr. Megan Palmer, the head of the iGEM biosafety committee. Dr. Palmer informed us that QS genes fall under a general category of “Dual Use Research of Concern (DURC)”. AHLs are not currently listed under formal US DURC policies (which is limited to a number of potentially pathogenic agents), but raising issues with safety committees and local safety experts for potential concern of AHLs is a first step to determining whether it should belong on such a list. Dr. Megan Palmer also connected us with the iGEM safety committee, which told us that, while under certain circumstances involving the correct combination of organisms AHLs could be classified as “dual-use research.” This suggests that AHL research provides potential benefits, and the dangers posed by them can be minimized by proper experimental design and execution.

Finally, the AHL safety data sheets of Sigma-Aldrich (a chief supplier of synthetic AHLs) were investigated in order to determine the safety information available from chemical companies. Of the six MSDS’s that we obtained (N-Dodecanoyl-L-homoserine lactone, N-Dodecanoyl-DL-homoserine lactone, *N*-(*p*-Coumaroyl)-L-homoserine lactone, *N*-(3-Oxotetradecanoyl)-L-homoserine lactone, N-Octanoyl-DL-homoserine lactone and N-Hexanoyl-L-homoserine lactone), none mentioned pathogenic activation as a potential biosafety concern. Five of the MSDS’s showed no data for ecological concerns, with N-Dodecanoyl-L-homoserine lactones being noted as a potential hazard in aquatic environments, but only as a toxic substance. Overall, these MSDS’s did not address the biological concerns around AHLs.

Are the standard protocols enough?

AHLs are not considered reagents, but as chemicals, often added to bacterial cultures. Because of this, AHLs are usually disposed of with the bacterial waste, and these disposal means are subject to Environmental Health and Safety (EH&S) protocols. There are two common methods of disposing *E. coli* waste, autoclaving and bleaching.

In regards to bleaching, S. A. Borchardt *et al.* (8), studied the interaction between oxidized halogens (bleach) and AHLs. It was discovered that bleach rapidly degraded synthetic 3-oxo AHLs, but did not affect non-3-oxo AHLs . This suggests that EH&S protocol of treating bacterial waste with bleach would be sufficient for some types of signaling molecules; however, while 3-oxo AHLs are the most commonly utilized AHLs in synthetic biology, many bacteria communicate with AHLs without the 3-oxo group. Additionally, AHLs may be produced or placed in bacterial media, and there is no evidence to suggest that AHLs will be degraded in a similar fashion under these different conditions. Standard EH&S-approved disposal methods may not be sufficient to prevent release of active-AHLs into the environment.

We conducted several experiments to explore whether bleach will continue to degrade 3-oxo-AHLs in bacterial waste. Figure 1-4 in the appendix demonstrate the induction of the LuxR producing *E. coli* strain with bleached AHLs. Figure 1 shows the LuxR Receiver (iGEM part F2620) induced with CerI media treated with various disposal methods.

