Contents

1 Designing new switches

1.1 Introduction

In the previous Chapters I studied the effect that adding positive feedback loops to the genetic toggle switch has on the robustness of the system. I found that adding two positive feedback loops to the simple toggle switch can increase its parametric robustness. The next step in this analysis ~~is to~~ test these predictions experimentally. Therefore, in this Chapter I provide the experimental design for the construction of the genetic toggle switch with single and double positive autoregulation. The newly constructed switches ~~can then be compared~~ to the simple Litcofsky et al. (2012) toggle switch experimentally. Their robustness can be tested by varying the experimental conditions, like temperature and pH, and measuring the response of the switch.

Structurally, this Chapter is organised as follows: First I provide an overview of the cloning plan, by listing the relevant BioBrick parts used and their interactions. Then I outline the experimental design for producing these switches.

1.2 Cloning overview

The Litcofsky et al. (2012) toggle switch plasmid, pKDL071, used in Chapter (XXX) is modified to construct three new switches. Two switches will have single positive autoregulation, one on each gene, and one switch will have positive autoregulation on both genes. An overview of the cloning stages to be carried out is shown in Figure 1.1. The three stages required for the cloning plan to be completed are outlined in the sections below.

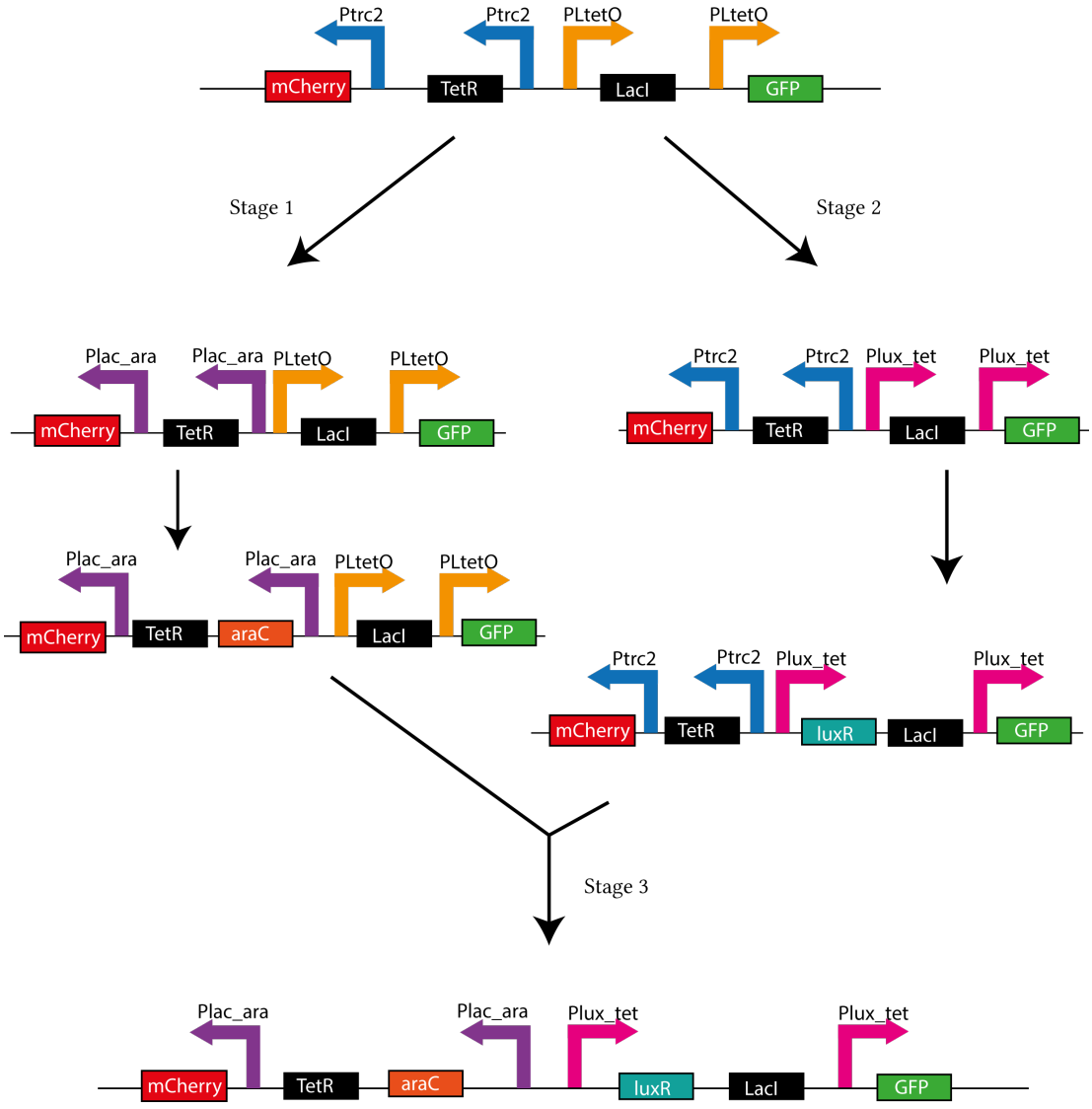

Figure 1.1 : An overview of the cloning plan to produce three new switches, two with single positive autoregulation and one with double positive autoregulation.

1.2.1 Resulting switches

