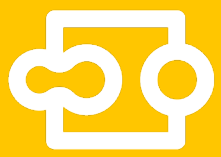

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Notebook

Destination vector experiments

Wednesday 6/06/18
Benchling design of the ccdB module (ccdB+chloramphenicol resistant gene) to adapt it to the Golden Braid grammar and pGrey_alpha1 assembly (Level 1 destination plasmid with ccdB as a negative selection marker).
Modules for the pGrey assembly:
GB0009 (M1- pGreen) (Amp)
GB0010 (M3-pGreen) (Amp)
GB0011 (Km)

A colony from each module is inoculated in LB medium with their respective antibiotic to do stock+miniprep. Incubate for 16 h at 37°C with vigorous shaking.

Colony from pGreen alpha 1 (Level 1 destination vector with lacZ as positive selection marker) plate is inoculated in LB medium with kanamycin. Incubate for 16 h at 37°C with vigorous shaking.

Thursday 7/06/18
Benchling modification of the ccdB module and pGrey_alpha1 vector to adapt them to the BioBrick grammar: https://benchling.com/s/seq-POF2bQSWCfTAmuK7mloW and https://benchling.com/s/seq-oP2ZBPxMvqudEs9J4EA6

Stock and miniprep (NucleoSpin® Plasmid EasyPure)

Name	260/280	DNA conc. (ng / uL)	DNA amount ug
M1-pGreen	1,88	460.451	23.02255
GB0011	1,92	497.582	14.92746
M3 pGreen	1,944	470.179	23.50895

Name	260/280	DNA conc. (ng / uL)	DNA amount ug
pGreen alpha 1	1,928	520.495	26.02475

Monday 11/06/18
Correct verification of the DNA integrity of modules M1, M2, M3, M4 and pGreen alpha1 vector by running an 1% electrophoresis gel.

Tuesday 12/06
Stock of DB3.1 (ccdB resistant strain).

Thursday 14/06/18
Golden Braid assembly of the destination vector modules.

Friday 15/06/18
Transformation in DB3.1 electrocompetent cells and plating in kanamycin. Incubate overnight 37°C.

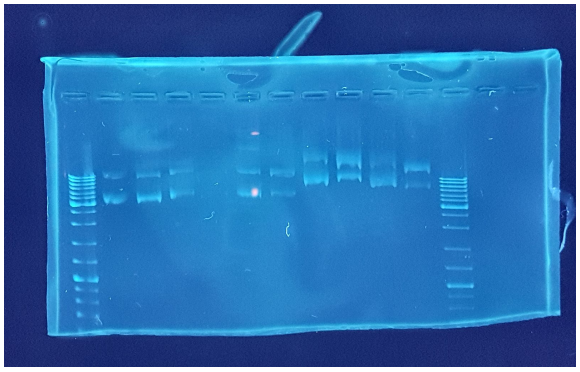
Monday 18/06/18
Pick 4 of the colonies from the plates.
Miniprep:

Name	A260/A280	DNA conc. ng/ uL	DNA amount ug

pGrey c.1	1,933	161.337	8,06685
pGrey c. 2	1,915	145.69	7,2845
pGrey c. 3	1,923	266.749	13,33745
pGrey c. 4	1,915	87.782	4,3891

Digestion assay with EcoRI and PstI. Run electrophoresis gel of the digested samples. Length fragments after digestion must be: 2570 pb and 1605 pb.

1: ladder, 2: mRFP1 3: mRFP2 4: mRFP3 5: sfGFP1 6: sfGFP2, 7: sfGFP3, **8: pGrey-alpha1 1, 9: pGrey-alpha1 2, 10: pGrey-alpha1 3, 11: pGrey-alpha1 4**, 12: ladder



pGrey was not correctly assembled, as the the digestion fragments have not the correct sizes.

Tuesday 18/06/18
Electroporation of DB3.1 electrocompetent cells with the Golden Braid reaction product. Plating in kanamycin, chloramphenicol and Km+Cm selection plates.

Wednesday 19/06/18
Transformation result: negative (no colonies). No colonies in the negative control and many colonies in the positive transformation control. As a result, individual modules are sent to sequencing.

