FSU iGEM
2017

Our Solution

Bacteria Aimed at Removing and Terminating Intestinal Invaders

BARTII is a cell-based therapeutic for Celiac Disease that contains three features:

Gliadin Cuff

Gliadin Degrader

Zonulin Cuff

BARTII

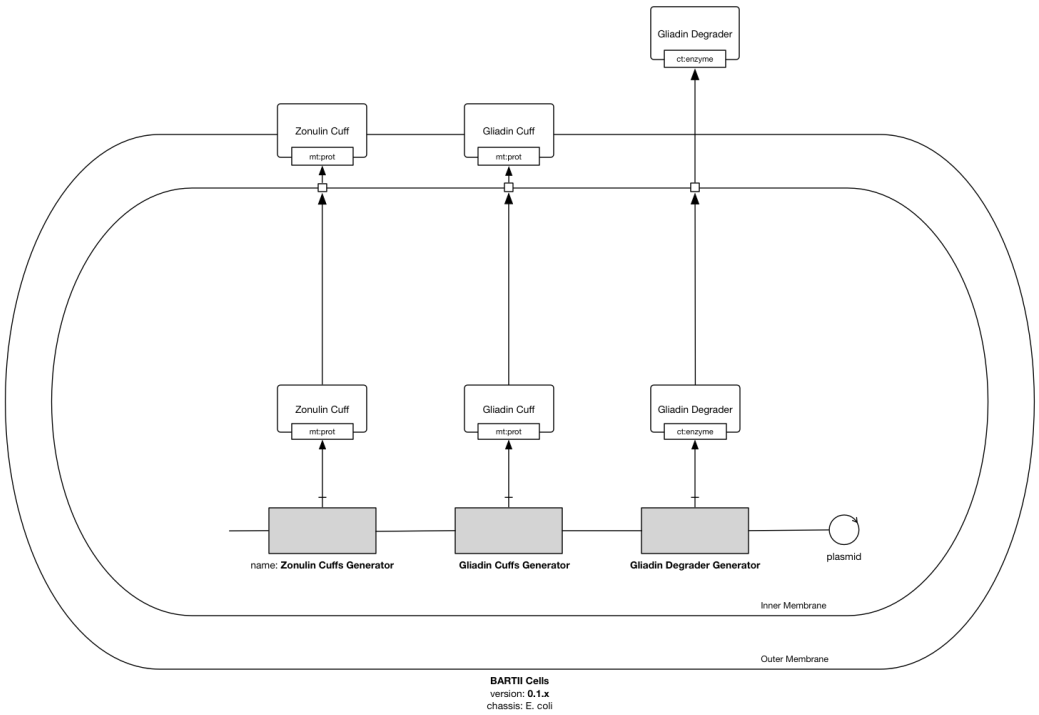
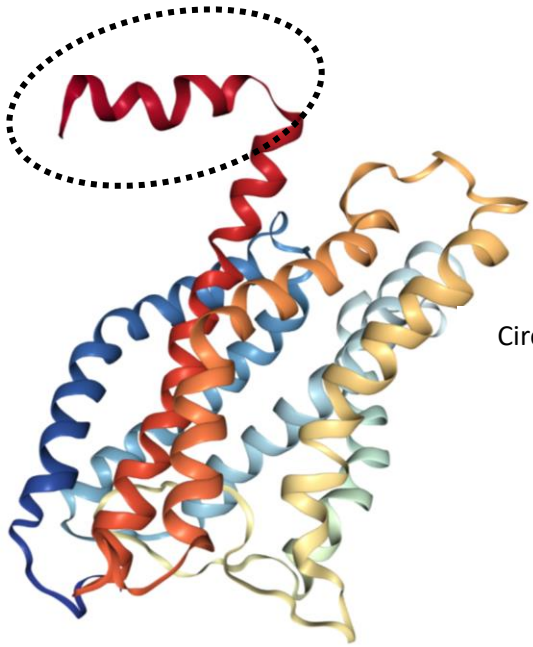


Figure 1: ***BARTII Design*** *The diagram uses symbols from the Systems Biology Graphical Notation (SBGN) and experimental symbols that are under development. From bottom-left: Zonulin Cuff Generator, Gliadin Degrader Generator, and Gliadin Cuff Generator, encoded in a plasmid. The generators, respectively, express the Gliadin Cuffs, Gliadin Degraders, and the Zonulin Cuffs. The Cuffs localize to the outer membrane of the chassis (also known as the host cell). The Gliadin Degrader is secreted into the extracellular space.*

BARTII is a prototype cell-based therapy for Celiac Disease. BARTII's design is depicted in Figure 1. It's designed to contain three generators that produce the Gliadin Cuff, Gliadin Degrader, and the Zonulin Cuff. The Gliadin Cuff is designed to bind gliadin. Gliadin is a peptide in gluten that triggers inflammatory responses in the small intestine of patients with Celiac Disease. The Gliadin Degrader is designed to be secreted by BARTII and is intended to degrade gliadin in the intestinal lumen. The Zonulin Cuff is designed to bind zonulin, a hormone that regulates the permeability of the small intestinal mucosa and can amplify the inflammatory response to gliadin. The three parts working together in BARTII are expected to decrease the inflammation associated with Celiac Disease.

