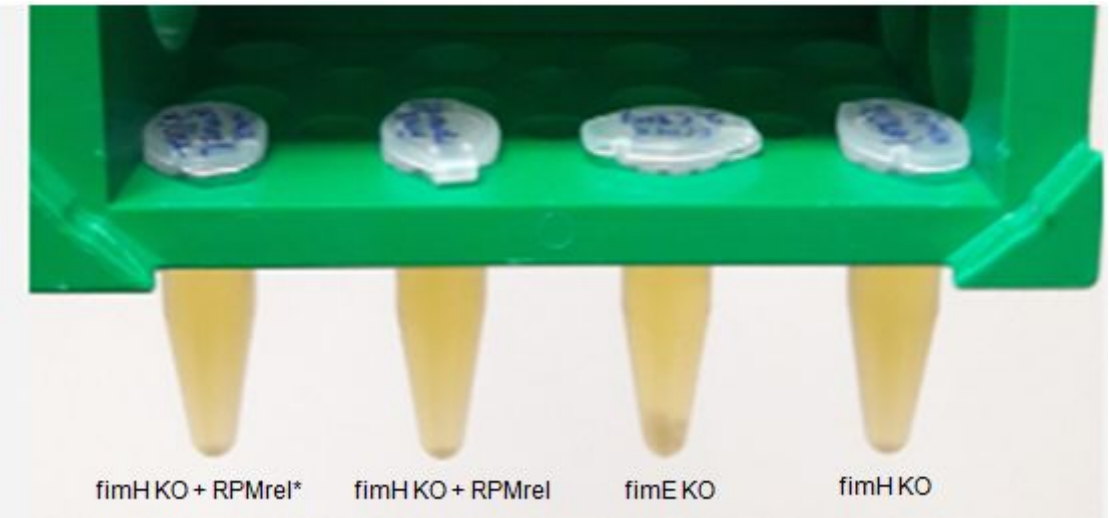
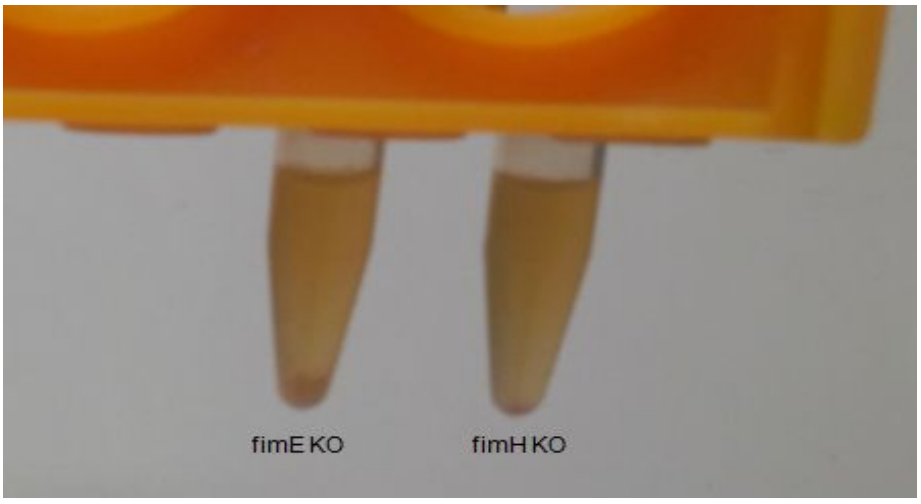
Agglutination Assay

In order to test our colorectal cancer-targeted delivery system, we tested three different strains of *E.coli* which were obtained from the Keio collection. We used fimE KO (JW4276-1) and fimH KO (JW4283-3) stains. The fimE KO is overproducing the type-1 pili which we expect to show extensive adhesion to mannose residues on glycoproteins found on human epithelial cells[1]. Type-1 pili adhesion relies on the fimH lectin and a fimH KO strain is expected to be unable to bind to mannosylated glycoproteins. We then transformed the fimH KO with the RPMrel peptide addition (rhamnose inducible part BBa_K1850011 from iGEM Harvard 2015) and tested all three constructs for their capability to bind to the yeast *S. cerevisiae* which according to van Asbeck et al. shows a high affinity to mannose binding lectins. The results show that, when mixed together with yeast, the fimE KO strains appeared to form clumps which precipitated to the bottom of the tubes, whilst fimH KO and the fimH KO with the BBa_K1850011 construct did not show any clumps.



