Lab Book Part 1

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Gene deletions for yeast strains construction

In order for our subpopulation system to work we had to construct two different yeast strains. One, which could not break down sucrose and consumes only glucose and fructose for ethanol production (for simplicity we call it strain A). The other strain unable to consume hexose sugars, but is able to consume ethanol and from that produce ethylene (let's call it strain B).

We used the sequential gene knockout method developed by Hegemann and Heick (2011). In the budding yeast we can knockout the unwanted gene in a single step. Because of the fact that homologous fragments can easily recombinate with high efficiency in yeast. We use this phenomenon to replace the target gene with linearized DNA which contains a selectable marker gene with homologous regions to those found in the target gene.

So, in strain A the following genes must be deleted: SUC2; AGT1; ADH2;. In strain B, on the other hand, we wanted to delete genes SUC2; AGT1; GAL2; HXT1-7. Because SUC2 and AGT1 genes have to be knocked-out in both strains we began with deleting these two from our starting yeast strain (CEN.PK 7D). CEN.PK are widely used *S. cerevisiae* strains in fermentation, that's why we chose it to as host strain.

Below we describe the process of strain construction week by week and provide sufficient information to ensure reproducibility.

Primers, Plasmids, Strains used in this Project

All the primers used in this iGEM project were designed using *Benchling.org* software and synthesized by Mycrosynth. In tables 1 and 2 the primers used in this project are listed, in table 3 the plasmids used for gene deletion are listed, in table 4 the host strain as well as all strains and intermediates we need to construct are listed. Their status indicate whether we manage to get the strain or not.

Table 1. Primers for deletion of genes from strain A

Number	Name	Sequence(5'->3')	Description
1.	OL5'_SUC2	CAAGCAAAACA AAAAGCTTTTC TTTTCACTAACG TATATGCAGCT	SUC2 gene deletion

		GAAGCTTCGTACGC	
2.	OL 3'_SUC2	CTTTTGAAAAAAATAAAAAAGACAATAAGTTTTATAACCTGCATAGGCCACTAGTGGATCTG	SUC2 gene deletion
3.	A_SUC2	GGTACGCCCGATGTTTGCCTATTACC	confirmation of SUC2 gene deletion
4.	D _SUC2	CAAATTCCAGGTAACTGGGGTCGGGAG	confirmation of SUC2 gene deletion
5.	KanMx B-M	GGATGTATGGGCTAAATG	Primer for deletion confirmation using plasmid pUG6 and pUG27 as disruption cassete. Use with primer A of the deleted gene
6.	KanMx C-M	CCTCGACATCATCTGCCC	Primer for deletion confirmation using plasmid pUG6 and pUG 27 as disruption cassete. Use with primer D of the deleted gene

7.	OL 5'_ADH2	TACAATCAACT ATCAACTATTA ACTATATCGTA ATACACACAGC TGAAGCTTCGT ACGC	ADH2 gene deletion
8.	OL 3'_ADH2	ATAATGAAAAC TATAAATCGTA AAGACATAAGA GATCCGCGCAT AGGCCACTAGT GGATCTG	ADH2 gene deletion
9.	A_ADH2	GCCGGAACACC GGGCATCTCC	confirmation of ADH2 gene deletion
10.	D_ADH2	CGAGGGAGACG ATTCAGAGGAG CAGG	confirmation of ADH2 gene deletion

Table 2. Primers for deletion of genes from strain B

Number	Name	Sequence (5'->3')	Description
1.	OL5 AGT1	TACATAGAAGA ACATCAAACAA CTAAAAAAATA GTATAAT CAGCTGAAGCT TCGTACGC	AGT1 gene deletion

2.	OL3 AGT1	TTCCTTATTTCT TCCAAAAAAAA AAAAACAACCC TTTTACGCATA GGCCACTAGTG GATCTG	AGT1 gene deletion
3.	A_AGT1	CGTACCGGGCT TGAGGGACATA CAGA	confirmation of AGT1 gene deletion
4.	D_AGT1	GCACCTGGTAC TTGTGCCTGGA	confirmation of AGT1 gene deletion
5.	KanMx B-M	GGATGTATGGGC TAAATG	Primer for deletion confirmation using plasmid pUG6 and pUG27 as disruption cassete. Use with primer A of the deleted gene
6.	KanMx C-M	CCTCGACATCAT CTGCCC	Primer for deletion confirmation using plasmid pUG6 and pUG 27 as disruption cassete. Use with primer D of the deleted gene
7.	OL5_HXT1	AAGATATCATAA TCGTCAACTAGT TGATATACGTAA AATCCAGCTGAA GCTTCGTACGC	HXT1 gene deletion

