



LAB JOURNAL GENE EXTRACTION



Prepared by

bioiqs.igem@gmail.com

Laboratory of Biochemistry, IQS School of Engineering

Strategy 1: Gene extraction, PCRs



In this strategy, we considered obtaining the HLA-DQ from scratch. Based on former studies, only the exons 2 and 3 form each chain (α and β) codify for the extracellular domain of the HLA-DQ that interacts with gluten epitopes. With this in mind, we designed a robust model for the extraction of the α and β chains of the HLA-DQ from the genomic DNA of a celiac patient. A set of primers were designed to conduct 3 different PCRS (including 10 reactions) to obtain the α and β chains flanked with restriction sites for further cloning.

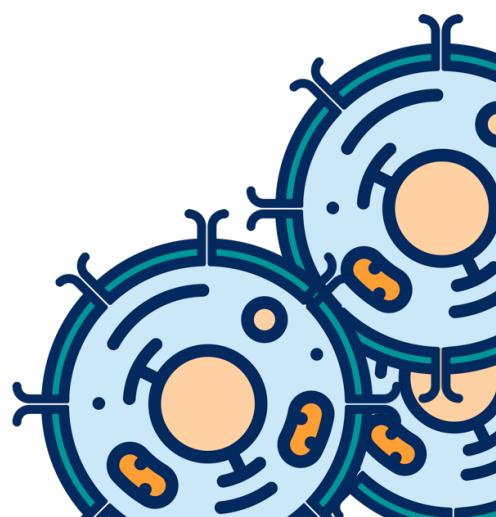
Strategy 2: Synthetic genes



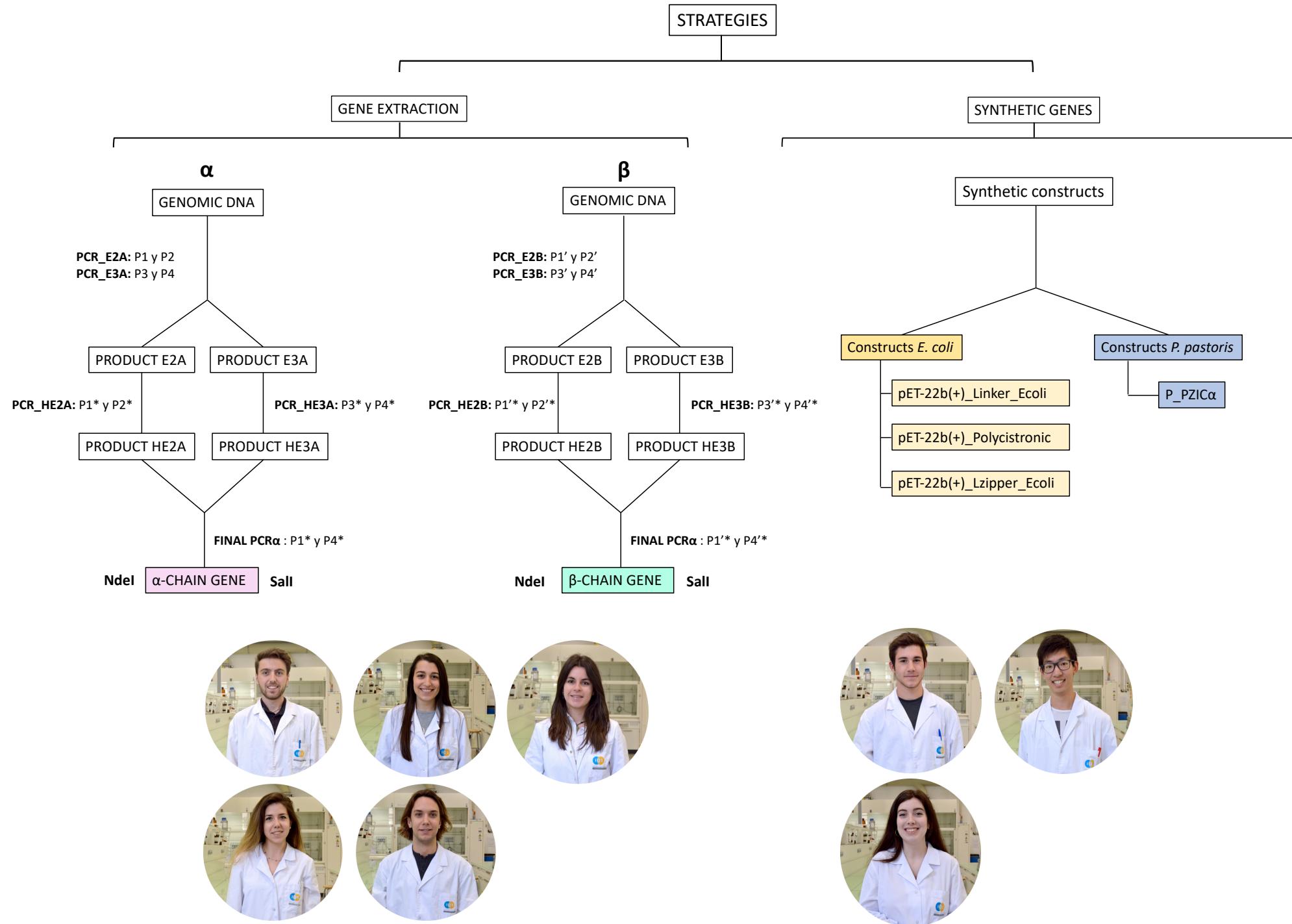
As one of the main objectives is to express the human HLA in a bacterial cell host, several approaches to conduct this were considered. Firstly, a structural analysis of the protein was made in order to visualize the different protein domains. Based on this analysis, we decided to express only the extracellular domain which was encoded by the exons 2 and 3 of the HLA gene. Next, as an attempt to improve the protein stability, we chose to add Leucine Zippers to the constructs. And finally, specific tags were added at the C-terminus of the gene sequences to enable the protein purification.