The three switches shown in Figure 1.2 will be constructed through this cloning process. The first switch, on plasmid pKDL071-plac/ara-araC is a toggle switch with positive autoregulation on the TetR/mCherry side of the switch. The second plasmid, pKDL071-pLuxTet-luxR consists of a toggle switch with positive autoregulation on the LacI/GFP side of the switch. Finally, the switch with positive autoregulation on both sides of the switch is on the pKLD0713a plasmid. The plasmid maps and a schematic of their components' interactions are shown in Figure 1.2.

Figure 1.2 : The plasmid maps of three new switches to be constructed. The first two switches have a single positive autoregulation on each side of the switch respectively. The switch on the far right has positive autoregulation on both genes.

1.3 Experimental design

The construction of the three switches shown in Figure 1.2 was broken down in three stages, one for the construction of each switch. In this section I will outline the necessary cloning steps that need to be carried out in order to construct each switch. The detailed methods for each cloning step are described in Section (XXX). All primer sequences are given in Appendix (XXX). Following the construction of each plasmid outlined below, competent *E.coli* cells were transformed following the method outlined in Section (XXX).

1.3.1 Stage 1 - Construction of pKDL071-plac/ara-araC

In order to construct plasmid pKDL071-plac/ara-araC with single positive autoregulation on the mCherry/TetR side, the P_{trc2} promoter was swapped for the $P_{lac\_ara\text{-}1}$ and AraC was added upstream of TetR. $P_{lac\_ara\text{-}1}$ is activated by arabinose (AraC) and repressed by LacI (Lutz & Bujard 1997), and is thus ideal. The $P_{lac\_ara\text{-}1}$ promoter is present in the pJS167 plasmid, which was ~~a gift from~~ Jeff Hasty (Addgene plasmid # 48881) (Stricker et al. 2008). This promoter is also present in the BioBrick registry of standard biological parts as BBa_K1713000.

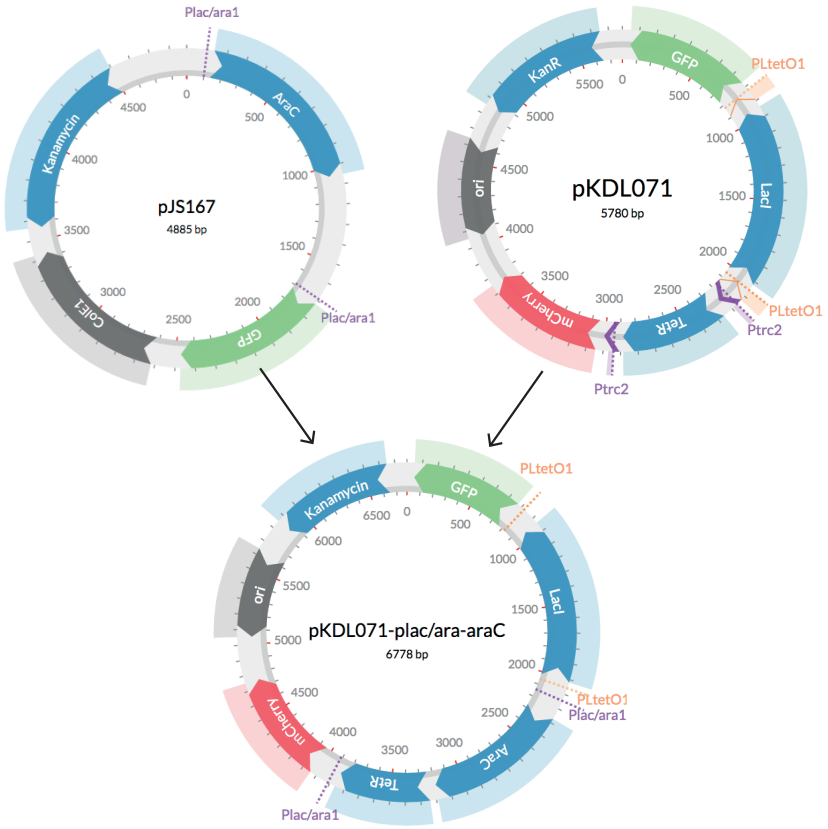

Figure 1.3 : Stage 1 cloning procedure. The pKDL071-plac/ara-araC plasmid is constructed via PCR cloning from the pJS167 and pKDL071 plasmids.

