BioBrick assembly

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BioBrick assembly

During the summer we have been able to construct the following BioBricks:

BBa_K2349000 – ADH1 promoter for yeast

BBa_K2349001 - CYC1 terminator from the yeast

BBa_K2349002 - SUC2 gene

BBa_K2349003 - EFE optimized for yeast

BBa_K2349004 - FCY2 promoter (BBa_K530015), SUC2 gene (BBa_K2349002), tFba1 terminator (BBa_K1829004)

BBa_K2349005 - ADH1 promoter (BBa_K2349000), EFE gene (BBa_K1065000), tENO2 terminator (BBa_K1829003)

BBa_K2349006 - ADH1 promoter BBa_K2349000, EFE optimized (BBa_K2349003), CYC1 terminator (BBa_K2349001)

BBa_K2349010 - TEF2 promoter (BBa_K165037), EFE gene (BBa_K1065000), tENO2 terminator (BBa_K1829003)

Protocols

Bacterial transformation

Materials:

Strain *E. coli* DH5α
Plasmid DNA
Ice
Thermostat 42C
Selection plates
LB liquid medium

Transformation:

1. Take the competent cells out of -80C fridge and put them on ice and let them melt
2. Take 1ul of plasmid DNA and put it in a separately labelled Eppendorf
3. Add 50ul of the cells, resuspend gently
4. Leave it on ice for 30 minutes
5. Heat shock at 42C for 2 minutes
6. Put them on ice for 2 minutes
7. Add 400ul of LB medium into the eppendorfs, put them all together in a flask and shake at 37C for 30 minutes
8. Take the selection plates from the cold room and leave them on the table (label them and add around 10 glass beads to each plate)
9. Take Eppendorf out and centrifuge them at 6000 rpm for 60 seconds
10. Remove supernatant and resuspend cells in the remaining liquid
11. Pipette the cells onto the selection plates and shake them until the surface is dry
12. Transfer the glass beads into the bid container containing ethanol
13. Let cells grow at 37C overnight

Digestion protocol

In our experiments, we have used both TermoFisher's and Neb's digestion kits and protocols!

TermoFisher's protocol:

DNA	500 ng
Enzyme 1	2ul
Enzyme 2	2ul
Fast digest GB 10x TermoFisher®	4ul
Mq water	up to 40ul
Total	40ul

Neb's protocol:

DNA	500 ng
Enzyme 1	1ul
Enzyme 2	1ul
10X NEBuffer 2.1 ®	5ul
Mq water	up to 50ul
Total	50ul

Leave the mixture at 37C for 60 minutes!

Ligation protocol

In our experiments, we have used both TermoFisher's and Neb's ligation kits and protocols!

TermoFisher's protocol:

Plasmid DNA	1ul
Insert	3ul/5ul
Ligation buffer	2ul
T4 Ligase TermoFisher®	1ul
Mq water	14ul/12ul
Total	20ul

Neb's protocol:

Plasmid DNA	1ul
Insert	3ul/5ul
10x T4 DNA Ligase Buffer	2ul
T4 Ligase NEB®	1ul
Mq water	14ul/12ul
Total	20ul

Leave the mixture on the bench for 60 minutes or at 14C overnight!

Gel purification and Minis

For our gel purification and minis, we used Favorgen®'s kit!

Colony PCR Protocol

Once we got colonies we wanted to locate the one that contained the insert of our interest, and to do this we used the colony PCR protocol.

Dream Taq TermoFisher® Polymerase	12.5ul
DNA	1ul
Verification primer VR_F	1.5
Verification primer VR_R	1.5
mQ water	8.5
	25ul

PCR cycle protocol

Step	Time	Temperature (ºC)	# of cycles
Initial step	3 min	95	
Denaturaturation	30 s	95	35
Anneling	30 s	52	
Extension	* s	72	
Final extension	10 min	72	

* *The extension time depended on the length of the segment of interest. We would usually allow 60s for 1kb!*

The list of primers that we used:

NAME	***EXPLANATION***	***SEQUENCE 5'-3'***
ADH1_F	Amplification of ADH1 promoter with the prefix	ATATATGAATTCGCGGCCGCTTCTAGAGATCCTTTTGTTGTTTCCGGGTG
ADH1_R	Amplification of ADH1 promoter with the sufix	CAAGCTATACCAAGCATACAATCAACTTACTAGTAGCGGCCGCTGCAGAATAAT
CYC1_F	Amplification of CYC1 terminator with the prefix	GAATTCGCGGCCGCTTCTAGAGTCATGTAATTAGTTATGTCACGCTTAC
CYC1_R	Amplification of CYC1 terminator with the suffix	CGCTCGAAGGCTTTAATTTGCTACTAGTAGCGGCCGCTGCAGATAATT
SUC2_BB1_start_for	amplification of SUC2 gene with prefix	atatatGAATTCGCGGCCGCTTCTAGATGCTTTTGCAAGCTTTCCTTTTCC
SUC2_BB1_stop_rev	amplification of SUC2 gene with suffix	CATTGACAAGTTCCAAGTAAGGGAAGTAAAATAGTACTAGTAGCGGCCGCTGCAGataatt
SUC2_BB1_mut_F	mutation of SUC2 gene for illegal restriction site removal	GTTTGACAATCAATCGAGAGTGGTAGATTTTGGT
SUC2_BB1_mut_R	mutation of SUC2 gene for illegal restriction site removal	GTTTGACAATCAATCGAGAGTGGTAGATTTTGGT
VR primer F	Verification primer	CCACCTGACGTCTAAGAAAC
VR primer R	Verification primer	GTATTACCGCCTTTGAGTGA

ADH1 Promoter and CYC1 Terminator Amplification

Week 1

ADH1 Promoter and CYC1 Terminator had to be amplified from the DNA template as they were not present in the iGEM's official kit.