Gliadin Cuff

Understanding the human receptor of Gliadin was the first step for this cuff as we needed to see what we could use as a 'cuff' to sequester the Gliadin. The first thought was to utilize nano-bodies however the library I had sought out from U-Lethbridge didn't have one that I could use. There was a full-length antibody however since E. Coli can't create full antibodies so we had to explore other avenues. Further research revealed that a receptor called **CXCR3** interacted with Gliadin and played a part in the autoimmune response in celiac patients. CXCR3 is a G-protein coupled receptor (GPCR) or a 7-transmembrane receptor which mean that there could be some problems incorporating those transmembrane domains into a gram-negative *E. coli.* Since E. coli is often used as an expression vector for GPCR crystallization experiments we understood that CXCR3 was easily expressed by *E. coli*. However, there were a few solutions to the transmembrane domain problem as we consulted our advisors and they suggested using solely the extra cellular domain as the receptor as we don't require the signal transduction part which includes much of the receptor. Now that we decided the methods we were going sequester gliadin we now had to find a way to anchor the receptor on the surface so it could do its job.



Circled portion is Extracellular Domain

Source: pdb.org

Figure 2: CXCR3

The research led to many ways to anchor and express this receptor in significant enough quantity so have an effect at gut concentrations of gliadin. Designs **0.0.1, 0.0.7, 0.1.1, and 0.1.7** use the truncated CXCR3 and all other designs utilizes the full-length receptor. The first seven designs utilized FLAG or His-Tags since they are cheap, small and have a high specificity. Designs **0.1.1, and 0.1.7** also were the designs that were decided to be the most sound, easiest to test, most cost effective and were therefore our primary designs.

0.0.1 *(OMPa - Trunc. CXCR3 - 3xFLAG)* utilizes a protein called OMPa which is a beta barrel structure that can transport fused proteins to the surface of an *E. Coli*. OMPa is also from a past iGEM team as well and we have improved the part by giving it the ability to bind to gliadin by fusing it to CXCR3. OMPa has shown to be effective with sfGFP and we are only using the extracellular domain of the receptor in this design since its anchored to the cell and a FLAG tag is used as well. **0.0.2** *(MBP – Linker – CXCR3 – 6xHis)* follows a design detailed in a paper that expressed GPCRs on the surface of *E. coli*. Maltose Binding Protein (MBP) acts as a solubilization agent to avoid inclusion bodies and the linker was to aid with folding of the receptor. However, this design was much larger than the others and the mechanism was not well documented therefore this was one of the risker designs. **0.0.3** *(PelB – Linker – CXCR3 – 6xHis)* the PelB leader sequence was pulled from the iGEM registry and has been shown to localize fused protein to the periplasm however it has not been shown to integrate receptors. The reason this was proposed was because it followed the same build as 0.0.2 which also has a protein that aids in periplasm localization (MBP) and this also faced the same problems as 0.0.2. **0.0.4** *(OMPa Signal seq – CXCR3 – 6xHis)*, **0.0.5** *(DsbA Signal seq – CXCR3 – 6xHis),* **0.0.6** *(PhoA Signal seq – CXCR3 – 6xHis)* all follow the same build with varying signal sequences that help localize the construct to the periplasm. Each of these signals originate from their respective protein that is typically localized to the periplasm in E. coli. These designs stemmed from 0.0.2 due to similar mechanisms as well. **0.0.7** (*BclA - Trunc. CXCR3 - 3xFLAG*) consists of a glycoprotein anchor, BclA, which presents the truncated receptor to the media. This would be test by utilizing the FLAG tag.

Figure 3.

Our finalized designs utilized a florescent protein called mNeonGreen for some testing protocols. The circled designs were the ordered constructs but that does not mean it was tested and data was collected. These designs stem from the original designs and therefore have the same mechanisms albeit with different testing methods. The main reason to use a FP was that we could better visualize where the receptor localized by using florescent microscopy and were still able to measure binding efficiency by Bradford assay. **0.1.1** *(OMPa – Trunc. CXCR3 – TEV Site – mNeonGreen)* utilizes OMPa as the anchoring motif and the same truncated receptor to bind to the excess gliadin. mNeonGreen is a monomeric green-yellow FP which performs very well as a fusion protein. The TEV site was added as a contingency if the mNeonGreen adversely affected the binding capability of the trunc. receptor due to its much larger size. **0.1.2** *(MalE (aka MBP) – GS Linker – CXCR3 – mNeonGreen)* This design was not pursued further due to its large size due to the fusion of mNeonGreen. This design stemmed from a paper that detailed how they were able to express and embed functional GPCRs into the E. coli outer membrane. **0.1.7** *(BclA – Trunc. CXCR3 – TEV Site – mNeonGreen)* Design 0.0.7 was improved by utilizing a FP instead of a FLAG tag. The mechanisms in this design are the same but the main difference being the FP and testing parameters.

Figure 4.

Gliadin Cuff – Performance

Confirmation of Gliadin Cuff Expression

The gliadin cuff was designed with an mNeonGreen protein attached so that expression could be confirmed microscopically. This does not confirm that the Gliadin Cuff is attaching to the outer membrane, but does confirm that our bacteria expresses the coding sequence inserted.

