Toehold Switch Characterisation

Methodology

Despite having amplified our switches earlier in the summer, before characterisation, we decided to reorder our switches and luciferase as linear DNA with 250 random base pairs before and after the prefix and suffix respectively on the advice of our TXTL kit supplier. We first suspended our gBlocks, consisting of a T7 promoter, upstream of the switch (containing an RBS), upstream of a firefly luciferase CDS, to reach an overall concentration of 6.7nM in TE buffer.

	Tube							
Component	**517 Switch (-)**	**517 Switch, miR-517-5p (9M)**	**517 Switch, miR-517-5p (2.25M)**	**517 Switch, miR-518f-5p (9M)**	**210 Switch (-)**	**210 Switch, miR-210-3p (9M)**	**210 Switch, miR-210-3p (2.25M)**	**210 Switch, miR-6867-5p (9M)**
Master Mix / ul	75	75	75	75	75	75	75	75
Linear DNA / ul	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8
miRNA 517 / ul	-	10	2.5	-	-	-	-	-
miRNA 210 / ul	-	-	-	-	-	10	2.5	-
miRNA 518 / ul	-	-	-	10	-	-	-	-
miRNA 6867 / ul	-	-	-	-	-	-	-	10
Mili Q / ul	10	-	7.5	-	10	-	7.5	-
P70a-T7rnap HP (2.4 nM) / ul	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Total	**110**	**110**	**110**	**110**	**110**	**110**	**110**	**110**

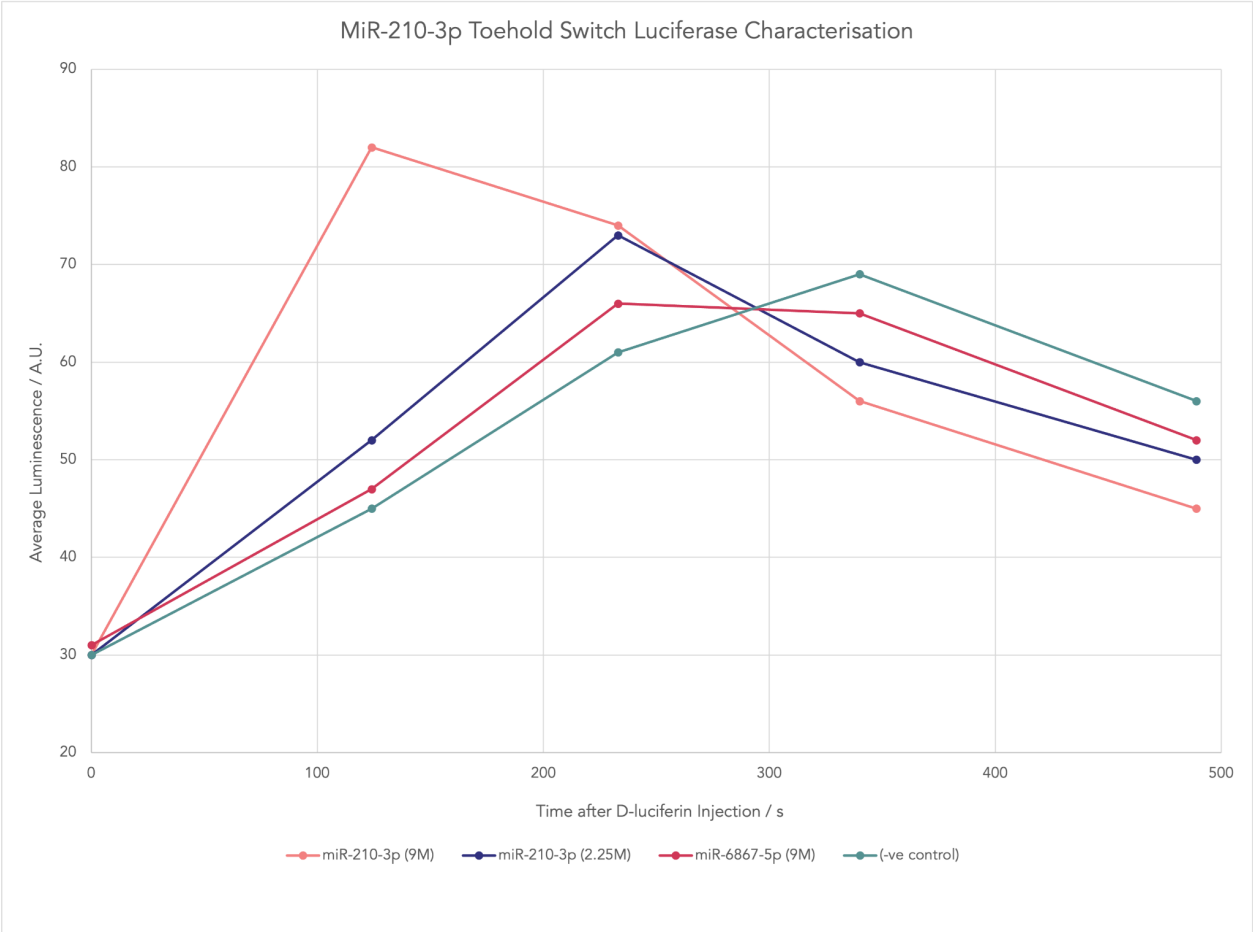
For each gen2 switch, we tested, at a constant DNA concentration, the target miRNA (miR-517-5p or miR-210-3p) at 9M and 2.25M to replicate the roughly 4-fold increase of expression levels in patients with preeclampsia [1] [2], as well as a homolog for each miRNA (miR-518f-5p for miR-517-5p and miR-6867-5p for miR-210-3p), which were determined to be the miRNAs have the highest probability of activating the switches aside from the target miRNAs themselves by our software tool (see software page). We then incubated the DNA, miRNAs with a plasmid containing a gene coding for T7 polymerase (to promote transcription of our toehold switch mRNAs) as well as a TXTL cell-free master mix for 1 hour, and then pipetted the contents of each tube into 8 wells of a 96 well plate, and took four measurements after D-luciferin in a buffer was injected, at different times. The luciferase assay kit told us to take a measurement 'immediately' after injection. But we found that taking the

