Design + Build + Test:

Wet Lab Design + Build:

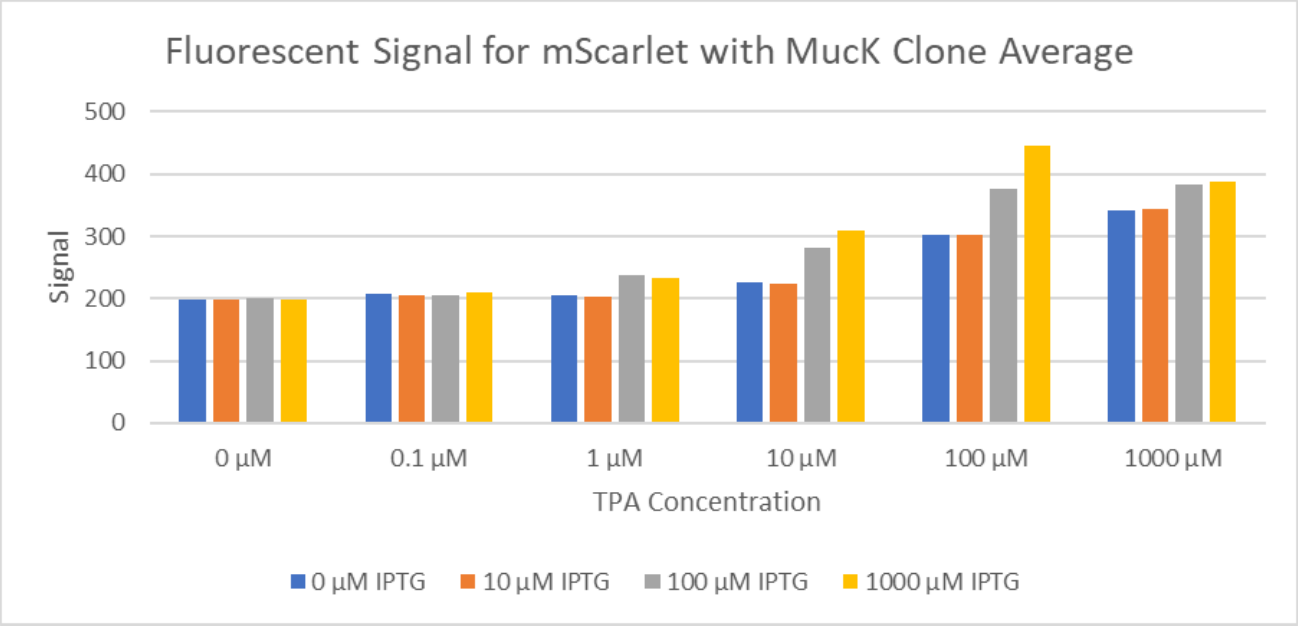
- The pathway that we will be studying is PET degradation into TPA and EG.
- We aim to create a biosensor that is sensitive to TPA to improve PET detection:
    - Use of biosensors is well-documented for PET degradation pathways
        - Examples are the use of a *Comamonas thiooxidans* strain (this is a Gram-negative bacteria that transports and metabolizes TPA) for sensing TPA at low concentrations [3]
- We will implement our design in a cell-free system and our design will be built using already assembled DNA parts.
- The plasmids we used are the following:
    - **pIGEM1**: mainly has mEmerald, a green fluorescent protein
    - **pAB132e18**: mainly has mScarlet, a red fluorescent protein
    - **iGEM2:** mainly has MucK [1], an importer
- **First Assay**: Our first goal is to test MucK[1] as a new importer, and our second goal is to determine whether mScarlet or mEmerald works as a better reporter. Plasmids with MucK with mScarlet and MucK with mEmerald will be tested.
    - Our biosensor is meant to detect TPA, and MucK (which allows the detection of TPA) is intended to only be expressed if IPTG, a small molecule that binds to the promoter that controls MucK expression, is present as well. As a result, this assay should test various concentrations of both TPA and IPTG, so our team decided on conducting a double titration with TPA and IPTG for 4 clones of each mScarlet with MucK plasmids and mEmerald with MucK plasmids in two 96 deep well plates.
    - Our team decided on using 10-fold increases, six different concentrations of TPA (0 µM, 0.1 µM, 1 µM, 10 µM, 100 µM, and 1000 µM), and four different concentrations of IPTG (0 µM, 10 µM, 100 µM, and 1000 µM) to fully utilize the 96 wells.
    - Below is the arrangement of the 96 deep well plate for each strain (one plate for mScarlet with MucK, a second plate for mEmerald with MucK). This can also be found in the lab journal under the entry on 9/15/23.

