



LAB JOURNAL CONSTRUCTS



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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.

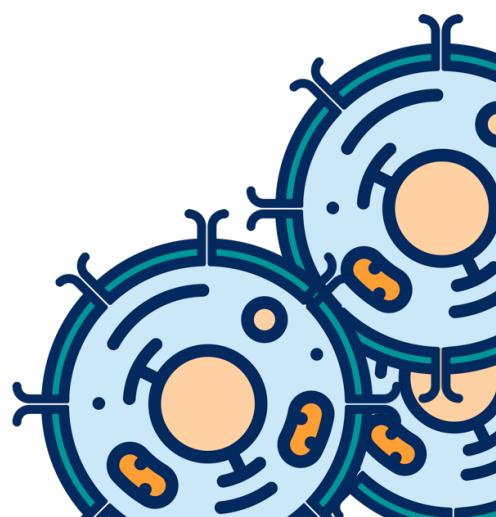
Strategy 2: Synthetic genes, Constructs.



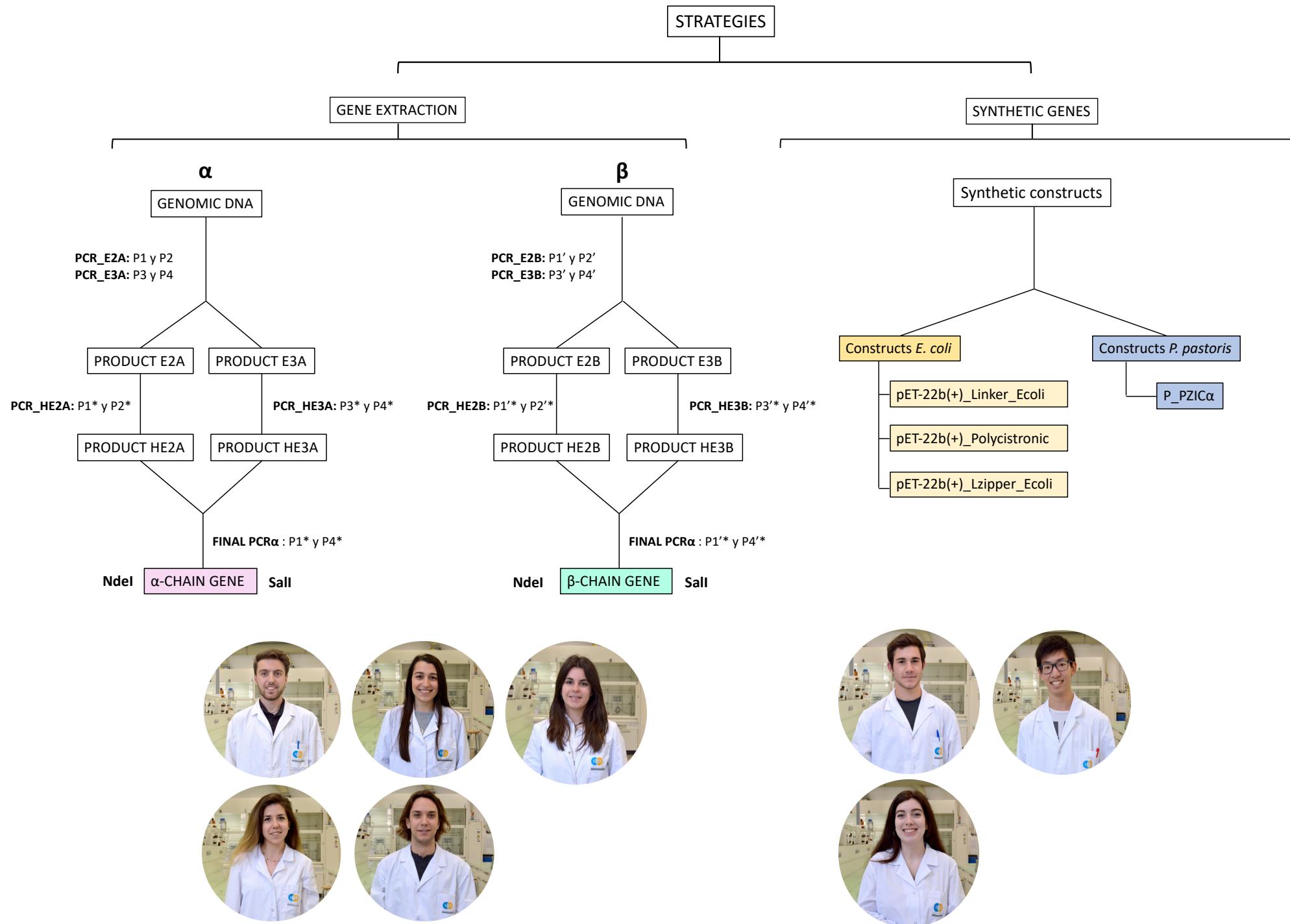
As one of the main objectives is to express the human HLA in a bacterial cell host, several approaches to conduct this were considered. Firstly, a structural analysis of the protein was made in order to visualize the different protein domains. Based on this analysis, we decided to express only the extracellular 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



NOTEBOOK: STRATEGY 2 CONSTRUCTS

Week 1: 20/08/18 – 24/08/18

- ***E. coli* DH5α electroporation with synthetic genes 2b, 4b, 6d, 7**
- **Miniprep of 2b, 4b, 6d, 7**

Preparative Digestion: With NdeI and Sall enzymes and Cutsmart Buffer. Digestion time: 3.5h

-> **gBlocks 2a, 3a, 3b and 2b:** 15 µL ~ 300 ng. Final volume: 20 µL.

Cutsmart: 2µL

DNA: 15µL

NdeI: 1µL

Sall: 1µL

H₂O: 1µL

-> **pET22b(+)·BsPdaC:** Final volume: 40 µL. (This vector has a gene of 0.7kb between the restriction enzymes NdeI and Sall)

Cutsmart: 4µL

DNA: 30µL

NdeI: 2µL

Sall: 2µL

H₂O: 2µL

- **Preparative agarose gel (2%) of 2b, 2a, 3a, 3b and band extraction at ~ 1kb** using QIAquick Gel Extraction kit (QIAGEN).
- **Preparative agarose gel (1%) of pET22b and band extraction at ~ 5kb** using QIAquick Gel Extraction kit (QIAGEN). Elution: 30 µL Elution Buffer.