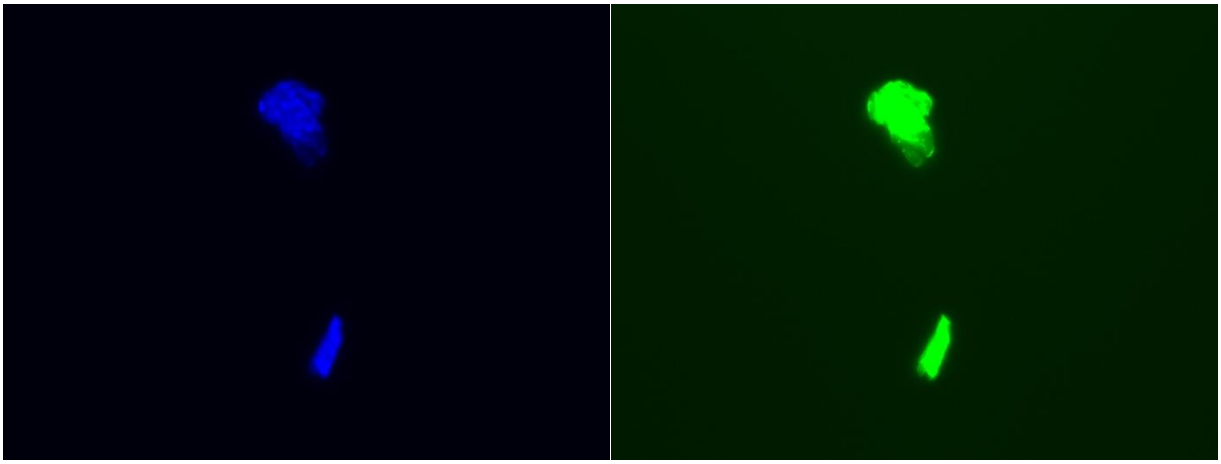


Figure 5. DAPI stain (in blue) and the same bacteria expressing green fluorescence. This confirms our cells are expressing the inserted coding sequences.

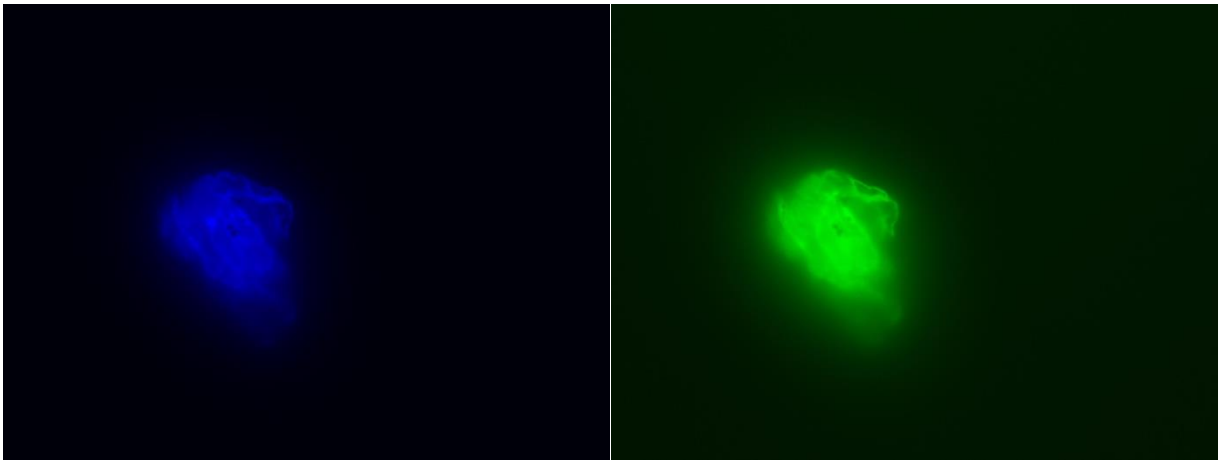


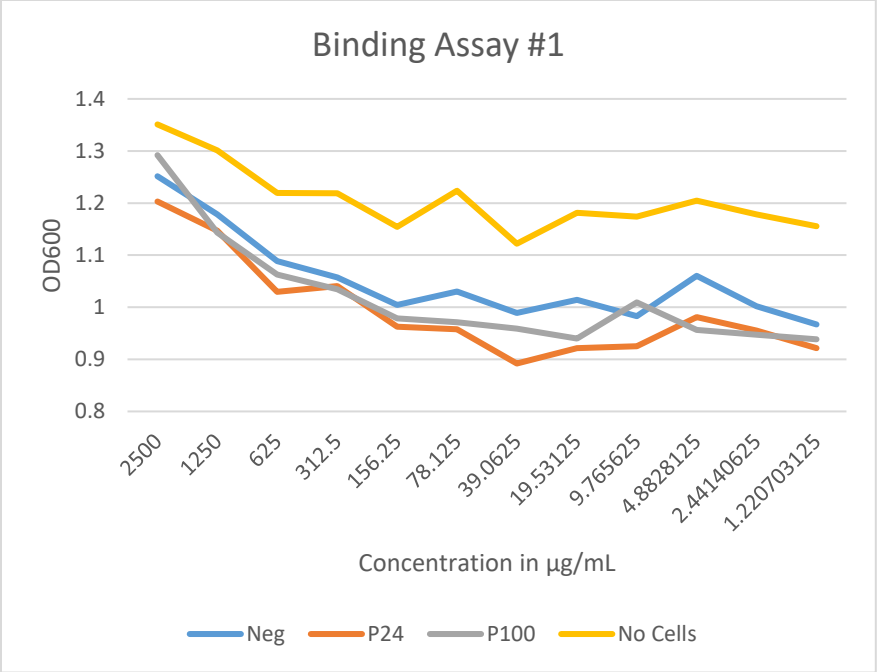
Figure 6. DAPI stain (in blue) and the same bacteria expressing green fluorescence. This confirms our cells are expressing the inserted coding sequences.