With these ideas, our team designed different synthetic genes following three strategies for the heterologous expression of the human HLA in *E.coli*.

- Expression of both α chain and β chain as a unique gene by using a fusion linker.
- Expression of the α and β chain as two separate genes in the same constructs by establishing a RBS for each gene.
- Expression of the α and β chain as two separate genes but adding the Leucine Zippers to increase its stability.



STRATEGY MAP



Constructs for *Pichia pastoris*

Week 1

Vectors

In order to obtain this expression systems, two different *E. coli* DH5 α were acquired on a LB zeocin plate from the Universitat Autònoma de Barcelona (UAB). Then, each strain (*E. coli* DH5 α – pPICZ α and *E. coli* DH5 α – pGAPZ α) were cultured O/N at 37°C in LB medium with and μ g/ml of zeocin, **Table 1**.

Table 1: Suggested concentrations of Zeocin™ to use for selection in mammalian tissue culture cells, yeast, and *E. coli*.

Organism	Zeocin™ Concentration and Selective Medium
<i>E. coli</i>	25-50 μ g/ml in Low Salt LB medium*
Yeast 50	50-300 μ g/ml in YPD or minimal medium
Mammalian cells	50-1000 μ g/ml (varies with cell line)

On the next, a standard miniprep assay was performed in order to obtain the different vectors using a final elution volume of 30 μ l.

Preparative digestion

To clone the different constructs into two different expression systems, a double digestion using XbaI and EcoRI was carried out using CutSmart® as digestion buffer.

For each vector sample:

- 30 μ l of miniprep eluted volume
- 4 μ l of digestion buffer
- 2 μ l of EcoRI
- 2 μ l of XbaI
- 2 μ l of Water

Then, both digested vector samples were incubated 3h at 37°C.

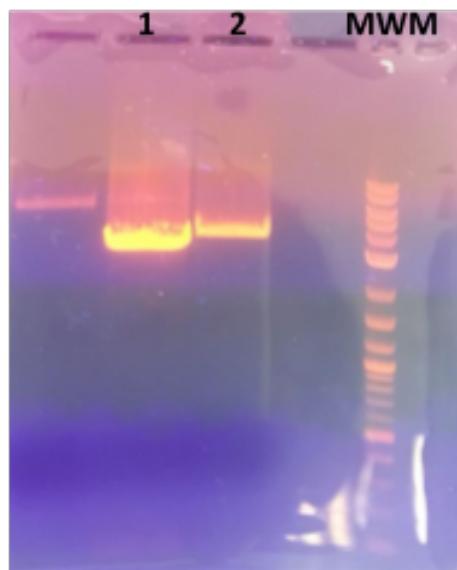
Band purification

Once pPICZ α and pGAPZ α were digested, the next step was to purify the samples to obtain the digested vectors. Both samples were run on a preparative Agarose 1% at 120 V using 48 μ l as final volume.