8.	OL3_HXT1	TGTATAAGTCATTAAAATATGCATATTGAGCTTGTTTAGTGCATAGGCCACTAGTGGATCTG	HXT1 gene deletion
9.	HXT1-A	CCGACAAGCCAGGAAACTCCACCAT	confirmation of HXT1 gene deletion
10.	HXT1-D	CGGGCGTTTGAACTATGTATAGCGCC	confirmation of HXT1 gene deletion
11.	OL5’ HXT2	ACAACAAATTAAATTACAAAAAGACTTATAAAGCAACATACAGCTGAAGCTTCGTACGC	HXT2 gene deletion
12.	OL3’ HXT2	TTAGCCTTAAAAAAATCAGTGCTAGTTTAAGTATAATCTCGCATAGGCCACTAGTGGATCTG	HXT2 gene deletion
13.	HXT2 A	GGCCACGCAACTGGCGTGGAC	confirmation of HXT2 gene deletion

14.	HXT2 D	GTGTCAATACCT CGAAGCAGCGTT TCAAG	confirmation of HXT2 gene deletion
15.	OL5’ HXT3	ATAGAATCACAA ACAAAATTTACA TCTGAGTTAAAC AATC CAGCTGAAGCTT CGTACGC	HXT3 gene deletion
16.	OL3’ HXT3	TAAAATACACTA TTATTCAGCACT ACGGTTTAGCGT GAAAGCATAGGC CACTAGTGGATC TG	HXT3 gene deletion
17.	HXT3-A	GGGGGTTGCATA TAAATACAGGCG C	confirmation of HXT3 gene deletion
18.	HXT3-D	CCTGTTCGGCTC TCGCCGATGG	confirmation of HXT3 gene deletion
19.	OL5’ HXT4	GTTTGGTTTTGA AACACTTTTACA ATAAAATCTGCC AAAACAGCTGAA GCTTCGTACGC	HXT4 gene deletion
20.	OL3’ HXT4	TTATTCCTTGAA GGAAGTCTATAT	HXT4 gene deletion

		TATTTAATTAAC TGACGCATAGGC CACTAGTGGATC TG	
21.	A_HXT4	CAATTAGTGGTG AAAAGCTTCAAC ACTGGGG	confirmation of HXT4 gene deletion
22.	D_HXT4	GCCATCGTTAAG TGGAGAATTCGG CCTA	confirmation of HXT4 gene deletion
23.	OL5’ HXT5	ATTTTTCTAGAA AAAAGAATATAT TAGAGGTAAAGA AAGACAGCTGAA GCTTCGTACGC	HXT5 gene deletion
24.	OL3’ HXT5	TGCAAGTATGCG AAAATAGTTGAT CCTACACTACAA GAGAGCATAGGC CACTAGTGGATC TG	HXT5 gene deletion
25.	HXT5-A	GCTAGTCGAACG GTTCTCCCTCTA AG	confirmation of HXT5 gene deletion
26.	HXT5-D	GGCGTAGCAACC CTTTCTCCCC	confirmation of HXT5 gene deletion

27.	OL5’ HXT6	AAACACAAAAA CAAAAAGTTTTT TTAATTTTAATC AAAAACAGCTGA AGCTTCGTACGC	HXT6 gene deletion
28.	OL3’ HXT6	AATTAGAGCGTG ATCATGAATTAA TAAAAATGTTCG CAAAGCATAGGC CACTAGTGGATC TG	HXT6 gene deletion
29.	HXT6 A	TTCAGATGCCCT CCGTGCCTTCAT TG	confirmation of HXT6 gene deletion
30.	HXT6 D	GCGCCTACTTCG CTTCTAGCGC	confirmation of HXT6 gene deletion
31.	OL5’ HXT7	AAACACAAAAA CAAAAAGTTTTT TTAATTTTAATC AAAAACAGCTGA AGCTTCGTACGC	HXT7 gene deletion
32.	OL3’ HXT7	AATTAGAGCGTG ATCATGAATTAA TAAAAGTGTTCG CAAAGCATAGGC CACTAGTGGATC TG	HXT7 gene deletion

33.	HXT7-A	CCCCACCATCTTTCGAGATCCCCTG	confirmation of HXT7 gene deletion
34.	HXT7-D	GCGGTCGGTAAACAACTGACTTCTTCCC	confirmation of HXT7 gene deletion
35.	OL 5'_GAL2	AACACAAGATTAACATAATAAAAAAAATAATTCTTTCATACAGCTGAAGCTTCGTACGC	GAL2 gene deletion
36.	OL 3'_GAL2	AAAATTAAGAGAGATGATGGAGCGTCTCACTTCAAACGCAGCATAGGCCACTAGTGGATCTG	GAL2 gene deletion
37.	A_GAL2	GCCCTTCCCATCTCAAGATGGGGAGC	confirmation of GAL2 gene deletion
38.	D_GAL2	TCGGTGAACAAAGGATGGCAGAGCATG	confirmation of GAL2 gene deletion
39.	KIURA3 B-M	CTAATAGCCACCTGCATTGG	Primer for deletion confirmation using plasmid pUG72 as disruption