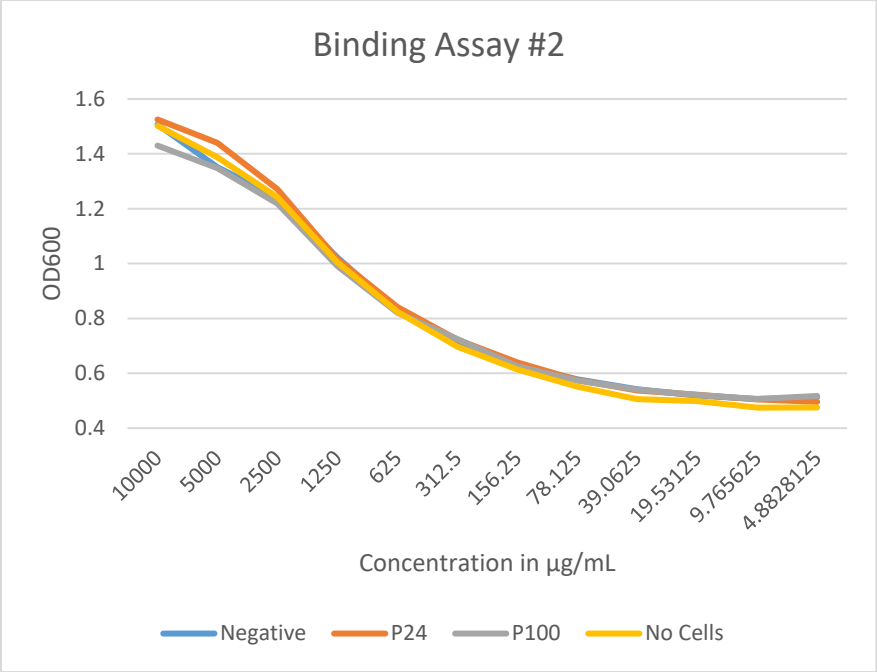
As seen in Figures 5 and 6, initial results indicate the cells are expressing the inserted coding sequences.

Gliadin Cuff Binding Assay

Gliadin is not water soluble and so stock solutions of gliadin in DMSO at 25 mg/mL and 100 mg/mL were made. Initially, multiple assays were conducted using PBS as a buffer. However, gliadin still has limited solubility in PBS and so different buffers were tested that could solubilize more gliadin while still not causing adverse reactions with the Bradford. Eventually, a PBS solution with 0.01% Triton X-100 was settled on as it appeared to solubilize the most gliadin and was still compatible with the Bradford reagent.

For each assay 2 plates were run and the values of each row of a device was averaged together to form the following graphs.

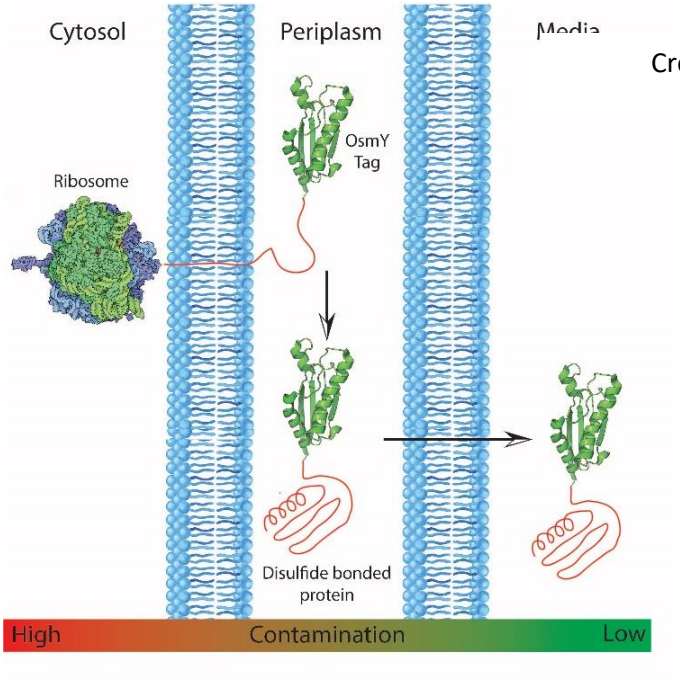




The first assay suggests that gliadin is binding nonspecifically. Meaning there was no significant different between OD values in a row that had cells with the gliadin cuff versus cells without the gliadin cuff. The second assay suggests no difference between rows with cells and without. Both assays suggest that the cells are not binding gliadin as expected. This could be caused by the cuff not being inserted onto the outer membrane or it could be that the protein is on the outer membrane but is misfolded. We are awaiting confocal microscopy to confirm whether the cuff is inserted onto the outer membrane.

