UNAM_genomics Mexico Notebook: Mariana

Goals

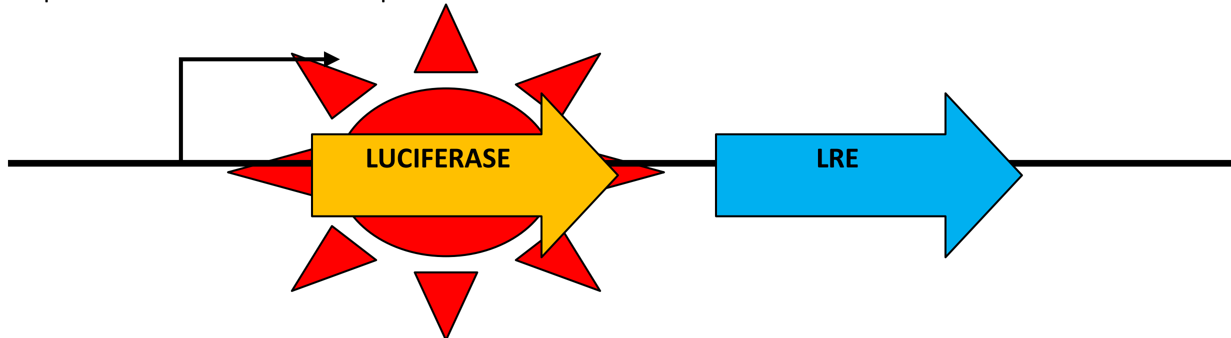
*During my participation in the project, I was focused on constructing the luciferase and Luciferin Regenerating Enzyme (LRE) of the red emission module.

Objectives

*To obtain a functional red luciferase.

*To assemble the synthesized LRE.

*To prove that LRE worked as expected.



Methodology and Results

Mutated Firefly Luciferase

The original intention was to use the *Photinus pyralis* luciferase biobrick (BBa_I712019) as a base and to increase its efficiency by using the primer BBa_K360114 (as registered) due to previous design notes as referred in the part design section of this briobrick. After achieving this, a punctual mutation S284T (also known as S851T) was induced to change its emission spectrum from green to red by means of a PCR with special primers (which are also referred in the biobrick part design). The biobrick name is BBa_K360115; unfortunately, we were never able to find out why it was not functional.

Click Beetle Luciferase

By the middle of the summer, we decided to try the luciferase from another organism: Click Beetle, specifically the Promega Chroma-Luc™ Reporter Vectors pCBG99-Basic Vector Restriction Sites and the pCBR-Basic Vector Restriction Sites, where the first one has a green emission and the second one red. The intention was to work with these vectors as a means of having a previously-

known-to-be functional luciferase, so that once the LRE biobrick was ready, characterization could be feasible. The first step was to extract the coding sequence of the luciferases from these vectors by means of PCR and also to standardize them by adding the prefix and suffix with the following primers:

>Forward Click Beetle luciferase
GAATTCGCGGCCGCTTCTAGAGATTAAAGAGGAGAAAATGGTGAAGCGTGAGAAAAAT
>Reverse Click Beetle luciferase
CTGCAGCGGCCGCTACTAGTATTATTAACCGCCGGCCTTCT

The second step was to ligate it into the BBa_J61002 plasmid with a J23101 promoter, and to transform it, having as an output several colonies which were then grown overnight to check whether the luciferases showed any brightness. The protocol followed for the assay is mentioned in BBa_K216015 biobrick, in the Experience part. In this first assay, no luminometer was used; so even though we added 1μL of 100mM luciferin and waited in the dark room for ~30min taking pictures with a SLR camera with high ISO (~400), for about 30secs to 1 min exposure, nothing was observed :'(.

After the initial disappointment, I talked to Cambridge team and they help us ever since then by sending us some of their constructions (BBa_K325909, BBa_K325109, BBa_K325209, and 2 other biobricks).

