



Our Solution



Bacteria Aimed at Removing and Terminating Intestinal Invaders

We developed 3 cell-based therapeutic approaches for Celiac Disease, each inserted separately in an E.Coli chassis.



Gliadin Cuff



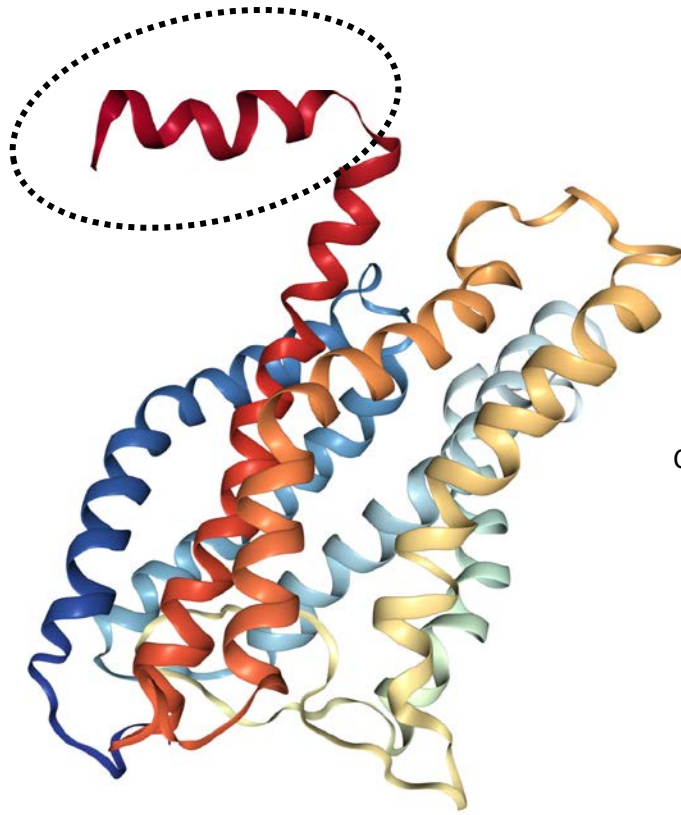
Gliadin Degradator



Zonulin Cuff

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

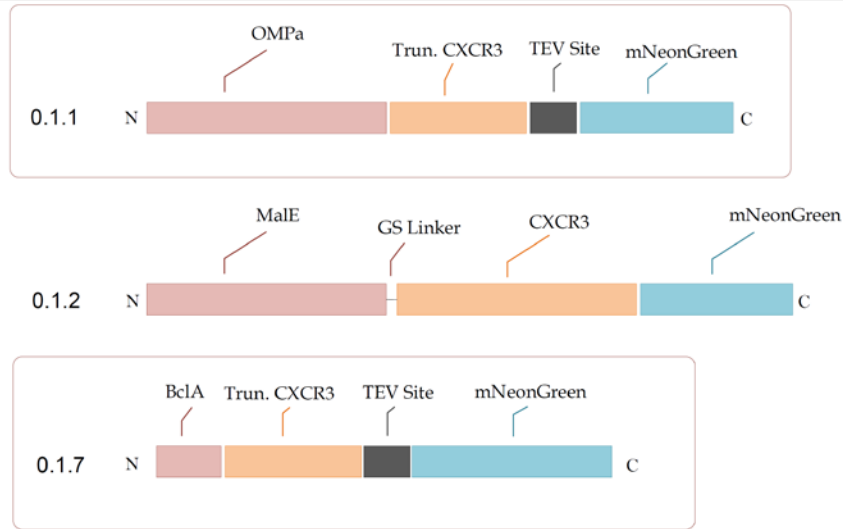
Source: PDB.org

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 (*OMP_a* - *Trunc. CXCR3* - *3xFLAG*) utilizes a protein called OMP_a which is a beta barrel structure that can transport fused proteins to the surface of an *E. Coli*. OMP_a 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. OMP_a 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 riskier designs. **0.0.3** (*PeIB* - *Linker* - *CXCR3* - *6xHis*) the PeIB 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** (*OMP_a 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.

