

Introduction

Our biothermometer is an *Escherichia coli* that changes color at different temperatures. Such a biological thermometer can for example be applied as a temperature reporter system in large-scale fermentations, or as a temperature-inducible protein production system. A class of known biological temperature sensitive switches are called RNA thermometers. Below a certain temperature

the secondary structure of an RNA thermometer¹ masks the Shine-Dalgarno (SD) sequence in a hairpin. Above this temperature the hairpin opens up, allowing the ribosome to bind (Fig.1). The input temperature is then converted to a color output by switching on the isoprenoid biosynthesis pathway (Fig.5). Ethical issues of this project were considered during the design and production phase.

Color Output

For producing enough farnesyl diphosphate (FPP) to produce clearly visible colors, FPP needs to be overexpressed. To achieve this an isoprenoid biosynthesis pathway, part of the *Saccharomyces cerevisiae* mevalonate pathway combined with *E. coli* enzymes, should be engineered into *E. coli*. It has been shown that this combination of enzymes can overproduce FPP in *E. coli*.⁵ This pathway is combined with several Edinburgh BioBricks to produce a red, orange and yellow color, with the phytoene desaturase, lyopene B-cyclase, and 3,3'-B ionone hydroxylase enzymes, respectively (Fig.5).



Figure 5. Enzymes (blue) and metabolites (black) of the isoprenoid biosynthesis pathway.

Temperature Input

Parts Design

An RNA thermometer (Fig.1) is a temperature sensitive regulation that switches translation at a certain temperature. This post-transcriptional regulatory system is used as the input for our system. Three RNA thermometers from literature^{2,3,4} have been turned into BioBricks.



Figure 1. The principle of an RNA thermometer. When the temperature rises above a certain threshold the SD sequence becomes exposed leading to initiation of translation.

Two other RNA thermometers have been designed that theoretically switch at a lower temperature. In order to achieve this, the RNA sequence of an existing RNA thermometer is changed to destabilize the temperature sensitive hairpin. This will cause it to unfold and thereby initiate the translation at a lower temperature.

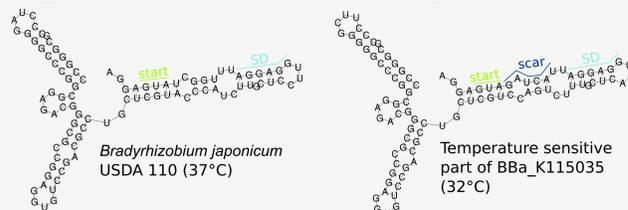


Figure 2. A designed RNA thermometer with a theoretical switching point of 32°C based on an existing RNA thermometer from *Bradyrhizobium japonicum* with the original switching point of 37°C. The temperature sensitive hairpin, containing the SD sequence, is destabilized in order to lower the switching temperature.

Theory

Translation of proteins without the regulation of an RNA thermometer gradually increases with increasing temperature. RNA thermometers suppress translation of the protein below a threshold temperature. A steep increase in translation can be observed above this threshold (Fig.3). Constructs were made that have the luciferase gene under control of the designed RNA thermometers. Upon addition of substrate, luciferase catalyses a chemoluminescent reaction. Luminescence can be measured to visualize protein expression.

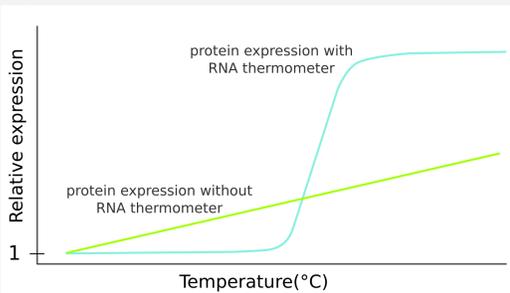


Figure 3. Idealized representation of non-RNA thermometer protein expression vs. RNA thermometer protein expression.

Experimental Results

The effect of the designed RNA thermometers on translation were analyzed by luciferase assays. Cultures of *E. coli* bearing the luciferase gene under regulation of different RNA thermometer sequences were grown at several temperatures. BBa_K115035 is supposed to switch at 32°C. We have shown a steep luminescence increase as expected for temperature induced gene expression regulated by an RNA thermometer (Fig.4).

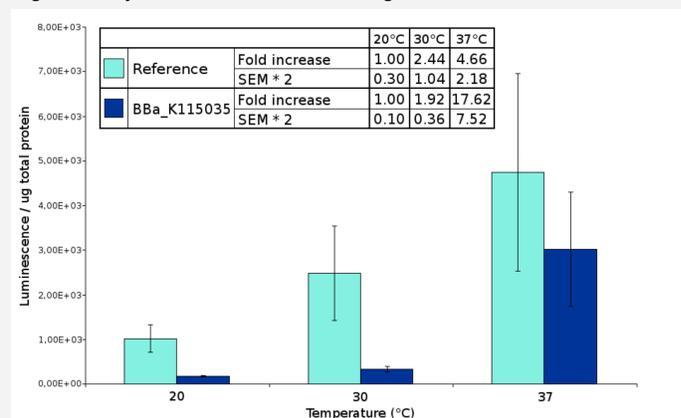


Figure 4. An overview of the luminescence measured per µg protein for the reference (BBa_K115012) and BBa_K115035 constructs. The table indicates the ratio of luminescence to the amount of luminescence measured at 20°C.