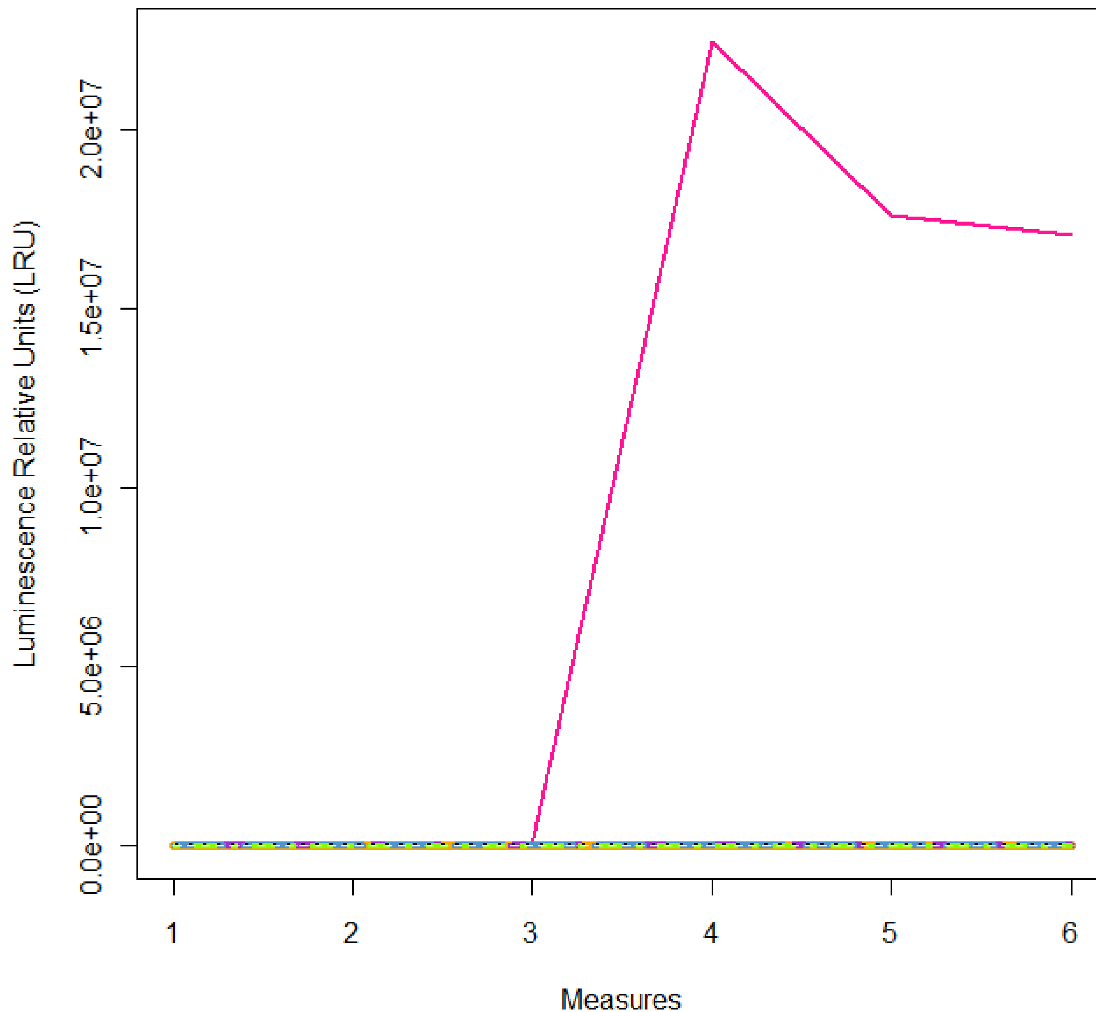
Luciferase assay

After having worked with luciferases from both firefly and click beetle, another measurement was performed, but this time I was guided by the kind help of Chris Wood Phd., and the willingness of Susana López, Phd. to lend us her luminometer. The measurements were taken as follows (with and without luciferase, triplicate for each one):