Using PCR cloning, the $P_{lac\_ara\text{-}1}$ promoter was cloned from pJS167, with added XmaI and KasI restriction enzyme sequences on each end. Both pKDL071 and the PCR product were digested with XmaI and KasI restriction enzymes. After gel electrophoresis and gel extraction, the two were subsequently ligated. The resulting plasmid should have the $P_{lac\_ara\text{-}1}$ promoter instead of the P_{trc2} promoter upstream of mCherry.

Following that, PCR cloning was used to clone $P_{lac\_ara\text{-}1}$ and AraC from the pJS167 plasmid. EagI and SalI flanking sequences were added via PCR on the 5' and 3' ends. Plasmid pKDL071 and the PCR product were digested using EagI and SalI. Following gel electrophoresis and gel extraction, the two products were ligated in order to complete the pKDL071-plac/ara-araC plasmid. The detailed methods for each cloning technique mentioned here can be found in Section (XXX). All PCRs and digestions described here were completed successfully (data not shown). The ligated products were transformed into thermocompetent *E.coli*, but following plasmid sequencing, the cloning was found to be unsuccessful.

1.3.2 Stage 2 - Construction of pKDL071-pluxtet-luxR

In order to construct the plasmid pKDL071-pluxtet-luxR, the $P_{Lux/tet}$ promoter is necessary. The $P_{Lux/tet}$ promoter is present in the BioBrick registry of standard biological parts as BBa_K934024. $P_{Lux/tet}$ is a hybrid promoter activated by LuxR and repressed by TetR. This promoter is added in exchange of $P_{LtetO\text{-}1}$ to the pKDL071 plasmid. The LuxR gene is also added upstream of LacI, in order to construct a switch with positive autoregulation on the LacI/GFP side.

First, the $P_{Lux/tet}$ promoter was synthesised. The reverse complement of BBa_K934024 with added flanking sequences of EcoO1091 and SphI on the 5' side and AclI and Eag1 at the 3' side. These were added to aid with further cloning steps. The sequence synthesised is given below:

5'- TTGGGACCTGCATGCTAATCTCTATCACTGATAGGGATAATACGAGTATCTC
TATCACTGATAGGGAGTAAACCTGTACGATCCTACAGGTAACGTTCGGCCG -3'

This sequence was added to an pIDTSMART-KAN plasmid backbone. Synthesis was carried out by Integrated DNA Technologies, Inc. (Leuven, Belgium, http://eu.idtdna.com/CodonOpt). *E.coli* Dh5α was transformed with the synthesised plasmid. The method is outlined in Section(XXX).

The pLux/tet and pKDL071 plasmids were subsequently digested with SphI and AclI. Following gel extraction and ligation, the $P_{Lux/tet}$ promoter was added upstream of GFP, replacing the $P_{LtetO-1}$ promoter. Then, the plux/tet and pKDL071 plasmids were digested with EcoO109I and EagI restriction enzymes. Following gel extraction and digestion, the $P_{Lux/tet}$ promoter was added upstream to LacI, replacing the $P_{LtetO-1}$ promoter.

The final stage of constructing the pKDL071-pluxtet-luxR plasmid consists of PCR cloning of the pTD103aiiA(Cm) plasmid with added BsGI flanking sequences at both ends. The pTD103aiiA(Cm) was ~~a gift from~~ Jeff Hasty (Addgene plasmid # 48886) (Prindle et al. 2012). The plasmid constructed in the previous step and the PCR product are digested with BsGI restriction enzyme. Following gel extraction and ligation, the pKDL071-pluxtet-luxR should be complete. The ligated products were transformed into thermocompetent *E.coli*, but following plasmid sequencing, the cloning was found to be unsuccessful.