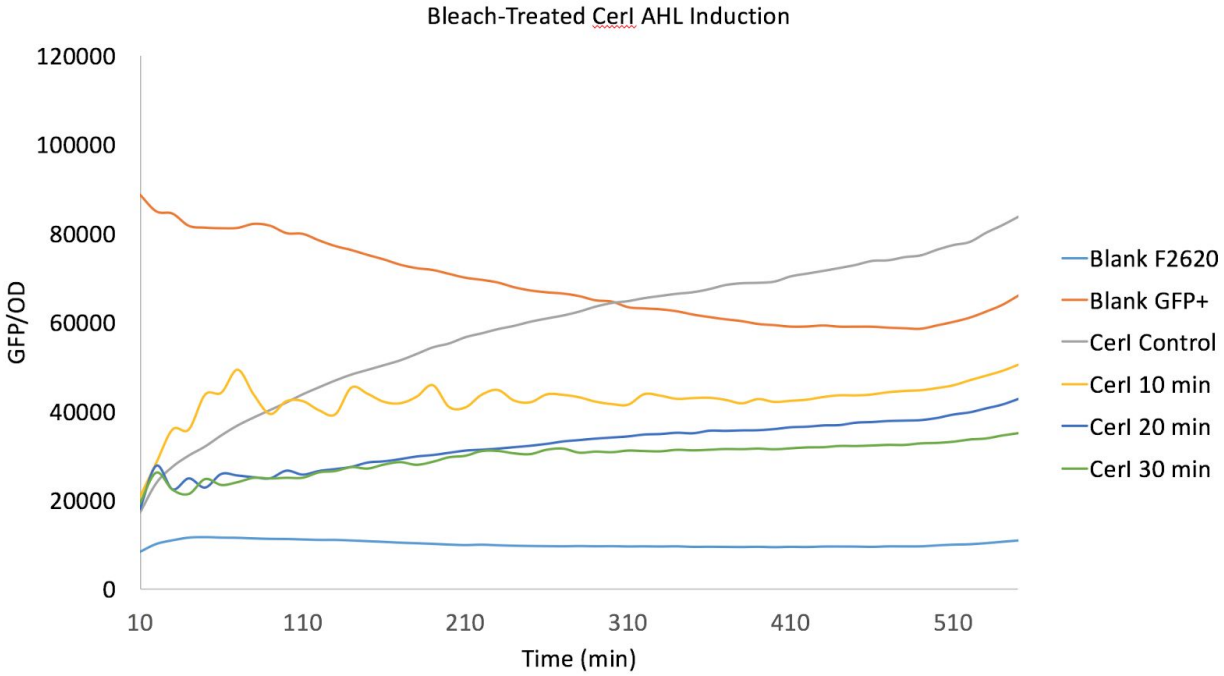

Figure 1 LuxR Receiver (iGEM part F2620) induced with CerI-media treated with various disposal methods.

Our results suggest a different conclusion concerning the degradation of AHLs. In general, bleach decreases the activity of both the LuxI AHL (3-oxohexanoyl-homoserine

lactone) and abolishes the activity of the LasI AHL (3-oxododeanoyl-homoserine lactone). The Borchardt paper suggested a rapid decrease in activity after only 2.5 minutes, however here a 3-oxo AHL retains its ability activate even after 30 minutes. Additionally, in accordance with the Borchardt paper, the non-3-oxo AHLs (RpaI and CerI) did not degrade after being exposed to bleach, as seen in Figure 3-4. Figure 5 shows induction of bacteria with AHLs in bleach, not extracted, and these still show induction. This suggests that 3-oxo AHL degradation is not as rapid in bacterial waste media, and that if the bleached liquid is poured down the drain containing AHLs, the bleach may become diluted, and AHLs may retain activity for a period of time.

Disposal Recommendations

To resolve the difficulty of continued activity of AHLs, another EH&S standard protocol, autoclaving, was attempted to determine whether it would be sufficient to degrade the AHLs. For each case depicted in Figure 6-10, the samples were autoclaved at 120°C for 15 minutes.

The results suggest a new method of properly disposing of AHLs, especially for non-3-oxo AHLs. Activity of all signaling molecules decreased after the autoclaving procedure. Although activity was still detected, the much lower activation is a promising sign. While most EH&S protocols require liquid waste to be bleached at 10% for 20 minutes, it might be necessary for the waste to also be autoclaved. This would simply mean autoclaving the liquid before the addition of bleach, to both properly dispose of the AHLs, and the the bacterial waste.

Discussion

The information presented here does not show evidence of exactly how dangerous AHLs are, or what effect they may have on the environment. However, AHLs are stable molecules, and are known to induce virulence factors in a wide variety of pathogens, which infect humans, plants and marine life. The increased use and production of AHLs could lead to exposure of much higher, more concentrated doses than commonly found in nature. Additionally, there are no regulations currently in place to control the purchase of AHL synthase genes. This potential for harm has not been properly considered, but should be studied before AHLs become more widespread and commonly utilized. There is no well-studied method for properly destroying AHLs. As already shown, both autoclaving and bleach standard protocols do not completely eliminate the activity of all AHLs. While this is pending further study, the results so far suggest a need for further research concerning the inactivation and disposal of AHLs.

As our project adds five new AHL synthases to the registry, we felt that it was vital to address these concerns as part of our project. In accordance with this, we are adding Safety Information sections to our parts pages. As we heard from Genewiz, the responsibility is currently on the user to understand the safety of the genetic parts they are ordering; however, as synthetic biology is a highly interdisciplinary field, we cannot be sure that all users of QS systems will

have the microbiology background to understand hazardous crosstalk potentials. We are using Safety Information sections in our AHL synthase parts pages to address this issue and create a culture of conscious usage around QS.

