U of T iGEM Design Competiton 2014: Team C

Plasmid Loss Genetic Safeguards for Biocontainment

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Abstract

Conventional genetic safeguards of biocontainment often rely on various suicide mechanisms. However, these “kill switches” suffer from a fundamental flaw, in that they impose selective pressure on synthetic organisms to evolve inactivation of the genetic safeguard. We propose a new approach: plasmid loss genetic safeguards that remove genetically engineered traits from a population of synthetic organisms without imposing selective pressure against the safeguard itself. We aim to demonstrate the different evolutionary outcomes of kill switch versus plasmid loss systems, as well as to develop two implementations of plasmid loss genetic safeguards: one using the I-SceI restriction enzyme, and the other using Cre recombinase.

Introduction

The need for biocontainment

Synthetic biology has the potential for a variety of applications outside the limited confines of a lab-grown culture. For example, many biosensor and bioremediation systems developed using synthetic biology entail the release of genetically modified organisms into an outdoor environment. In addition, even synthetic organisms meant only for lab or industrial use could potentially escape into the environment though accidental release.

The potential impact of synthetic organisms on the natural environment is difficult to predict. However, as demonstrated by the devastating impact of invasive species on ecosystems around the world today, the introduction of new organisms into the environment poses significant risks. Thus, the development of reliable means of biocontainment will be essential for synthetic biology to be considered safe for routine use.

Conventional genetic safeguards for biocontainment

Many of the biocontainment systems being developed today are genetic safeguards that work by conditionally expressing a gene that leads to the host cell's death (Moe-Behrens et al. 2013). Such systems are called induced lethality, suicide, or "kill switch" systems. In 2012, the Paris Bettencourt iGEM team conducted a review of biosafety ideas in iGEM projects from 2006 to 2011 (http://2012.igem.org/Team:Paris_Bettencourt/Human_Practice/WikiScreen, last accessed April 15, 2014). Out of 32 records, 17 mentioned kill switches or some other sort of suicide mechanism.

The latest example of an iGEM project focused on biocontainment was Paris Bettencourt 2012's bWARE containment module. As part of their project, the team designed a system in which a population of *E. coli* cells are able to persist only for a limited amount of time. The mechanism for population-wide suicide at the end of the programed time limit is the expression of Colicin, a toxic protein that degrades *E. coli*'s chromosomal DNA.

The fundamental flaw of kill switch systems

At first glance, the use of kill switches seems to be a simple and effective solution to the problem of biocontainment. However, when considered from an evolutionary perspective, kill switches may not be as effective in practice as they are conceptually. This is because the lethality of a kill switch imposes a selective pressure on the population of synthetic organisms and incentivises the evolution of the population towards resistance to the kill switch. There has been evidence that in many implementations of kill switch systems, a small (but not insignificant) proportion of individuals in a population are able to survive the activation of the kill switch due to spontaneous mutations that render the kill switch ineffective (Knudsen and Karlström, 1991). This presents a significant challenge to the reliability of kill switches as genetic safeguards.

A new approach: Plasmid loss genetic safeguard system

We propose that a more effective genetic safeguard for biocontainment would remove genetically engineered traits (ie a biobrick plasmid) from a population of synthetic organisms, without otherwise harming the organisms themselves. The main difference between our proposal, which we call a plasmid-loss genetic safeguard system, and the conventional kill-switch genetic safeguard systems is that the plasmid-loss system does not impose a selective pressure on the synthetic organisms to evolve inactivation of the genetic safeguard.

The immediate effect of a plasmid loss system, upon turning it on (ie with an inducible promoter), would be that most individuals in a population of synthetic organisms would lose the genes responsible for the engineered trait and revert to a wild-type genotype and phenotype. As in the kill-switch systems, a small proportion of individuals could still retain the engineered trait due to mutation inactivation of the genetic safeguard system.