Gliadin Degrader

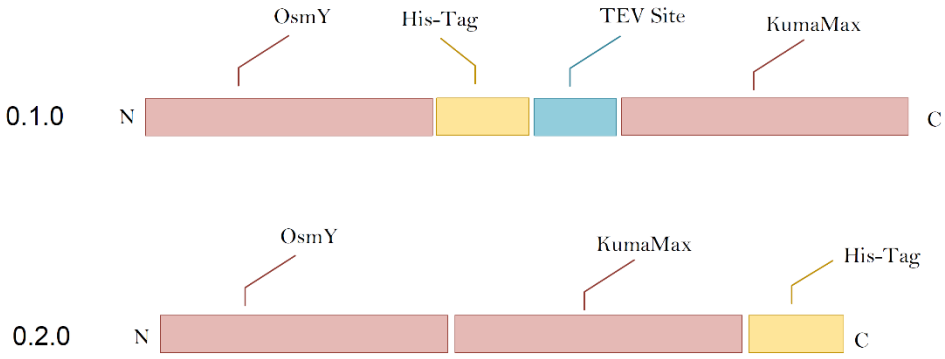
The first thing to do was determine an enzyme that would 'break down' gliadin and one of the first ones we came across was something called KumaMax which was developed by WashU's iGEM team in 2011. We decided to use this enzyme because it was shown to be quite effective but now the next step was to improve it for our use. Searching for a way to secrete this enzyme was the first battle as we had to retain biological activity after it is secreted. We determined that a protein called OsmY, which has been shown in past iGEM projects, could be used to transport a fusion construct through both the inner and outer membrane. This method relies on a ABC transport system and that system is what recognizes the signal peptide that OsmY possesses.

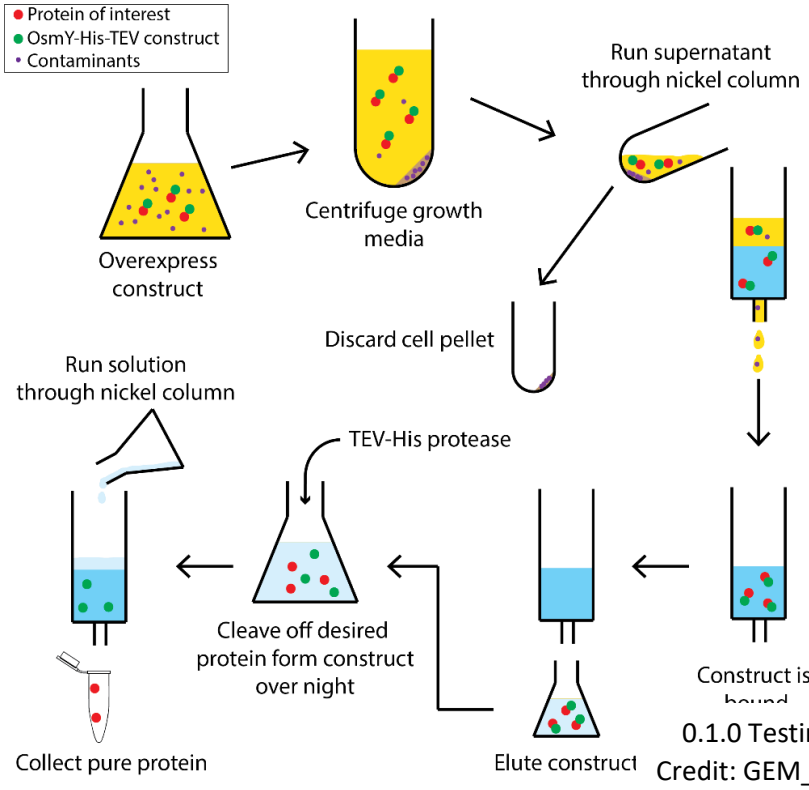


Credit: iGEM_UMich_2014

We finalized two designs that are very similar on paper but serve different purposes. 0.1.0 would be used to test the effectiveness of OsmY as a transporter and give us the ability to test the activity of the enzyme. It would be tested by running a Nickel column that binds to the his-tag and then the construct would be eluted. You would then cleave the construct with TEV protease and it would cut at the designed TEV Site. Running the solution through a nickel column again would allow the pure enzyme to pass straight through since there isn't a his-tag attach to it, only the OsmY. 0.2.0 is simpler in that we plan to just produce the protein and see how much could be autocatalytically cleaved as OsmY has been shown cases depending on the fusion protein. Purification would be done with a nickel column and a SDS-PAGE would be done to determine the extent of auto-cleaving.

CDS Breakdown





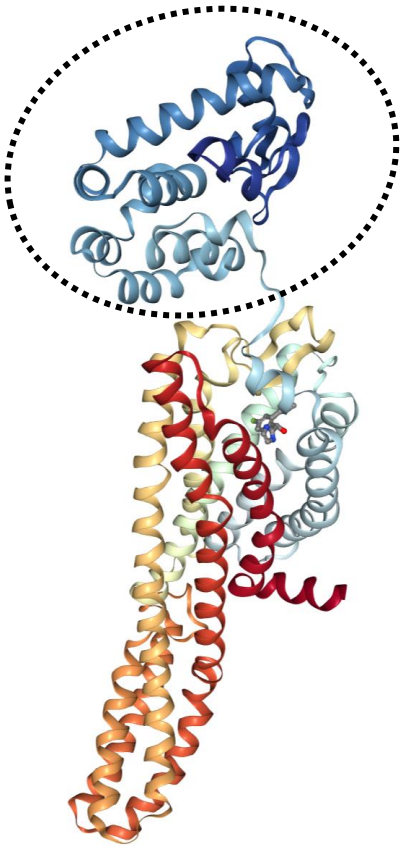
0.1.0 Testing Protocol
Credit: GEM_UMich_2014