References

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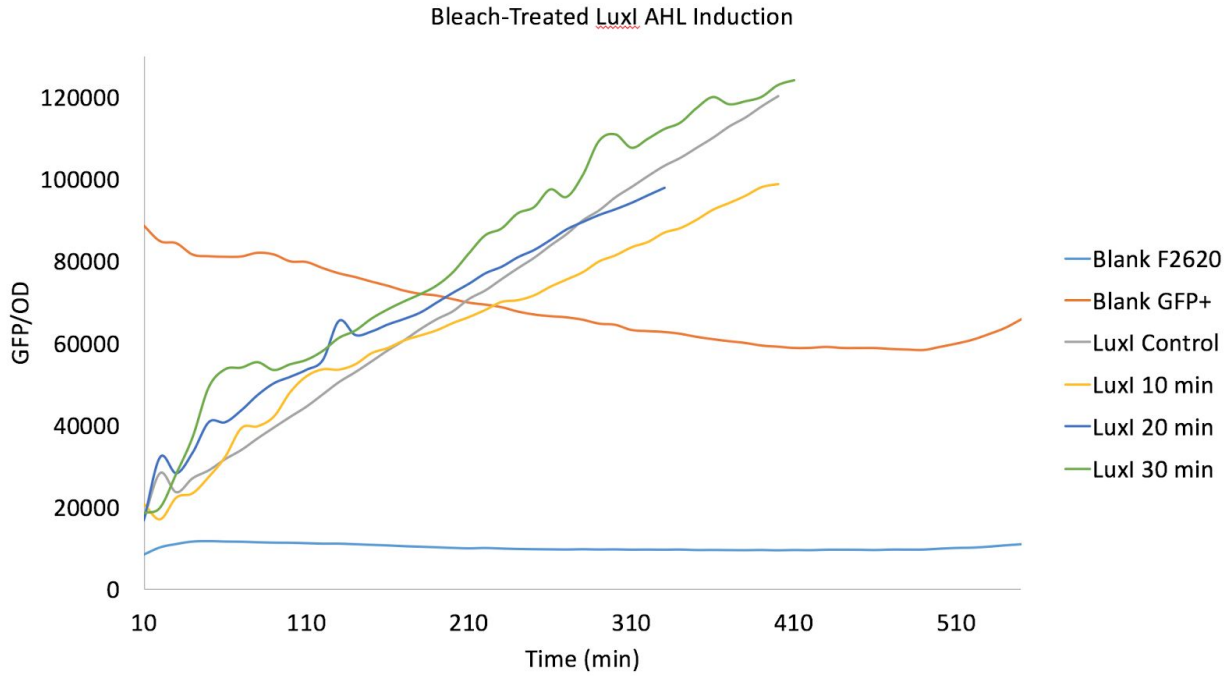
Appendix:

Figure 2:



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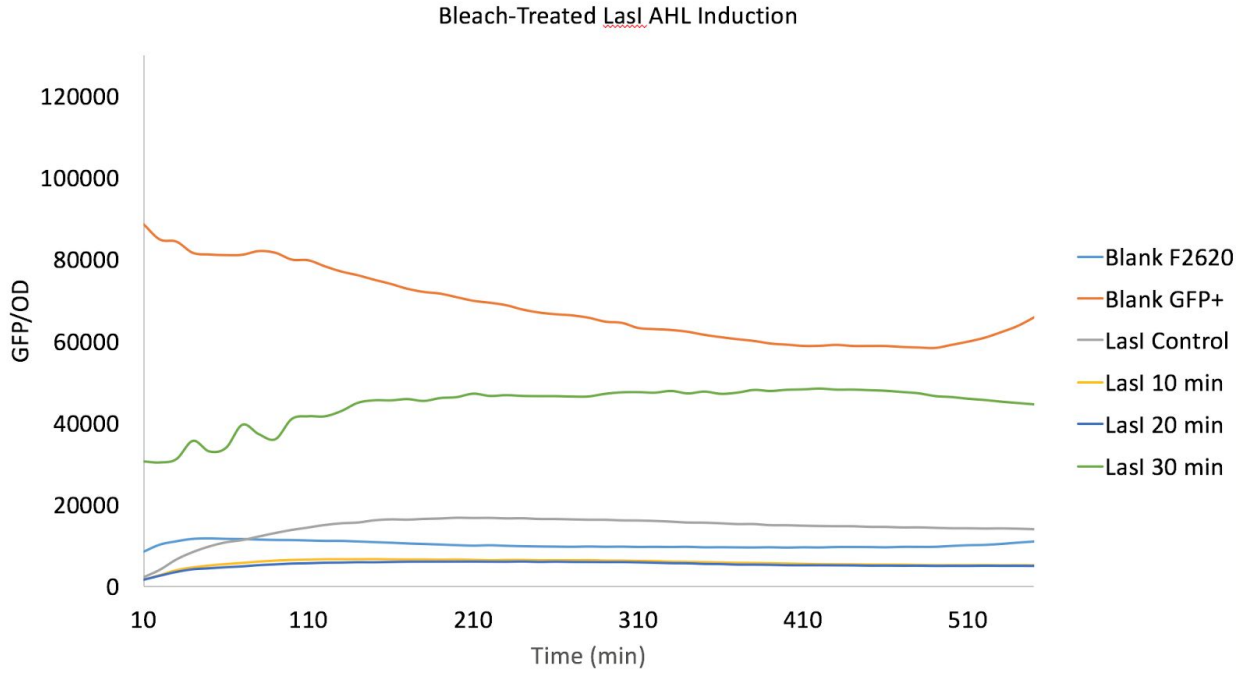