For each sample:

- 40 μ l of digested sample.
- 8 μ l of loading buffer 6x.

Total volume (48 µl) was charged into two different lines corresponding to each vector sample. As expected, sample on line 1 (pGAPZ α) showed a lower size than sample 2 (pPICZ α), with a molecular weight of 3.1 kb and 3.6 kb respectively.



A band purification protocol using gel extraction kit (QIAGEN®) was carried out eluting both bands separately in a final volume of 30 µl. The eluted volume was stored at -20°C.

Week 2

Next to do was to quantify the vector concentration for each sample. Eluted products from purification steps were thawed at room temperature. Nextly, 2 µl of each sample were loaded in a 1% Agarose gel. Sample concentration was detected and quantified using band intensity method.

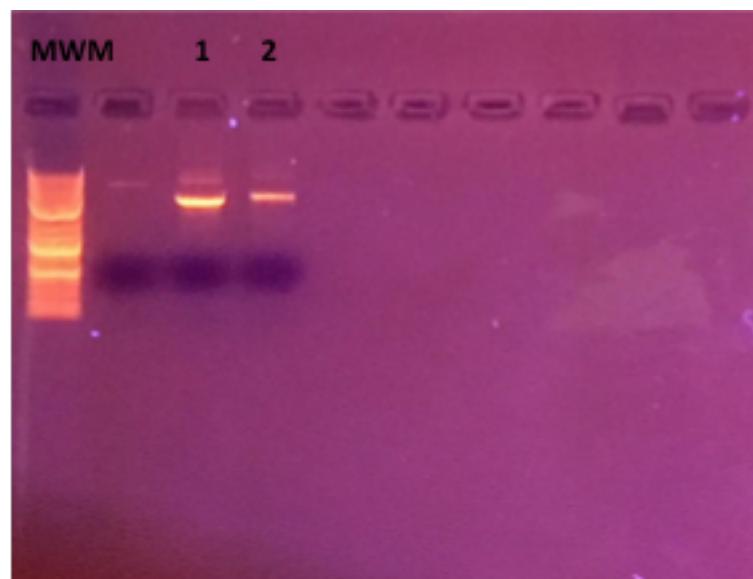


Figure 2: Analytic 1% agarose gel with 2µl of ethidium bromide. **1** pGAPZ α . **2** pPICZ α .

In figure 2 it can be observed that the purification of different vectors pPICZ α and pGAPZ α was successful with a good correlation of intensity and size.

Inserts

The three available constructs for *P. Pastoris* are 4b, 6d and 7.

- **4b. Yeast_2A_2Tags_EcoR1XbaI_PichiaOptimised**
- **6d. Yeast_LZ_2A_2Tags_EcoR1XbaI_PichiaOptimised**
- **7. Linker_EcoR1NdeI_XbaISall_Pichia_Ecoli_Optimized**

All different constructs were received inside a plasmid with Ampicillin resistance. The different plasmids were transformed in *E. coli* DH5 α strain and cultured at 37°C O/N in separated cultures for each plasmid construct (4b, 6d and 7). On the next day, a miniprep assay was performed to extract the different constructs with a final 30 μ l of elution volume.

Preparative digestion

In order to clone the constructs into two different expression systems, the three samples (plasmids with 4b, 6d and 7) were digested with same restriction enzymes than vectors. A preparative digestion using XbaI and EcoRI was performed using CutSmart® as digestion buffer.

For each sample:

- 30 μ l miniprep
- 4 μ l Digestion buffer
- 2 μ l EcoRI
- 2 μ l XbaI
- 2 μ l Water

Reactions were incubated 3h at 37°C and products were stored at -20°C.

Week 3

Band purification

Once the different constructs were digested, a purification assay was performed.

A preparative Agarose 1% gel was carried out at 120 V using 48 μ l as final volume.