Analytical gel after purification

- **pET22b Vector and inserts 2b, 2a, 3a, 3b ligation.** Final Volume: 10 µL. Ligation time: 1h starting at 16°C and reaching 37°C using the thermocycler, then heat inactivation at 65°C for 10min

	2a	2b	3a	3b
Buffer 10X (µL)	1	1	1	1
Vector (µL)	1	1	1	1
Insert (µL)	4	1	1	3
T4 Ligase (µL)	1	1	1	1
H2O (µL)	3	6	6	4

NOTEBOOK: STRATEGY 2 CONSTRUCTS

- ***E. coli* DH5 α electroporation with ligation products.** Ratio: 5 μ L ligation product in 50 μ L *DH5 α* cells.

Results: High Background.

	# Colonies (100 μ L plated)	# Colonies (plated all)
Pet22b	4	>100
Pet22b-2a	1	~20
Pet22b-2b	2	~20
Pet22b-3a	0	1
Pet22b-3b	0	2

- **Colony PCR:** 13 Samples. #1 to #5 are from construct 2a; #6 to #10 are from construct 2b; #11 is from construct 3a;

->Final Volume: 10 μ L

Colony TaqPol mix 2x: 5 μ L

Primers: T7F: 0.5 μ L (0.3 μ M)
T7R: 0.5 μ L (0.3 μ M)

Water: 4 μ L

-> Program:

94°C 10min

94°C 30s

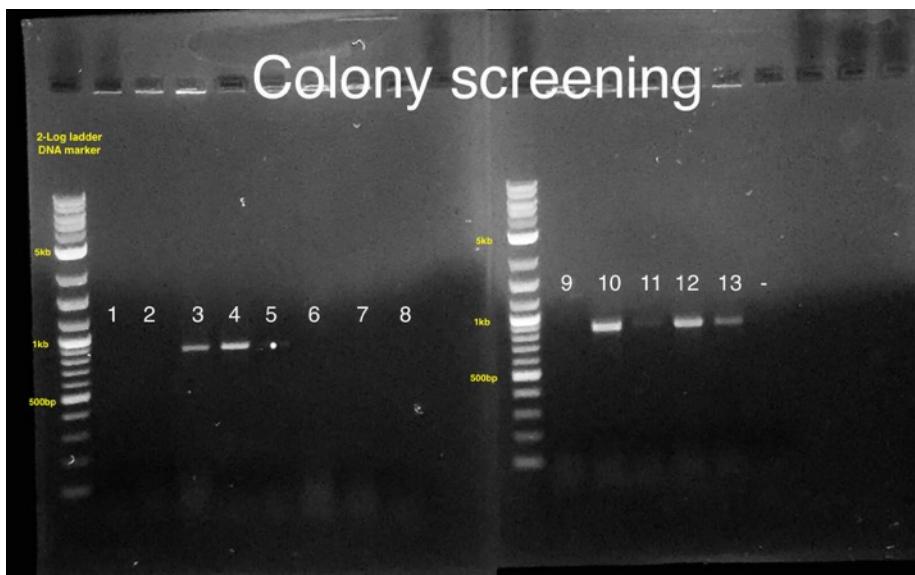
50°C 30s (Annealing)

72°C 1.5min (Extension)

72°C 10min (Final Extension)

4°C — 29 cycles (from 2 to 4)

Results were not as expected. Results show that the analyzed colonies were from the undigested vector.



- **pET22b vector digestion.** Final Volume: 40 μ L. With NdeI i Sall enzymes in Cutsmart buffer. Digestion time: 3.5h at 37°C
- **DH5 α Competent cells preparation** as described in Protocol 4.

NOTEBOOK: STRATEGY 2 CONSTRUCTS

Week 2: 27/08/18 – 31/08/18:

- **pET22b Vector and inserts 2b, 2a, 3a, 3b ligation.** Final Volume: 10 µL. 16°C O/N

	2a	2b	3a	3b
Buffer 10X (µL)	1	1	1	1
Vector (µL)	3	3	3	3
Insert (µL)	5	3	3	4
T4 Ligase (µL)	1	1	1	1
H2O (µL)	0	0	0	0

- **E. coli DH5α electroporation with ligation products.** Ratio: 5µL ligation product in 50µL DH5α cells.

	2a	2b	3a	3b	Vector
Time (ms)	2.8	2.8	2.9	2.9	4.6

Results: No Colonies

- **Preparative Digestion of pET22b and Construct nº7 with NdeI, SalI and Cutsmart.** Final Volume: 40µL.



- **Miniprep Construct nº7**

Week 3: 03/09/18 – 09/09/18

- **pET22b Vector and inserts nº 7 ligation.** Final Volume: 10 µL. 16°C O/N

	7 (6/2)	7 (4/4)
Buffer 10X (µL)	1	1
Vector (µL)	6	4
Insert (µL)	2	4
T4 Ligase (µL)	1	1
H2O (µL)	0	0

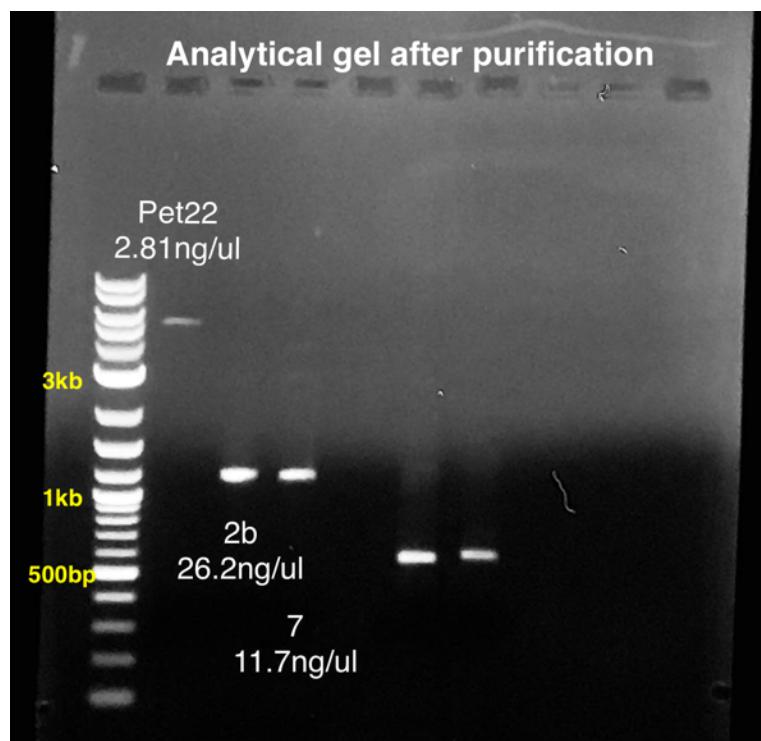
- **E. coli DH5α electroporation with ligation products.** Ratio: 10µL ligation product in 50µL DH5α cells.