measurement a few seconds after injection caused no luminescence to be detected, so we took measurements at later times, to analyse how luminescence changed over time.

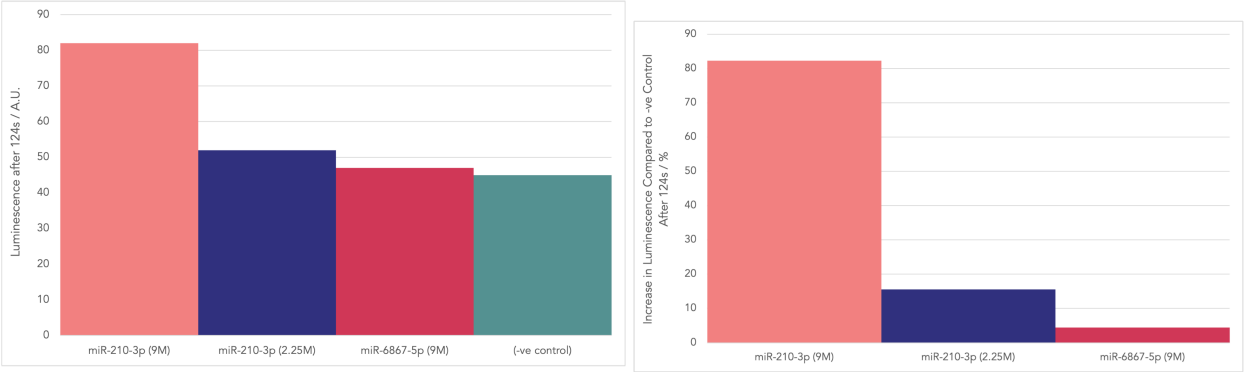
	Tube				
Component	**(-)**	**miR-210-3p (9M), miR-517-5p (9M)**	**miR-210-3p (2.25M), miR-517-5p (9M)**	**miR-210-3p (9M), miR-517-5p (2.25M)**	**miR-210-3p (2.25M), miR-517-5p (2.25M)**
Master Mix / ul	75	75	75	75	75
Linear DNA (THS) / ul	10.4	10.4	10.4	10.4	10.4
Linear DNA (aRNA) / ul	10.4	10.4	10.4	10.4	10.4
miRNA 517 / ul	-	10	10	2.5	2.5
miRNA 210 / ul	-	10	2.5	10	2.5
Mili Q / ul	20	-	7.5	7.5	15
P70a-T7rnap HP (2.4 nM) / ul	4.2	4.2	4.2	4.2	4.2
Total	**120**	**120**	**120**	**120**	**120**

For the gen3 switch, we tested, at a constant DNA concentration, the target miRNAs (miR-517-5p and miR-210-3p) at 9M and 2.25M combinations to replicate the roughly 4-fold increase of expression levels in patients with preeclampsia [1] [2]. We then incubated the DNA for the switch and luciferase, the DNA for the antiRNA (aRNA) (See Design page), miRNAs with a plasmid containing a gene coding for T7 polymerase (to promote transcription of our toehold switch mRNAs) as well as a TXTL cell-free master mix for 1 hour, and then pipetted the contents of each tube into 8 wells of a 96 well plate, and took a measurement after 155s as this was in the region of high expression for high miRNA concentration samples observed in the single trigger switches.