We started off with the PCR reaction for amplification of both ADH1 promoter and CYC1 terminator. *The protocols are presented below*!

Amplification PCR for ADH1 :

Reagents	1 reaction (uL)	4 reactions (uL)
Dream Taq mix	25	100
Primer ADH1_F (10uM)	2.5	10
Primer ADH1_R (10 uM)	2.5	10
template DNA x50 dilution	1	4
ddH20	19	76
Total	50	200

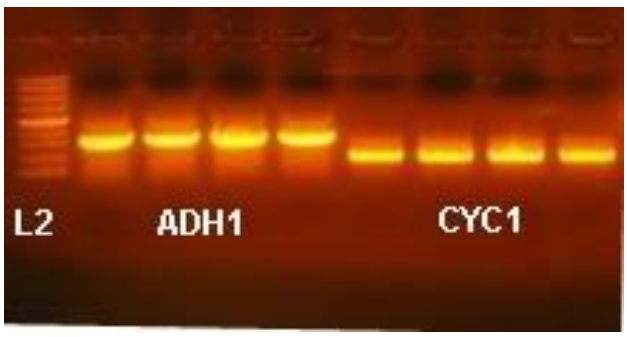
Amplification PCR for CYC1 :

Reagents	1 reaction (uL)	4 reactions (uL)
Dream Taq mix	25	100
Primer CYC1_F (10uM)	2.5	10
Primer CYC1_R (10 uM)	2.5	10
template DNA x50 dilution	1	4
ddH20	19	76
Total	50	200

The PCR protocol for amplification was the same and as follows:

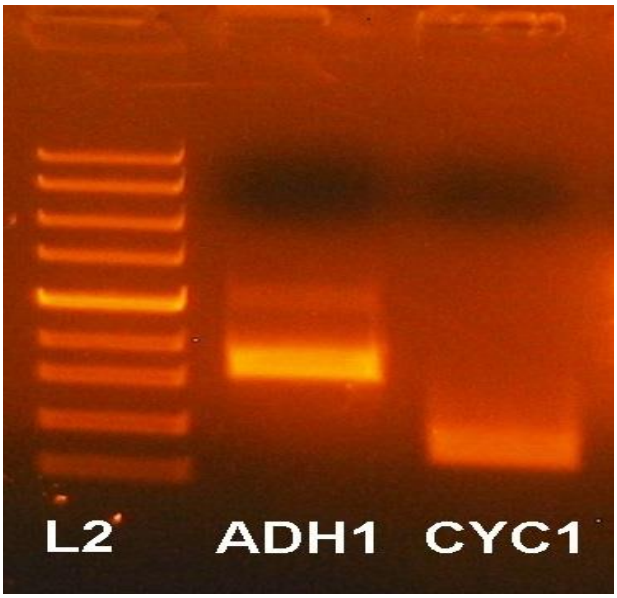
Step	Time	Temperature (ºC)	# of cycles
Initial step	3min	95	
Denaturaturation	30s	95	35
Annealing	30s	50	
Extension	40s	72	
Final extension	15min	72	

The expected length of the ADH1 promoter is 748bp and CYC1 terminator 291bp.



Since all of the fragments correspond to the appropriate lengths we proceeded with gel purification.

We have also digested the purified fragments with EcoRI and PstI restriction enzymes *(See the digestion protocol ThermoFisher above)*



As the fragments we of the corresponding length they were purified and ligated into already linearised pSB1C3 (digested with EcoRI and PstI) vector.

The ligation followed the protocol attached above! *(See ligation protocol ThermoFisher)* We tried both 1:3 and 1:5 ratios.

Week 2

We transformed our ligation mixture into the DHα5 competent cells *(See Bacterial transformation protocol)*

As the transformation worked, we decided to put minis to grow!

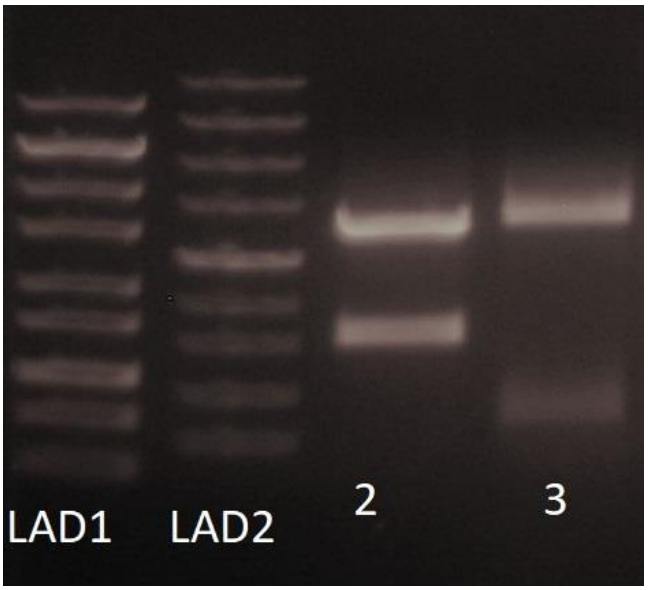
Before we sent them for sequencing we decided to digest the product in order to see if the corresponding bends will be obtained!