Zonulin Cuff

Zonulin also plays a large part in the autoimmune response in that it breaks down tight junctions when levels are high but this protein does circulate at much lower levels than Gliadin. It also goes by the name of Pre-Haptoglobin 2 (Pre-HP2) not to be confused with Haptoglobin 2 which plays a slightly different role. Zonulin being released is believed to be a result of gliadin binding to its receptor and exerting downstream effect where Zonulin is one of those. Zonulin will continue the downstream effects by binding to its respective receptor which is believed to a shared mechanism with **PAR2** and **EGFR.** Both receptors are in different classes so we had to take into account which would be the best to use in the case of E. coli.

EGFR (Epidermal growth factor receptor) is a receptor tyrosine kinase (RTK) which rely dimerization to bind to a ligand which presented some problems as it was also much larger than a GPCR and still suffered from the same problem of being integrated into the outer membrane. The RTK could be possible if we anchored both extracellular domains to a presenting motif but we were skeptical on its effectiveness with a diminished ability to dimerize.

PAR2 (protease-activated receptor 2) is a GPCR so we could utilize the same design of using the extracellular portion that would be expected to bind to the ligand. We had the same concern in that there is some role that the transmembrane domain has in ligand binding so a reduced ligand binding could be observed. The GPCR was also comparatively smaller than the RTK which was favorable for cost and build reasons.

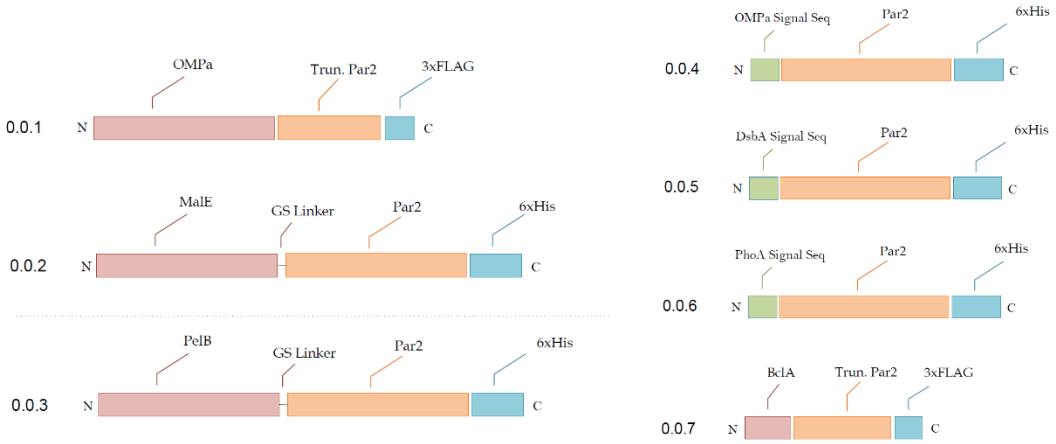


Circled portion is Extracellular Domain

Disclaimer: Complexed with AZ8838

Source: PDB.org

All designs follow the same reasoning as the gliadin cuff and the only changes are the receptor which is PAR2. It is the orange block in all designs for ease of location. Refer to each respective design number designations (i.e. 0.0.4) in the Gliadin cuff for function and reasoning.

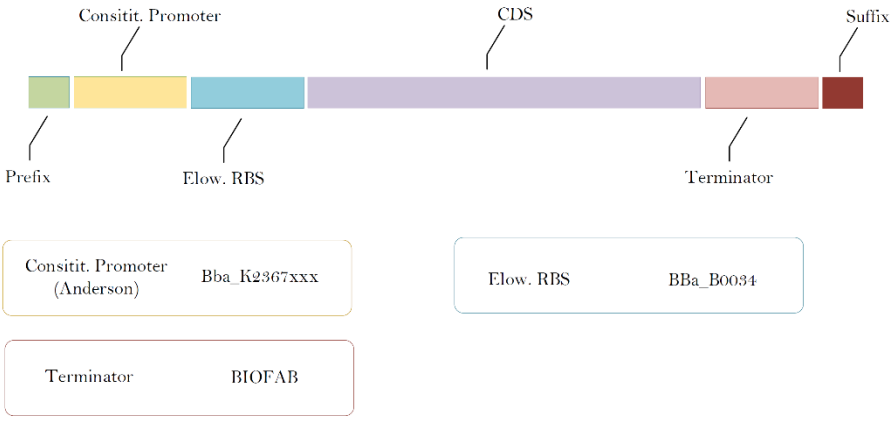


The following are the FP constructs that only differ from the gliadin cuff in respects to the receptor. Please refer to the Gliadin Cuff for more an in-depth breakdown of each design. Again, the circled designs are the ones that were ordered but does not necessarily mean they were tested. Design number designations (i.e. 0.0.4) are the same and match to each description.