			cassette. Use with primer A of the deleted gene
40.	KIURA3 C-M	CAGACCGATCTTCTACCC	Primer for deletion confirmation using plasmid pUG72 as disruption cassette. Use with primer D of the deleted gene
41.	KILEU2 B-M	AGTTATCCTTGGATTTGG	Primer for deletion confirmation using plasmid pUG73 as disruption cassette. Use with primer A of the deleted gene
42	KILEU2 C-M	ATCTCATGGATGATATC	Primer for deletion confirmation using plasmid pUG73 as disruption cassette. Use with primer D of the deleted gene

Table 3. Plasmids used in this project.

Number	Name	Description	Reference	Euroscarf catalog Number
1.	pUG6	Plasmid contains a disruption cassette which consists of a selection marker KanMX and LoxP recombination site. This disruption cassette is used as a template for PCR amplification	[1]	P30114

2.	pUG27	Plasmid contains a disruption cassette which consists of a selection marker His+ and LoxP recombination site. This disruption cassette is used as a template for PCR amplification	[1]	P30115
3.	pUG72	Plasmid contains a disruption cassette which consists of a selection marker Ura+ and LoxP recombination site. This disruption cassette is used as a template for PCR amplification	[1]	P30117
4.	pUG73	Plasmid contains a disruption cassette which consists of a selection marker Leu+ and LoxP recombination site. This disruption cassette is used as a template for PCR amplification	[1]	P30118

Table 4. Strains.

Number	Name	Genotype	Reference	Status
1.	CEN.PK1-7D	MATa URA3 TRP1 LEU2 HIS3 SUC2		
2.	CEN.PK1-2C	MATa ura3-52 trp1-289 leu2-3,112 his3 Δ SUC2		in stock

3.	CEN.PK1-2C**Δ**SUC2	MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 suc2Δ	This work	couldn't construct
4.	CEN.PK1-2C**Δ**SUC2**Δ**AGT1	MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 suc2Δ agt1Δ	This work	couldn't construct
5.	CEN.PK1-2C**Δ**SUC2**Δ**AGT1**Δ**GAL2	MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 suc2Δ agt1Δ gal2Δ	This work	couldn't construct
6.	CEN.PK1-2C**Δ**SUC2**Δ**AGT1**Δ**ADH2	MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 suc2Δ agt1Δ adh2Δ	This work	couldn't construct

Deletion diary

Week 1

First of all, we wanted to remove SUC2 gene, because it must be knocked-out from both strains we needed to construct . We inoculated CEN.PK 7D yeast strain in 5 mL of YPD liquid media and put it to grow to 30ºC 200 rpm shaker.

The disruption cassette for deletion of SUC2 gene was amplified using primers OL5'_SUC2 and OL 3'_SUC2 (number 1 and 2 in Table 1, respectively), and pUG6 plasmid as a template. In our project we have used DreamTaq Green PCR Master Mix (Thermo Scientific, Cat no: K1081), PCR reaction and Program were as follows:

PCR reaction mix

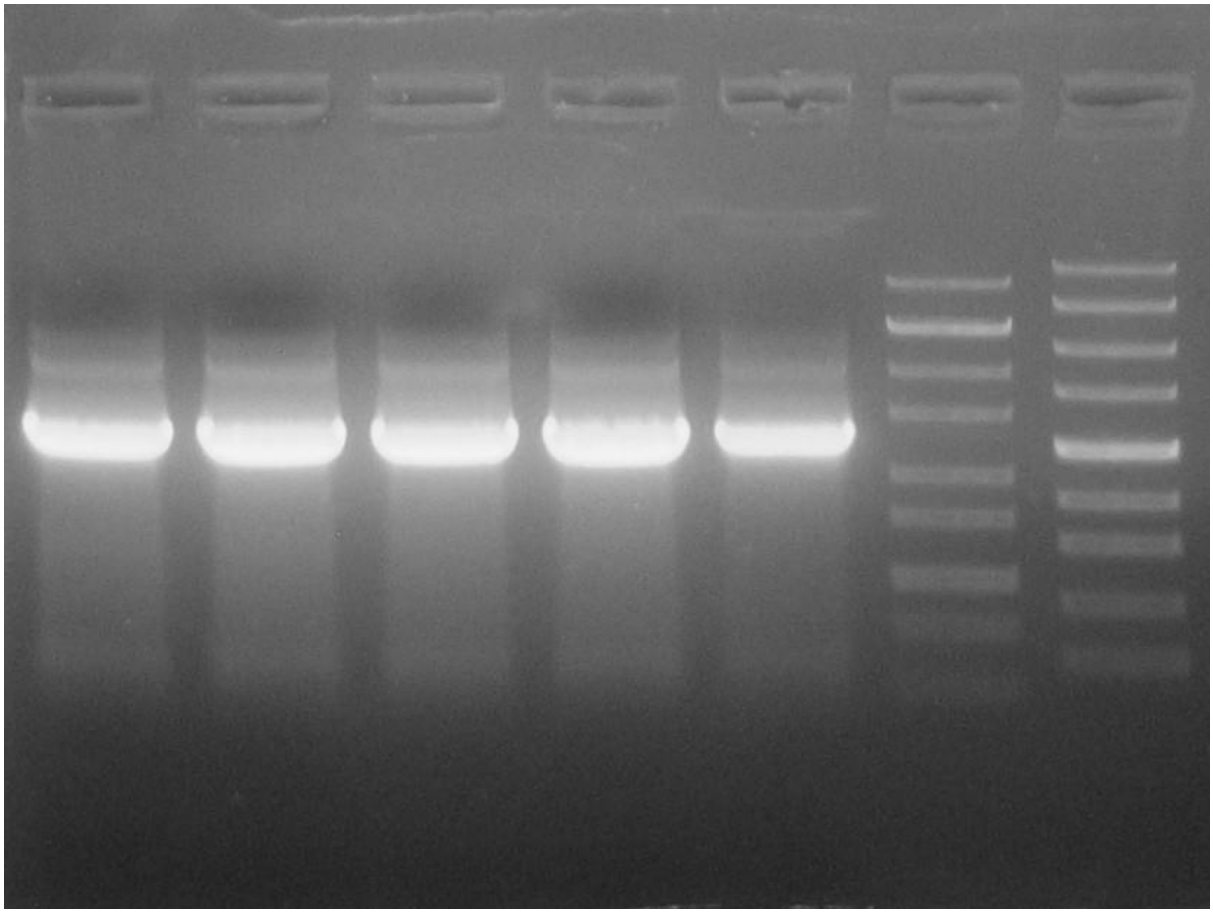
Reagent name	**Quantity (µL)**
DreamTaq Green PCR Master Mix (2X)	25
OL5'_SUC2	1
OL 3'_SUC2	1
pUG6	1
H_2O	22
Total	**50**

Program

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	95	
Denaturaturation	1 min 30 s	95	35

Anneling	30 s	58	
Extension	2 min	72	
Final extension	5 min	72	
Hold	∞	15	

The expected PCR product size was: 1.7 kb and after running the PCR product through 1% agarose gel for 30 min we got this result:



Lane 1-5. SUC2 disruption cassette

Lane 6. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Lane 7. ZipRuler Express DNA ladder 2 (Cat. No: SM1373)

The PCR product size was as expected (1.7 kb), so we purified it from gel with FavorPrep GEL/PCR Purification Mini Kit (Cat. No: FAGCK001-1) according to manufacturer's protocol. This PCR product was then used on the next day for transformation.

On the next day we diluted the overnight CEN.PK 7D culture to OD600 of 0.2 in 25 mL of YPD and let it grow till the mid-log phase (OD600 = 0.7) at 30ºC 200 rpm. Then we have transformed SUC2 disruption cassette into CEN.PK 2-1C strain using Li-Ac yeast transformation technique[1]. Transformed cells were plated on YPD+G418 plates and incubated at 30ºC for 24 h. After 24 h, the cells were replica plated to the new YPD+G418 plates and incubated 24 h more at 30ºC. Unfortunately, no colonies were formed.

Since we did not succeed with SUC2 deletions in the CEN.PK7D and this strain lacks free auxotrophic markers we opted to use a different and auxotrophic *S.cerevisiae* CEN.PK 2-1C strain, which would allow us to use disruption cassettes with amino acids markers.

So we inoculated CEN.PK 2-1C yeast strain in 5 mL of YPD liquid media and put it to grow overnight at 30ºC 200 rpm.

We repeated the transformation the following day with the same disruption cassette, but in a different strain. We diluted the overnight CEN.PK 2-1C culture to OD600 of 0.2 in 25 mL of YPD and let it grow till the mid-log phase (OD600 = 0.7) at 30ºC 200 rpm. Then we have transformed SUC2 disruption cassette into CEN.PK 2-1C strain using Li-Ac yeast transformation technique[1]. Transformed cells were plated on YPD+G418 plates and incubated at 30ºC for 24 h. After 24 h, the cells were replica plated to the new YPD+G418 plates and incubated 24 h more at 30ºC. Unfortunately, there was no colony growth observed again.

Week 2

We inoculated CEN.PK 2-1C yeast strain in 5 mL of YPD liquid media and put it to grow to overnight at 30ºC 200 rpm.