We used the following digestion protocol:

Reagents	**Amounts 1 rxn**
Plasmid	1ug
Fast digest green buffer	2ul
EcoRI	1ul
Pstl	1ul
Water	16-plasmid(ul)
TOTAL	20ul

Incubated for 1hr at 37C.

The results:



Number 2 – ADH1

Number 3 – CYC1

As both fragments were of the corresponding length we sent them for sequencing!

Week 3

After we have sequenced ADH1 and CYC1, we learnt that the sequence corresponds perfectly to the expected sequence. Therefore, we can say that we have successfully managed to amplify, digest and ligate ADH1 promoter and CYC1 terminator into the pSB1C3 vector!

SUC2 BioBrick Construction

SUC2 gene is a central part of our project and as it was not present in iGEM's official kit, we decided to amplify it ourselves. As the gene itself contained an illegal restriction site, we had to mutate the site by performing a two-step PCR reaction.

Week 1

PCR mixture for amplification of 1-800bp

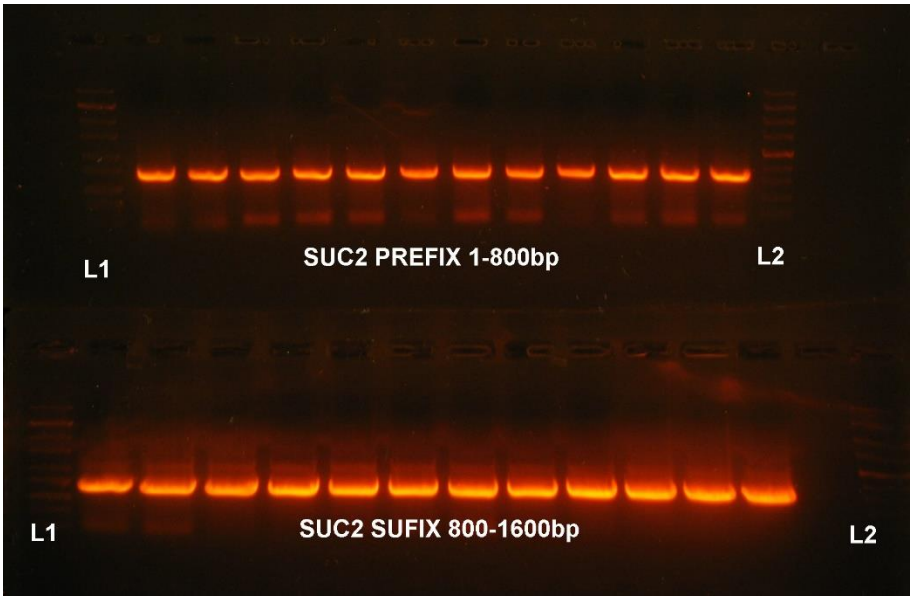
Reagents	1 reaction (uL)	2 reactions (uL)
HF buffer	10	20
oligo SUC2_BB1_start_for 10uM	2.5	5
oligo SUC2_BB1_mut_R 10uM	2.5	5
genomic DNA	2	4
phusion	0.5	1
dNTPS 25mM	0.5	1
ddH20	32	64
Total	50	100

PCR mixture for amplification of 800-1600bp

Reagents	1 reaction (uL)	2 reactions (uL)
HF buffer	10	20
oligo SUC2_BB1_stop_rev 10uM	2.5	5
oligo SUC2_BB1_mut_F 10uM	2.5	5
genomic DNA	2	4
phusion	0.5	1
dNTPS 25mM	0.5	1
ddH20	32	64
Total	50	100

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	98	
Denaturaturation	20 s	98	30
Anneling	20 s	60	
Extension	30 s	72	
Final extension	5 min	72	

Electrophoresis:



All the fragments were of the corresponding length, but due to a small misunderstanding within the team, we were only able to save one sample of each.
The samples were purified and quantified.

A(prefix) - 51 ng/uL
B(sufix) - 117 ng/uL

The new reaction was done in order to assemble both parts into one gene (Suc2)

PCR reaction protocol:

Reagents	1 reaction (uL)
HF buffer	10
1-800 SUC2 A	1
800-1600 SUC2 B	0.5
primers SUC2_BB1_start_for and SUC2_BB1_start_rev*	5(2.5 each)
phusion	0.5
dNTPS 25mM	0.5
ddH20	32.5
Total	50

*The primers were added after the first cycle was done (after about 10 minutes)

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	98	
Denaturation	40s	98	
Annealing	1min	65	
Extension	3min	72	
Add primers			
Denaturaturation	40s	98	32
Anneling	20 s	65	

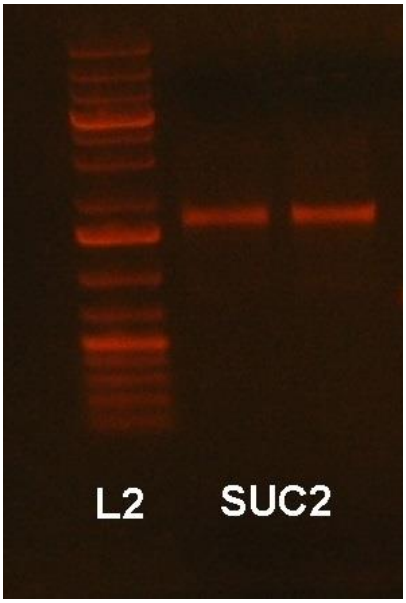
Extension	30 s	72	
Final extension	10min	72	

Electrophoresis:

As all fragments corresponding to the 1.6kb (which is the length of our gene) we can infer that the PCR worked. The PCR product will further be purified, quantified, and digested so that it can be inserted into the linearized pSB1C3 plasmid and sent for sequencing!