	7 (6/2)	7 (4/4)	Vector
Time (ms)	1.5	1.5	4.3

- **pET22b Vector and Construct nº7 (4µL/4µL) ligation.** Final Volume: 10 µL. 1h at thermocycler using a temperature ramp from 16°C to 37°C

NOTEBOOK: STRATEGY 2 CONSTRUCTS

- **E. coli DH5α electroporation with 4μL/4μL ligation product.** 2μL Transformation. Time: 3.9ms. Results: No Colonies.
- **Miniprep of pET22b vector and Constructs #2b and #7**
- **Preparative Digestion of pET22b and Constructs 2b and 7 with NdeI, SalI enzymes and Buffer 3.1.** Final Volume: 40μL. Proportions as described before.
- **Band extraction:** pET22b Vector and 2b, 7 Constructs were purified from agarose gel using QIAquick Gel Extraction kit (QIAGEN).



- **pET22b vector and 2b, 7 Constructs Quantification from gel purification with Qubit.**

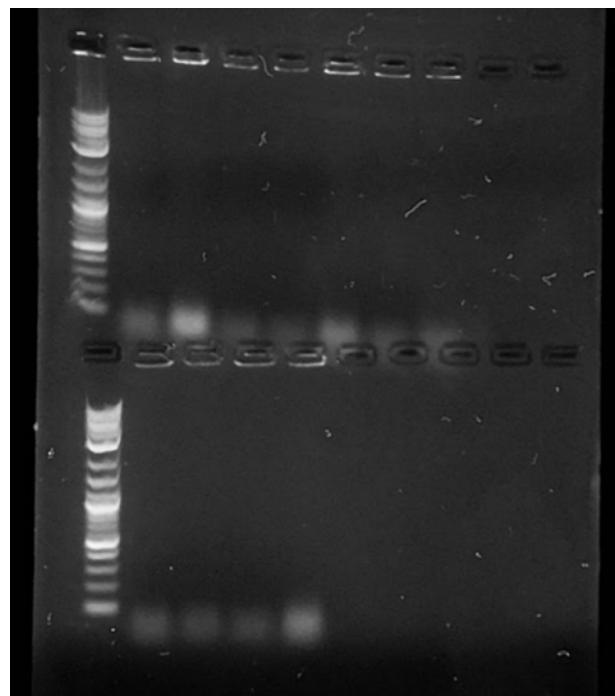
	Vector	2b	7
Concentration (ng/μL)	2.81	26.2	11.7
- **pET22b Vector and 2b, 7 Constructs ligation.** 37°C 1h at thermocycler using a temperature ramp from 16°C to 37°C

	2b	7
Buffer 10X (μL)	1	1
Vector (μL)	7	7
Insert (μL)	1	1
T4 Ligase (μL)	1	1
H2O (μL)	0	0

- **E. coli DH5α electroporation with ligation products.** 2μL Transformation. Results: Some colonies were observed.

	# Colonies (100µL plated)	# Colonies (plated all)
Pet22b-2b	1	21
Pet22b-7	0	7

- **Colony PCR:** 11 Samples. Samples #1 to #5 are from pet22b-7 (all); #6 to #9 are from pet22b-2b (all) and #10 is from pet22b-2b (100µL). Used primers from T7-promoter and T7-terminator
Results: No insert amplification caused by technical error). Annealing temperature was set at 50°C



Week 4: 10/09/18 – 14/09/18

- **Colony PCR (Repetition):** The plates from Week 3 were frozen accidentally and most of the colonies were lost. Colony PCR repetition using Colonies 4,7,8,9 (Same nomenclature as week 3). Results: 1 band amplified at well 8 corresponding to construct 2b (1380pb).



- **Streak plates with colonies 4,7,8,9**
- **Colony PCR (Repetition) using streak plate's colonies**
- **Colony #8 pre-inoculum:** Miniprep and Analytic Digestion. Final volume: 10 µL. 1h at 37°C.