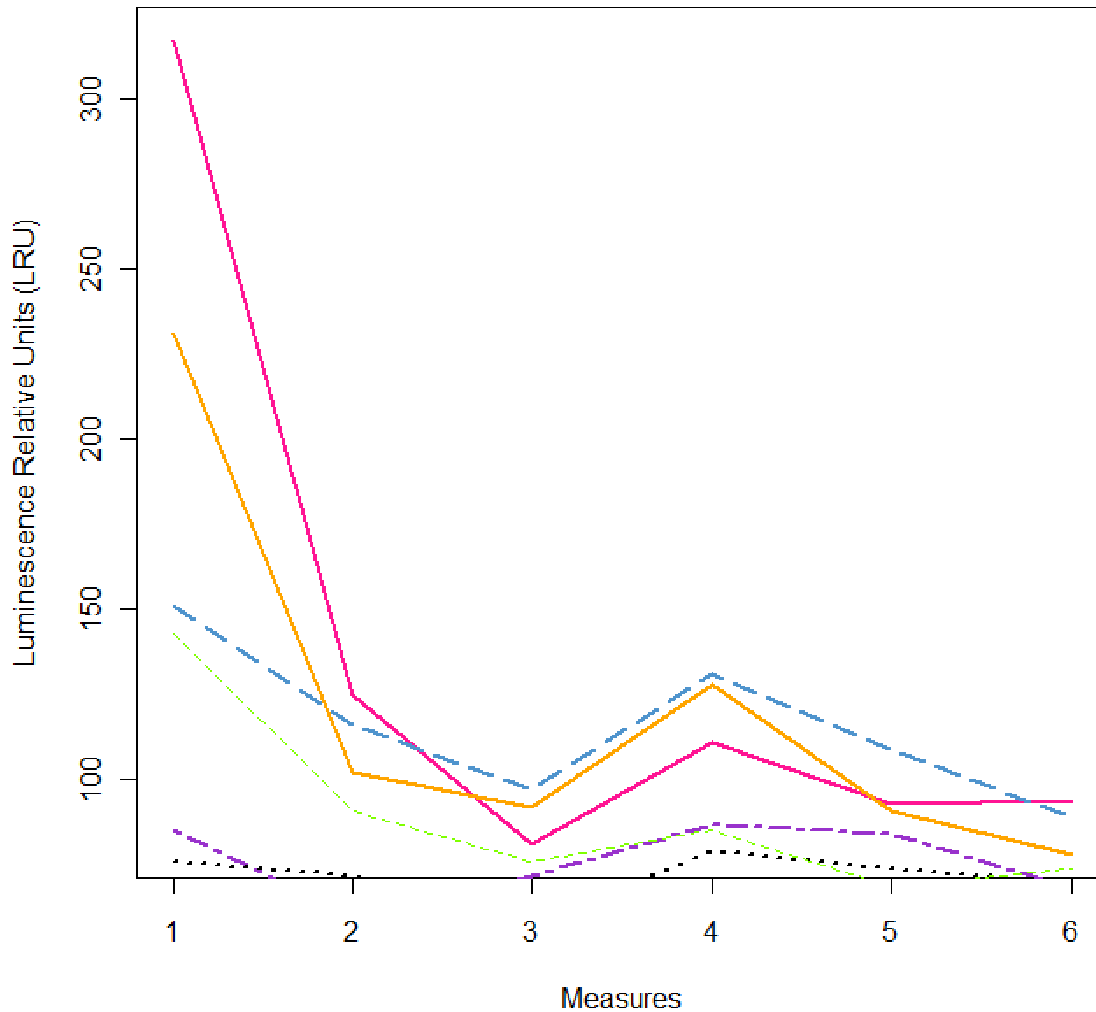
	control	medium	bacteria	CBG	CBR	PPlucwt	PPmluc	PPmlucJ23102
1	85	151	317	2181	76	143	231	241
2	60	116	125	4144	72	91	102	107
3	72	97	81	7748	58	76	92	79
Luci1	87	131	111	22443367	79	85	128	109
Luci2	84	109	93	17592397	74	69	91	77

Luci3	68	89	94	17037342	70	74	78	82

Control = Citratum sodium medium 1mM, Medium = LB, Bacteria = DH5-? without transformation, CBG = Click beetle green, CBR = Click beetle red, PPlucwt = Firefly luciferase (BBa_I712019), PPmluc = red mutated luciferase, PPmlucJ23102 = red mutated luciferase under J23102 promoter in BBa_J261002.



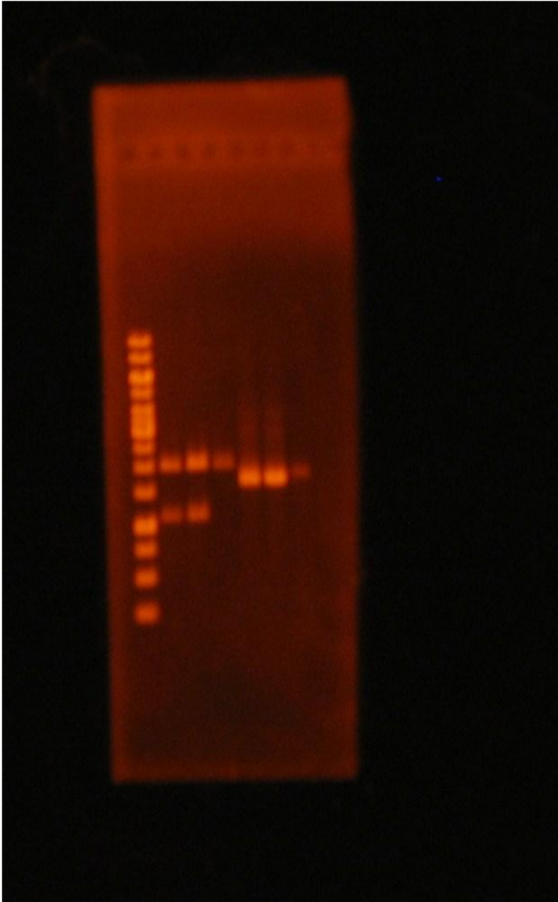
The image shows the data, where a clear burst of emission can be observed in the CBG (pink) luciferase. As the other data can't be distinguished in this graph, the next one shows the dynamic for every sample with the exception of CBG.