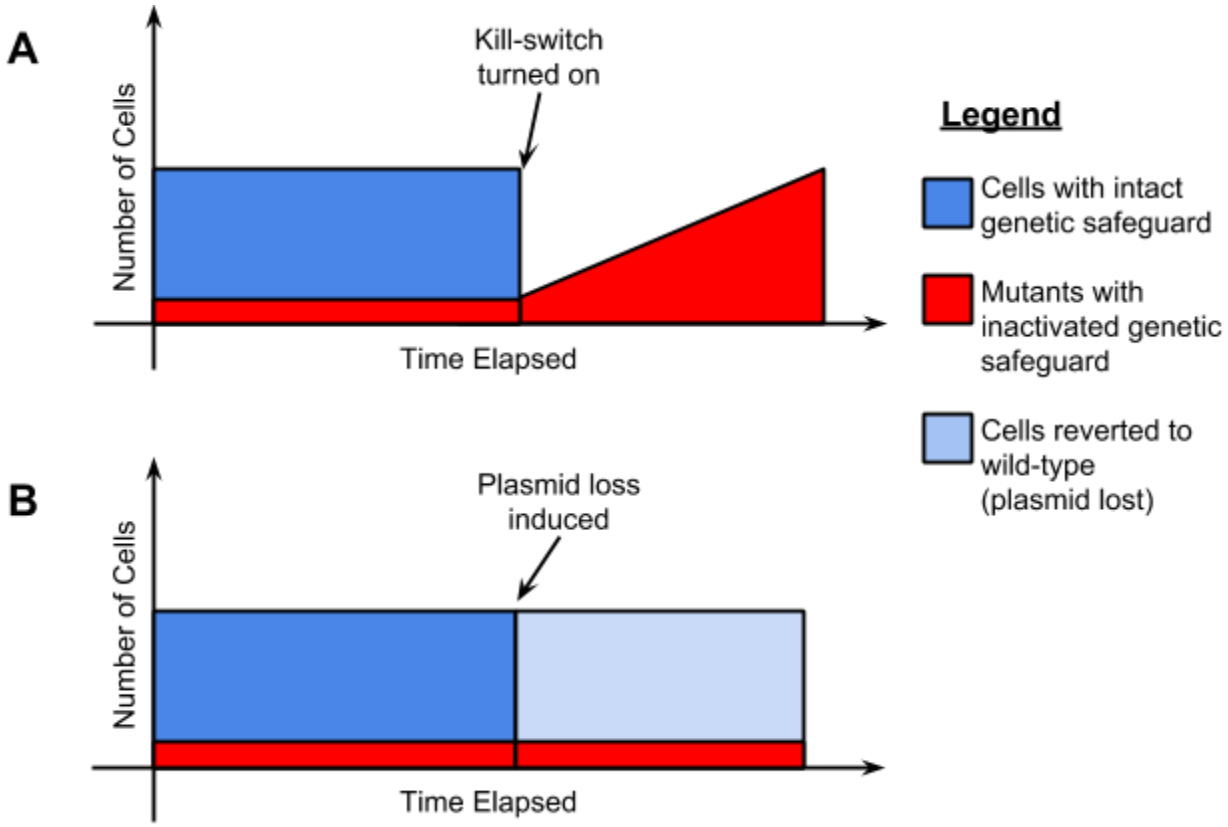

Figure 1: *The effect of (A) kill-switch and (B) plasmid loss genetic safeguard systems on a hypothetical population of synthetic organisms, containing a small proportion of mutant individuals resistant to the effect of the genetic safeguard. Not shown: the effect of genetic drift on the mutant sub-population over time.*

Although resistant mutants are present in both the kill switch and plasmid loss systems, their evolutionary outcomes differ:

- **Kill-switch system**: Once the system is turned on, all individuals with an intact genetic safeguard die, leaving a new population consisting entirely of resistant individuals. In evolutionary terms, the resistance allele has gone from a very low allele frequency to fixation (100% allele frequency).
- **Plasmid loss system**: The allele frequency of the inactivated genetic safeguard does not change. This is because the individuals with an intact genetic safeguard do not die, they simply revert back to the wild-type genotype and phenotype. In the new population, the proportion of resistant individuals in the population remains small.

The effect of genetic drift

A population consisting entirely of resistant individuals (the survivors of a kill-switch), even if it starts out small, could grow and spread, releasing a large amount of synthetic organisms into the environment.

On the other hand, in a population with only a small proportion of resistant individuals, the resistant subpopulation is crowded out by the rest of the wild-type population (after plasmid loss), and will not be able to monopolize all the resources available. Over time, the inactivated genetic safeguard allele, along with the engineered trait, is likely to be lost through genetic drift.

Of course, it is still possible that the resistant individuals, carrying the engineered trait, could have enough of a fitness advantage to out-compete the wild-type (plasmid-loss) individuals in the population. However, this outcome is no worse than the worst-case scenario of kill-switch systems. We expect that this worst-case scenario will be unlikely to occur. Even if the engineered trait confers a slight fitness advantage to the resistant individuals, it may not be enough of a selective advantage to overcome the effect of genetic drift.