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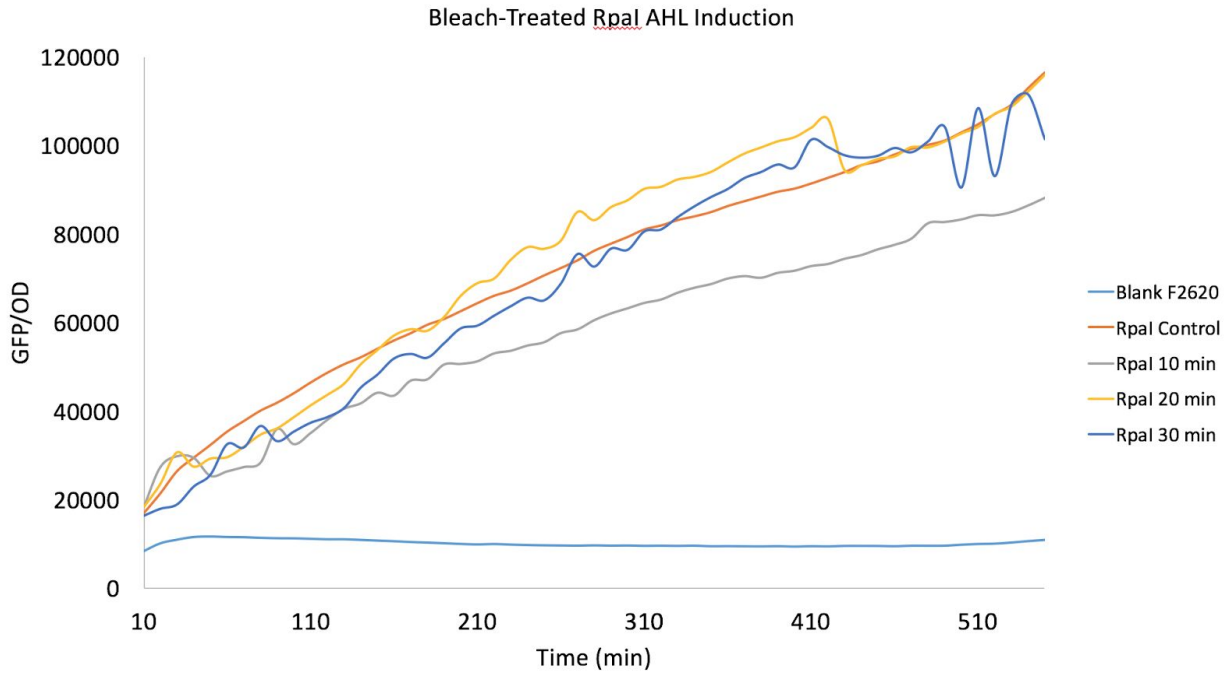