H₂O: 5 µL

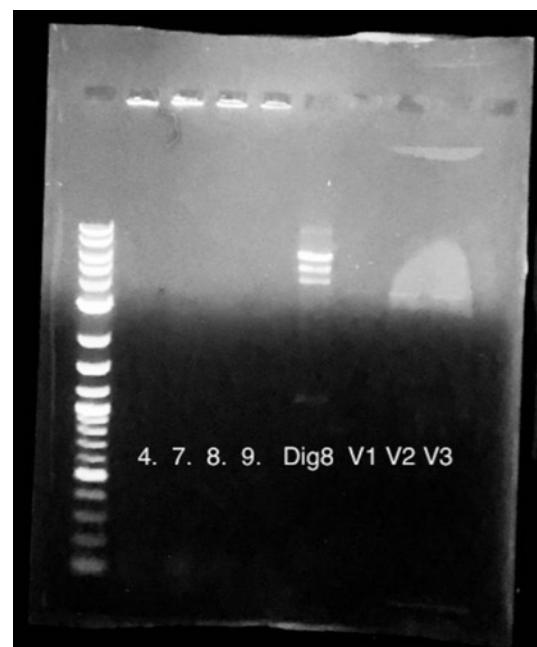
DNA: 3 µL

Buffer 3.1: 1 µL

NdeI: 0.5 µL

Sall: 0.5 µL

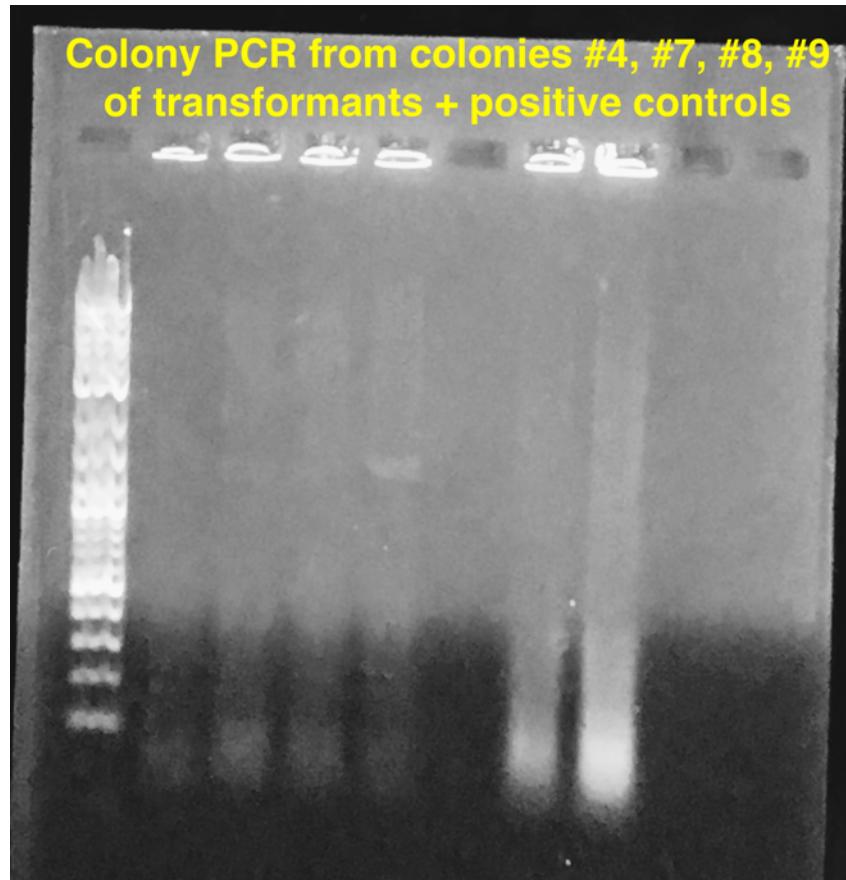
Gel results from colony PCR and digestion: No amplified bands in Colony PCR. Good results for Miniprep Digestion.



- **Transformed miniprep from colony #8 (pET22b-2b) in DH5a and BL21 using electrocompetent cells**
- **Preinoculum and miniprep of the transformed cells.**

Week 5: 17/09/18 – 21/09/18

- ***E. coli DH5α* and *BL21* electroporation with pET22b-2b obtained from Week 4.** Results: *DH5α* and *BL21* colonies with pET22-2b have grown. **Made preinoculum from both pET22b-2b (DH5α and BL21) for glycerinates**
- ***E.coli DH5α* electroporation of ligation of construct #7.** No colonies were formed.
- **Streak plates with pet22 (vector).**
- **Colony PCR** using 4, 7, 8, 9 samples and positive controls (pET22-2b *DH5α* and *BL21*). No bands were observed in the gel so we supposed that either the TaqPol mix or the primers were working properly.



- **Miniprep of pET22b (x3)**
- **Miniprep (x3) and pET22-2b plasmid Quantification with Qubit.** Results:

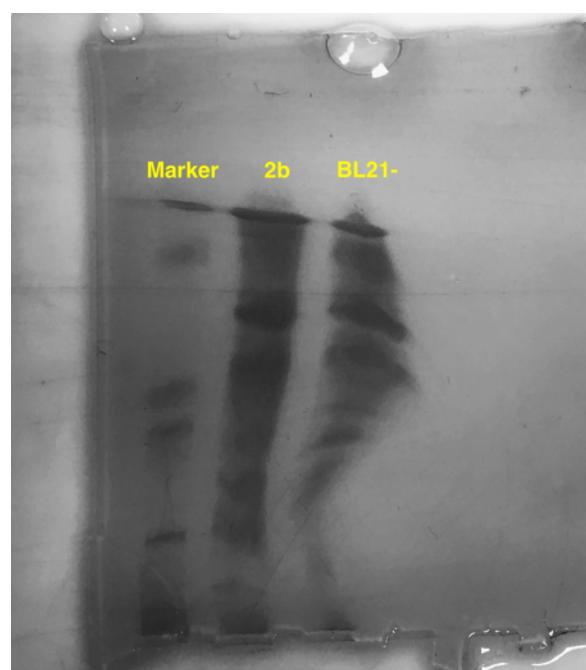
	Pet22b (1)	Pet22b (2)	Pet22b (3)	Pet22b-2b
Concentration (ng/µL)	12.9	15.6	16.3	35.2

- **Streak plates of *DH5α* and *BL21* for competent preparation**
- **Preparative digestion of vector pET22b (3).** Final volume: 40 µL. Proportions as described before, using Buffer 3.1
- **Gel purification of vector pET22b vector and Quantification using Qubit.** Results: 2.77 ng/µL.

- pET22b, pET22b-2b (*DH5α*) pre-inoculum for miniprep
- Minipreps Quantification using Qubit. Sent Pet22-2b(3) to sequence

	Pet22b-2b (1)	Pet22b-2b (2)	Pet22b-2b (3)	Pet22b (1)	Pet22b (2)
Concentration (ng/μL)	30.5	32.1	34.4	27.3	24

- pET22b-2b (*BL21*) and untransformed *BL21* pre-inoculum for protein expression
- pET22-2b (*BL21*) culture in 250mL Erlenmeyer. Results: OD_{600nm}(time = 0h) = 0.1. Culture induction with 0.5mM IPTG when OD_{600nm} = 0.3, then incubate O/N.
- SDS gel of O/N inoculums: 500 μL of O/N cultures (OD_{600nm} = 6) were centrifuged and resuspended in 50 μL SDS buffer 1x. Samples were boiled at 95°C 10' and centrifugated (max. rpm, 10'). 20 μL of each sample were charged in SDS gel. The gel was unclear. Pellets from the culture were kept at -20°C.