The disruption cassette for deletion of SUC2 gene was amplified using primers OL5'_SUC2 and OL 3'_SUC2 (number 1 and 2 in table 1, respectively), and pUG27 plasmid as a template. In our project we have used DreamTaq Green PCR Master Mix (Thermo Scientific, Cat no: K1081), PCR reaction and Program were as follows:

PCR reaction mix

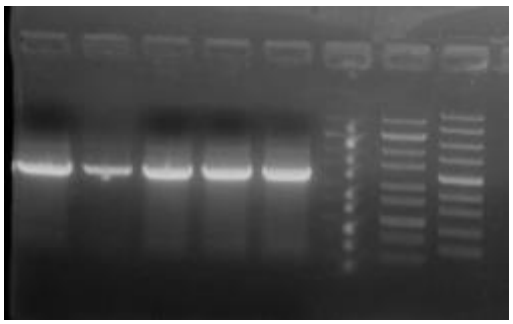
Reagent name	**Quantity (µL)**
DreamTaq Green PCR Master Mix (2X)	25

OL5'_SUC2	1
OL 3'_SUC2	1
pUG27	1
H_2O	22
Total	**50**

Program

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	95	
Denaturaturation	1 min 30 s	95	35
Anneling	30 s	58	
Extension	2 min	72	
Final extension	5 min	72	
Hold	∞	15	

The expected PCR product size was: 1.6 bp and after running the PCR product through 1% agarose gel for 30 min 150V 300A we got this result:



Lane 1.-5. SUC2 disruption cassette (pUG27)

Lane 6. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Lane 7. ZipRuler Express DNA ladder 2 (Cat. No: SM1373)

The PCR product size was as expected, so we purified it from gel with FavorPrep GEL/PCR Purification Mini Kit (Cat. No: FAGCK001-1) according to manufacturer's protocol. This PCR product was then used on the next day for transformation.

On the next day we diluted the overnight CEN.PK 2-1C culture to OD600 of 0.2 in 25 mL of YPD and let it grow till the mid-log phase (OD600 = 0.7) at 30ºC 200 rpm. Then we have transformed SUC2 disruption cassette into CEN.PK 2-1C strain using Li-Ac yeast transformation technique[1].Transformed cells were plated on -HIS/Glc plates and incubated at 30ºC for 48 h. There were colonies formed, so thirteen of them were chosen for colony verification PCR.

The chosen colonies were lysed to extract the genomic DNA (gDNA) in 30 µL of 20mM NaOH and kept in 100ºC thermostat for 10 minutes, vortexed, cooled down on ice for 2 minutes and centrifuged at 13 000 rpm for 15 s. These samples' supernatants were used as a template for colony verification PCR.

PCR reaction mix

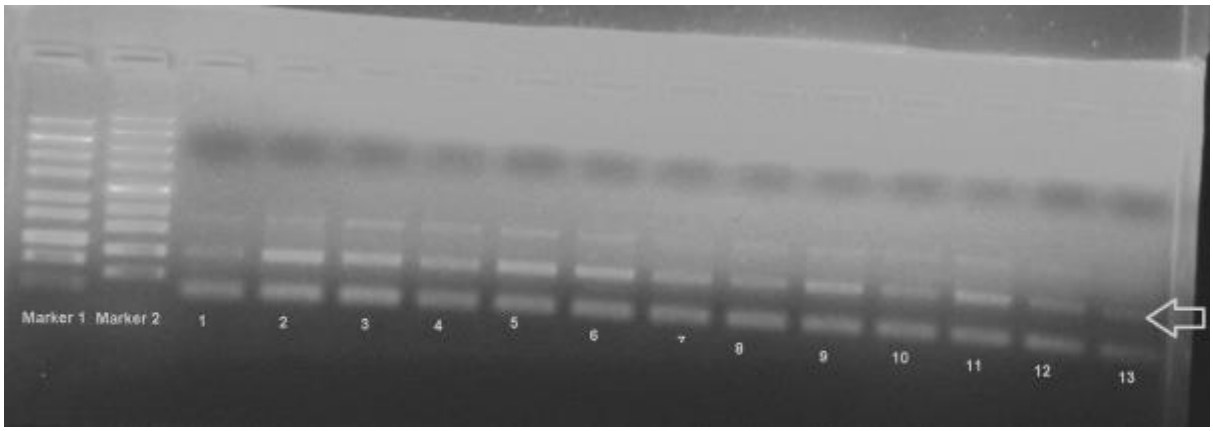
Reagent name	**Quantity (µL)**
DreamTaq Green PCR Master Mix (2X)	25
A_SUC2	1
KanMx B-M	1
gDNA	1
H_2O	22
Total	**50**

Program

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	95	

Denaturaturation	1 min 30 s	95	35
Anneling	30 s	50	
Extension	2 min	72	
Final extension	7 min	72	
Hold	∞	15	

The expected PCR product size was: 468 bp and after running the PCR product through 1% agarose gel for 30 min 150V 300A we got this result:



Lane 1. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Lane 2. ZipRuler Express DNA ladder 2 (Cat. No: SM1373)

Lane 3.-15. PCR product from colonies

Fragments of appropriate lengths were observed, hence, the transformation worked.