Design criteria for plasmid loss genetic safeguard systems

An effective plasmid loss genetic safeguard system will need to meet the following three design criteria:

1. Ability to inactivate/destroy plasmid, or increase rate of plasmid loss
    - If the efficiency of plasmid loss is low, the system will not be effective at biocontainment
2. Affects the plasmid only, no other significant costs on cell.
    - It is essential that fitness advantage of developing a mutation against the plasmid loss system be kept as low as possible. If the system is too costly, populations will evolve resistance to it.
3. Inducible/repressible expression
    - During culturing in the lab, plasmid loss is undesirable. If the system causes plasmid loss even in a lab environment, selection for plasmid retention (as is standard lab procedure) will result in selection for individuals resistant to the effects of the plasmid loss system.
    - Therefore, the system must be turned off during lab culturing, and must only become active when the synthetic organism is released into the environment
    - For some applications, it may also be desirable that the activation of the plasmid loss system occur on a time-delay, so that the synthetic organism can perform its intended function outside the lab.

Implementation

Our project will pursue three main goals:

1. Demonstrate the different evolutionary outcomes of kill-switch versus plasmid loss genetic safeguards
2. Implement a plasmid loss genetic safeguard using the I-SceI restriction enzyme
3. Implement a plasmid loss genetic safeguard using the Cre recombinase system

Work on each of these three goals can proceed in parallel.

Goal 1: Kill-switch versus plasmid loss genetic safeguards

We will experimentally simulate the outcome of each genetic safeguard using an *E. coli* strain with an antibiotic resistance plasmid (+P strain), and a wild-type *E. coli* strain (-P strain).

A population consisting only of the +P strain represents a population after the activation of a kill-switch. The individuals in this population represents mutants that were able to survive the kill-switch due to mutation-inactivation of the kill-switch genes.

A population consisting of both +P and -P strains represents a population after the activation of a plasmid loss genetic safeguard system. The -P individuals represents the individuals that had lost the plasmid due to the plasmid loss system, while the +P individuals represents mutants that were able to retain the plasmid.

The evolutionary outcome of each genetic safeguard system will be observed by growing each population separately in continuous-flow liquid cultures over a period of time (possibly several days). A continuous-flow liquid culture is used instead of a conventional (finite) liquid culture because the periodic subculturing required to maintain a liquid culture over time may interfere with our ability to manipulate genetic drift (see below). In addition, the transitioning between lag/log/stationary phases of bacterial growth may introduce confounding factors, such as physiological changes in response to nutrient depletion, and decreasing antibiotic concentrations (changes the strength of selection for P+ individuals).

If the equipment/facilities for a continuous-flow liquid culture are not available, it may also possible to run this experiment by manually removing old media and adding fresh media to a liquid culture on a set schedule.

In each culture, samples will be taken at regular intervals. The cell density (population size) will be measured by using absorbance readings, and colony counts on serially diluted plates. In addition, the proportion of +P individuals in each population will be determined by plating on both antibiotic and non-antibiotic plates. This is necessary even for the kill-switch population, because we also want to measure the natural rate of plasmid loss.

The variables we will be manipulating in this experiment are as follows:

- Initial population size of the kill-switch population
    - Represents kill-switch efficiency
- Initial proportion of +P individuals in the plasmid loss population
    - Represents the efficiency of the plasmid loss system
- Concentration of antibiotic in the culture medium
    - Represents the strength of selection for the engineered trait on the plasmid
- Frequency of taking a sample from the culture and starting a new culture with it
    - Represents the impact of genetic drift; each subculture is a bottleneck event

We hypothesize that the kill-switch populations will remain almost entirely composed of +P individuals over time, regardless of its initial population or genetic drift. There may be some plasmid loss in the cultures with low/no antibiotics, but this will occur at a relatively slow rate.

In the plasmid-loss populations, we expect to see a decrease in the proportion of +P individuals over time in the cultures where the initial proportion of +P individuals is low, strength of selection for P+ trait is low, and genetic drift is high.

Goal 2: I-SceI restriction enzyme

One possible way to implement a plasmid loss genetic safeguard system is to incorporate a restriction enzyme gene as well as its restriction site into a plasmid with an engineered trait (such as antibiotic resistance). When the restriction enzyme is expressed, it cuts the plasmid at the restriction site, resulting in linear DNA that is degraded by the bacterial host cell. This causes the loss of the plasmid carrying the engineered trait.

