NESTED Results

Establishing a reliable, time-saving single-reaction PCR method

Establishing a reliable and fast method to generate our sensor is required for the testing of the many potential switch designs. In our original plan, the manufacture of the toehold-switches is performed by two sequential polymerase chain reactions (PCR), which build our design through extending the PCR product via 5' primer extension. Each forward primer (P1 for the first extension step, P2 for the second extension step) aligns to a conserved region in the previous DNA template. The process of preparing two extension PCRs, analyzing the proper length by gel elelectrophoresis in between reactions and eventually purifying the intermediate and final products requires a total of about 10 hours concentrated work in the lab. This usually means, that the production stretches over two days.

Increasing the scale of our test pipeline, while reducing the time and costs spent during the generation of a single sensor, is essential to a successful future of our project. Thus, we focused on establishing a reliable Nested PCR method by which the switch production could be performed within a single reaction. We estimated, that this would reduce the time to generate a sensor to approximately 4 hours.

Using the single-tube nested PCR method, we add both extension primers and the template simultaneously to the PCR mastermix. First, the primer P1 will add the individual first half of the toehold loop and the conserved loop region onto the reporter gene sequence. The second extension primer P2 anneals to the conserved loop region that was added through the P1 primer. The P2 primer extends the PCR product by the second half of the individual toehold switch design flanked by at T7 promoter at the 5' end. This creates a full dsDNA template containing 5' a T7 promoter for RNA synthesis followed by the toehold switch and its loop structure that is ending into the reporter gene 3' end. Considering this, we hypothesized that:

- The forward primer P1 should be used in a smaller amount then the second forward primer P2, skewing the PCR reaction towards exponentially amplifying only full toehold switches after the P1 reaction has constructed first templates.
- The reverse primer should be used in the same amount as forward primer P2

In the original pipeline using two sequential PCR reactions, the Zika Switch (Pardee et al 2016) was used as a DNA template for the first extension PCR. When we used a full toehold switch plasmid in the Nested PCR approach, we found that the P2 primer annealed at the conserved region of the Zika template, rather than the freshly amplified P1 products that contained the essential counterpart for a functional switch. To prevent such an interference, we decided to use a DNA template that only contains our reporter gene. Thus, the P2 primer can only bind onto the PCR products containing the other correct half of the toehold switch loop created through P1 extension.

In our study, we performed first trials of the nested PCR method on two toehold switch designs. The size of the amplified switches of the first PCR was evaluated on an agarose gel (Fig. 1). All the PCR products were of the expected length (aprox. 3.2 kb). Having this auspicious result in mind, we performed a second attempt with 7 other switch designs. This time 5 out of 7 nested attempts showed the desired outcome (data not shown).

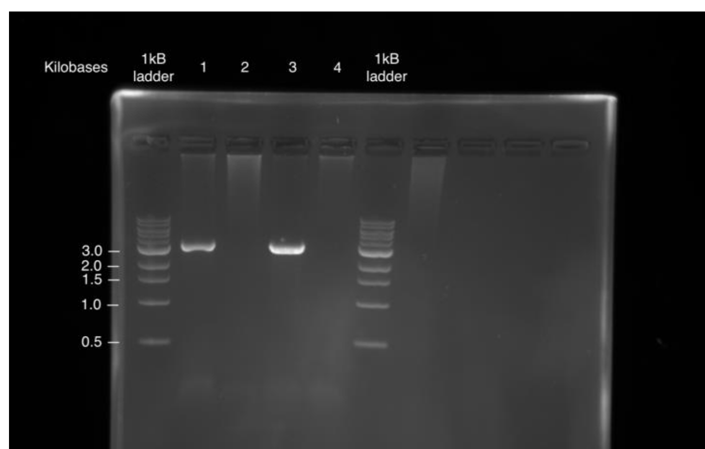

Fig. 1: Agarose gel of the first nested PCR attempt with the nested template and two toehold primers. PCR products 1 and 3 show the expected length, while lane 2 and 4 show, as anticipated, the failure of the negative controls. 1-nested PCR of switch Tso31_898 2-negative control of switch 898 (without P1) 3-nested PCR of switch 16 4-negative control of switch 16 (without P1)

In conclusion, the nested PCR is a promising prospect for reliable and fast production of toehold riboswitches. It reduces the amount of manual labor and materials used. Therefore, this method allows the production of a wide range of toehold switches, which then could be tested in sensitivity for trigger DNA in the cell free system.