Glycerol stocks positive CEN.PK 2-1C **Δ**SUC2 colonies (2, 3, 9, 11) were prepared according to protocol.

Week 3

We have moved on to the next deletion step, which is to remove the AGT1 gene, because it must be knocked-out from both strains we needed to construct . We inoculated CEN.PK 2-1C **Δ**SUC2 yeast strain in 5 mL of YPD liquid media and put it to grow to 30ºC 200 rpm.

The disruption cassette for deletion of AGT1 gene was amplified using primers OL5 AGT1 and OL3 AGT1 (number 1 and 2 in table 2, respectively), and pUG73 plasmid as a template. In our

project we have used DreamTaq Green PCR Master Mix (Thermo Scientific, Cat no: K1081), PCR reaction and Program were as follows:

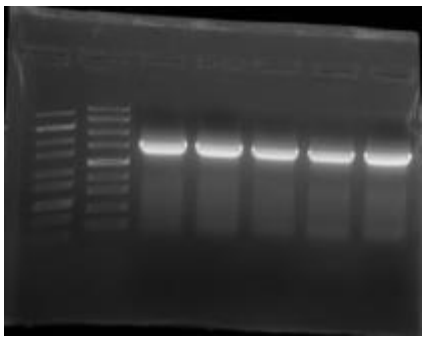
PCR reaction mix

Reagent name	**Quantity (µL)**
DreamTaq Green PCR Master Mix (2X)	25
OL5 AGT1	1
OL3 AGT1	1
pUG73	1
H_2O	22
Total	**50**

Program

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	95	
Denaturaturation	40 s	94	25
Anneling	1 min	58	
Extension	1 min	68	
Final extension	2 min	68	
Hold	∞	15	

The expected PCR product size was: 2.5kb and after running the PCR product through 1% agarose gel for 30 min 150V 300A we got this result:



Lane 1. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Lane 2. ZipRuler Express DNA ladder 2 (Cat. No: SM1373)

Lane 3.-7. AGT1 disruption cassette (pUG73)

The PCR product size was as expected, so we purified it from gel with FavorPrep GEL/PCR Purification Mini Kit (Cat. No: FAGCK001-1) according to manufacturer's protocol. This PCR product was then used on the next day for transformation.

On the next day we diluted the overnight CEN.PK 2-1C **Δ**SUC2 culture to OD600 of 0.2 in 25 mL of YPD and let it grow till the mid-log phase (OD600 = 0.7) at 30ºC 200 rpm. Then we have transformed AGT1 disruption cassette into CEN.PK 2-1C **Δ**SUC2 strain using Li-Ac yeast transformation technique[1]. Transformed cells were plated on -LEU/Glc plates and incubated at 30ºC for 48 h. Single colonies were observed.

The chosen colonies were lysed to extract the genomic DNA (gDNA) in 30 µL of 20mM NaOH and kept in 100ºC thermostat for 10 minutes, vortexed, cooled down on ice for 2 minutes and centrifuged at 13 000 rpm for 15 s. These samples' supernatants were used as a template for colony verification PCR.

PCR reaction mix

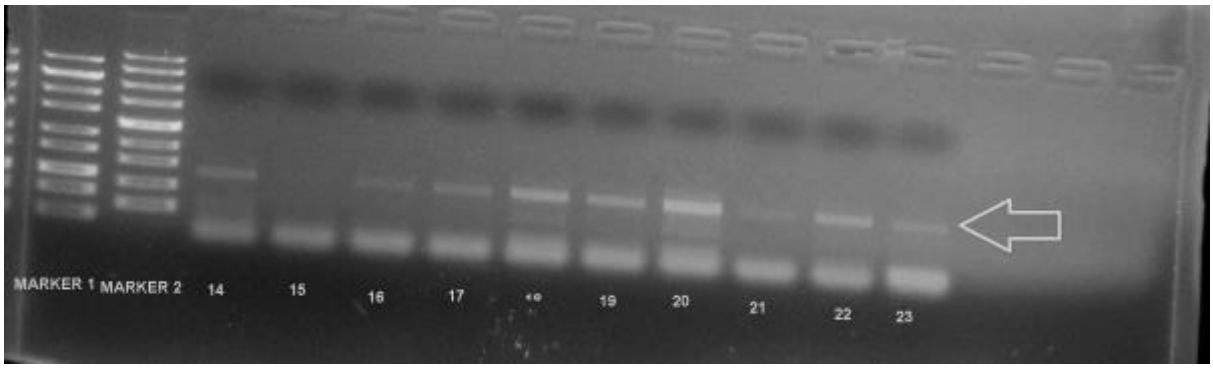
Reagent name	**Quantity (µL)**
DreamTaq Green PCR Master Mix (2X)	25
A_AGT1	1
KILEU2 B-M	1

gDNA	1
H_2O	22
Total	**50**

Program

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	94	
Denaturaturation	1min 30 s	94	35
Anneling	30 s	50	
Extension	2 min	72	
Final extension	7 min	72	
Hold	∞	15	