MiR-210-3p Toehold Switch

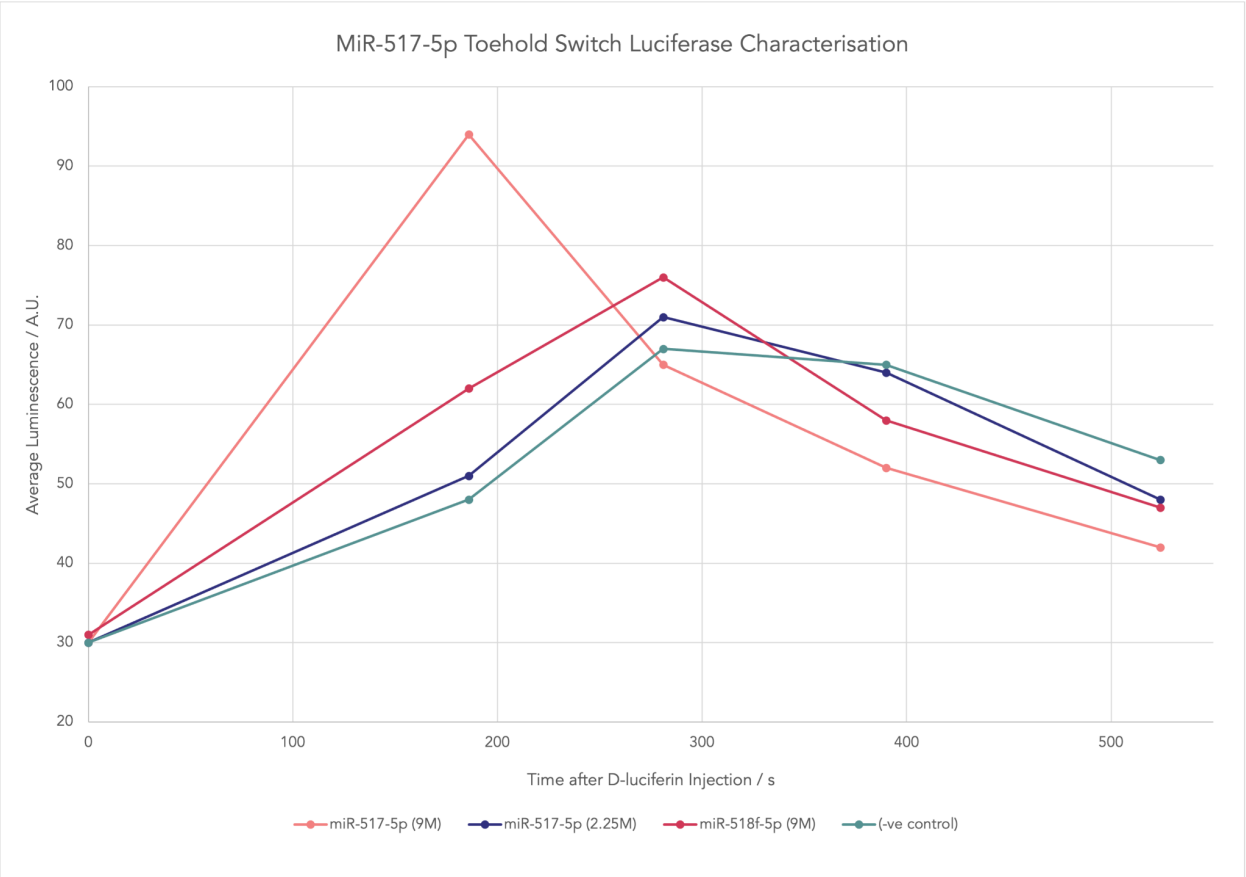


The results show a brief, high peak in luminescence for a high concentration of miR-210-3p. This is because, during the incubation time, more luciferase is produced due to an increased rate of translation, so there is a higher concentration of luciferase, so more luciferase-luciferin complexes form at the beginning, converting luciferin to oxyluciferin, producing luminescence. The results show that, compared to the negative control, the homolog ‘miR-518f-5p’ activates the switch more, causing an higher peak, sooner on, and there is only a small increase in luminescence between the control and the target RNA at reduced concentration. Based on this data, we decided that our kit should detect luminescence at some point between 100 and 200 seconds.