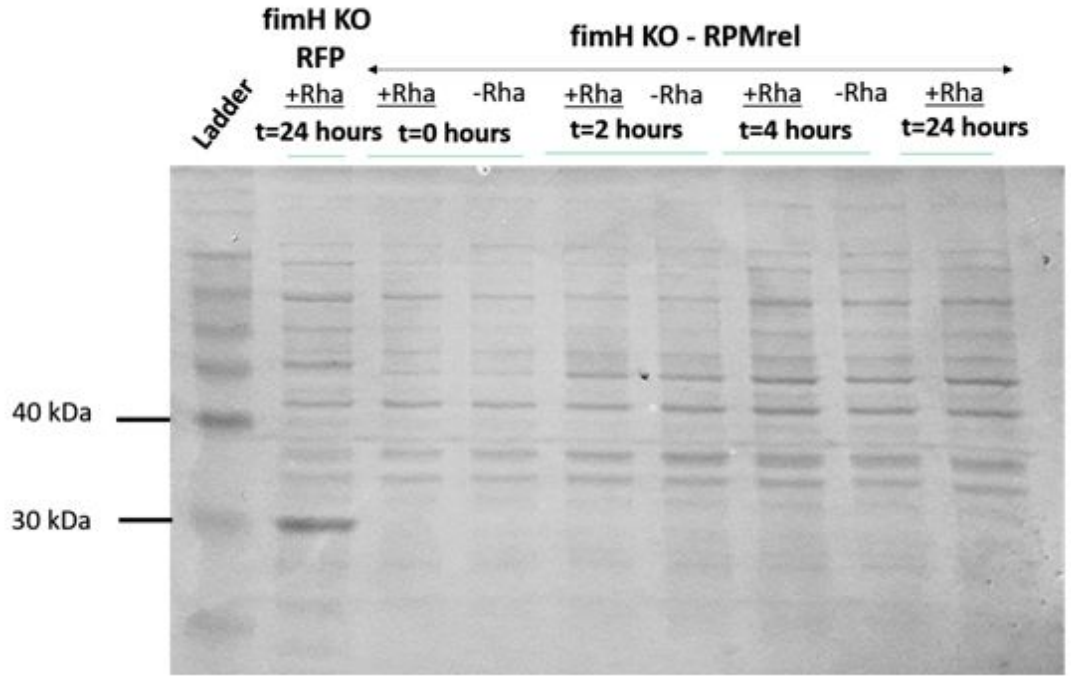
Picture 1. Results seen after 1 hour of mixing 500µl of yeast and *E.coli*, both grown for 24 hours to saturation. Some minor precipitation has formed in all tubes due to bacterial cells sitting down due to lack of turbulence. * indicates induction with 0.05% rhamnose for 3 hours.

Since the pSB1C3 backbone, in which we received the part BBa_K1850011, is a high copy number plasmid, we wanted to avoid excessive expression and the possibility of inclusion body formations by transferring the part to a low copy backbone (pSB1T3) obtained from the part BBa_J04450. This way, our transformed strain possesses both Kanamycin (due to the Keio mutation) and Tetracycline (from pSB1T3) resistance. These bacterial strains were grown in LB Medium with both kanamycin and tetracycline antibiotics and the saturation OD was half of the one of fimH KO and fimE KO strains. In order to have comparative results, we diluted the culture in half (OD_{600} 1.4) and the results were the same.