Quanify:
SUC2 A - 17.5 ng/uL
SUC2 B - 113.6 ng/uL
SUC2 C - 110.0 ng/uL
SUC2 D - 136.6 ng/uL
SUC2 E - 92.3 ng/uL

We have also digested purified fragments with EcoRI and PstI restriction enzymes. *(See digestion protocol TermoFisher®)*

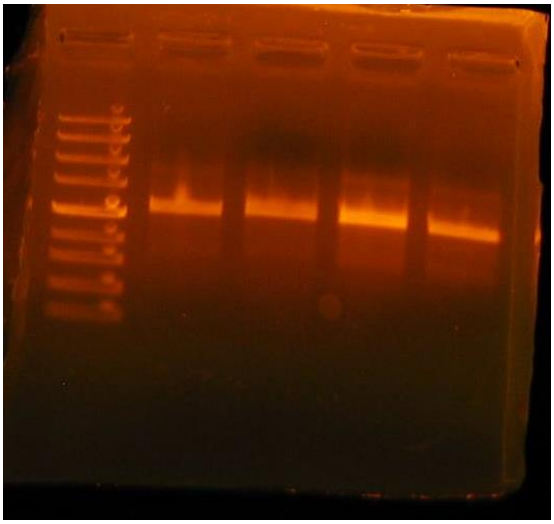


As the bands were of the corresponding length we ligated the purified mixture into the pSB1C3 linearised vector. *(See Ligation protocol TermoFisher®)*

Week 2

Ligation mixture has been transformed into DH5α competent cells by using *BACTERIAL TRANSFORMATION* protocol. The plates have been incubated at 37C overnight

As the transformation did not work we decided to digest the SUC2 gene with EcoRI and PstI one more time! – We prepared 4 reactions! *(See Digestion protocol TermoFisher®)*



As the bands were of the corresponding length we ligated the purified mixture into the pSB1C3 linearised vector. *(See Ligation protocol TermoFisher®)*

Ligation mixture has been transformed into DH5α competent cells by using *BACTERIAL TRANSFORMATION* protocol. The plates have been incubated at 37C overnight

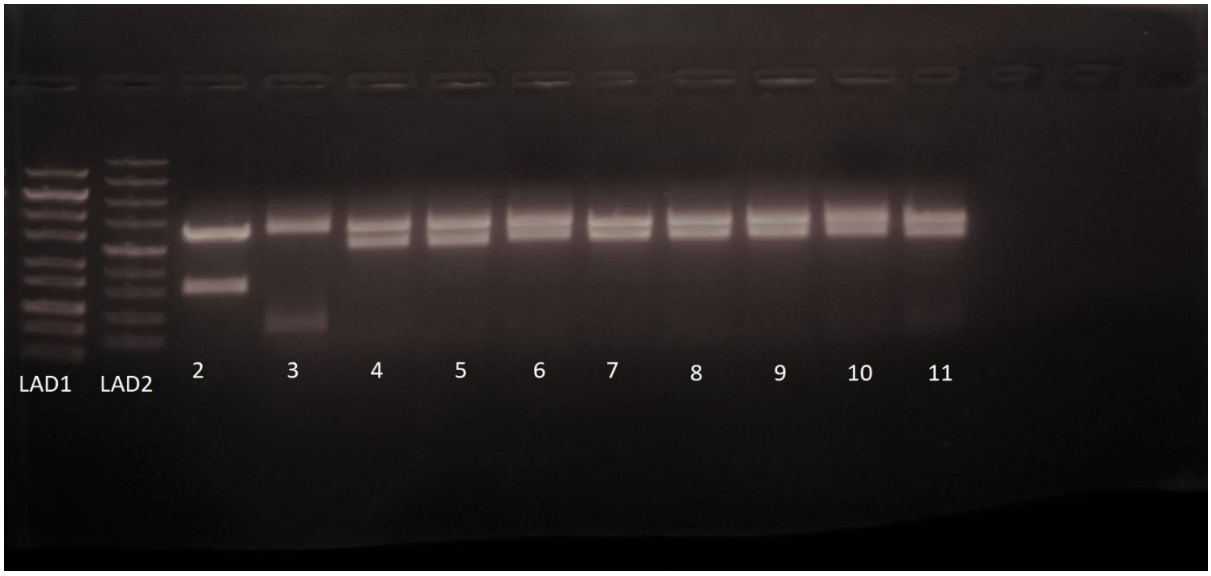
The transformation worked ☺ The minis were put to grow!

Before we sent minis for sequencing we decided to digest the product in order to see if the corresponding bends will be obtained!