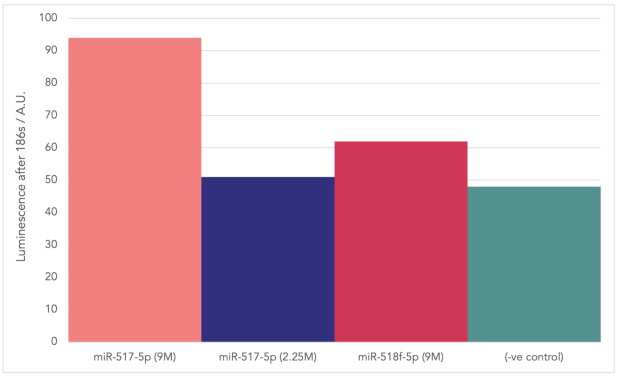


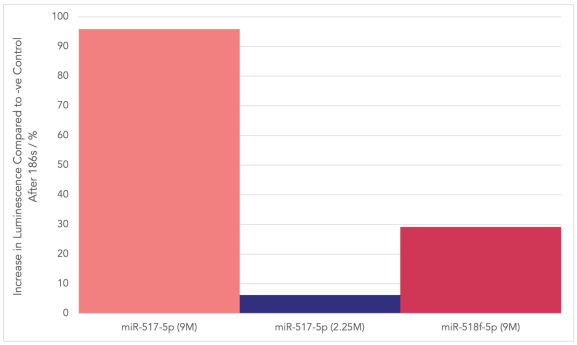
The graph on the left shows the luminescence when measurement was taken at 124 seconds, and the graph on the right shows % increase in luminescence compared to the negative control (no miRNAs added). This shows an 82% increase in expression for the target miRNA at 9M concentration, which drops to just 16% for a 2.25M concentration, showing how our toehold switch can clearly discriminate between these percentage differences in miRNA concentrations, and these concentrations are based on what we'd expect in patients with and without the condition at ten weeks [1]. However, there is no data on actual concentrations of these miRNAs in the serum and the data we collected shows that if concentrations in the blood are lower than 2.25M, then an amplification technique would be necessary to discriminate between low concentrations of miRNA and leaky luciferase expression.

MiR-517-5p Toehold Switch



The results for the miR-517-5p switch were very similar to that of the miR-210-3p switch. However, it showed that the difference in luciferase concentration after incubation was lower for the lower concentration of miR-517-5p than that of miR-210-3p. Furthermore, the homolog for this trigger miRNA, miR-518f-5p gave a higher concentration of luciferase than the homolog for miR-210-3p, suggesting that it induces translation better. This is what we would expect given it's closer similarity.

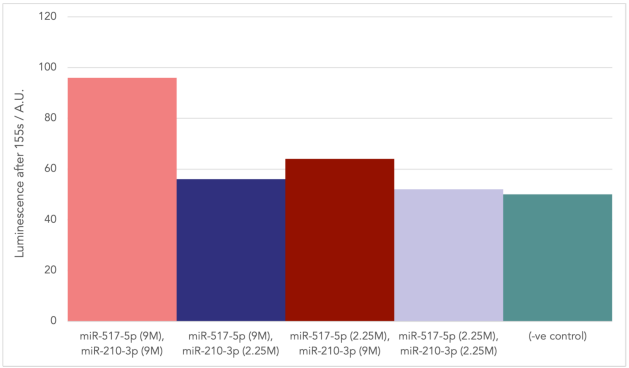


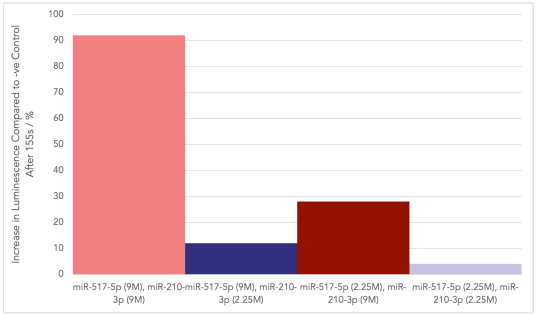


The above diagrams are also similar to those of the miR-210-3p switch, and show how the toehold switch can discriminate clearly between miR-517-5p at 9M and 2.25M.