The expected PCR product size was: 568 bp and after running the PCR product through 1% agarose gel for 30 min 150V 300A we got this result:



Lane 1. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Lane 2. ZipRuler Express DNA ladder 2 (Cat. No: SM1373)

Lane 3.-12. AGT1 gene + pUG73 plasmid

Fragments of appropriate lengths were observed which means that the transformation worked.

Glycerol stocks positive CEN.PK 2-1C **Δ**SUC2 **Δ**AGT1 colonies (18, 19, 20) were prepared.

Week 4

We wanted to remove the GAL2 gene, because it must be knocked-out from strain B we needed to construct. We inoculated CEN.PK 2-1C **Δ**SUC2 **Δ**AGT1 strain in 5 mL of YPD liquid media and put it to grow overnight at 30ºC 200 rpm.

The disruption cassette for deletion of GAL2 gene was amplified using primers OL 5'_GAL2 and OL 3'_GAL2 (number 35 and 36 in table 2, respectively), and pUG72 plasmid as a template. In our project we have used DreamTaq Green PCR Master Mix (Thermo Scientific, Cat no: K1081), PCR reaction and Program were as follows:

PCR reaction mix

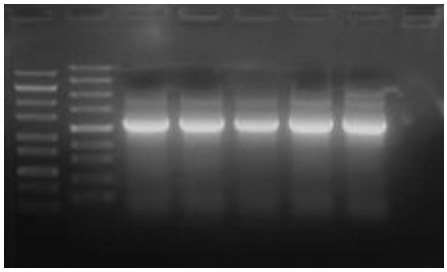
Reagent name	**Quantity (µL)**
DreamTaq Green PCR Master Mix (2X)	25
OL5'_GAL2	1
OL 3'_GAL2	1
pUG72	1
H_2O	22
Total	**50**

Program

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	95	
Denaturaturation	40 s	94	25

Anneling	1 min	58	
Extension	2 min	68	
Final extension	15 min	68	
Hold	∞	15	

The expected PCR product size was: 1.7 kb and after running the PCR product through 1% agarose gel for 30 min 150V 300A we got this result:



Lane 1. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Lane 2. ZipRuler Express DNA ladder 2 (Cat. No: SM1373)

Lane 3.-7. GAL2 disruption cassette

The PCR product size was as expected, so we purified it from gel with FavorPrep GEL/PCR Purification Mini Kit (Cat. No: FAGCK001-1) according to manufacturer's protocol. This PCR product was then used on the next day for transformation.

On the next day we diluted the overnight CEN.PK 2-1C **Δ**SUC2 **Δ**AGT1 culture to OD600 of 0.2 in 25 mL of YPD and let it grow till the mid-log phase (OD600 = 0.7) at 30ºC 200 rpm. Then we have transformed GAL2 disruption cassette into CEN.PK 2-1C **Δ**SUC2 **Δ**AGT1 strain using Li-Ac yeast transformation technique [1]. Transformed cells were plated on -URA/Glc plates and incubated at 30ºC for 48 h. Some colonies were observed.

The chosen colonies were lysed to extract the genomic DNA (gDNA) in 30 µL of 20mM NaOH and kept in 100ºC thermostat for 10 minutes, vortexed, cooled down on ice for 2 minutes and centrifuged at 13 000 rpm for 15 s. These samples' supernatants were used as a template for colony verification PCR.

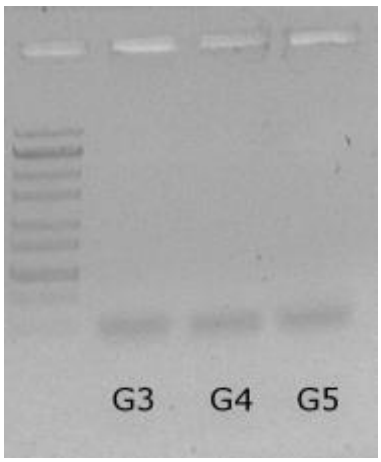
PCR reaction mix

Reagent name	**Quantity (µL)**
DreamTaq Green PCR Master Mix (2X)	25
A_GAL2	1
KIURA B-M	1
gDNA	1
H_2O	22
Total	**50**

Program

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	94	
Denaturaturation	20 s	94	44
Anneling	30 s	50	
Extension	20 s	72	
Final extension	7 min	72	
Hold	∞	15	