We used the following digestion protocol:

Reagents	**Amounts 1 rxn**
Plasmid	1ug
Fast digest green buffer	2ul
EcoRI	1ul
Pstl	1ul
Water	16-plasmid(ul)
TOTAL	20ul

Incubated for 1hr at 37C.



Numbers 6-11 represent different colonies containing the SUC2 plasmid! As the digestion worked we sent them to sequencing.

The sequencing results showed that the colony number 9 contained SUC2 gene with the correct sequence!

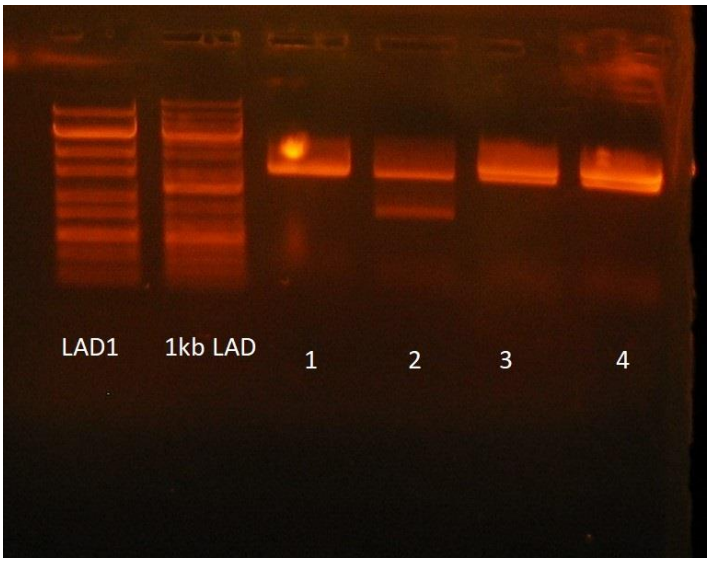
Week 3

We wanted to ligate our newly synthesised SUC2 gene with the FCY2 promoter (BBa_K530015) and tFba1 terminator (BBa_K1829004), both of which we have obtained from the official iGEM's kit.

SUC2 was digested with XbaI and PstI enzymes whereas the FCY2 promoter part was digested with SpeI and PstI enzymes!*(See Digestion protocol TermoFisher®)*

The two were ligated using the *Ligation protocol TermoFisher®* and transformed into DH5α competent cells by using *bacterial transformation* protocol.

As the colonies were observed we decided to do the digestion prior to sequencing! The digestion was done with EcoRI and PstI enzymes.*(See Digestion protocol TermoFisher®)*

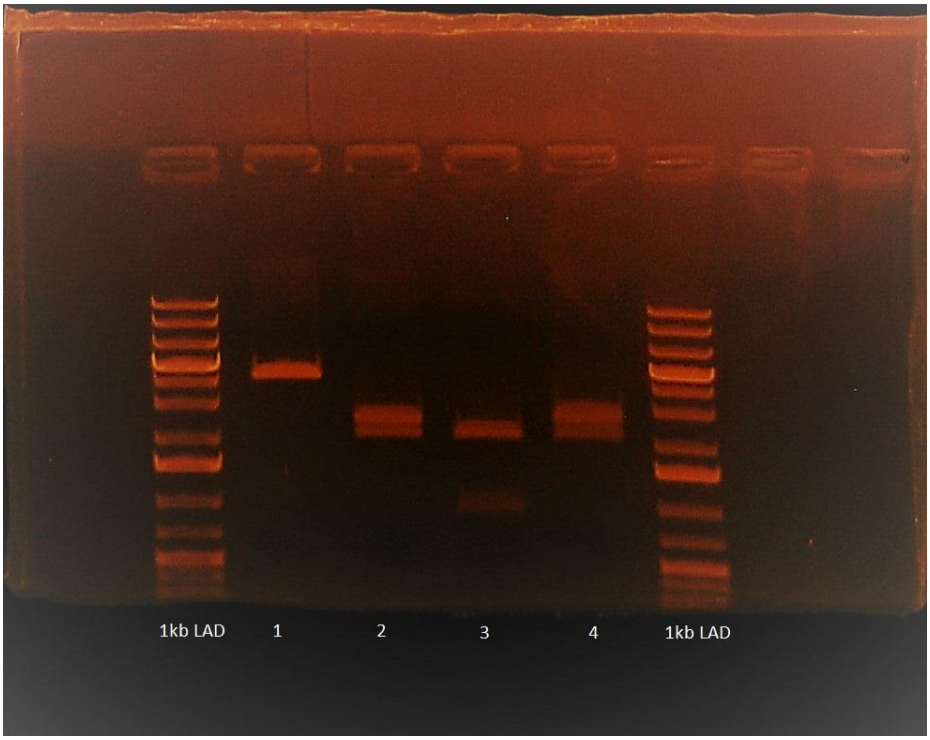


The length of FCY2 + SUC2 is around 2.5kb, therefore, colonies 3 and 4 seem to have the corresponding length. The samples were sent for sequencing!

Week 4

The sequencing results showed that no mutations took place so we were ready to ligate FCY2+SUC2 into the tFba1 terminator.