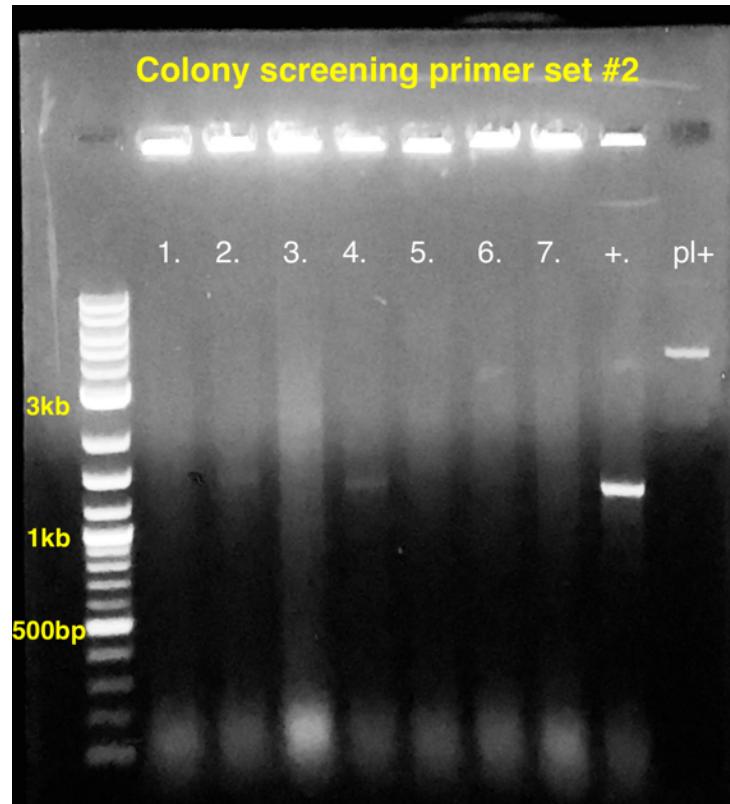
- Gibson assembly with pET22 and 2a, 3a and 3b Constructs. 50°C 30'

	Gibson mix	Vector	Insert
Volume (μL)	10	9	1

- *E. coli* *DH5α* and *BL21* electroporation with Gibson assembly products. Results:

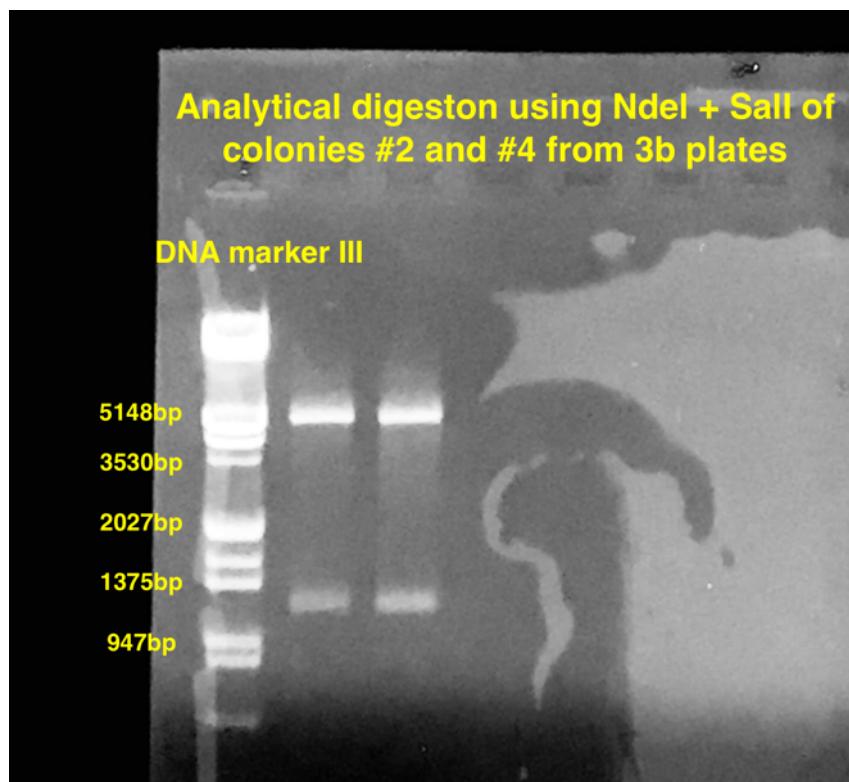
	2a	3a	3b	7
Time (ms)	4.7	4.9	4.9	4.2

- *E. coli* electroporation with 3b (6μL in 120 μL *E. coli*). Results: Some colonies were formed
- Colony PCR of 7 samples, positive control and vector. Results: Colonies 2 and 4 may be positive but the bands should be higher.



Week 6: 24/09/18 – 29/09/18

- 3b (colonies #2 and #4 from Week 5) pre-inoculum for miniprep and streak plates
- Minipreps of 3b (2 and 4) followed by analytic digestion with NdeI and Sall enzymes. Results: Some bands were observed at ~ 1300 bp. However, they don't correspond to the vector or insert size.

