Response module

Description

Our response system is based on a fluorescence emission signal and uses the fusion protein GFP-Dark Quencher linked together by a TEV cleavage site, as the key component of the module. We characterized an intein mediated reconstitution of the Split TEV as the system's input; when the association by the intein mechanism occurs, the two split TEV bind together into a functional TEV protease. This active protease is then capable of triggering our response module by catalyzing the proteolytic cleavage of the GFP-Dark Quencher linker, leading to an increase in fluorescence emission. With this system, we established a new intein-split TEV tool approach.

Advantageous Response

Our response module is mediated by the enzymatic reaction of the reconstituted split TEV. One single detection event will guarantee one functional TEV, which could target multiple linker sites. This key feature based on the reconstitution of split TEV assures the amplification of the response making it easy to be detected and quantified. This response module and the split TEV approach are ideal to set a detection threshold.

Background

1. TEV Activation

We based our response system's strategy on a split TEV approach, where the TEV sequence was separated into two fragments, which can be fused together by a reaction. The original TEV sequence has a S219V mutation to reduce its autoproteolysis and the integration position picked out to generate the split fragments was the amino acid 118, a functional split site already described (Sonntag & Nootz, 2011). This split TEV is already part of the iGEM repertory and based on the principle that there is no basal activity of the protease, we selected it as our response system's trigger.

Nevertheless, a second regulated reaction needs to take place to activate the TEV protease. Sonntag and Nootz (2011) mention that reconstitution of the TEV activity by a conditional protein splicing, as mediated by inteins, is a powerful tool due to its high specific activity and artificial regulation within the cell. We consider protein splicing by inteins the proper strategy to regulate the reconstitution of the split TEV in a post-translational reaction, specified in figure_.

The intein mechanism provide the reconstitution of the TEV mediated by a post translational reaction, where the two split inteins must find each other to start the reaction. Protein trans-splicing

consist in the reaction of two split inteins fragments expressed separately; however when they find each other they make a four-step reaction to fuse the exteins components and reconstituted the intein at the same time (Topilina & Mills, 2014; Stevens et al., 2015). We decided to use this approach due to the fact that the reaction of the inteins will we limited by the release of one intein fragment associated to a split TEV from the dCas9 chassis, nonetheless the intein will ensure the reaction upon release.

Upon releasing the split TEV from the dCas9, we needed a fast reaction leading to a reduction on the signal detection time. Sonntag and Nootz (2011) argue that the intein mechanisms required for the split TEV reconstitution is a trans-splicing intein; the natural existing split DnaE is highly specific and has a spontaneous activity leading this split intein into a powerful tool for inner cell regulation. Using this strategy, we wanted to make a two-component system with the lower time between detection and the cleavage of the TEV linker site fussing the GFP with the quencher.

2. Quenched-GFP

Our response system is based on Förster resonance energy transfer (FRET), which is a mechanism that describes energy transfer between two chromophores; a donor chromophore is able to transfer its excitation energy to a second chromophore or acceptor (Lemke & Deniz, 2016). This system highly depends on the distance between the donor and the acceptor molecule and an ideal FRET couple should have a large spectral overlap between donor emission and acceptor absorption (Ganesan et.al, 2006).

We use a Green Fluorescent Protein (GFP) as the donor chromophore of our system, which generates high fluorescence yields and low autofluorescence because of its absorption and emission characteristics. Since Genevan et.al (2006) modified an Enhanced Yellow Fluorescent Protein (EYFP) to obtain an optimized acceptor for GFP in FRET systems, we used this Resonance Energy-Accepting Chromoprotein (REACh) as the aceptor chromophore of our response mechanism.
REACh was designed by mutating an EYFP in random amino acid residues. These mutations eliminated the protein's fluorescence without altering its absorption characteristics, which resulted in a non-fluorescent chromoprotein that acts as a dark quencher for the donor chromophore in a FRET mechanism (Ganesan et.al, 2006). Because of its absorption wavelength, REACh is a suitable aceptor for GFP, our system's donor, and one of its most advantageous features is its absence of fluorescence, which enables a selective detection of GFP's florescence emission (Ganesan et.al, 2006).

3. No more Quenched-GFP

We decided to fuse our donor chromophore (GFP) with our acceptor molecule (REACh) by a recognition peptide for TEV protease. Ganesan et.al (2006) tested this linker sequence and proved that FRET is lost upon cleavage of the linker. When the linker is cut, GFP is separated from

REACh and this increase in distance prevents REACh from interacting with GFP, which results in fluoresce emission.

As we previously explained, in our system, after the Split-TEV is released from the dCas9, an intein reaction occurs that enables the reconstitution of an active TEV. Once this protease is activated, it is able to cleavage the GFP-REACh linker, resulting in a strong GFP fluorescence emission. This fluorescence emission is the final response of our system and it indicates the detection of a highly specific DNA or RNA molecule; in our case, the presence of prostate cancer.

Design

For the split intein approach we created two construct, each of them having one section of the intein:

The first BioBrick was formed with the split TEV N - split Intein - Double Terminator based on the iGEM parts: BBa_K1159318, BBa_K1362400, BBa_B0015.
For the second BioBrick, split TEV N-split Intein N - Double Terminator, we used the following parts: BBa_K1362401, BBa_K1159102, BBa_B0015.

For this module we designed the BioBrick GFP-TEV Site-Dark Quencher-Double Terminator based on iGEM parts: BBa_E0040, BBa_K1159318 and BBa_K1319001 and BBa_B0015 respectively. Then we assembled the construct with the Anderson Promoter J23101 and the RBS Elowitz BBa_B0034 to generate our response module device, and we registered this complete device as a BioBrick too.

We designed a positive control device expressing a TEV protease to cleavage the linker between the GFP and the Dark Quencher. We based this BioBrick on the following parts: BBa_R0010, BBa_B0034, BBa_K1319004, BBa_B0015.

References

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