		[TPA] (µM)											
[IPTG] (µM)		0	0.1	1	10	100	1000	0	0.1	1	10	100	1000
	0												
	10												
	100												
	1000												
	0												
	10												
	100												
	1000												

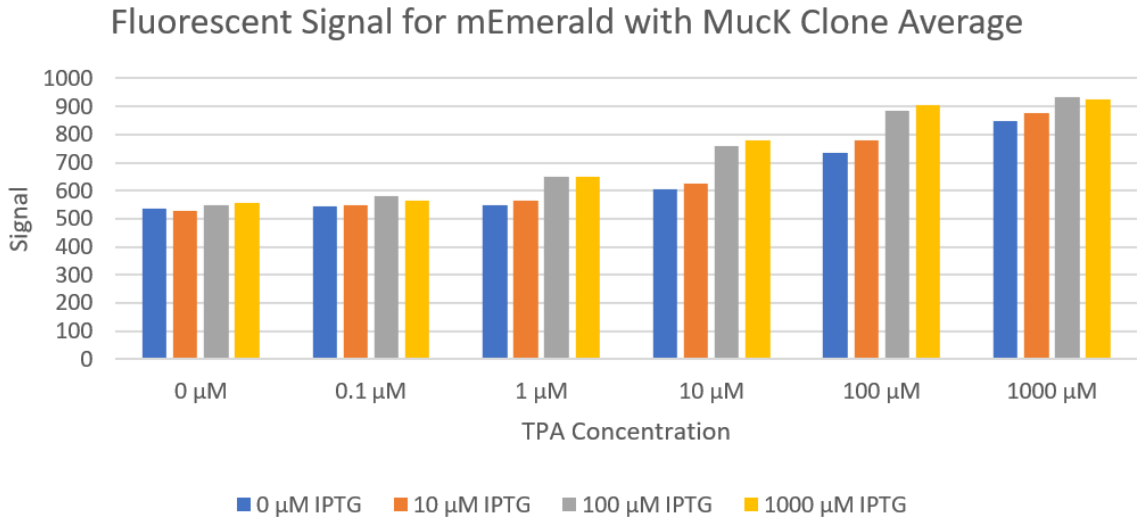
- Clone 1 represented by green
- Clone 2 represented by blue
- Clone 3 represented by orange
- Clone 4 represented by purple

- The assay was conducted properly according to the arrangement above, and below are the graphs for the data.

Graph 1: Average mScarlet signal/OD for all 4 clones



Graph 2: Average mEmerald signal/OD for all 4 clones



- The largest fold-increases that can be observed from the above graphs are 2.24 for mScarlet with MucK and 1.77 for the mEmerald with MucK.
- While these fold-increases are noticeable, they are not large enough for a proper biosensor. Therefore, we concluded that mScarlet, mEmerald, and MucK are likely not the major parts inhibiting the detection of TPA or don't work as effectively as we believe they do.
- As a result, we decided to examine another part to see if another piece of the detection process was the hindrance. We focused on our promoter, the TpaR-Regulated Promoter (see reference 2), as we found in a research paper a different promoter called pTPA3 (see reference 1) that appeared to give a larger fold-increase. We compared the TpaR-Regulated Promoter with pTPA3, and found three major modifications to potentially increase the efficiency of the TpaR-Regulated Promoter or test whether pTPA3 would solve our issue. These modifications were tested in the second assay. To properly test the differences in promoters, the fluorescent signal would need to have been constant, so our team picked mEmerald. mEmerald and mScarlet exhibited no large difference in efficiency however, so this choice wouldn't have altered our future experiments.

- **Second Assay:**
  - The three modifications to the TpaR-Regulated Promoter[2] are as follows:
    - Removal of a native ribosomal binding site in the current promoter, and this modified promoter was called iGEM1a
    - Changing the promoter's -35 and -10 sigma factors to match those pTPA3 [1], and this modified promoter was called iGEM1b