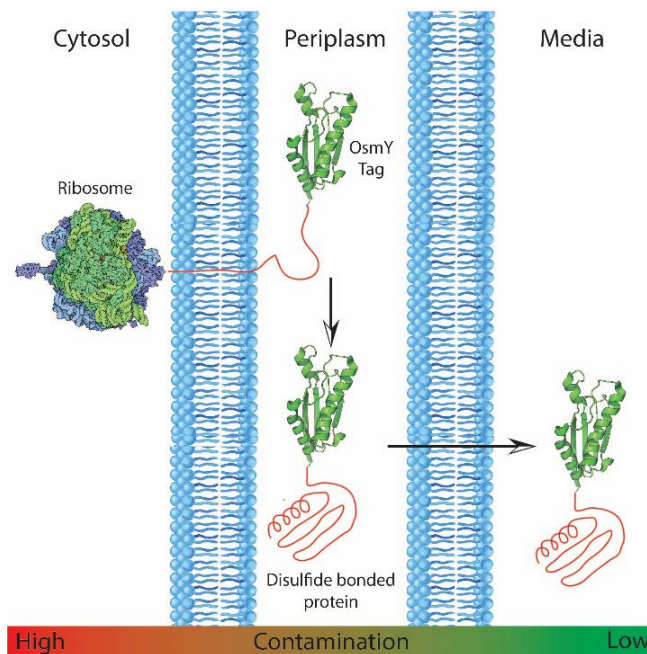


Our finalized designs utilized a fluorescent protein called mNeonGreen for some testing protocols. The circled designs were the ordered constructs but that does not mean it was tested and data was collected. These designs stem from the original designs and therefore have the same mechanisms albeit with different testing methods. The main reason to use a FP was that we could better visualize where the receptor localized by using fluorescent microscopy and were still able to measure binding efficiency by Bradford assay. **0.1.1** (*OMP_a – Trunc. CXCR3 – TEV Site – mNeonGreen*) utilizes OMP_a 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.



Gliadin Degradator

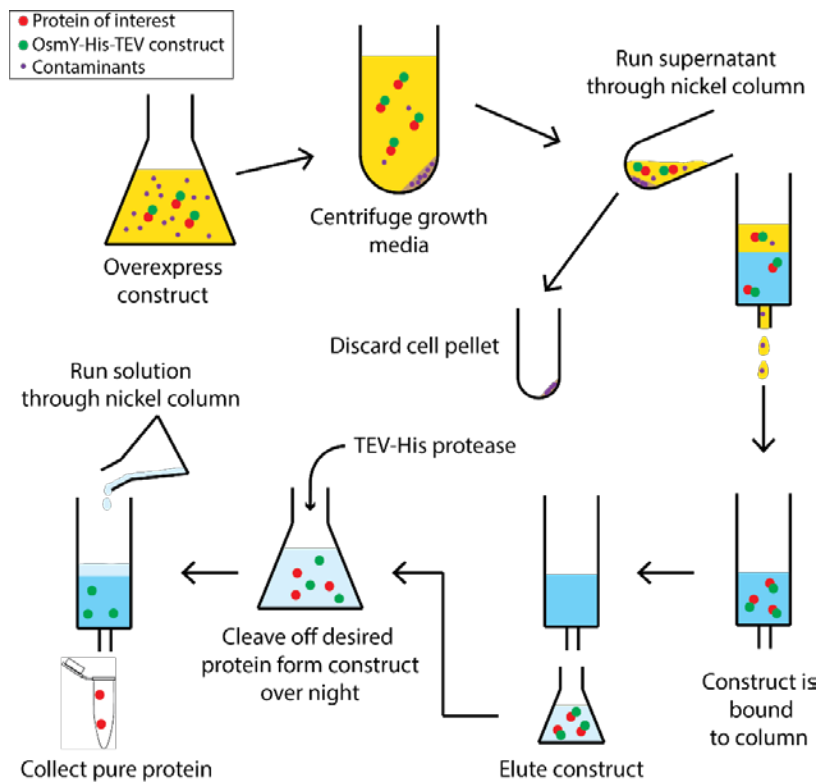
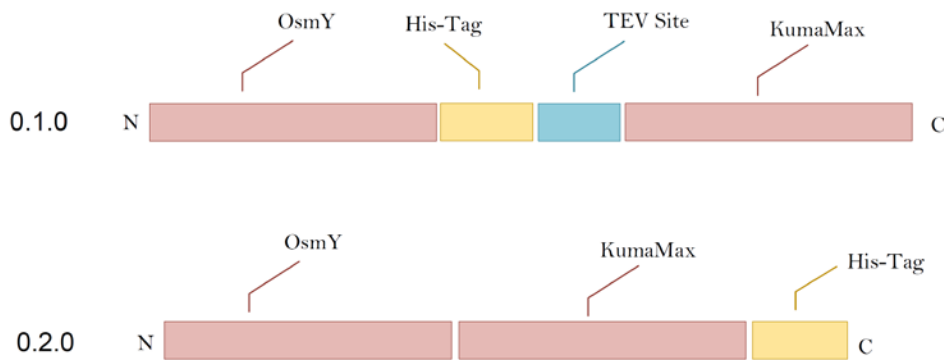
The first thing to do was determine an enzyme that would ‘break down’ gliadin and one of the first ones we came across was something called KumaMax which was developed by WashU’s iGEM team in 2011. We decided to use this enzyme because it was shown to be quite effective but now the next step was to improve 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