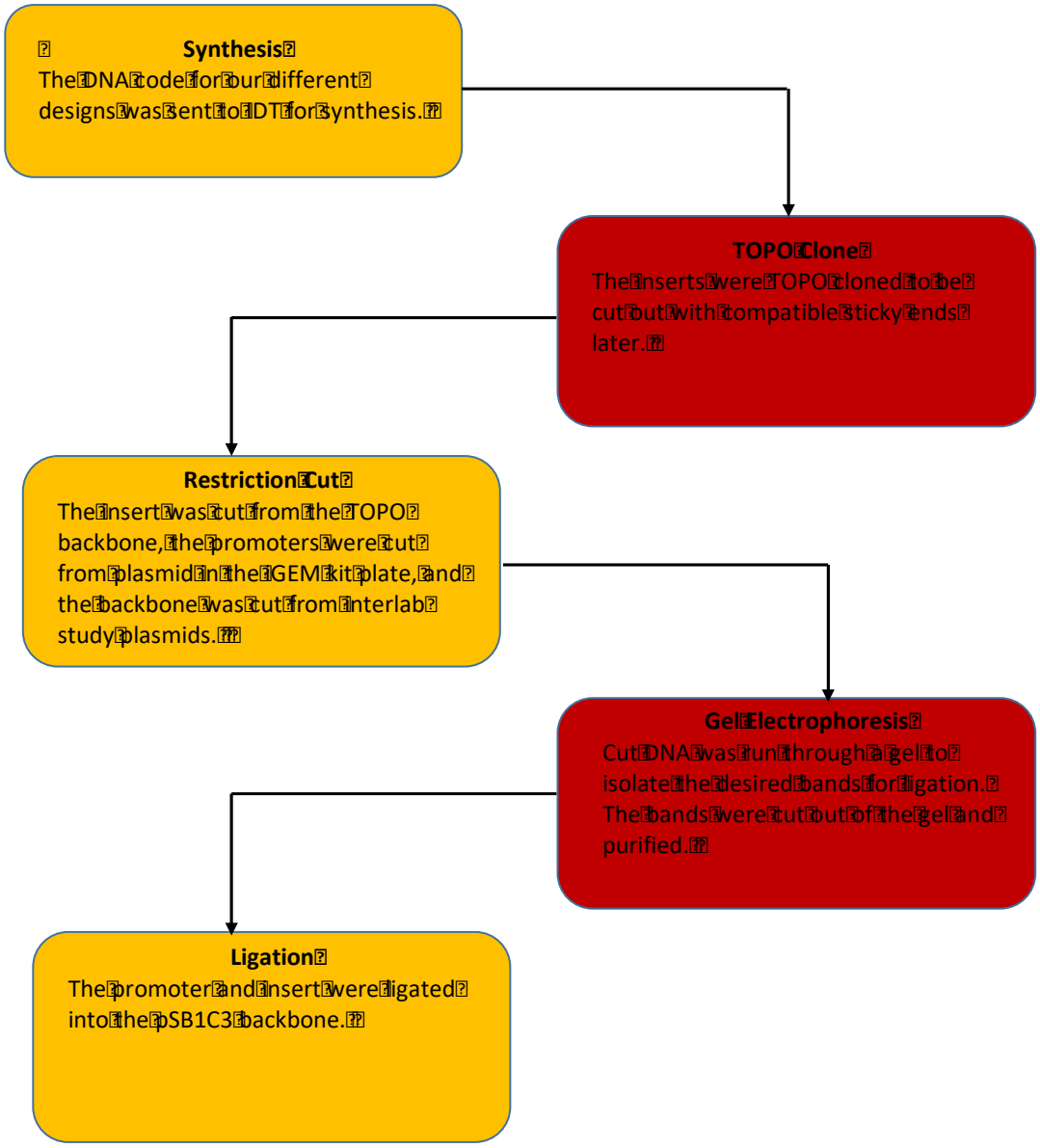
Design Pattern

gBlocks were used for the majority of the project.

- **Promoters:** from the Anderson library which are all varying strengths of constitutive (always on) promotors. **Each design used the same library** where they were **tested with Strong, Medium, and Weak** promotors in order to determine the most efficient strength.
- **RBS:** The RBS used for **all the designs** is the Elowitz RBS due to it proven track record and it's medium strength works well for our designs.
- **Terminator:** The terminator used in **all our designs** came from BIOFAB as we understood those to be the best characterized therefore we went with it to reduce any uncertainty with the iGEM terminators.
- **Prefix/Suffix:** The Prefix and suffix were **the same for all design**s. They differed from the iGEM ones in that there was an overhang on each side to aid with R/L of the gBlocks to a iGEM approved backbone.



The following flowchart summarizes the build process for the gliadin cuff generators.