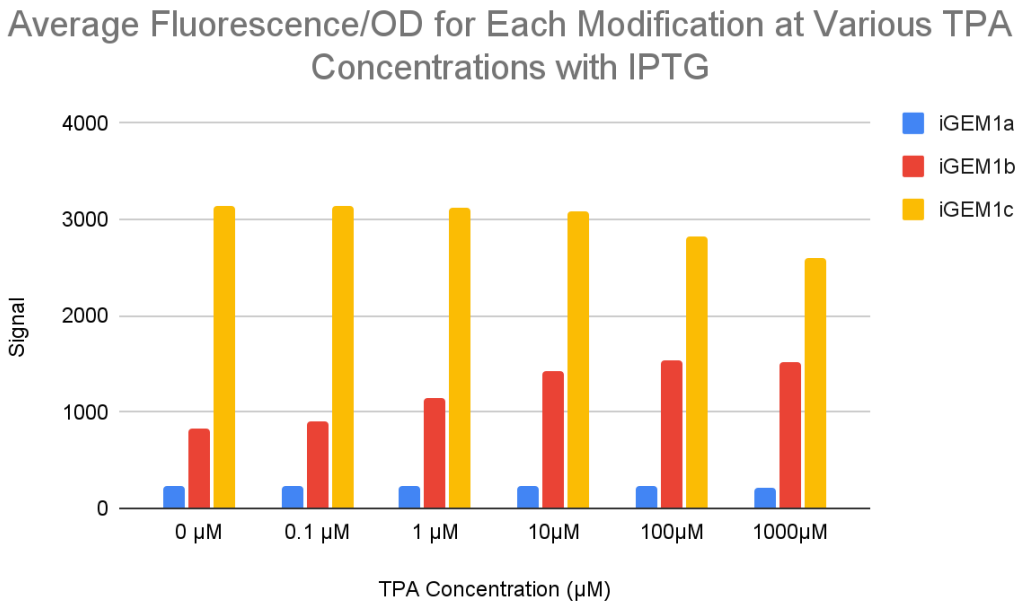
        - Taking out the entire promoter and replacing with pTPA3 [1], and this modified promoter was called iGEM1c
    - Similar to the first assay, this biosensor is being tested to detect TPA, so a titration for TPA would need to be done. Unlike the first assay, we decided to not titrate for IPTG. Despite the background signal and increase in signal without IPTG as the concentration of IPTG increased seen in the first assay, the difference between signal with and without IPTG are still noticeable. Therefore, IPTG and MucK likely works as intended, so we decided to only test the plasmids with and without IPTG without intermediate concentrations.
    - Our team decided on using the same six concentrations of TPA (0 µM, 0.1 µM, 1 µM, 10 µM, 100 µM, and 1000 µM), but set the two IPTG concentrations to 0 µM for without IPTG and 1 mM for with IPTG. We used two 96 deep well plates, with one for testing with IPTG and the other for testing without IPTG.
    - Below is the arrangement of the 96 deep well plate (both plates have the above three modifications and four clones each.) This can also be found in the lab journal under the entry on 9/28/23.

	Modification											
[TPA] (µM)		iGEM1a				iGEM1b				iGEM1c		
	0											
	0.1											
	1											
	10											
	100											
	1000											
	N/A											
	N/A											

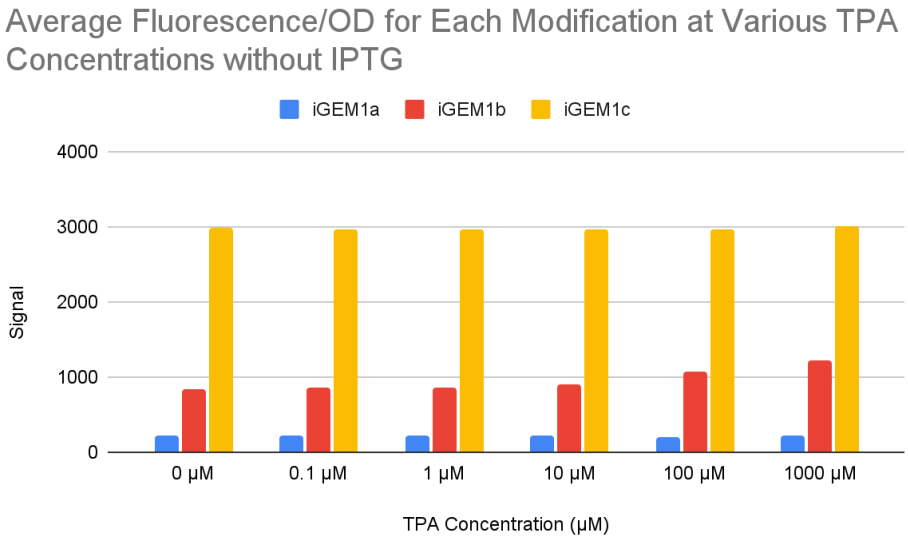
- iGEM1a clone 1 is light red, clone 2 is light orange, clone 3 is light green, and clone 4 is light blue
- iGEM1b clone 1 is red, clone 2 is orange, clone 3 is green, and clone 4 is blue
- iGEM1c clone 1 is dark red, clone 2 is dark orange, clone 3 is dark green, and clone 4 is dark blue

- The assay was conducted properly according to the arrangement above, and below are the graphs for the data and further comparisons.