Three digestions samples (4b, 6d and 7) were prepared using:

- 40 μ l of digest product
- 8 μ l loading buffer 6x.

Final volume was charged into three different lines as shown in **Figure 3**. As expected, same backbone (vector with Ampicillin) can be observed as the upper band for each well.

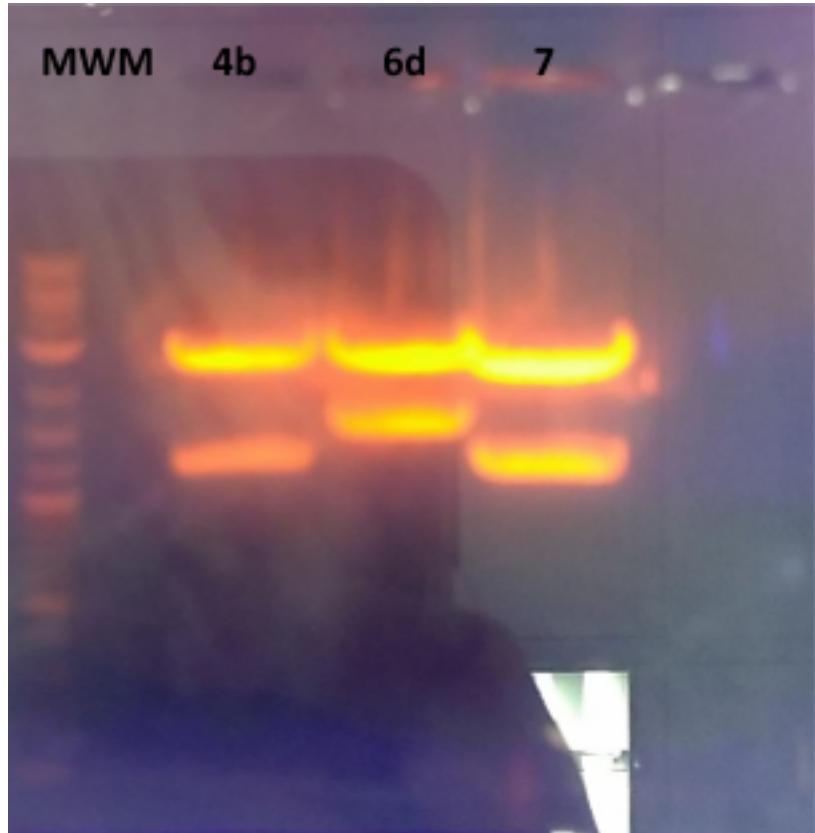


Figure 3: Preparative 1% agarose gel with 2 μ l of ethidium bromide. 48 μ l of three different digestion products were charged and analysed with transilluminator.

The theoretical size of the different inserts are:

- 4b → 1229 pb
- 6d → 1517pb
- 7 → 1156 pb

The gel showed that digestion was successfully for all three vectors.

Following this, a band purification protocol using gel extraction kit (QIAGEN®) was carried out eluting the samples in a final volume of 30 μ l. Finally, 2 μ l of each sample were taken to be loaded into a 1% Agarose gel and determine the insert concentration using band intensity method.