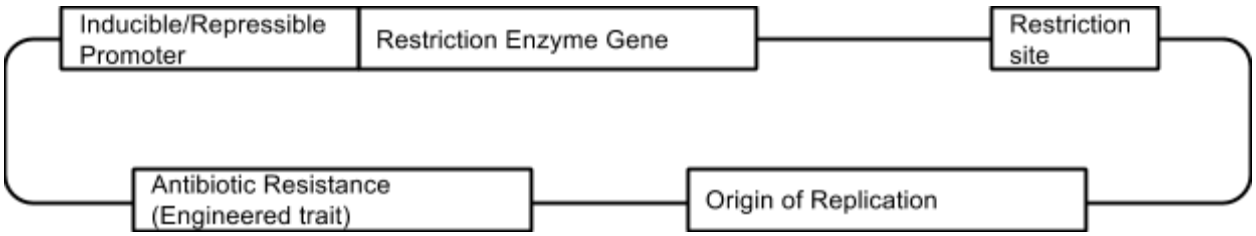

Figure 2: *A plasmid incorporating a restriction enzyme plasmid loss genetic safeguard system (AKA a self-restricting plasmid)*

One of the requirements for a plasmid loss genetic safeguard is that such a system must not harm the cell (otherwise, it is no different from a kill-switch). Thus, it is essential that the restriction enzyme cut only the plasmid and not the host cell's chromosomal DNA.

Previous iGEM teams (TUDelft 2009, Paris Bettencourt 2012) have attempted to create such a self-restricting plasmid using the I-SceI meganuclease. The I-SceI restriction enzyme was chosen because the restriction site it recognizes is 18-bp long. Statistically, a specific 18-bp sequence will only occur by random chance one in every 6.9×10^{10} base pairs, 20 times the size

of the human genome (Paris Bettencourt 2012). Since it is unlikely that an I-SceI restriction site occurs in the host cell's chromosomal DNA, I-SceI expressed from a self-restricting plasmid will cut only the plasmid itself and not the host cell's chromosomal DNA.

Previous attempts by other iGEM teams

The first attempt to engineer a self-restricting plasmid using I-SceI was done by TUDelft 2009. The team biobricked the restriction site recognized by I-SceI and showed that it could be cleaved by I-SceI in vitro. However, the self-restricting plasmid they created did not show self-restricting activity. This was because the I-SceI gene sequence they were using included an LVA degradation tag, which may have decreased the lifespan of the protein within the cell or interfered with its normal folding.

The next iGEM team to use I-SceI was Paris Bettencourt 2012. Unlike TUDelft, Paris Bettencourt started with the gene sequence of I-SceI without the LVA degradation tag. The main promoter candidate to be used to control the I-SceI gene expression was pRha (an L-rhamnose inducible promoter), although the team also tested pLac and pBad. pRha was chosen because it can be tightly repressed in the absence of L-rhamnose or in the presence of glucose.

In their experiment, Paris Bettencourt 2012 transformed two plasmids into *E. coli*:

- Plasmid 1 (iGEM parts: K914005, K914007, K914008)
    - Contained the I-SceI gene under the control of an inducible promoter (either pRha, pLac, or pBad)
    - Chloramphenicol resistance
- Plasmid 2 (iGEM part: K175027)
    - Target plasmid, contained an I-SceI restriction site
    - Ampicillin and Kanamycin resistance

The team then plated the transformed cells onto Chloramphenicol, Ampicillin, and Chloramphenicol + Ampicillin plates. They found that even with pRha repressed by glucose, no colonies grew on the Chloramphenicol + Ampicillin plates. After doing further experiments to show that these results were not due to the incompatibility of the two plasmids, and that pRha does show a response to induction with L-rhamnose (repressed in the absence of L-rhamnose), Paris Bettencourt concluded that I-SceI may have been so efficient that even a tiny amount of leaky expression resulted in the cleaving and degradation of the target plasmid.

Paris Bettencourt 2012 also measured the efficiency of the I-SceI plasmids made by TUDelft 2009. Paris Bettencourt found that TUDelft's plasmids that expressed I-SceI were able to cleave a target plasmid with the I-SceI restriction site within the cell. However, the efficiency was not very high (probably due to the LVA degradation tag). When I-SceI expression was induced, 9% of cells were still able to retain the target plasmid.

Building upon previous work

In our project, we will build upon the work done on an I-SceI self-restricting plasmid by previous iGEM teams.

First, we will confirm the conclusion of Paris Bettencourt 2012 (that I-SceI works, but has problems with leaky expression). We will replicate Paris Bettencourt's experiment, with the addition of an additional negative control: a plasmid with the I-SceI gene but no promoter. This plasmid should have no leaky expression, since it does not contain a promoter.