AND-Gate Toehold Switch

Having tested the single trigger switches, and thereby demonstrating how our concept of a toehold switch works as hypothesised, the main purpose of testing the AND-Gate Switch was to see whether it could discriminate between a high concentration of one miRNA, and a low concentration of another, and a high concentration of both. This was in case a patient had increased levels of one miRNA and not the other, which would not be indicative of Preeclampsia.





The graphs show how, compared to the negative control, which shows leaky expression of the toehold switch, the luminescence output of the sample with 9M concentration of both miRNA triggers is 92% higher. Interestingly, the percentage increase in luminescence of the miR-210-3p at 9M and miR-517-5p at 2.25M is twice more than twice as high as the miRNAs at flipped concentrations - 28% compared to 12%. This could be because the miR-210-3p binding site is further downstream than the miR-517-5p binding site, so miR-210-3p is able to bind and partially unfold the

switch in the absence of miR-517-5p, increasing the rate of translation. However, as the miR-517-5p binding site is further upstream, it is completely bound up in hydrogen bonds, so it is not accessible to the miRNA unless it binds as the switch is being transcribed.

Concluding points

- The gen2 toehold switches cause a noticeable increase in translation of luciferin with increased miRNA concentration, and, with software, they would be able to binarily discriminate between levels of miRNA at concentrations indicative of the condition, given they are above 2.25M in ‘normal’ patients.
- The gen3 toehold switch also causes a noticeable increase in translation when both miRNAs are at a high concentration (9M), compared to when one of both of them is at a low concentration (2.25M), which gives strong evidence that our AND-gate design works.
- However, to prove the concept of the test as a whole, we should design a method of amplification to ensure concentration reaches above 9M and 2.25M respectively.
- Furthermore, the fact that homologous RNAs were able to increase translation, compared to the negative controls, provided evidence that the algorithm in our software tool can predict miRNAs likely to unfold switches.
- Further research could involve testing more homologous RNAs.

miRPA Characterisation

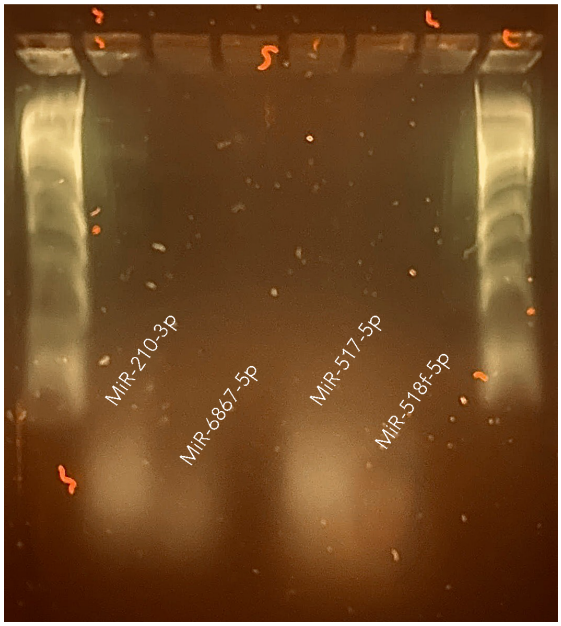
Based on results from our toehold switch characterisation, we decided to find an isothermal amplification strategy. Read more about it on our Design page.

We were supplied miRPA characterisation protocols by Dr O'Sullivan (see Integrated Human Practices page) after we told her about our project. To show that miRPA can discriminate between our miRNAs and homologous miRNAs, we tested our trigger miRNAs with their respective closest homologs, as determined by our software tool.

In order to design probes for miRPA, we designed a python script using Nupack's design functions in its API to find probes which would bind to the miRNAs, but have overhangs which didn't bind within themselves, to ensure primers could easily anneal to them.

We ordered our probes and primers on IDT and then resuspended them in a TE buffer and added them to our trigger and homologous miRNAs, with DNA ligase to bind the probes together. We amplified the miRPA product with PCR.

miRPA Product with Target miRNAs and Homologs



We performed a gel electrophoresis on the PCR products. We did not have access to gel ladders which could discriminate between <100bp DNA strands, but our gel showed that the target miRNAs miR-210-3p and miR-517-5p were amplified more than the homologs. Furthermore, the fact that the homologs moved further through the gel suggested that the probes had not bound together, which would suggest they did not bind to the homologs.

Using miRPA in our test would allow us to ensure there is an adequate miRNA concentration, and our characterisation of the technique showed it is specific to our miRNAs.