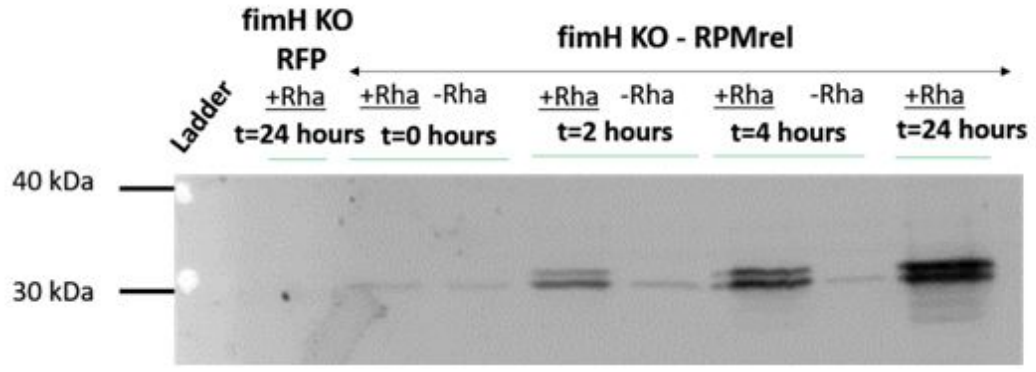
Confirmation of fimH + RPMrel expression in the fimH KO strain

To validate the expression of the fimH KO 49 – RPMrel we've grown two E. coli K12 fimH KO cultures, transformed with the fimH KO – RPMrel part but only one was induced with rhamnose and one E. coli K12 fimH KO culture transformed with an RFP insert in the same vector as a negative control. Samples were collected at specific time points from each culture, then sonicated and after centrifugation we separated the supernatant that contains the correctly folded proteins from the pellet. We did a Bradford assay to determine the protein concentration of each supernatant sample. We loaded on SDS-Page 25 µg of total protein from each sample. The image below: SDS-Page stained with Coomasie G250.



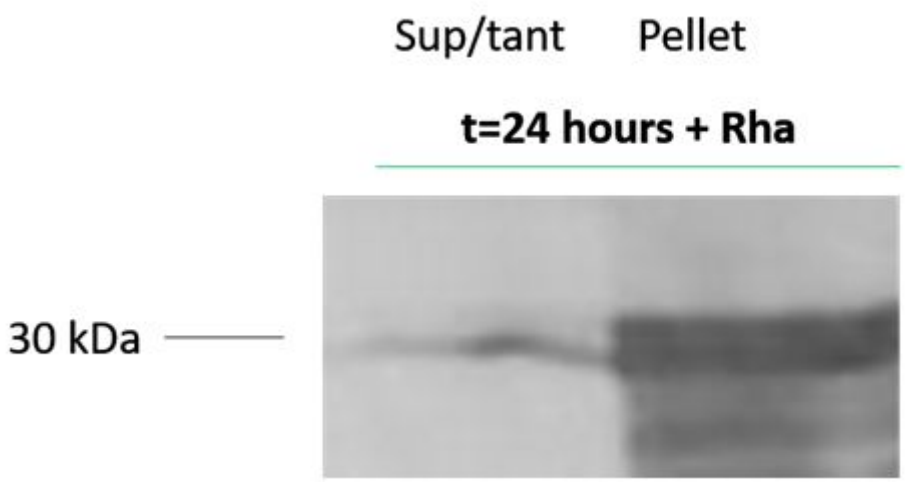
We see a strong band at approximately 30 kDa in fimH KO – RFP sample that is not present in the remainder of the samples. The protein that corresponds to this band is RFP. However the intensity of specific bands appearing at the same molecular weight indicates that all samples are evenly loaded even though collected at different time points.

We expect the fimH KO protein to be appearing at 30 kDa according to literature. Because the pRha is a constitute promoter a low intense band is most likely to appear also in the non-inducible samples. We also want to prove that increasing the time of Rhamnose induction will result in more fimH KO – RPMrel production. To do so, we performed an a-His Western Blot taking advantage of the his-tag located in the C-terminus of our construct. The fimH KO - RFP sample was used as a negative control. The image below shows the results after ECL staining.