Modeling

For the kinetic model of this pathway we used kinetics based on Michaelis-Menten equations. The activities of the last three enzymes are set under control of the temperature switch. The equation used for modeling the enzyme kinetics, including its dependency on temperature, shown in Eq.1, is based on Hill-type equations. Moreover, we introduced switching behavior at different temperatures. Parameters of the model are estimated

$$[e/e_0] = \alpha \frac{T^m}{T^m + K^m} \frac{1}{T^p + K_p^p} + 1$$

Equation 1. The kinetic model for the last three enzymes.

by using genetic algorithms. Finally the *in vitro* analyzed enzyme concentrations have been used to estimate the general kinetics for the pathway. The switching behavior is shown in Fig.6. Sensitivity analysis proved the importance of the inhibition function and the role of enominator's power in the estimations. The Bifurcation analysis showed the stability of the model for all estimated parameters.

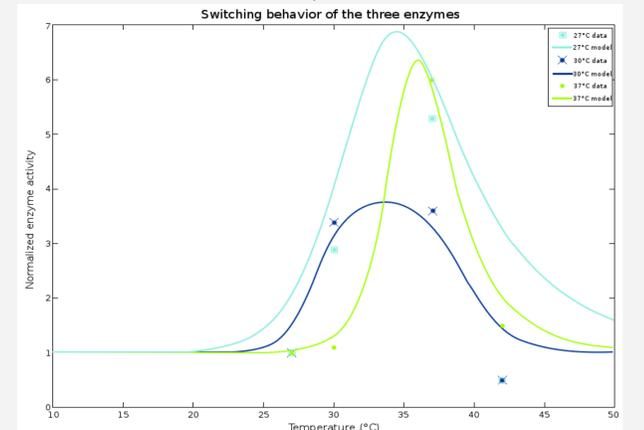


Figure 6. The switching behavior of the three enzymes at 27°C, 30°C and 37°C plus comparison with the lab results.

Ethics

By considering ethical issues early in the project, i.e. in the design phase, ethical issues can be incorporated in the design instead of considered in hindsight. Moral obligations and considerations of the team members played a central role.

- 1) Value sensitive design** A Value Sensitive Design session was organized to help the team consider ethical issues in biological design, even at the very beginning of the project.
- 2) Literature survey – Macro ethics** To learn which ethical issues play in the field of Synthetic Biology, a literature survey was carried out. Fig.7 depicts the issues identified and investigated.
- 3) Questionnaire – Micro ethics** A questionnaire (based on the survey and VSD session) was developed by which all team members were individually interviewed, to learn their opinions, to generate awareness of the ethical issues and to reflect on these issues.
- 4) Question of the week** After the interviews, ethical questions were asked during the weekly meetings, to stimulate discussion on ethical issues relevant to the project.

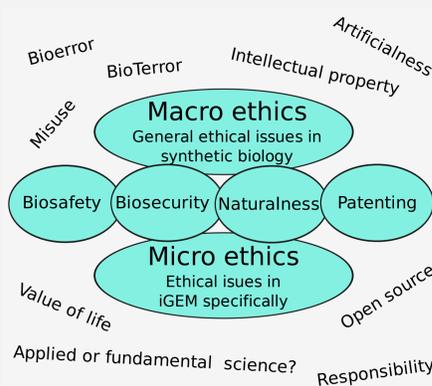


Figure 7. Ethical issues identified and discussed during the competition.

References

1. F. Narberhaus *et al.* RNA thermometers. *FEMS Microbiol Rev*, 30(1):3-16, 2006.
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3. T. Waldminghaus *et al.* FourU: a novel type of RNA thermometer in *Salmonella*. *Molecular Microbiology*, 65(2):413-424, 2007.
4. J. Johansson *et al.* An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell*, 110(5):551-561, 2002.
5. V. Martin *et al.* Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnology*, 21(7):796-801, 2003.

Conclusions

We were able to confirm the functionality of one RNA thermometer (BBa_K115035) experimentally. Furthermore, mathematical models describing the biological thermometer were set up. Also software with a graphical user interface was made to design RNA secondary structures that can act as an RNA thermometer. Our results show that a combined approach of laboratory work and modeling can be implemented successfully for the design of RNA thermometers. During the project the team became more aware of ethical issues in synthetic biology. This was achieved by implementing value sensitive design, interviews and discussing ethical questions regularly.