Figure 6

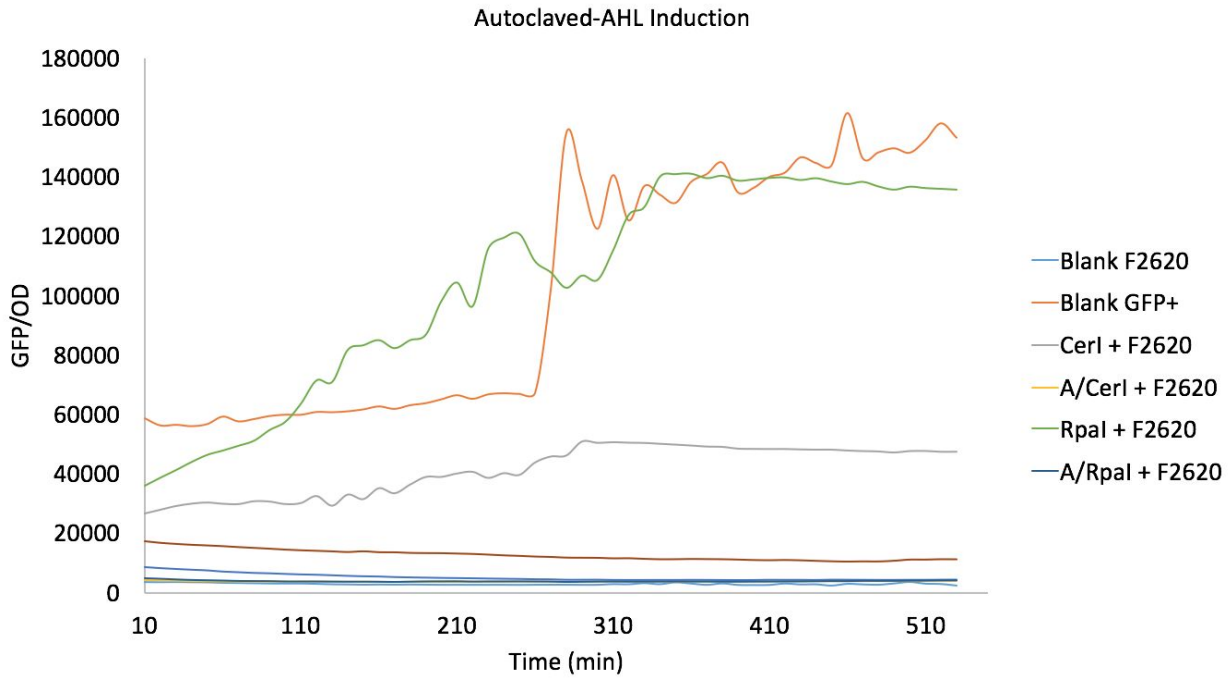
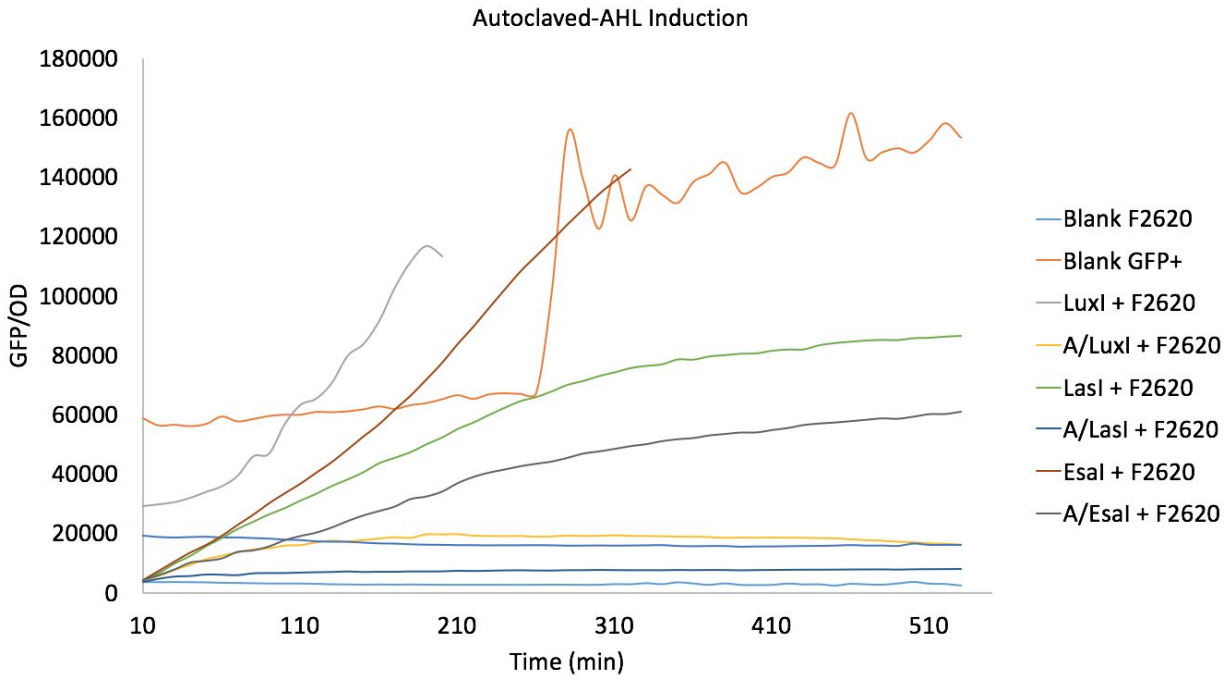


Figure 7


Autoclaved-AHL Induction
GFP/OD
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
10
110
210
310
410
510
Time (min)
Blank F2620
Blank GFP+
LuxI + F2620
A/LuxI + F2620
LasI + F2620
A/LasI + F2620
EsaI + F2620
A/EsaI + F2620