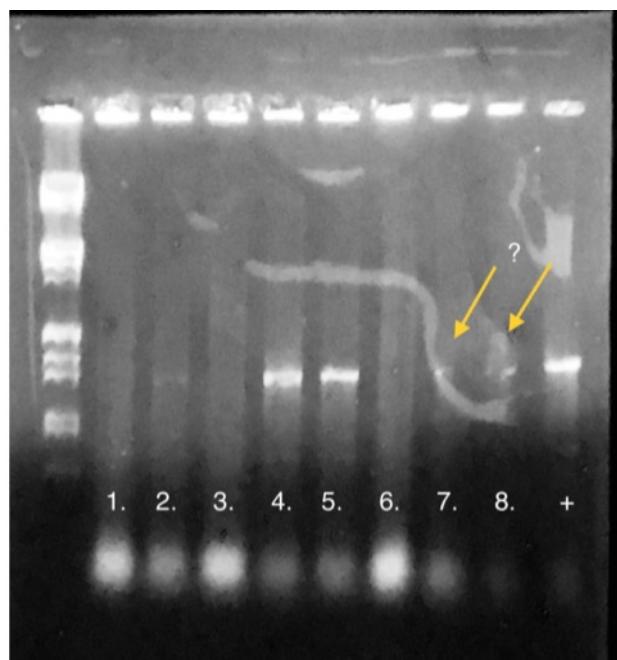


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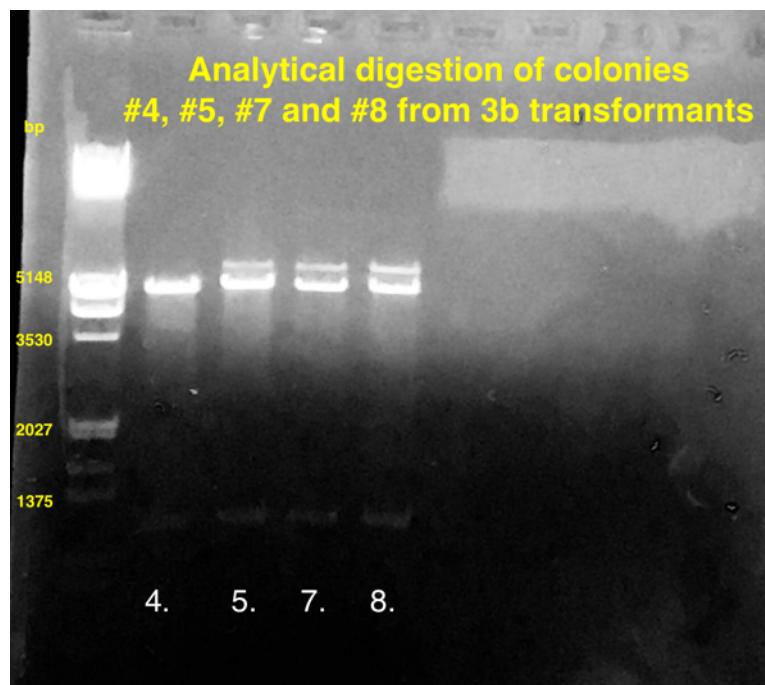
- 3b minipreps (colonies #2 and #4) were quantified using Qubit. Sent Pet22b-3b (#2) to sequence.

	Pet22b-3b (#2)	Pet22b-3b (#4)
Concentration (ng/ μ L)	32.4	26.8

- Colony PCR of 3b plates. 8 samples and 1 positive control. Results: Bands were again lower than expected.
Took colonies #4, #5, #7 and #8 and streak them in a new plate.



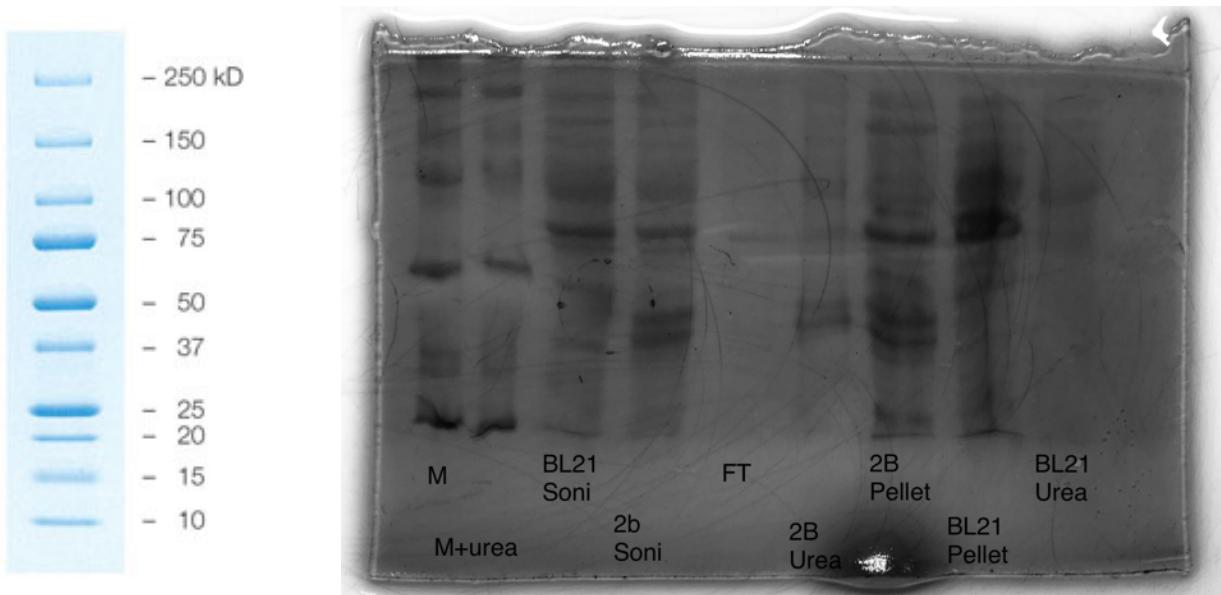
- *E. coli* electroporation with 3a construct (6 μ L in 120 μ L *E. coli*).
Results: No colonies were observed.
- Miniprep of 3b colonies: 4, 5, 7, 8 and Analytic digestion. Results: Bands are still lower than insert theoretical size.



- Buffers preparation for Strep-tag AKTA:

- Lysis buffer (50mM Tris-HCl, 100mM NaCl, 1mM PMSF; pH=7.5)
- Binding buffer (50mM NaH₂PO₄, 300mM NaCl; pH=8.5)
- Ethanol 20%
- NaOH 0.5M
- 1mM HABA in Binding Buffer
- 2.5mM Desthiobiotin in Binding Buffer

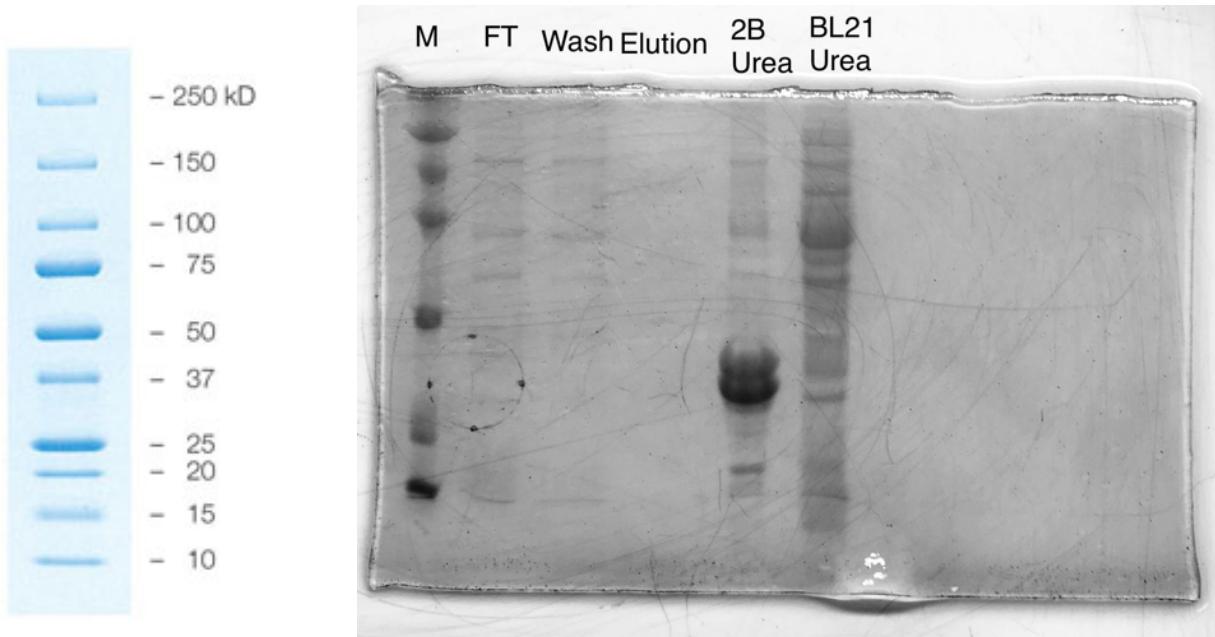
- Pellets from week 5 culture were sonicated at 50% amplitude, 2s ON and 5s OFF for 2min; then centrifuged (4°C 30' 12000 rpm). Supernatant was filtered and purified with Strep-tag column. No peak was detected. Stored pellet at -20°C. Pellets were washed twice with Binding Buffer and solubilized (20 mL Urea 8M-Tris 25mM pH 8). The mix was centrifuged (12000rpm 4°C 30min) and a sample was taken for SDS-Page.