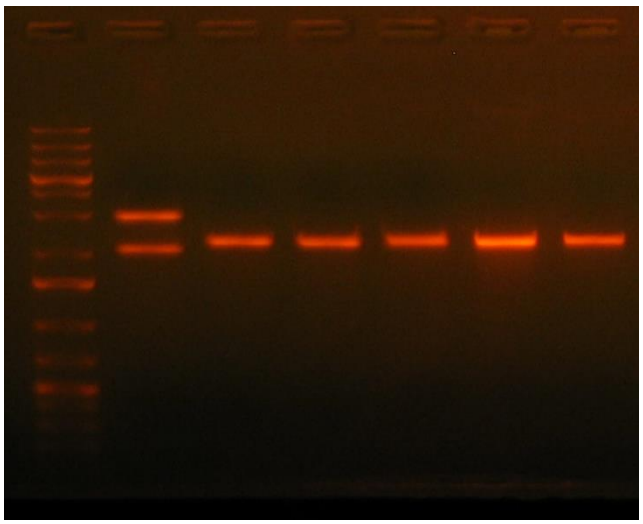
Firstly, we digested the FCY2+SUC2 part with SpeI and EcoRI enzymes and tFba1 terminator was already predigested with EcoRI and XbaI enzymes. *(See Digestion protocol NEB®)*



Number 4 corresponds to the FCY2+SUC2 part and it can be seen it is of a corresponding length. The part was ligated into the pSB1C3 vector containing the tFba1 terminator. *(See Ligation protocol NEB®)*

The ligation mixture was transformed into DH5α competent cells by using *bacterial transformation* protocol.

The transformation worked so we made Minis ☺

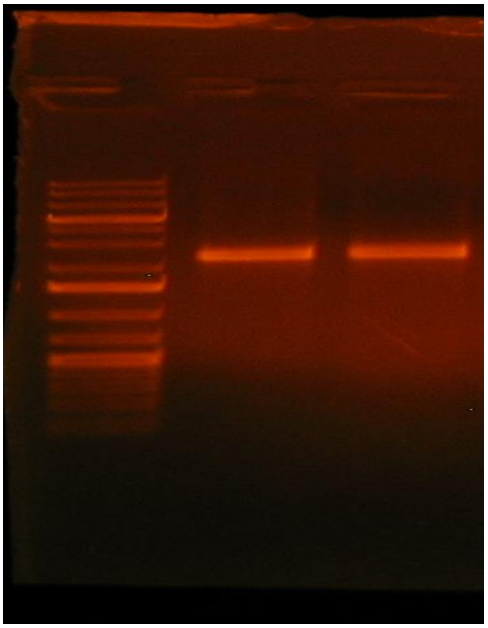


The digestion confirmed the right size 3kb, so a sample was sent to sequence!

Sequencing results show that we have successfully managed to construct FCY2+SUC2+tFba1 BioBrick! ☺

Week 5

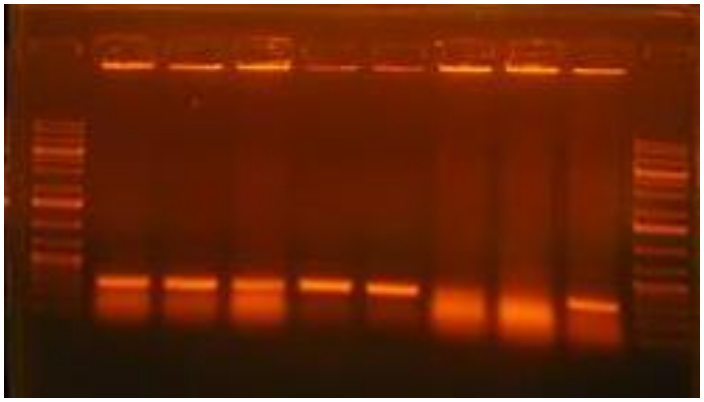
Our second SUC2 biobrick came already synthesised thanks to IDT company! We followed their instructions and digested the TEF2+SUC2+CYC1 with EcoRI and PstI enzymes using *Digestion protocol NEB®.*



The corresponding length of 2.3kb was observed and we proceeded on with the ligation of the vector into both pSB1C3 vector and a yeast pRS305 vector. *(See Ligation protocol NEB®)*

We transformed both of the ligation mixtures into DH5α competent cells by using *bacterial transformation* protocol.

Unfortunately, we have not been able to, even after a couple of attempts, to clone this BioBrick into the pSB1C3 vector. We would either get colonies with empty plasmid (pic below) or for positive colonies the sequencing showed that the sequencing was missing the terminator.

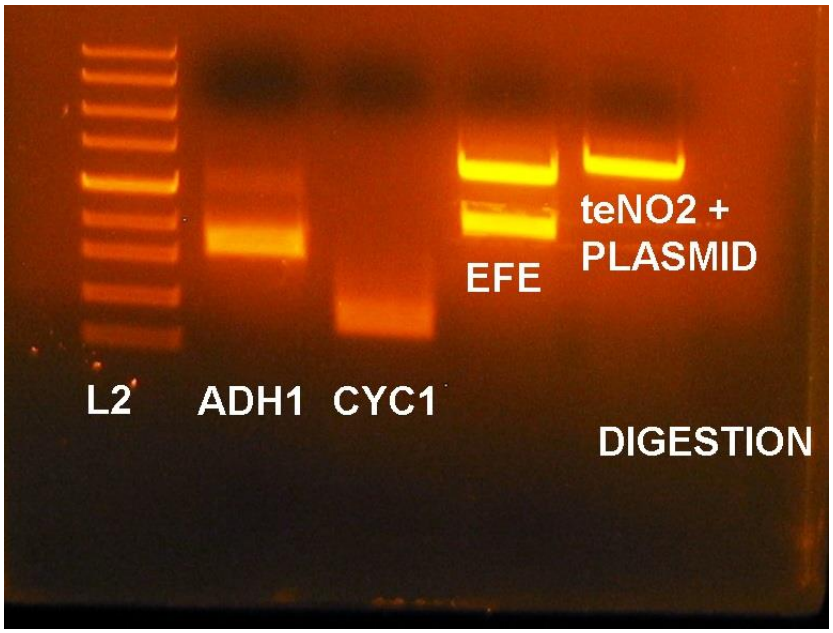


We have, however, been able to successfully clone it into the yeast vector. This part was very useful as it allowed us to fully characterise the SUC2 gene!