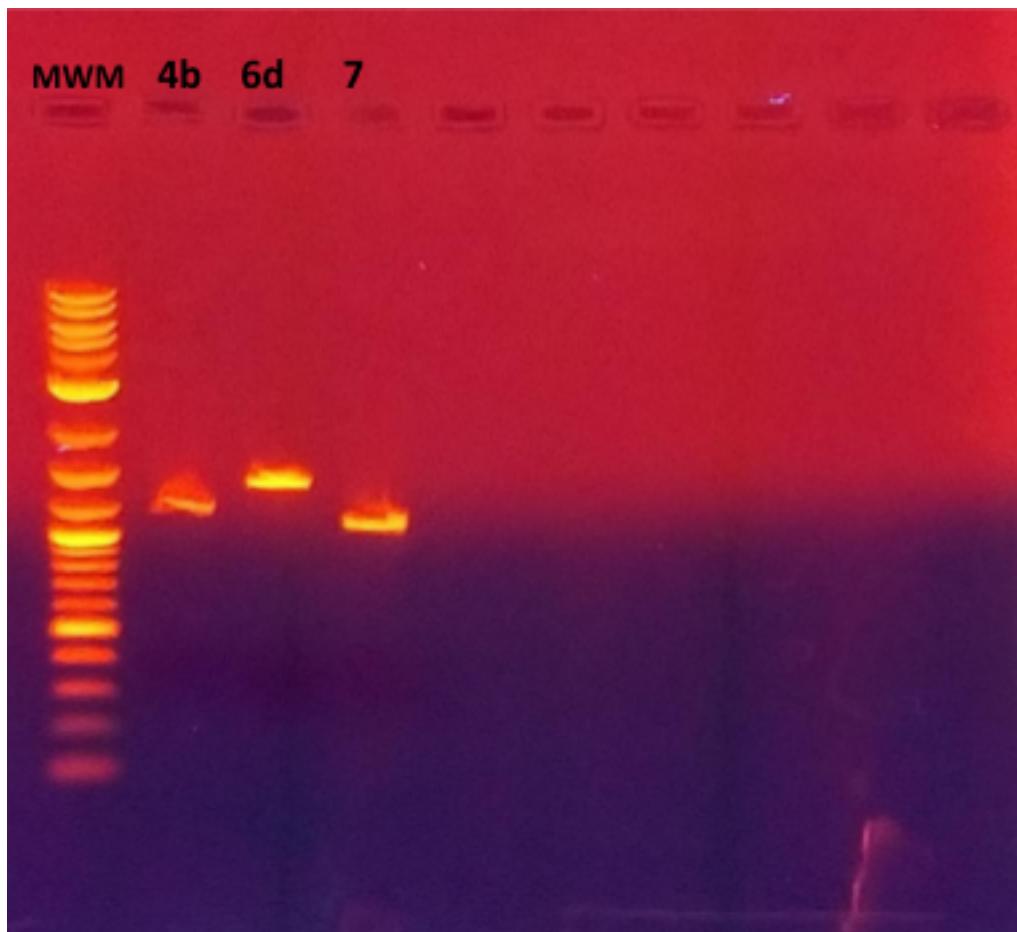


Figure 4: Analytical 1% agarose gel with 2 μ l of ethidium bromide. 2 μ l of three different digestion products were charged and analysed with transilluminator.

Finally, the inserts samples were stored at -20°C.

Week 4

Ligation (1)

As explained before, two different vectors (pPICZ α and pGAPZ α) and three different constructs (4b, 6d and 7) were digested with same restriction enzymes (EcoRI and XbaI). Following this, a ligation assay using T4 DNA ligase was performed.

Six different samples were ligated and transformed into *E. coli* DH5 α electrocompetent cells. A construct sample insert:vector with a 1:3 relation. And a negative control with the same proportion using water instead insert:

- pPICZ α -4b
- pPICZ α -6d
- pPICZ α -7
- pGAPZ α -4b
- pGAPZ α .6d
- pGAPZ α -

Each sample was prepared using:

- 1 μ l insert

- 3 µl vector
- 2 µl T4 DNA ligase Buffer
- 3 µl water
- 1 µl T4 DNA ligase

Then all samples were incubated at 16°C O/N.

To transform the electrocompetent cells, the different ligation samples were taken as well as electrocompetent DH5 α cells. 2µl of the ligation reaction were mixed in 50µl of the electrocompetent cells in a cuvette (previously chilled). The electroporation voltage transferred to the cuvette was 1700V t 5 msec.

- | | | |
|----------------------|---|----------|
| • pPICZ α -4b | → | 3.4 msec |
| • pPICZ α -6d | → | 3.7 msec |
| • pPICZ α -7 | → | 1.2 msec |
| • pGAPZ α -4b | → | 3.9 msec |
| • pGAPZ α .6d | → | 4.0 msec |
| • pGAPZ α -7 | → | 2.9 msec |

Once the pulse charge was finished, 1 ml of LB was added to the cells to transfer to a small tube. Subsequently, there were incubated for 1h at 37°C with 200rpm. Thereupon, 100µl and 900 µl of each sample were seeded in two different plate of LB-agar supplemented with zeocin (30 µg/ml). 12 plates were incubated at 37°C O/N.

