Purification of FimE and Hbif

In order to purify FimE or Hbif we will grow cells in LB medium at 37°C in a rotating incubator. We will take samples from the medium to measure the optical density. At mid-log phase (between 3.0 and 4.0 OD) we will slow the growth by decreasing the temperature in the medium to 20°C. Once the temperature in the incubator is stable we will induce the cells with 0.1 mM IPTG.

The IPTG will activate the T7 promoter that is downstream of a sequence coding for the desired enzyme (FimE or Hbif) attached to a complex containing a his-tag and a SUMO tag (figure 1). The his-tag will help us in our purification because histidines bind to Nickel. The SUMO tag is useful because it make the protein more soluble and is 12kDa, therefore making it easier for us to identify on protein gels.

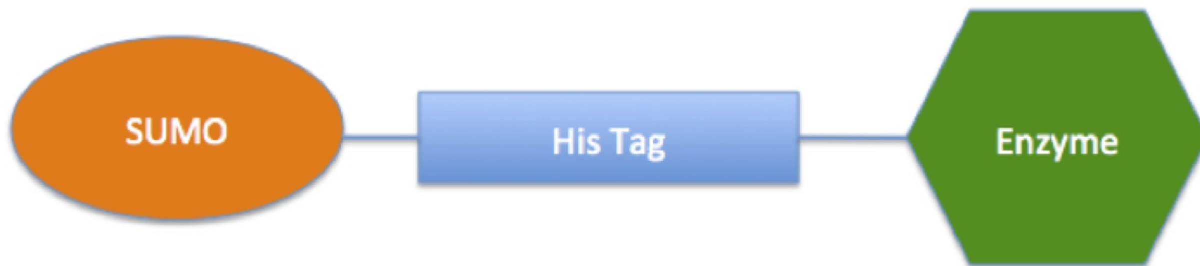

Figure 1: protein complex expressed in the cell.

The next day the medium will be spun down in order to collect the cells in a pellet. This pellet will then be re-suspended in enriched lysis buffer, which helps in lysing the cells (see protocol for recipe). The cells will be lysed once they are fully re-suspended with the help of a French Press (other instruments could be used for lysing). The lysed solution will then be centrifuged to separate cell debris and proteins. Once spun, the supernatant will be collected and put in a nickel column.
Through the nickel column, only the desired enzymes linked to the protein complex should bind to the column (with the exception of a few undesired protein that bind to nickel naturally). The column will then be washed with additional buffer in order to separate the maximum debris from our protein complex. Then a buffer containing ~200 mM imidazole will be used to elute our complex in a different flask than the one we used for the debris.
In order to separate the tag from the enzyme, a restriction enzyme will be added to the solution containing the complex that would cleave the linker between the enzyme and the tags (figure 2).

Figure 2: The restriction enzyme cuts the linker between the his-tag and the enzyme.

After letting the cleavage take place over night we will pass the solution through the nickel column again. This time the desired enzyme will pass through the column whereas the SUMO-his tag will stay bond to the column.
The elution containing the enzyme will then be passed through a cation affinity column. We will choose a cation affinity column for the purification of both enzymes because they have an isoelectric point above 9 and our buffers have a pH of 8, therefore both enzymes would be positively charged in solution (and bind to the column). After washing the column with buffer while the desired protein is bound to it, we will elute the protein by using a buffer with high salt concentration. In order to have the purest protein, it will be nice to do an elution gradient where the concentration of salt increases gradually in the column and a different vial would be used to collect the elution for each different salt concentrations.
We expect that after all these steps the enzyme would be pure.

In vitro assay

In this experiment, we will add pure Hbif or FimE to a solution only containing the plasmid with the switch. After reaction, we plan to undergo PRC purification in order to inactivate the proteins. The DNA will then be PCR amplified and run through a gel in order to identify if the switch worked. The results should be similar to those in figure 3. This experiment will tell us if the switch only needs these two enzymes in order to flip or if it requires additional cytoplasmic interactions.

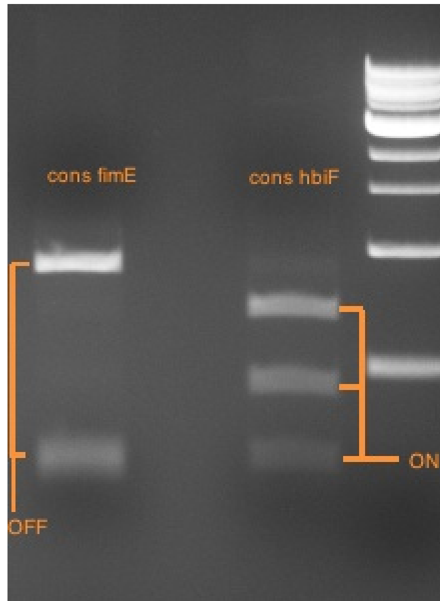

Figure 3: differences in bands depending on the enzyme used.

Phi-80 CRIM Chromosome Integration

The nature of our project altering the state of a transcriptional reporter construct from the “on” to “off” state raises problems with cells expressing both states. Because we are utilizing a plasmid based reporter construct, strains can possibly be expressing both the “on” and “off” states simultaneously if inducers are not binding the promoters at a sufficient rate. This problem could result in mixed state cells, introducing a variable that cannot be controlled for when using a plasmid based reporter strain.

To remove the problem presented by copy number with our reporter, we plan to integrate our reporter into the chromosome of *E. coli* to allow for single copy regulation of the locus. By integrating the reporter at a single site, there will be a forced phenotype of either “on”, expressing a fluorescent protein, or “off” expressing either no fluorescent proteins or a second color of protein.

Integrating our reporter at a single site will be done with the CRIM plasmid system, a three plasmid system used for chromosome integration. The system is composed of one plasmid, J72008, which expresses the phi-80 integrase protein, ampicillin resistance and a heat sensitive ORI, allowing the second plasmid, pAH153, containing the attP integration site to integrate DNA into the chromosome. A third plasmid, pCP20, is utilized to remove the antibiotic marker used for selection of successful transformants after integration of your DNA.

To accomplish this integration, we plan to use a protocol similar to that used by Anderson et. al,

in their 2010 publication in the Journal of Biological Engineering.
Protocol (Anderson, et. al, 2010): The target *E.coli* strain is to be transformed the J72008 and transformants will be selected on ampicillin plates at 30° C. Successful transformants will be made competent, transformed with the CRIM plasmid, containing chloramphenicol resistance, at 30°. Transformants surviving both selections will be grown overnight in LB supplemented with chloramphenicol at 37° C and then grown on plates at 43° C, allowing for the activation of the heat sensitive ORI and the integration of our construct. Genomic DNA will be utilized to identify successful transformants via PCR.