As a result, a functional luciferase was obtained, and even though its emission was not among the red spectrum, it was useful for the LRE characterization.

LRE

Luciferin Regenerating Enzyme (LRE) was first reported by Gomi, K. and Kajiyama, N. [(2001) Oxyluciferin, a Luminescence Product of Firefly Luciferase, Is Enzymatically Regenerated into Luciferin. *The Journal of Biological Chemistry*, Vol. 276, No. 39. This enzyme proved to recycle the luciferase product oxyluciferin. We decided to synthesize LRE with Mr. Gene and then to ligate it



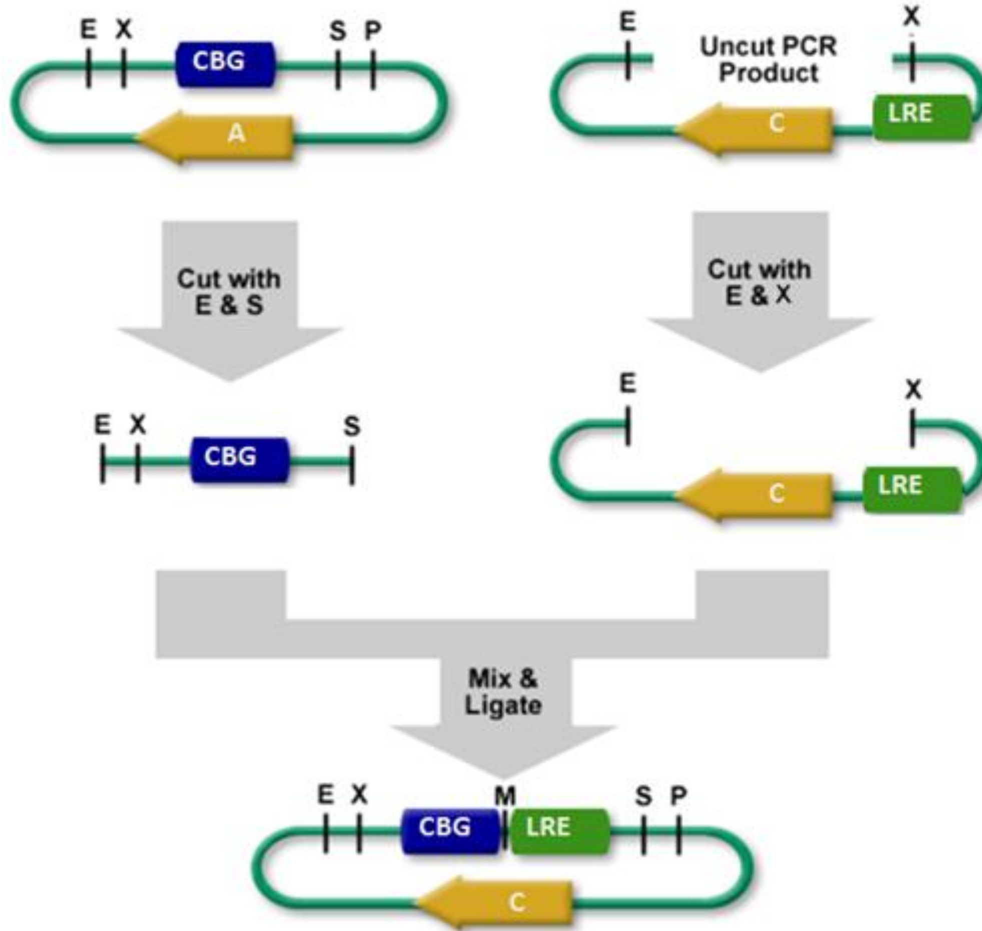
into the DNA submission plasmid pSB1C3 (BBa_K360113), and also into a constitutive promoter (J23100). The gel shows both, LRE and luciferases in the next order:

1. Ladder.
2,3,4. LRE + pSB1C3 restriction.
5. Green CB luciferase from purified PCR product.
6. Red CB luciferase from purified PCR product.
7. Mutated firefly luciferase from purified PCR product.