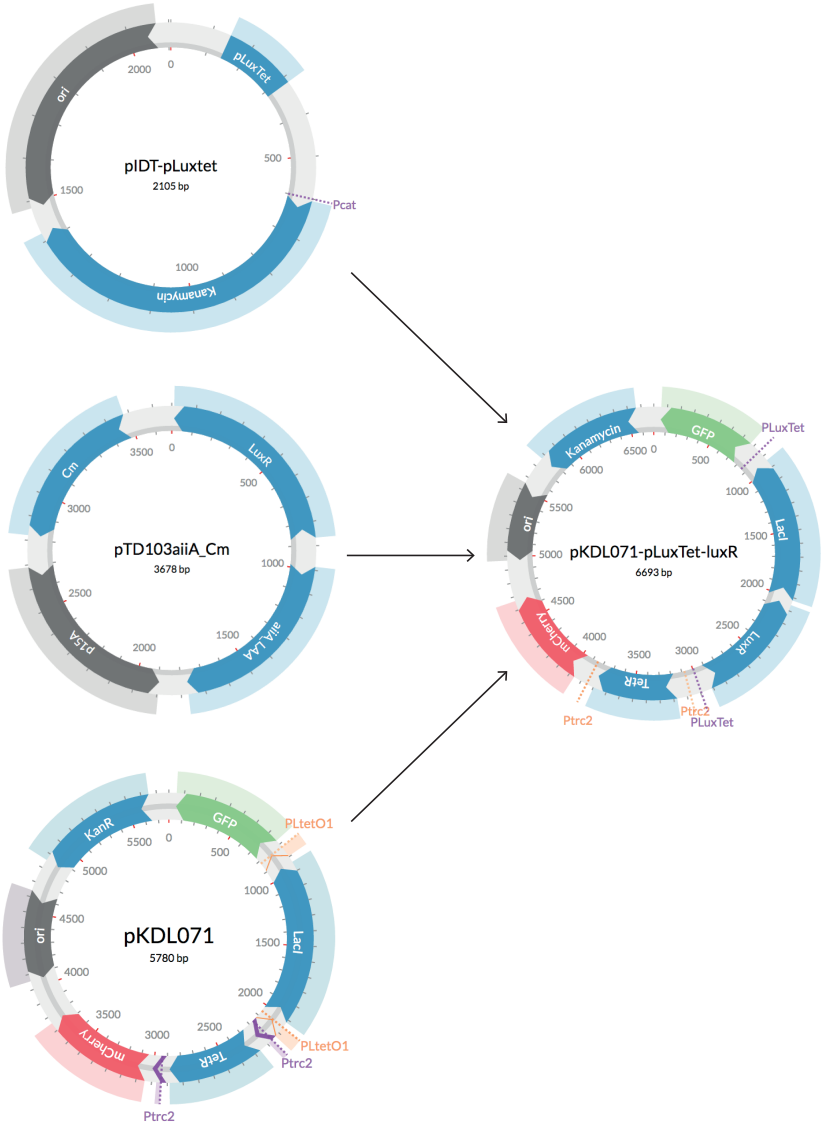

Figure 1.4 : Stage 2 cloning procedure. The pKDL071-pluxtet-luxR plasmid is constructed via PCR cloning from the synthesised $P_{Lux/tet}$ promoter, pKDL071 and pTD103aiiA(Cm) plasmids.

1.3.3 Stage 3 - Construction of pKDL0713a

The final construction stage requires the complete pKDL071-plac/ara-araC plasmid, as well as the synthesised $P_{Lux/tet}$ promoter and pTD103aiiA(Cm) plasmid used in Stage 2. Since the construction of the plasmids was unsuccessful in Stages 1 and 2, the following experimental plan has not been executed.

The plux/tet plasmid and pKDL071-plac/ara-araC are digested with SphI and AclI restriction enzymes. This is followed by gel extraction to isolate the fragments of interest. These are then ligated to result in the modified pKDL071-plac/ara-araC plasmid, (pKDL071-plac/ara-araC-pluxtetA) with $P_{Lux/tet}$upstream of GFP instead of $P_{LtetO-1}$.

Then, the plux/tet plasmid and the plasmid created above (pKDL071-plac/ara-araC-pluxtetA) are digested with EcoO109I and EagI. Following gel extraction and ligation, the $P_{Lux/tet}$ promoter is added upstream of LacI instead of $P_{LtetO-1}$ to make a new plasmid, pKDL071-plac/ara-araC-pluxtet. Subsequently, the PCR product produced above of pTD103aiiA_Cm with BsGI flanking sequences and pKDL071-plac/ara-araC-pluxtet are digested using BsGI. The framgments of intertest are then extracted following gel electrophoresis of the digested products and ligated. The ligates are screened for the correct orientation of the insert.