- Started a second round of pET22-2b BL21 and BL21(-) pre-inoculum
- pET22-2b BL21 and BL21(-) inoculum in 50mL initial OD 0.1
- Cultures induction with 0.5mM IPTG at OD_{600nm} = 0.4. Incubation at 30°C 6h 200rpm until OD_{600nm} = 4. Took 1mL from the cultures for further SDS-page. The sample was centrifuged and stored at -20°C. Cultures were centrifuged (4500 rpm 15') and pellets were also stored at -20°C.

Week 7: 01/10/18 – 05/10/18

- Sonication of pellets from 2nd inoculum. Same conditions as before
- AKTA: Purification of 2b. Results: Small peak but it was diluted in Binding Buffer
- Pellet resuspension with Urea
- Polyacrylamide gel



HLA-DQA and HLA-DQB biobrick construction

- **PCRs from inserts (DQA and DQB) and pSB1C3 vector.** Inserts were amplified from template from construct pET22b-2b in which the two chains (DQA and DQB) were correctly cloned using the primers iAF, iAR, iBF, iBR. Vector was amplified from linearized template pSB1C3 provided from iGEM using the primers vAF, vAR, vBF, vBR.

Primers sequences

Primer	Sequence 5'-3'
iAF	TTCTGGAATT CGCGGCCGCTTCTAGATGGCAGATCACGTTGCTTC
iAR	GGCCGCTACTAGTATTATTACTCCAATGTTTAACAACGGC
vAF	TAAAACATTGGGAGTAATAATACTAGTAGCGGGCGCTGCAG
vAR	GAAGCAACGTGATCTGCCATCTAGAAGCGGCCGCGAATT
iBF	TTCTGGAATT CGCGGCCGCTTCTAGATGGAAGACTTGTGTACCAGTTAAGGG
iBR	GGCCGCTACTAGTATTATTACCGCCACTCCACAGTAATTGG
vBF	CTGTGGAGTGGCGGTAAATAATACTAGTAGCGGGCGCTGCAG
vBR	TGGTACACAAAGTCTTCATCTAGAAGCGGCCGCGAATT

- **PCR reaction Mix:**
 - iProof mix 2X
 - Forward primer: 0.25µM
 - Reverse primer 0.25µM
 - Template DNA: 50ng
 - H₂O: fill up to 20µL for analytical PCR or 50µL for preparative PCR.

- **PCR reaction conditions**

1. Initial denaturalization: 98°C for 3min
2. Denaturalization: 98°C for 30s

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3. Annealing: X °C* for 30s
4. Extension: 72°C for Y s* (15-30s/kb)
5. Final extension: 72°C for 10min
6. Hold: 4°C

Repeat steps 2-4 29 times

	X (°C)	Y (s)
pET22b-2b (insert)	57	10 (600bp)
pSB1C3 (vector)	61	40 (2kb)

- PCR products were treated with DpnI and purified with QIAQuick purification kit following the manufacturers protocol. Following, samples were quantified using Qubit.

Sample	iA	iB	vA	vB
Concentration (ng/μL)	57	57	32.8	19.7

- PCR products were ligated using CPEC. Used 100ng and an equimolar quantity of the insert. CPEC mix:

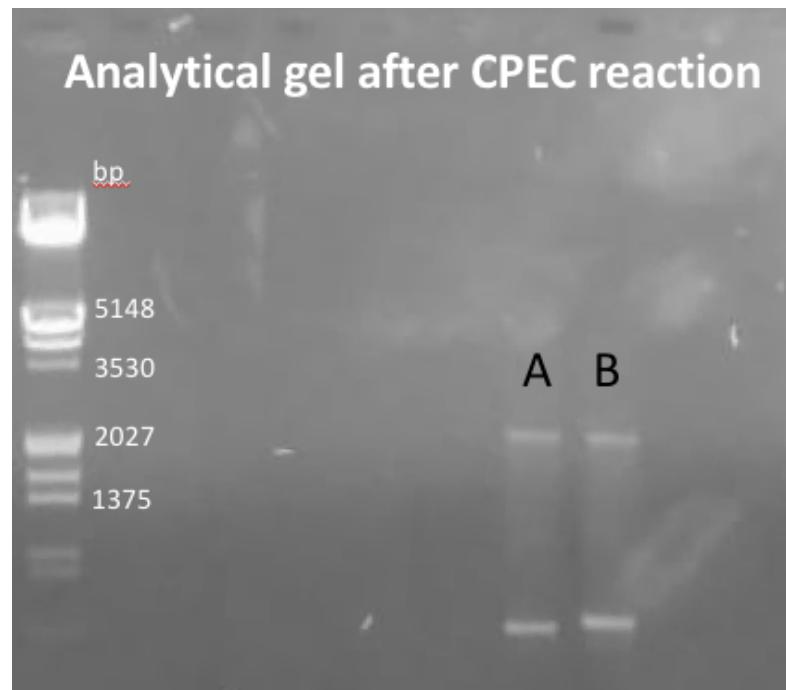
	A	B
Vector (pSB1C3) (μL)	3	5.1
Insert (pET22b-2b) (μL)	0.5	0.5
DMSO (3%)	0.75	0.75
iProof 2X	12.5	12.5
H ₂ O	8.25	6.15
Total volume (μL)	25	25