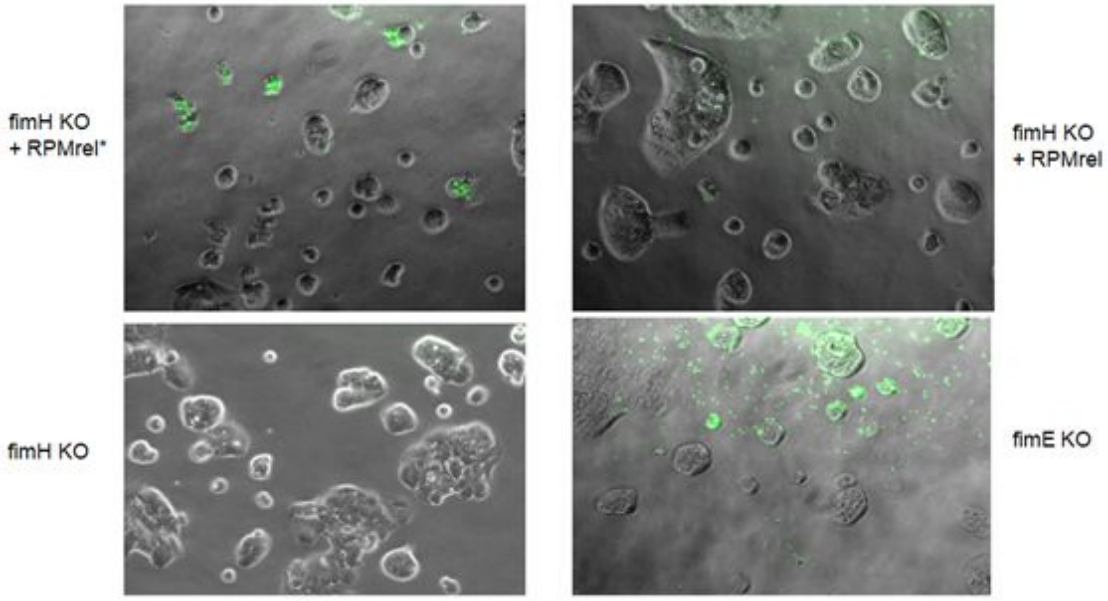
Samples taken at 4 timepoints show increasing expression of fimH in the induced culture. As expected the non-induced culture also appears a low intense band in all timepoints due to leakiness of the promoter. There is no band appearing on the negative control sample. The double bands appearing on the induced samples correspond to fimH, because fimH has to be unstable in SDS and may look like two bands close to each other as shown above.

Our next goal was to examine whether fimH KO – RPM precipitates after the induction with Rhamnose. The formation of inclusion bodies containing unfolded protein was observed after a second western blot in which we notice a very intense band at 30 kDa in the pellet as shown in the picture bellow.



Co-culture

After showing that the fimH + RPMrel construct did not bind to mannose and therefore healthy cells, while also having expression confirmation from our Westerns, our team wanted to take the adhesion test to the next level by showing the adhesion ability of the construct by co-culturing the transformed cells with Caco-2 (human epithelial colorectal adenocarcinoma cells). The RPMrel peptide has been shown by Kelly et al. to bind to 5 specific cancer cell types (HT29, CaCo-2, RKO, SW480 and DLD-1) and the one we worked with was Caco-2. Again we used the same strains as the agglutination assay. We expected fimE to play the role of the positive control as Caco-2 cells do not lack the natural mannosylated glycoproteins shown in epithelial cells[4]. Again we did not expect the fimH KO mutants to not attach to the cells, whilst the fimH KO with the BBa_K1850011 in a low copy backbone was expected to attach. Unlike iGEM Harvard which used a fimB KO mutant in order to stop the expression of the entire fim operon, we only wanted to control the expression of the fimH gene, as it was the only gene of interest in the adhesion system, thus reducing the plasmids that our strains had to bare with, as well as the risk of large scale expression differences. Expression control of only one construct is much safer and easier to calibrate along with the natural expression system of the cell. The co-culture protocol was a modified version of Tatsuno et al. and after several trials with different MOI and cell confluence, along with co-culture incubation times, we manages to standardize the co-culture where the both fimE and fimH KOs serve their role perfectly as positive and negative controls, whilst the fimH KOs with the fimH+RPMrel peptide, showed visible and comparable to the positive control adhesion.



The fimE KO strains show visible rings around the Caco-2 cells. In the wells were fimH KO were inserted, there were little to no bacteria remaining in the well. The fimH KO with the fimH gene and the RPMrel peptide, without rhamnose induction, showed some attachment to the cells (still many more than the unstransformed) but if induced with rhamnose for 3 hours, clumps of bacteria (even bigger than the Caco-2 cells) showed strong adhesion which despite all the washing steps did not detach of the cells. Several pictures were taken from all around the 24-wells and all showed similar results to the ones shown!