EFE BioBrick assembly

Week 1

EFE gene has been taken from the official iGEM's kit (BBa_K1065000). We decided to digest it and ligate into the pSB1C3 vector containing tEno2 terminator.
EFE has been digested with EcoRI and SpeI enzymes and tEno2 with XbaI and EcoRI.*(See Digestion protocol TermoFisher®)*



EFE size – 1.1kb

tEno2 + pSB1C3 – 2.5KB

As we got the expected sizes, we ligated the EFE gene into the vector containing the terminator. *(See Ligation protocol TermoFisher®)*

Ligation mixture has been transformed into DH5α competent cells by using *BACTERIAL TRANSFORMATION* protocol. The plates have been incubated at 37C overnight.

Week 2

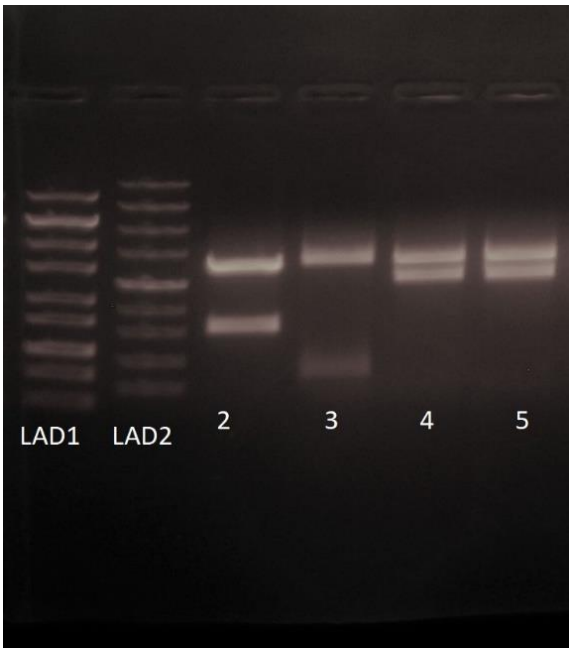
The transformation worked ☺ The minis were put to grow!

Before we sent minis for sequencing we decided to digest the product in order to see if the corresponding bends will be obtained!

We used the following digestion protocol:

Reagents	Amounts 1 rxn
Plasmid	1ug
Fast digest green buffer	2ul
EcoRI	1ul
Pstl	1ul
Water	16-plasmid(ul)
TOTAL	20ul

Incubated for 1hr at 37C.



4 and 5 - tEno2+EFE
As it can be seen from the gel, the digestion did succeed.

The sequencing showed that we got the right sequence ☺

As we have successfully ligated EFE and teNO2 inside of a pSB1C3 plasmid, we are going to ligate this insert into the plasmid with TEF2 and AHD1 promoter. Therefore, we did the digestion of all the parts.

Digestion was done with the following protocol:

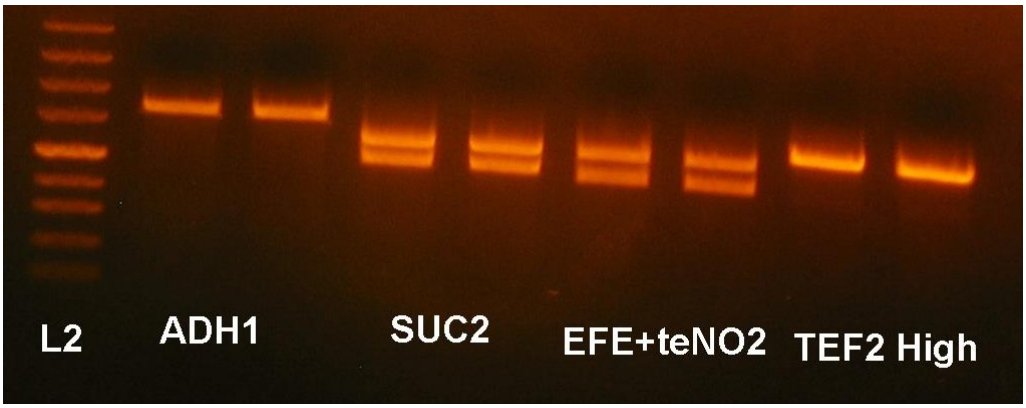
Reagents	Amounts 1 rxn
EFE+teNO2	5
Fast digest green buffer	4
XbaI	2
Pstl	2
ddH20	27
TOTAL	40

Reagents	Amounts 1 rxn
TEF2	5
Fast digest green buffer	4
SpeI	2
Pstl	2
ddH20	27
TOTAL	40

Reagents	Amounts 1 rxn
ADH1	5
Fast digest green buffer	4
SpeI	2
Pstl	2
ddH20	27
TOTAL	40

Digestion took place at 37C for 60 minutes.