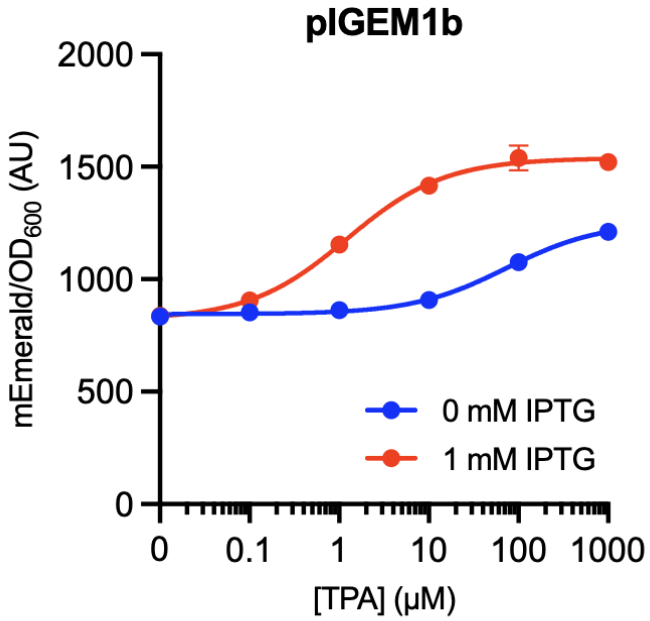
Graph 3: Average Fluorescence/OD for Each Modification at Various TPA Concentrations with IPTG)



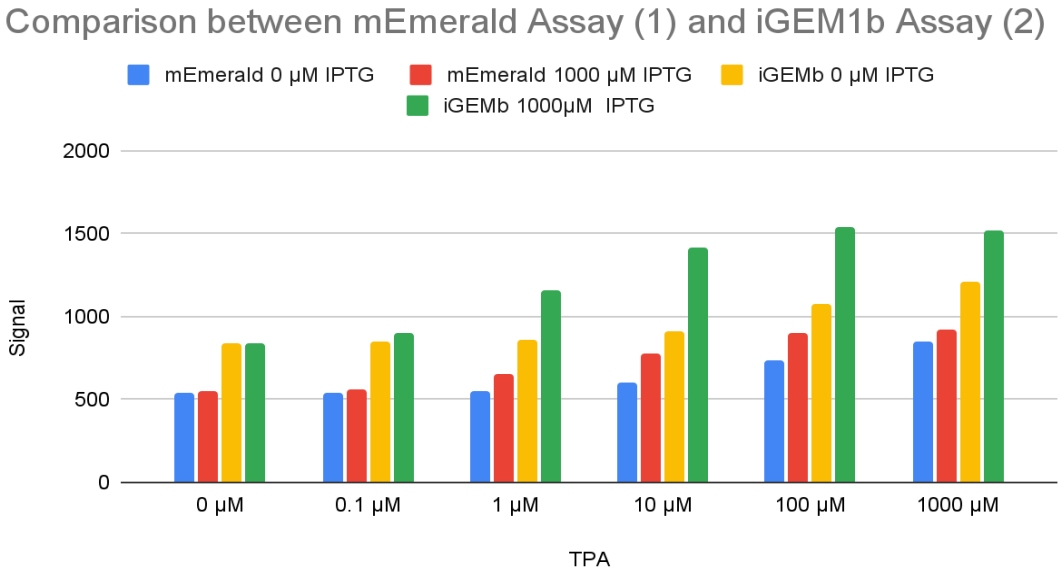
Graph 4: Average Fluorescence/OD for Each Modification at Various TPA Concentrations without IPTG



Graph 5: Comparing the results of iGEM1b With (+) and Without (-) IPTG



Graph 6: Comparison Between mEmerald From First Assay and iGEM1b From Second Assay



- When comparing graph 3 and graph 4, modification iGEM1b clearly had the largest increase in signal when IPTG was present among all three modifications. Graph 5 compares iGEM1b with and without IPTG, and it is clear that the signal increased with TPA concentration and is IPTG-dependent. This suggests that MucK is functional as the results align with our predictions. Graph 6 compares the promoter in the first assay, where the promoter was not modified, to the iGEM1b promoter modification in the second assay. The change to promoter

did increase the background signal and signal without IPTG, but the peak in signal with IPTG was noticeably larger.
    - The fold-increase for iGEM1b came out to roughly 1.82, which is slightly higher than the fold-increase without the promoter modification of 1.77.