The next day, an unexpected result had been shown → No colonies grew.

Week 5

Ligation (2)

Six different ligation reactions were performed with an insert:vector ratio of 1:4. A negative control with the same proportion using water instead insert.:

- pPICZ α -4b
- pPICZ α -6d
- pPICZ α -7
- pGAPZ α -4b
- pGAPZ α .6d
- pGAPZ α -7

Each sample was prepared using:

- 1 µl insert
- 4 µl vector
- 2 µl T4 DNA ligase Buffer
- 2 µl water
- 1 µl T4 DNA ligase

Then, all samples were incubated at 16°C 3h.

We repeated the same transformation protocol as explained before by adding 2µl of the reaction to 50µl of the electrocompetent cells in a cuvette (previously cooled). The electroporation voltage transferred to the cuvette was 1700V t 5 msec.

- pPICZα-4b → 3.3 msec
- pPICZα-6d → 3.2 msec
- pPICZα-7 → 2.9 msec
- pGAPZα-4b → 2.7 msec
- pGAPZα.6d → 3.2 msec
- pGAPZα-7 → 3.4 msec

Once the pulse charge was finished, 1 ml of LB was added to the cells to transfer to a small tube. Subsequently, there were incubated for 1h at 37°C with 200rpm. Thereupon, 100µl and 900 µl of each sample were seeded in two different plate of LB-agar supplemented with zeocin (30 µg/ml). 12 plates were incubated at 37°C O/N.

The next day, an expected result had been shown → No colonies were growth.

Ligation (3)

Six different ligation reactions were performed with an insert:vector ratio of 2:5. And a negative control with the same proportion using water instead insert.:

- pPICZα-4b
- pPICZα-6d
- pPICZα-7
- pGAPZα-4b
- pGAPZα.6d
- pGAPZα-7

Each sample was prepared using:

- 2 µl insert
- 5 µl vector
- 2 µl T4 DNA ligase Buffer
- 0 µl water
- 1 µl T4 DNA ligase

Then all samples were incubated at 16°C 3h.

The same transformation protocol was used and transformants were plated in LB-agar + zeocin and incubated at 37°C O/N

- pPICZα-4b → 2.4 msec
- pPICZα-6d → 2.2 msec
- pPICZα-7 → 2.4 msec
- pGAPZα-4b → 2.1 msec

- pGAPZ α .6d → 1.8 msec
- pGAPZ α .7 → 2.5 msec

The next day, an expected result had been shown → No colonies were growth.

Week 6

Ligation (4)

Six different ligation reactions were performed with an insert:vector ratio of 3:1. A negative control with the same proportion using water instead insert.:

- pPICZ α -4b
- pPICZ α -6d
- pPICZ α -7
- pGAPZ α -4b
- pGAPZ α .6d
- pGAPZ α .7

Each sample was prepared using:

- 1 μ l insert
- 4 μ l vector
- 2 μ l T4 DNA ligase Buffer
- 2 μ l water
- 1 μ l T4 DNA ligase

Then all samples were incubated at 16°C 3h.

The same transformation protocol was used and transformants were plated in LB-agar + zeocin and incubated at 37°C O/N

- pPICZ α -4b → 3.4 msec
- pPICZ α -6d → 3.3 msec
- pPICZ α -7 → 3.3 msec
- pGAPZ α -4b → 3.4 msec
- pGAPZ α .6d → 3.1 msec
- pGAPZ α .7 → 3.4 msec

The next day, an unexpected result had been shown → we obtain colonies.

A PCR colony assay was done using a Pre-mix that contained the Taq polymerase and the polymerase buffer.

However, no band amplification was obtained.

- One hypothesis was that the primers used to PCR-colony were wrong.

To obtain the different constructs, each ligation product (pPICZ α -4b, pPICZ α -6d, pPICZ α -7, pGAPZ α -4b, pGAPZ α .6d, pGAPZ α -7) were cultivated ON at 37°C in LB medium with 30 µg/ml of zeocin.

Once cultivated ON, a miniprep assay was performed to extract the different ligations products in 30 µl as elution volume.

Finally, 2 µl of each sample of vector purification were taken to perform 1% Agarose gel to detect the vector concentration using band intensity method. However, the intensity of each band were was lower than detection limit.