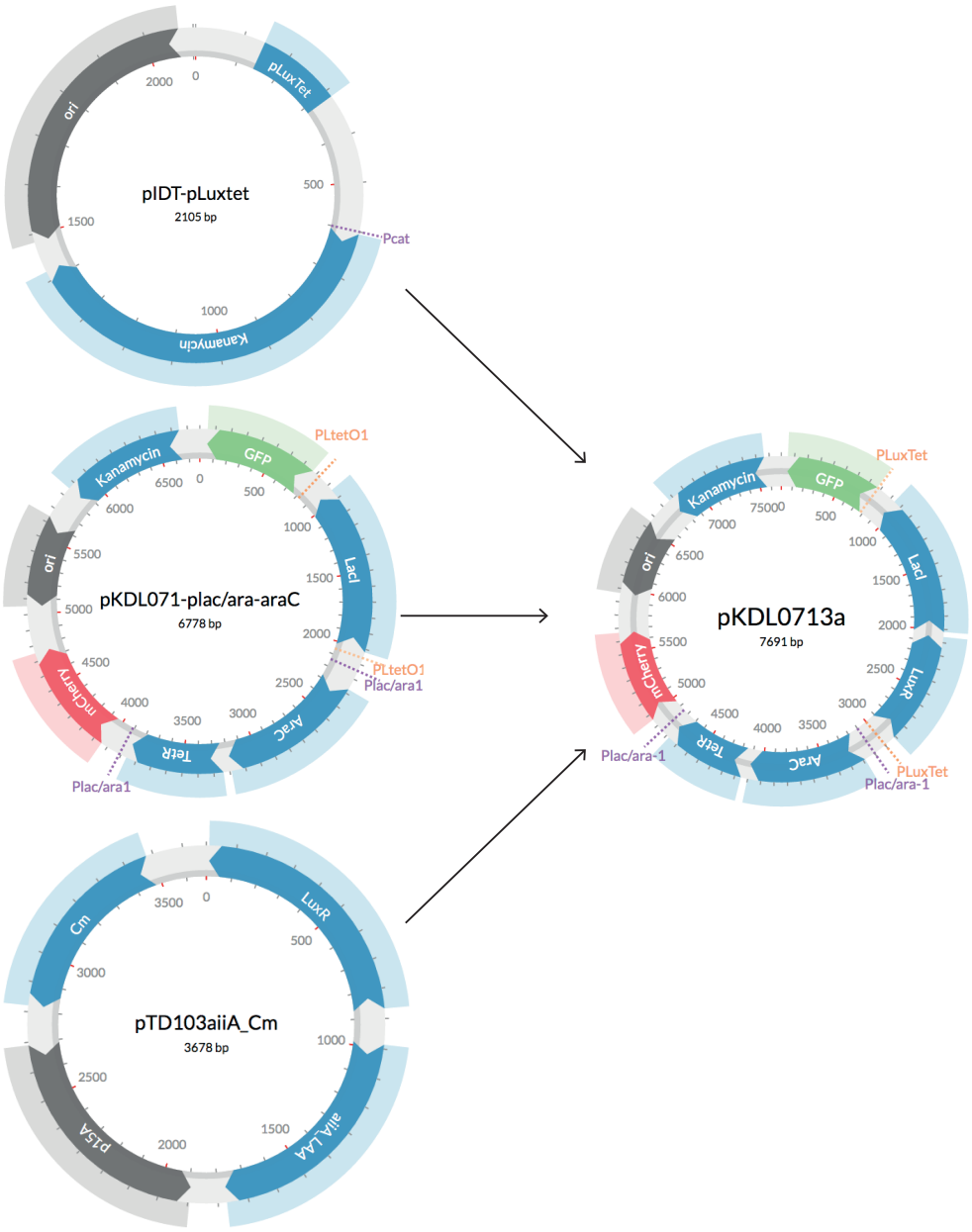

Figure 1.5 : Stage 3 cloning procedure. The pKDL0713a plasmid is constructed via PCR cloning from the synthesised $P_{Lux/tet}$ promoter, pKDL071-plac/ara-araC and pTD103aiiA(Cm) plasmids.

1.4 Summary

In this Chapter I designed the experimental protocol to be followed in order to construct three novel switches. These switches can be used in the future in synthetic biology applications. The execution of the eperimental protocol described has not been completed to date but constitutes the future directions of this project.

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