As the ligation with the BBa_J61002 went well, the next step was to insert the LRE with the constitutive promoter J23100 into the pSB1C3. The resulting biobrick was the part BBa_K360111.

Vector Containing both Click Beetle Luciferase and LRE

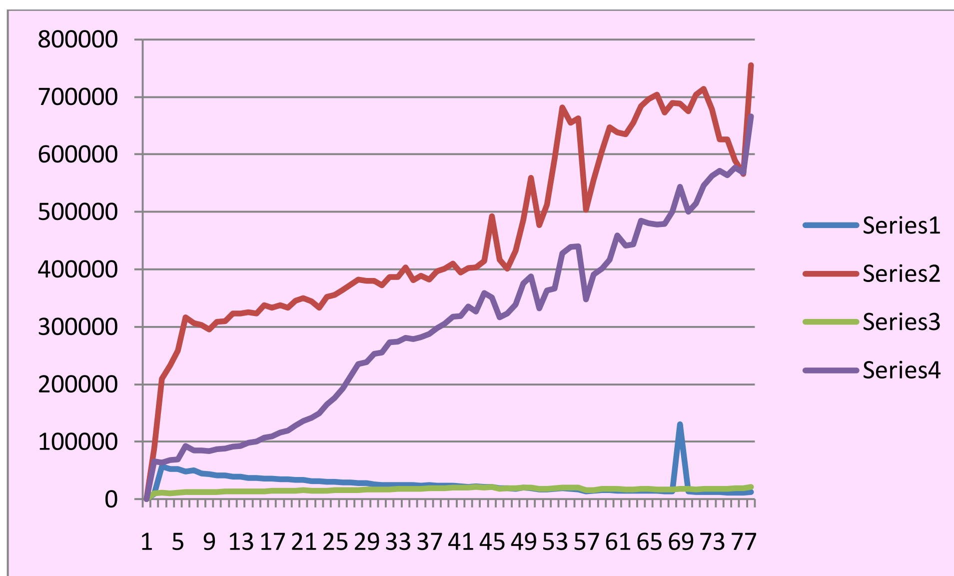
Our goal here was to couple both luciferase and LRE to be able to make the construction autonomous after the first and single input of luciferin. We tried two approaches where we intended to ligate either the Click Beetle Luciferase + promoter J23102 into the plasmid containing the LRE (pSB1C3), or the LRE used as an insert and ligate into the CBG luciferase plasmid (BBa_J61002) and just one of the resulting colonies was used to assess the functionality of LRE. The successful construction was the first one, which is represented in the following image (original image taken from http://partsregistry.org/Image:3AAssembly.png).



Once the construction was ready, Amhed ran an assay, where cells were normalized to an $OD_{(620nm)}$ = 0.3 for the following samples:

1) Negative control = Sodium citrate 1mM
2) Medium without bacteria
3) Bacteria without transforming (DH5-α)
4) Series4 (Positive control) = EPIC + LRE (BBa_K325909) from Cambridge Team
5) Series1 = BBa_K360113
6) Series2 = Click Beetle Green Luciferase
7) Series3 = L. cruciata luciferase + LRE from Cambridge Team

The results are graphed next (after 78 measurements):



The assay followed the design referred previously by Edinburgh (BBa_K216015) using 1µl of luciferin 100mM in 0.5ml of culture in Sodium citrate medium. Results demonstrate the fact that a better protocol must be designed and it will be useful to check out how diverse variables affect the dynamics. Agitation (oxygenation) must be necessary, as the increase in LRUs observed in Series1 (by measure 69) was due to shaking of the assay tube. Another explanation may be attributed to diffusion of luciferin. Up to now, we have no exact answer to these questions.

Conclusions

According to the objectives proposed at the beginning of the competition, I was able to achieve a functional luciferase as reported by the assay referred in the upper part of this report. This luciferase was then ligated with the LRE synthesized biobrick and intended to be characterized, though results demonstrate that some parameters must be improved to have a better output.

Future Perspectives

*Continue to improve the LRE assay to be able to characterize it before the competition.

*Once characterization of LRE is achieved; work with the Click Beetle Red luciferase is needed to check whether the spectrum change makes any difference in the effectiveness of LRE.

*Sequence the mutated red firefly luciferase to check whether the punctual mutation was performed correctly and, to our possibilities, try to make it functional.

My iGEM experience

iGEM came out to be a very enriching experience in various aspects of my life, ranging from learning how to work with any available media when you are left alone at a lab; how to record my work daily; how to work as a team; and also to question the results of an experiment. It is a very stressful, tiring, amazing experience which I wouldn’t change for anything ☺.