Taking into account the results from the agglutination assay, where the fimH KO with the BBa_K1850011 construct on a low copy backbone did not agglutinate to mannose, while at the same time our co-culture showed clear adhesion to Caco-2 cells, we are now confident that our targeted delivery system is functional!

In order to confirm that miR-145 and miR-143 have a much lower expression than miR-21, miR-372 and miR-373 we performed a Real-Time PCR to quantify the levels of the selected miRNAs for our pANDORRA circuit. We used as an endogenous control the RNU6 gene to compare the difference on the Ct values between the RNU6 and our target miRNAs. MiR-143 is detected last, so we determine the fold change between this miRNA and the others as it is shown in the table below. We did the same procedure also for the HEK cell line.

	SAMPLE	CT	MEAN CT	DCT	DDCT	FOLD CHANGE [2^-DDCT]	Expected CT Value	Deviation
CaCO								
	T143	27.77	27.50	13.23	0.00	1.00	29.88	0%
		27.22						
	T145	23.94	24.07	9.81	-3.42	10.73	25.76	-9%
		24.21						
	T21	15.84	16.08	1.82	-11.41	2729.98	17.04	18%
		16.33						
	T372	17.65	17.65	3.39	-9.84	918.56	18.99	40%
		17.66						
	T373	16.15	16.30	2.04	-11.19	2341.65	17.38	60%
		16.46						
	RNU6	14.23	14.26	0.00				
		14.30						
HEK								
	T143	30.16	29.82	14.47	0.00	1.00		
		29.48						
	T145	23.49	23.47	8.12	-6.35	81.48		
		23.45						
	T21	20.03	20.12	4.78	-9.70	828.98		
		20.22						
	T372	26.33	26.56	11.22	-3.25	9.54		
		26.79						
	T373	27.58	27.82	12.47	-2.00	4.01		
		28.05						
	RNU6	15.35	15.35	0.00				
		15.35						

To determine the change in miRNA expression in the two different cell lines, we compared the expression of T143 in CaCO to HEK. More specifically, in Caco-2 cells, for RNU6 CT equal to 14.26 the expression of T143 is at 27.50. Thus, assuming analogous expression relations between T143 and RNU6 in both cell lines, we calculated the expected CT value of T143 for RNU6 expression at 15.35. Then, the difference between the expected value and the experimentally determined value in HEK results in a percentage difference (Deviation) which determines the difference in miRNA expression between the two cell lines. The same procedure could be applied for the rest of the miRNAs. Bear in mind that there needs to be a common normalization factor in both cell lines (e.g. RNU6).

Classifier Results

A BD FACSCalibur analyzer was used for flow cytometry 3 days post transfection with Lipofectamin 3000 (2 days post doxycycline induction). For each sample we performed the following analysis:

We calculated the geometric mean of DsRed fluorescence intensity as well as the percentage of DsRed expressing cells. We also used a GFP plasmid as a transfection control and calculated the percentage of GFP expressing cells as well.

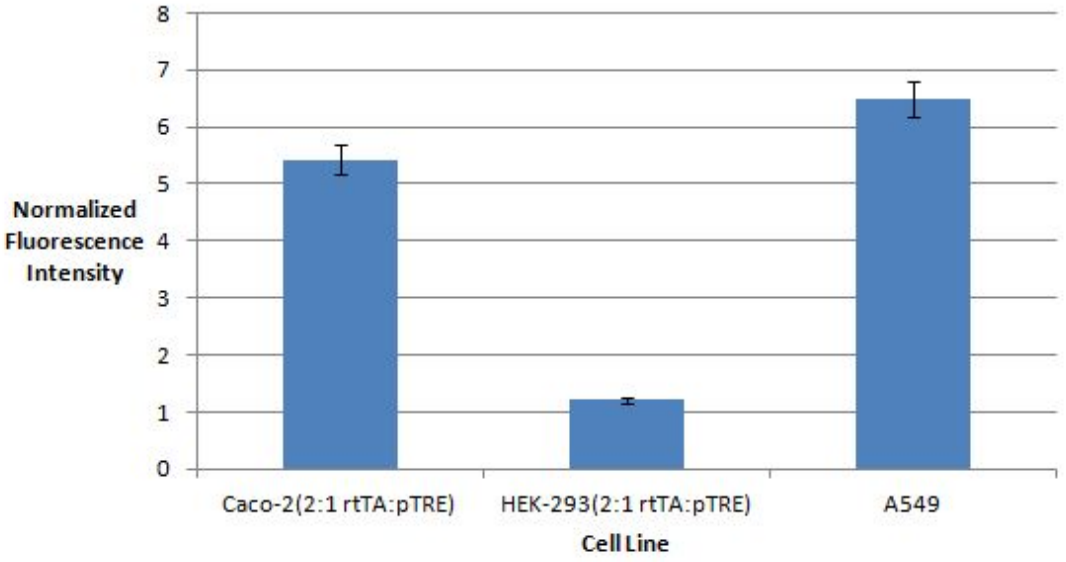
We calculated the cellular fluorescence intensity (NFI) for every cell in a sample as follows:

$$CFI = Ds\,\mathrm{Re}\,d_{sample}\frac{\%\,Ds\,\mathrm{Re}\,d+}{\%\,GFP+}$$

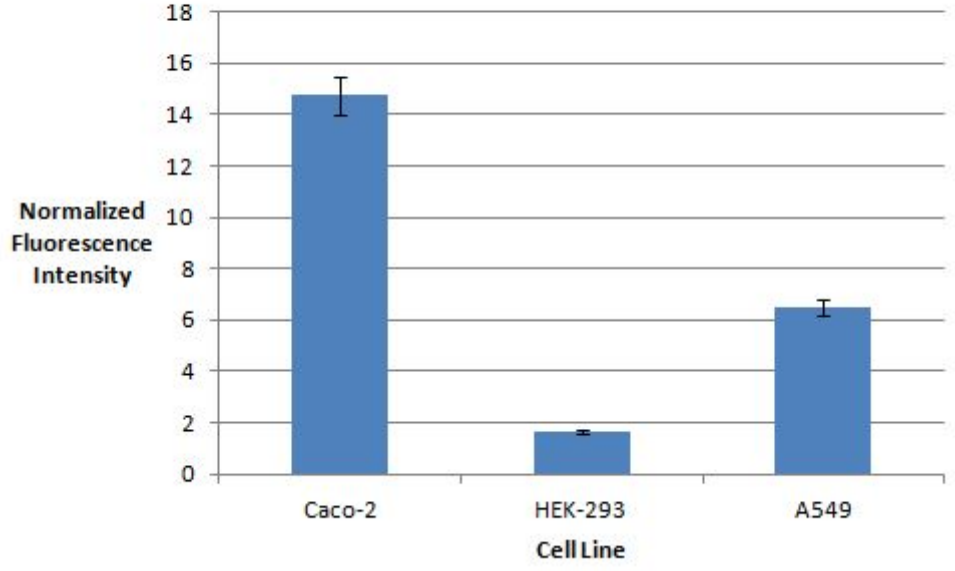
This normalization using the percentages of the cells expressing our plasmid of interest and the control, serves to compensate for transfection efficiency differences across various cell lines. Afterwards, we performed an additional normalization step to account for differential expression strength of the promoters used in the output plasmid across the cell lines, by dividing the CFI of each sample with the CFI of a predetermined sample (pCMV-DsRed-sfGFP-SV40) to obtain the Normalized Fluorescence Intensity (NFI),which we used as the output measure.