We will also perform additional assays to quantify the amount of leaky expression that is occurring. These assays include reverse transcriptase quantitative PCR, SDS-PAGE, and Western blot.

Reducing leaky expression with T7 phage promoter

If leaky expression of the pRha promoter is indeed the explanation for Paris Bettencourt 2012's results, we will try using the T7 phage promoter to reduce leaky expression. The T7 phage promoter is useful for this purpose because it is highly orthogonal to the host cell's RNA polymerases (Iyer S, Karig DK et al. 2013). Using the T7 promoter would effectively insulate the expression of our gene of interest (I-SceI) from the host cell's transcriptional machinery.

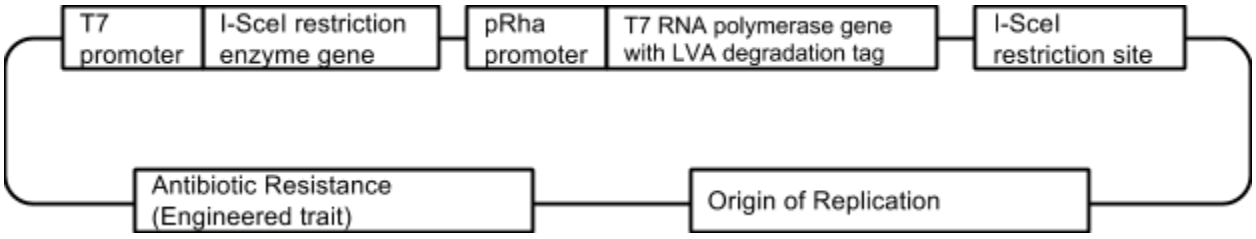

Figure 3: *A self-restricting plasmid, where the I-SceI restriction enzyme gene is controlled by a T7 phage promoter.*

Since only the T7 RNA polymerase recognizes the T7 promoter, one way of regulating the expression of the T7 promoter is by regulating the level of T7 RNA polymerase (RNAP) in the cell. This can be done by incorporating the gene encoding T7 RNAP into the plasmid, under the control of an inducible/repressible promoter such as pRha. Although the T7 RNAP may itself be subject to leaky expression (due to the pRha promoter), we can minimize the effect of any expression that does take place by adding an LVA degradation tag to the T7 RNAP gene sequence. As demonstrated by the problems encountered by TUDelft 2009, the LVA degradation tag is capable of reducing the lifespan and activity of an enzyme.

If regulating the level of T7 RNAP cannot reduce leaky expression of the I-SceI gene to sufficiently low levels, it is also possible to use T7 promoter variants that are engineered to be repressible by transcription factors such as LacI and TetR (Iyer S, Karig DK et al. 2013).

Goal 3: Cre recombinase

Another way to implement a plasmid loss genetic safeguard system is to use Cre recombinase to remove the origin of replication from a plasmid. Cre recombinase is a site-specific recombinase that recognizes loxP sites (Nagy 1999). A loxP site is 34 bp long, even longer than the I-SceI restriction site, so it is highly unlikely that a loxP site would occur randomly in the host cell's chromosomal DNA.

Cre recombinase is capable of excision, integration, or inversion of the DNA sequence between two loxP sites, depending on the orientation of the loxP sites. If the two loxP sites are in the same orientation, the DNA between them will be excised. For our purposes, we are interested in excising the origin of replication, so our origin of replication will be flanked with loxP sites facing the same orientation.