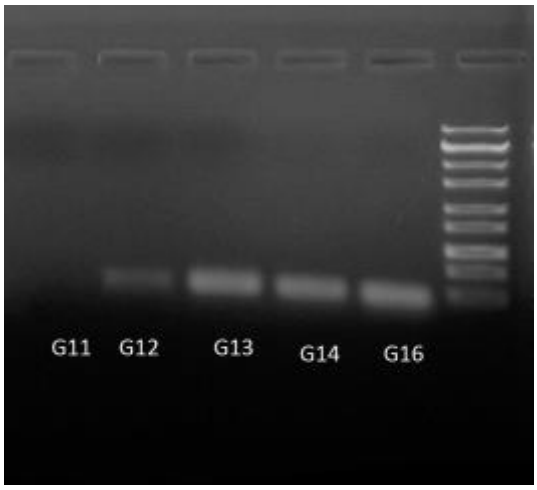
The expected PCR product size was: 444 bp and after running the PCR product through 1% agarose gel for 30 min 150V 300A we got this result:



Lane 1. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Lane 2.-4. Colony PCR products

All colonies checked were false positive. So screening of new colonies was performed and colony validation PCR was repeated.



Lane 1.-5. Gcolony PCR products

Lane 6. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Colonies were false positive.

Week 5

The ADH2 gene has to be knocked out in the strain A in order to make it unable to produce acetaldehyde and CO2. We inoculated CEN.PK 2-1C **Δ**SUC2 **Δ**AGT1 strain in 5 mL of YPD liquid media and put it to grow to 30ºC 200 rpm.

The disruption cassette for deletion of ADH2 gene was amplified using primers OL 5'_ADH2 and OL 3'_ADH2 (number 7 and 8 in Table 2, respectively), and pUG72 plasmid as a template. In our

project we have used DreamTaq Green PCR Master Mix (Thermo Scientific, Cat no: K1081), PCR reaction and Program were as follows:

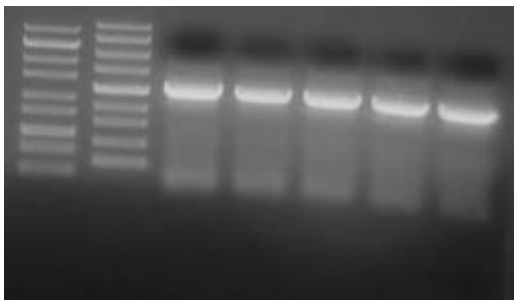
PCR reaction mix

Reagent name	**Quantity (µL)**
DreamTaq Green PCR Master Mix (2X)	25
OL 5'_ADH2	1
OL 3'_ADH2	1
pUG72	1
H_2O	22
Total	**50**

Program

Step	Time	Temperature (ºC)	# of cycles
Initial step	5 min	95	
Denaturaturation	1 min 30 s	95	35
Anneling	1 min	58	
Extension	2 min	68	
Final extension	5 min	68	
Hold	∞	15	

The expected PCR product size was: 1.7 kb and after running the PCR product through 1% agarose gel for 30 min 150V 300A we got this result:



Lane 1. ZipRuler Express DNA ladder 1 (Cat. No: SM1373)

Lane 2. ZipRuler Express DNA ladder 2 (Cat. No: SM1373)

Lane 3.-7. ADH2 disruption cassette

The PCR product size was as expected, so we purified it from gel with FavorPrep GEL/PCR Purification Mini Kit (Cat. No: FAGCK001-1) according to manufacturer's protocol. This PCR product was then used on the next day for transformation.

On the next day we diluted the overnight CEN.PK 2-1C **Δ**SUC2 **Δ**AGT1 culture to OD600 of 0.2 in 25 mL of YPD and let it grow till the mid-log phase (OD600 = 0.7) at 30ºC 200 rpm. Then we have transformed ADH2 disruption cassette into CEN.PK 2-1C **Δ**SUC2 **Δ**AGT1 strain using Li-Ac yeast transformation technique[1]. Transformed cells were plated on -URA/Glc plates and incubated at 30ºC for 48 h. Unfortunately, no colonies were formed. The transformation was repeated, but no colony growth was observed for the second time either.

Because 5 weeks were wasted and no strains that we need were constructed, we decided to search in a literature for the appropriate strains. Luckily, we found them in two different papers[2][3]. The authors were kind to send as those strains and we got them 3 weeks later.

In conclusion,thanks to *Prof.* Eckhard Boles' research group from Heinrich-Heine-Universität and *Prof.* Antonius J.A. van Maris' research group from the University of Delft we got the strains we need for our system to work and could start making biobrikcs and characterizing them.

References

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[2] - Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C. P., & Boles, E. (1999). Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in Saccharomyces cerevisiae. FEBS letters, 464(3), 123-128.

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