Protocols

Plasmid Purification

Method adapted from Macherey-Nagel NucleoSpin®

1. Pour 1.5 mL bacterial cell culture to 2 mL microcentrifuge tube
2. Spin cells in a microcentrifuge for 30s at 11,000 rpm
3. Discard the supernatant, then resuspend pellet with 250 µL resuspension buffer. Resuspend by either vortexing or pipetting up and down. There should be no visible pellet after this step.
4. Add 250 µL 0.5%-1.0% sodium hydroxide solution. Mix by inverting tube 6-8x. **Do not vortex.** Incubate at room temperature for 5 min.
5. Add 300 µL 36%-50% guanidine hydrochloride solution. Invert tube 6-8x to mix. **Do not vortex.** If an indicator was used in the sodium hydroxide solution, invert until lysate is colorless.
6. Centrifuge for 5 min at 11,000 rpm. If supernatant is not clear, repeat this step.
7. Place a Macherey-Nagel NucleoSpin® silica column into a 2 mL collection tube. Decant the supernatant into the silica column or pipette a maximum of 750 µL.
8. Centrifuge for 1 min at 11,000 rpm. Discard the flow-through and place the silica column back into the collection tube.
9. Add 500 µL Macherey-Nagel wash buffer (AW) to silica column, and centrifuge for 1 min at 11,000 rpm.
10. Add 600 µL Macherey-Nagel buffer A4 (supplemented with ethanol) to silica column. Centrifuge for 1 min at 11,000 rpm. Discard flow-through, and put silica column back into 2 mL collection tube
11. Centrifuge for another 2 min at 11,000 rpm. Discard the remaining flow-through and collection tube.
12. Place silica column into 2 mL microcentrifuge tube. Add 50 µL of elution buffer to silica column, and incubate at room temperature for 1 min. Centrifuge for 1 min at 11,000 rpm. Discard silica column.
13. Nanodrop purified plasmid to determine concentration and purity. Store microcentrifuge tube containing purified plasmid in a -20°C freezer for long-term storage.

Restriction Digestion

1. Calculate volume of purified plasmid required to get between 1 and 2 nanograms of DNA. If concentration is too low to obtain 1 nanogram in 17 µL use 17 µL.
2. Pipette calculated volume of plasmid into 2 mL Eppendorf tube.
3. Pipette 2 µL of buffer into Eppendorf tube.
4. If necessary, pipette required volume of water to reach volume of 19 µL.
5. Pipette 1 µL total of enzyme into tube. If using two enzymes pipette 0.5 µL of each.
6. Spin tube for 30s at 11,000 rpm to ensure all liquid is at bottom.
7. Incubate at 37°C for one hour.

Gel Electrophoresis

Adapted from Addgene Gel Electrophoresis protocol

Precautions: Ethidium bromide (EtBr) is a known mutagen. Handle with appropriate PPE.

1. Depending on size of fragments needed to be separated, prepare a 0.7%-2% agarose/TAE solution. Mix agarose with 100 mL of TAE in flask and microwave in microwave-safe vessel. Microwave in pulses. Swirl flask every 30-45 seconds until agarose is completely dissolved.
2. Let flask cool to 50°Cor until flask can be comfortably handled, but do not allow it to solidify.
3. Add 2-3 µl ethidium bromide (EtBr) stock solution to agarose solution.
4. Put well comb into a gel tray, and pour agarose solution slowly into the tray.
5. Allow solution to completely solidify and place tray into gel electrophoresis box. Pour TAE over gel until submerged. Remove well comb(s).
6. Add 4 µL of loading buffer to the 20 µL digest samples.
7. Add a molecular weight marker (DNA ladder) to first lane, followed by samples in the proceeding lanes.
8. Plug the positive electrode (red) into the end of the gel electrophoresis box opposite of your samples. (DNA is negatively charged, and will move towards the positive end.)
9. Run at 80-150 V for 45 minutes to 1 hour.
10. After time has elapsed, turn off power and remove electrodes.
11. Remove tray and visualize bands in gel using a UV light.

Gliadin Binding Assay

1. Grow overnight cultures of cells with gliadin cuff and cells without to be used as a negative control.
2. Spin down culture tubes for 15 minutes at 1,000 rpm.
3. Discard supernatant and resuspend cells in buffer desired for assay. In our case, the best buffer for solubility of gliadin was PBS with 0.01% Triton X-100. Other buffers tested were PBS and RIPA buffer.
4. Measure the OD of the resuspended cells using a Nanodrop. Record the values.
5. Calculate volume of cell + buffer solution required to bring all ODs to chosen standard in 50 µL. Experiments were conducted with ODs of 0.5 and 2.
6. Dilute gliadin stock in a 1 in 10 dilution using the buffer used to resuspend cells. Our stock solution was 100 mg/mL.
7. Pipette 200 µL of diluted gliadin into wells in the first column.
8. Put 100 µL of buffer into wells in columns 2-11.
9. Do a serial dilution of gliadin by transferring over 100 µL at a time across each row.
10. Label each row according to the device to be tested. Our rows were labeled either P24 or P100 (for gliadin cuff generators with a promoter strength of 24 or 100), negative for cells without the gliadin cuff generators, and zero for rows with no cells and just buffer. Add 50 µL total of cells and buffer. For example, if earlier calculations found that 34 µL of cells were required of P24 cells in each row labeled P24 add 34 µL of resuspended cells and 16 µL of buffer.

11. Seal the well plate with tape or parafilm and put it in the incubator for at least 1 hour. Experiments were conducted at 1 hour and 3 hours.
12. Remove from the incubator and spin down for 15 minutes at 3000rpm.
13. Transfer 50 µL of the supernatant into a clear plate with flat bottom wells and add 200 µL of the Bradford reagent.
14. Take the OD values using a spectrophotometer.

Ligation

The iGEM ligation protocol was used.

1. Add 2ul of digested Plasmid Backbone (25 ng)
2. Add equimolar amount of promoter fragment (< 3 ul)
3. Add equimolar amount of insert (< 3 ul)
4. Add 1 ul T4 DNA ligase buffer. Note: Do not use quick ligase
5. Add 0.5 ul T4 DNA ligase
6. Add water to 10 ul
7. Ligate 16C/30 min, heat kill 80C/20 min
8. Transform with 1-2 ul of product