In Silico Design/Modeling:

- The modeling aspect of our experiment was designed to provide a deeper look into the binding patterns of our various biological components.
- We planned to visualize and dock the following components:
    - Interactions between the biological molecules of relevance.
    - Dock terephthalic acid (also known as P-Phthalate or TPA) on to both the TpaK and MucK importers.
    - Dock TPA on to three additional proteins that we have identified to be binding targets of the ligand, namely mono(2-hydroxyethyl) terephthalate hydrolase, terephthalate 1,2-dioxygenase, terminal oxygenase component subunit alpha 1, and terephthalate 1,2-dioxygenase, and terminal oxygenase component subunit alpha 2 [4].

Learning:

- Following the first assay, where we tested plasmids with MucK and mScarlet and plasmids with MucK and mEmerald, where the fold increase only roughly doubled (2.24 for the mScarlet and 1.77 for the mEmerald), we made many observations with regards to the parts tested.
    - Since many parts are involved with the detection of TPA, it was possible that these parts were not the main obstacle to the detection, so the signal would not drastically increase no matter if these parts were more effective than their counterparts. If this was true, then finding and testing new versions of other parts of the plasmid that are more effective should result in a much higher fold-increase.
    - Since both reporters gave a fold-increase that was relatively similar while all other parts were kept the same, mEmerald and mScarlet should have roughly the same effectiveness in our case.
    - It was also entirely possible that the parts simply did not work as predicted, but the difference in signal with and without IPTG suggests that this idea is not correct.
- Continuing the first observation from the first assay, we decided to try and experiment with other parts of the detection process to try and remove the main obstacle. From

reference 2, it was seen that promoter pTPA3 gave a fold-increase of roughly 6-7. We wondered if the promoter could be the major issue with our biosensor, so we decided to alter our TpaR-Regulated Promoter and test pTPA3 with our plasmids. We tested three modifications mentioned above in the second assay, and modification iGEM1b gave the largest fold increase at 1.82. From this, our team made more observations and learned more about our biosensor.

- Altering the -35 and -10 sigma factors created the best result and this modification caused the fold-increase to increase from 1.77 to 1.82, so it did help the biosensor detect TPA more effectively. Yet, this increase is very slight and does not reach levels suitable for a biosensor, so the promoter likely is not the main issue and other parts are likely inhibiting the detection. More research and experimentation with other parts would need to be done to improve our biosensor.
- The clear difference in signal in iGEM1b with and without IPTG further demonstrates that IPTG and MucK are functioning as expected.
- The native RBS that was removed in iGEM1a should have caused a large increase in signal as an unwanted RBS can ruin the entire detection process, but no large increase was observed. This suggests that the native RBS also has another purpose that was missed and warrants further investigation.

References

1. Pardo I, Jha RK, Bermel RE, Bratti F, Gaddis M, McIntyre E, Michener W, Neidle EL, Dale T, Beckham GT, Johnson CW. Gene amplification, laboratory evolution, and biosensor screening reveal MucK as a terephthalic acid transporter in Acinetobacter baylyi ADP1. Metab Eng. 2020 Nov;62:260-274. doi: 10.1016/j.ymben.2020.09.009. Epub 2020 Oct 1. PMID: 32979486.
2. Hara H, Eltis LD, Davies JE, Mohn WW. Transcriptomic analysis reveals a bifurcated terephthalate degradation pathway in Rhodococcus sp. strain RHA1. J Bacteriol. 2007 Mar;189(5):1641-7. doi: 10.1128/JB.01322-06. Epub 2006 Dec 1. PMID: 17142403; PMCID: PMC1855752.
3. Dierkes, Robert F, et al. “An Ultra-Sensitive *Comamonas Thiooxidans* Biosensor for the Rapid Detection of Enzymatic Polyethylene Terephthalate (PET) Degradation.” *Applied and Environmental Microbiology*, vol. 89, no. 1, 31 Jan. 2023, https://doi.org/10.1128/aem.01603-22. Accessed 11 Oct. 2023. PMID: 36507653; PMCID: PMC9888244
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