Electrophoresis:



As all the bends corresponded to the right size we ligated EFE+teNO2 into the vectors containing TEF2 and ADH1 promoter! *(See Ligation protocol ThermoFicher®)*

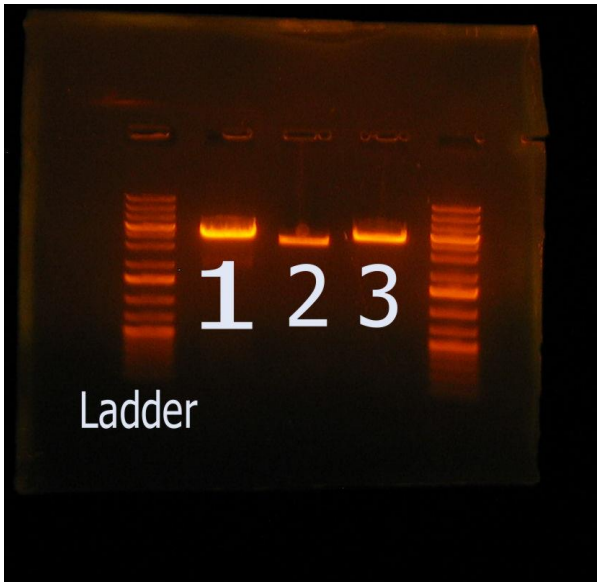
We transformed all of the ligation mixtures into DH5α competent cells by using *bacterial transformation* protocol.

Week 3

Colonies of both TEF2+ EFE+teNO2 and ADH1 + EFE+teNO2 were observed so we decided to the digestion before sending them off for sequencing! As the size of TEF2+ EFE+teNO2 is almost the same as of our pSB1C3 plasmid not much could have been inferred from the digestion. We proceeded on with the colony PCR which also failed.

Nonetheless, we decided to send it for sequencing!

For our ADH1 + EFE+teNO2 BioBrick, we did the digestion following the *(See Digestion protocol NEB®)*



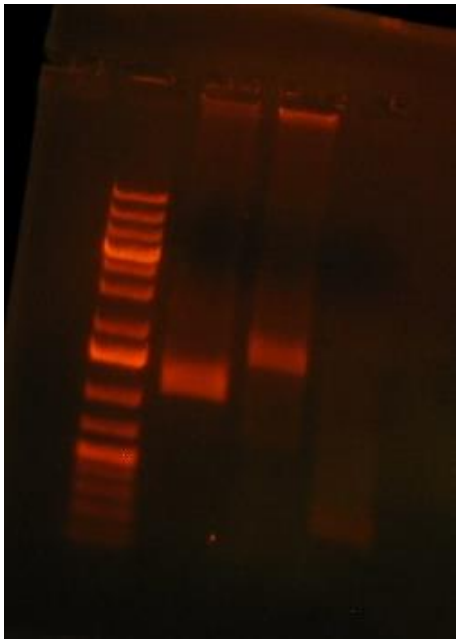
We used wrong enzymes and managed to linearize the vector. As it was of the corresponding size, the sent if for sequencing.

The sequencing results came and revealed that we have managed to produce two new BioBricks!

From IDT we got an EFE gene which has been codon optimized for yeast (we removed one illegal restriction site)

We have also received a full EFE BioBrick ADH1 promoter BBa_K2349000, EFE optimized (BBa_K2349003), CYC1 terminator (BBa_K2349001).

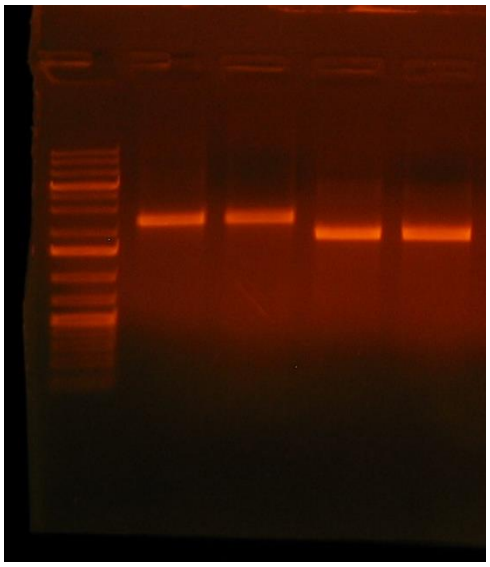
We followed their instruction and digested the both of our new parts in order to ligate them into the standard iGEM vector. Firstly, we digested them with EcoRI and PstI enzymes. *(See Digestion protocol NEB®)*



GeneRuler 1kb has been used. The first well corresponds to EFE optimized gene.

As it was of the right size we ligated it into the pSB1C3 vector.*(See Ligation protocol NEB®)*

Our ADH1 + EFE optimized + CYC1 has also been digested with EcoRI and PstI enzymes. *(See Digestion protocol NEB®)*



The corresponding wells are numbers 4 and 5. As it had the right size we ligated it into the pSB1C3 vector. *(See Ligation protocol NEB®)*

As we did not get a lot of colonies, all of them were sent for sequencing!

Week 4

We got the sequencing results and we inserted our EFE optimized and ADH1 + EFE optimized + CYC1 into the pSB1C3 vector ☺