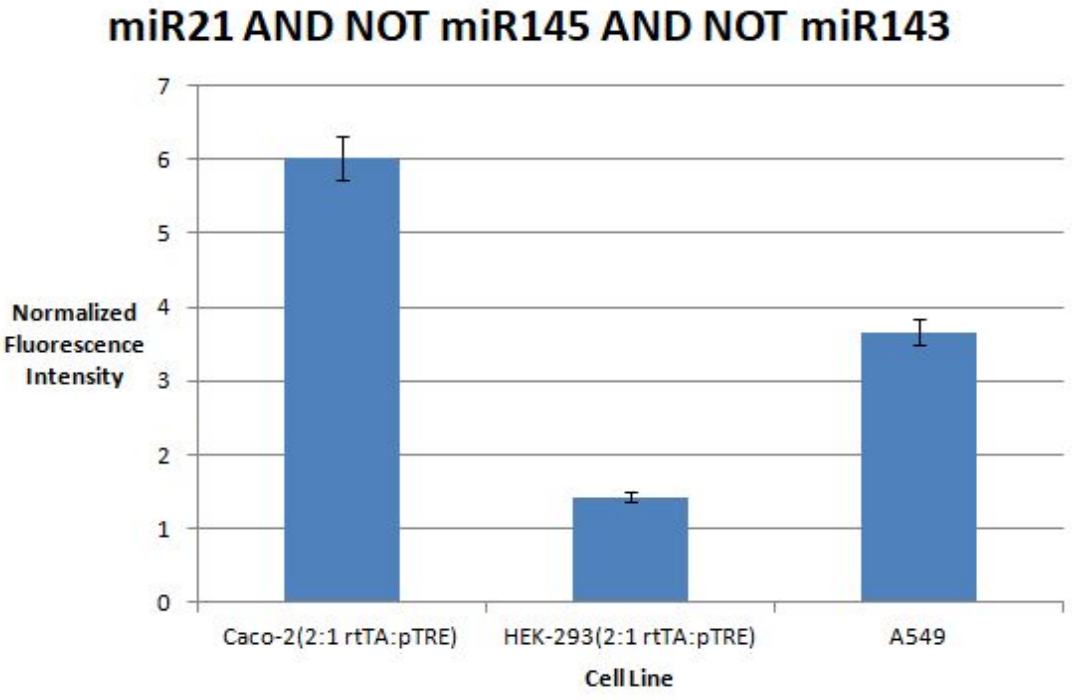
Since we did not have access to a colorectal epithelium cell line to accurately test our classifier circuit, we performed our experiments on two other cell lines, HEK 293 and A549, to gauge whether our circuit functions as predicted by our model.

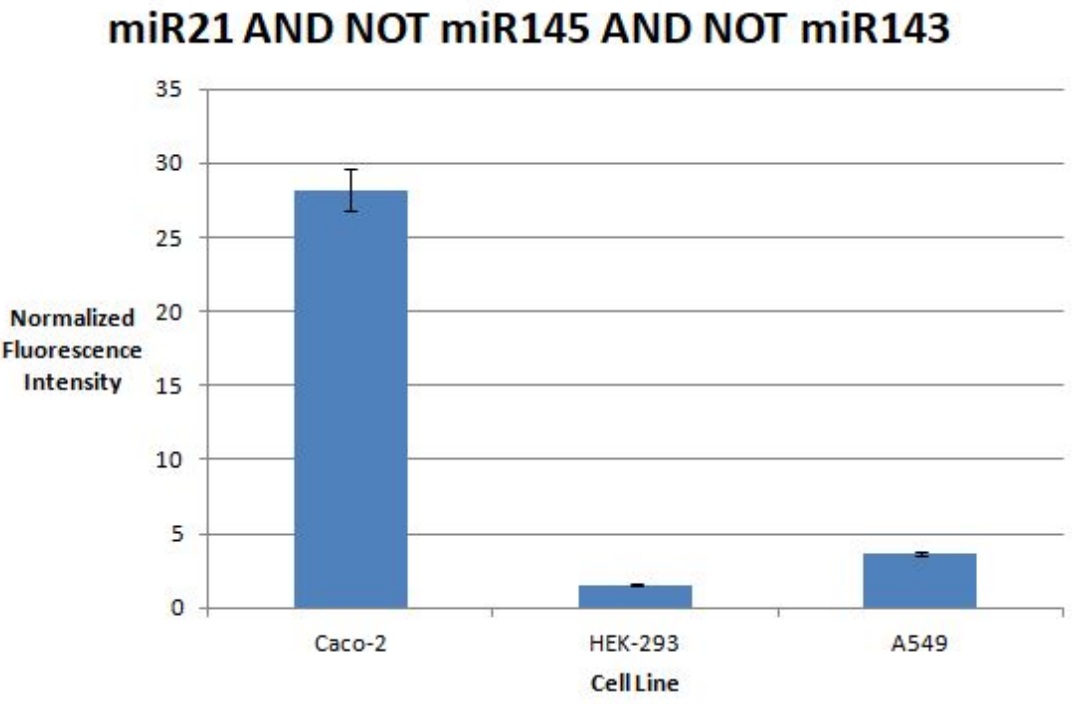
miR21 AND miR372 OR miR373 AND NOT miR145 AND NOT miR143