- CPEC reaction conditions

1. Initial denaturalization: 98°C for 3min
2. Denaturalization: 98°C for 30s
3. Annealing: 55°C for 30s
4. Extension: 72°C for 1min (15-30s/kb)
5. Final extension: 72°C for 10min
6. Hold: 4°C

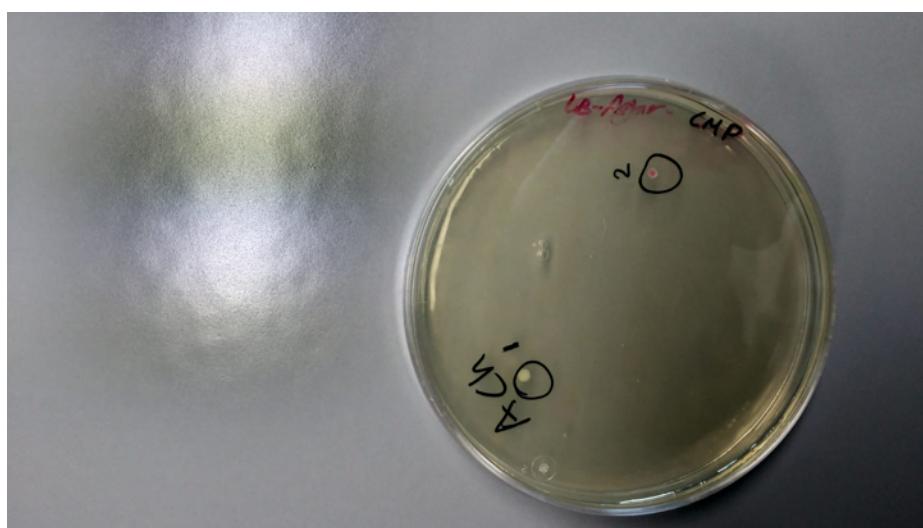
Repeat steps 2-4 29 times

- Ran an analytical gel after the reaction but forgot to put a control so the efficiency of the reaction could not be determined.



Week 8: 08/10/18 – 12/10/18

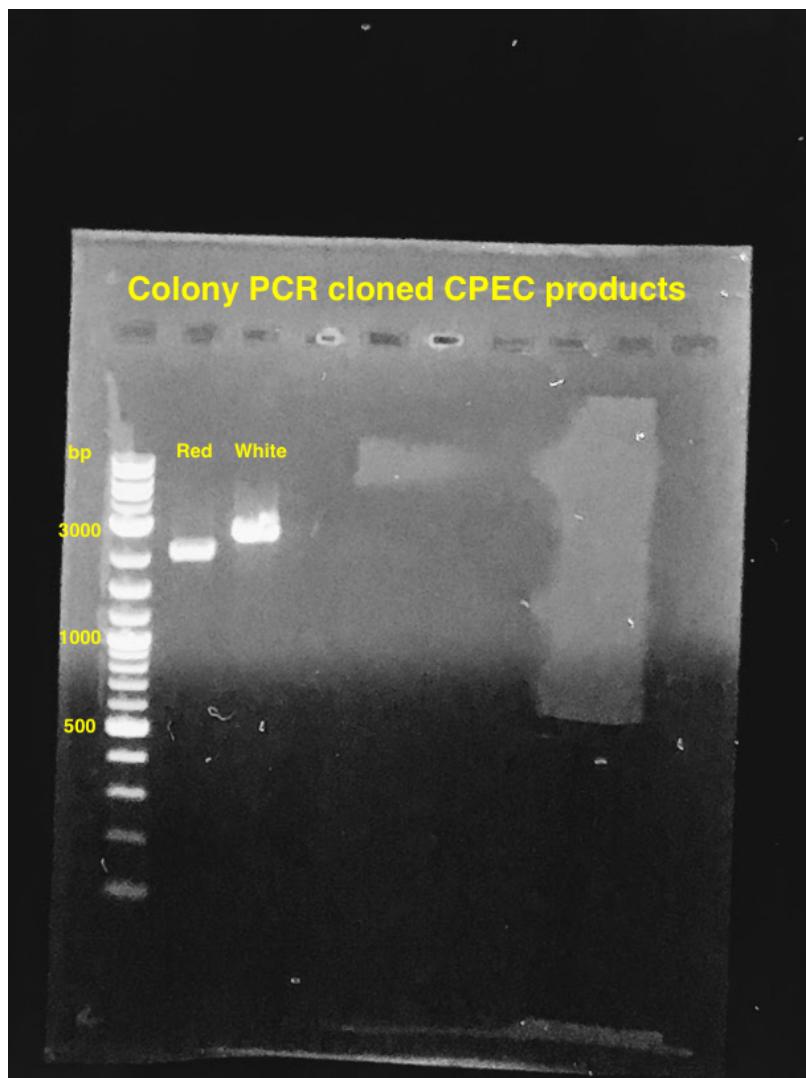
- Transformed 2 μ L of CPEC reaction into 50 μ L electrocompetent E.coli. Used an iGEM biobrick as a positive control for transformation. No colonies grown.
- Repeated CPEC using directly the linearized backbone provided by iGEM together with a 15X molar excess of the amplified insert and transformed 5 μ l of the reaction into 200 μ L CaCl₂ competent E.coli. Transformation of DQA resulted in the growth of red colonies (those that have no insert and have the RFP reporter intact), and also of white colonies (which have incorporated the insert and broke the RFP reporter gene). DQB transformation was unsuccessful



- Performed Colony PCR of the colonies using the insert primers. Annealing temperature were set at 52°C
- Made preinoculum from the colonies and miniprep. Following that, an analytical restriction was performed using the enzymes EcoRI HF and PstI in Buffer 2.1. Digestion results confirm that the red colony bears the intact vector since the DNA band is at 2kb. The white colony bears a plasmid but the digestion did not release

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any insert. We suspect that maybe one of the restriction sites has been lost during cloning and the band in the gel corresponds to the linear DNA of the DQA cloned gene.



- Quantified the correct insert using Qubit and prepared for biobrick shipment.