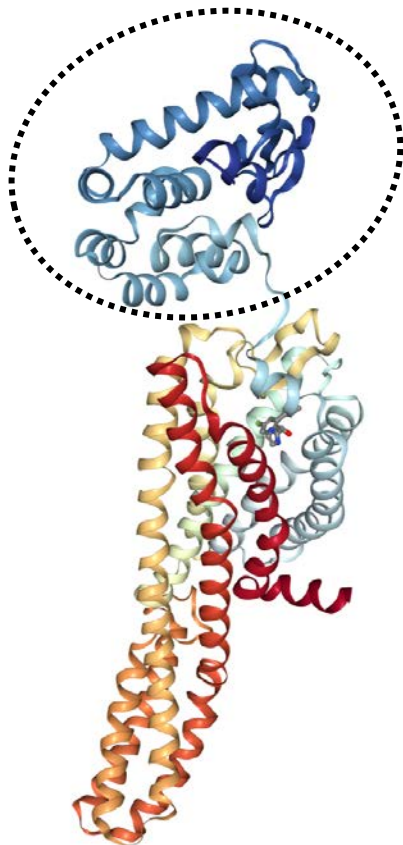
0.1.0 Testing Protocol
 Credit: GEM_UMich_2014

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 not 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 effects where Zonulin is one of those. Zonulin will continue the downstream effects by binding to its respective receptor which is believed to be 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 relies on 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.

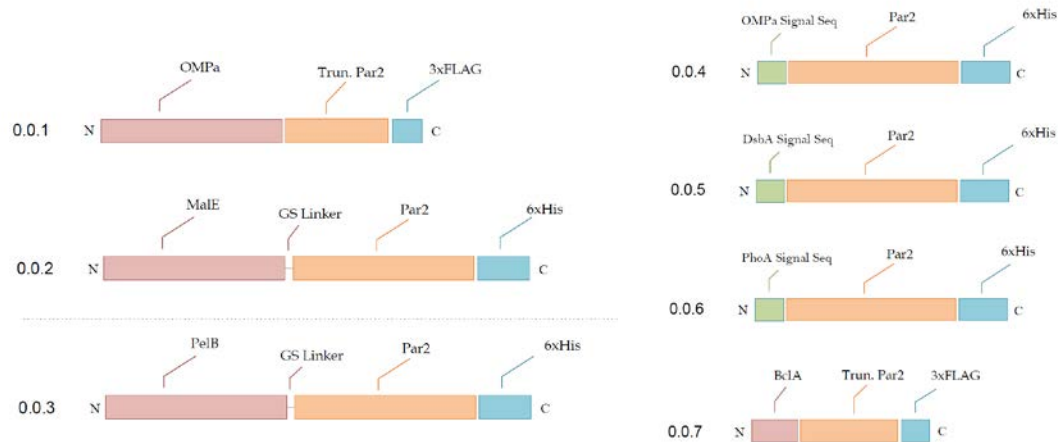


Circled portion is Extracellular Domain

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.



General Build Parameters

gBlocks were used for the majority of the project

-Promoters: from the Anderson library which are all varying strengths of constitutive (always on) promoters. **Each design used the same library** where they were **tested with Strong, Medium, and Weak** promoters in order to determine the most efficient strength.

-RBS: The RBS used for **all the designs** is the Elowitz RBS due to its proven track record and its 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 designs**. 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.