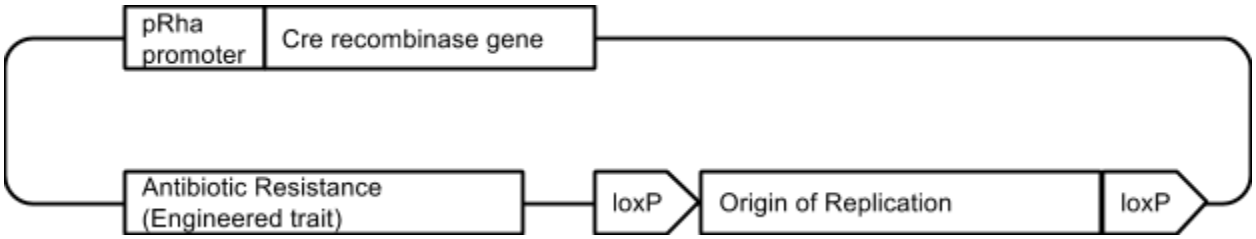

Figure 4: *A plasmid incorporating a Cre-recombinase genetic safeguard system*

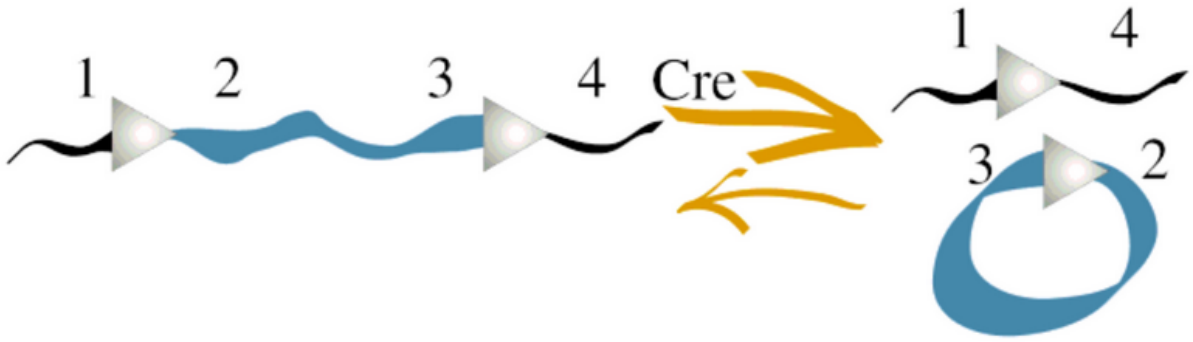

Figure 5: *A schematic of the Cre recombinase excision/integration reaction (reproduced from Nagy 1999)*

The result of the Cre recombination reaction is a plasmid carrying the engineered trait but without an origin of replication, and a very small plasmid consisting only of the origin of replication. Without an origin of replication, the engineered plasmid cannot undergo DNA replication and will not be maintained in the population. The small plasmid consisting only of the origin of replication may be retained, but this is not a problem since it does not carry the engineered trait.

Another potential problem with using Cre recombinase is the possibility of a reverse reaction (integration) occurring after the origin of replication has been excised. However, since the chemical equation is constantly being shifted to the right by the loss of the origin-less plasmids at each cell division, we expect that excision will be favoured over integration.

Summary of Methods

Goal 1: Kill-switch versus plasmid loss genetic safeguards

- Measure growth over time, E. coli with and without antibiotic resistance plasmid
    - Continuous-flow culture
    - At time-points, plate cells on plates with/without antibiotics, count colonies

Goal 2: I-SceI restriction enzyme

- Replicate Paris Bettencourt 2012's experiment (test for ability to induce plasmid loss)
    - Two plasmids
        i. (pLac/pBad/pRha) + I-SceI + CamR
        ii. I-SceI restriction site + AmpR, KanR
    - Double transformation
    - Grow in Cam plates, Amp plates, Cam+Amp plates (+ glucose to repress pRha)
    - See if any colonies grow on Cam+Amp plates
- Quantify leaky expression
    - Transform with pRha + I-SceI
    - Grow on plates with glucose
    - Measure using:
        i. Reverse transcription quantitative PCR
        ii. SDS-PAGE, Western blot
- T7 phage promoter
    - assemble plasmid/biobrick (see Figure 3)
    - quantify leaky expression, test for ability to induce plasmid loss (as above)

Goal 3: Cre recombinase

- Assemble plasmid/biobrick (see Figure 4)
- test for leaky expression, ability to induce plasmid loss (as above)

Feasibility

Except for the flow-culture apparatus, all of the equipment necessary for this project can be found in a typical molecular biology lab. As mentioned earlier, the flow-culture apparatus would be nice to have but it is not strictly necessary for this project.

Of the three goals of this project, we expect goal #1 to take the longest time, since each replicate of the experiment is labour intensive (at each time point, need to serially dilute, plate many dilutions, etc.) and may need to be run over several days. Fortunately, this experiment does not require advanced molecular biology techniques, so undergraduate students should be easily capable of doing the work required.

Goals #2 and #3 do not seem to require much time if all goes well. However, as demonstrated by the experience of previous iGEM teams (Paris Bettencourt 2012 and TUDelft 2009), it is likely that unexpected problems will arise during the implementation of these ideas. The molecular biology techniques required for these two goals are moderately advanced, so first and second year undergraduates may require some training. Once learned, these techniques are not expected to be beyond the capabilities of undergraduate students.

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