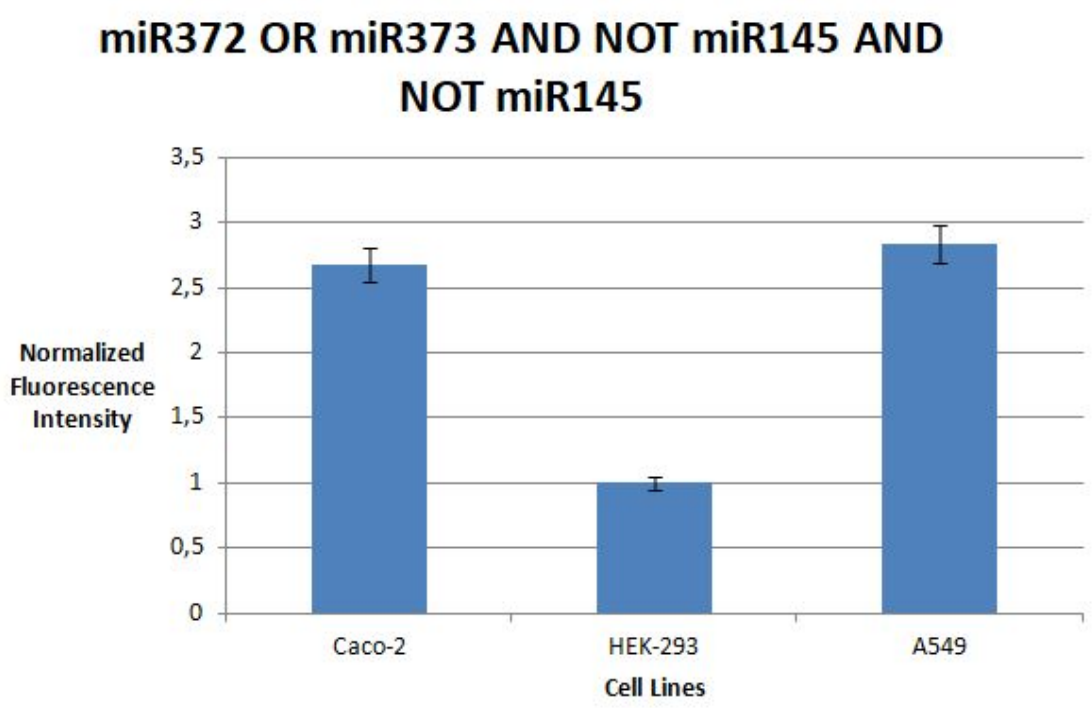


miR21 AND miR372 OR miR373 AND NOT miR145 AND NOT miR143




miR21 AND NOT miR145 AND NOT miR143
Normalized Fluorescence Intensity
7
6
5
4
3
2
1
0
Caco-2(2:1 rtTA:pTRE)
HEK-293(2:1 rtTA:pTRE)
A549
Cell Line


miR21 AND NOT miR145 AND NOT miR143
Normalized Fluorescence Intensity
35
30
25
20
15
10
5
0
Caco-2
HEK-293
A549
Cell Line



Indeed we see that our circuit behaves as predicted by our model (check our model page for more information), providing strong evidence that it is capable of achieving cell-type specific actuation!

References

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4. Sussman, M. (Ed.). (1997). Escherichia coli: mechanisms of virulence. Cambridge University Press.

5. Tatsuno, I., Nagano, K., Taguchi, K., Rong, L., Mori, H., & Sasakawa, C. (2003). Increased adherence to Caco-2 cells caused by disruption of the yhiE and yhiF genes in enterohemorrhagic Escherichia coli O157: H7. *Infection and immunity*, *71*(5), 2598-2606.