Friday 22/06/18
Preparing DB3.1 Mix & Go competent cells (ZymoResearch commercial protocol)

Monday 25/06/18
Sequencing result: modules that have been used previously were not the correct ones.

Monday 2/07/18
While we don't have the correct modules yet, we are going to try a 2nd approach: digestion of pYB06k30 (KmR vector backbone) and ligation with the ccdB module.
Bacteria carrying pYB06k30 plasmid from stock is inoculated in 5 mL LB medium+Km. Incubate for 16 h at 37°C with vigorous shaking.

Tuesday 3/07/18
Digestion of pYB06k30 and ccdB module with EcoRI and PstI. Incubate 20 min at 37°C.

Electrophoresis gel (1%) for pYB06k30 digestion and band purification of the backbone (DNA gel purification kit from Macherey-Nagel). Ligation reaction. 3:1 ratio (insert:vector).

Transformation of 50 uL DB3.1 electrocompetent cells with 5 uL ligation product. Plating in Km antibiotic medium. Incubate overnight at 37°C.

Tuesday 3/07/18
Result: no colonies in the plates. There are colonies in the positive control (pGreen alpha1), so the ligation step must have been inefficient.

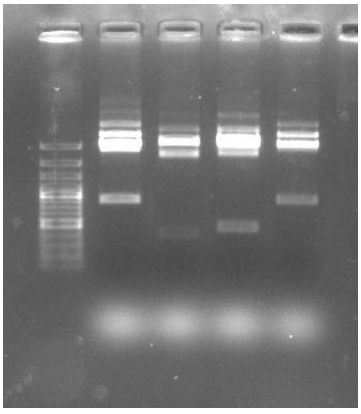
Wednesday 4/07/18
Correct modules for the GB assembly: pick a colony from a streaked agar plate of M1, M2, M3 and M4 and inoculate them in LB culture with Amp. Incubate for 16 h at 37°C with vigorous shaking.

Thursday 5/07/18
Miniprep (NucleoSpin® Plasmid EasyPure) of correct modules M1, M2, M3, M4.

Nombre	A260/A280	DNA conc. (ng/uL)
M1	1,96	117,222
M2	1,984	89,35
M3	2,02	98,181
M4	1,897	98,826

Friday 6/07/18
Preparation of DB3.1 electrocompetent cells following the electrocompetent cells protocol.

Monday 9/07/18
Primers design to amplificate the ccdB gblock (ccdB_module_FWD and ccdB_module_RV).
Making Mix&Go DB3.1 competent cells (Zymo Research protocol)
GB assembly, now with the correct modules M1, M2, M3 and M4.



1. Verification of the modules

Digestion with BbsI. Electrophoresis gel (1%).
Fragments size: M1: 890 + 3000 pb, M2: 393 + 3000 pb, M3: 467 + 3000 pb, M4: 900 + 3000 pb.
M1, M2, M3, M4 are correct.

2. Golden Braid assembly reaction

Transformation of the GB product in MixAndGo DB3.1 competent cells.
Plating with kanamycin antibiotic. Incubate overnight 37°C.

Tuesday 10/07/18
Negative result (no colonies in the plates).
Again: Transformation of the ligation product in MixAndGo competent and electrocompetent DB3.1 cells.

Wednesday 11/07/18
Negative result (no colonies in the plates). There are colonies in the transformation positive control (pGreen alpha1, KmR plasmid), so the negative result is not because of the transformation step.

As the GB reaction didn't work we will try again the 2nd approach (digestion of a BioBrick KmR vector and the ccdB module with E/P enzymes and ligation). Digestion reaction and ligation with ratio 3:1.

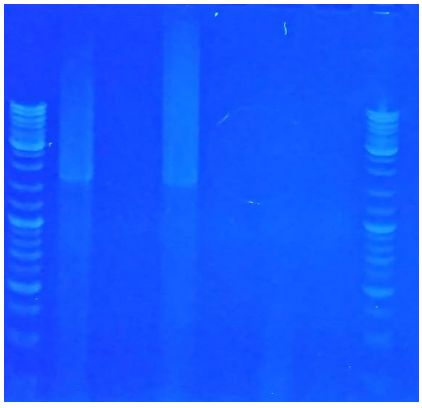
Inoculate pYB06k30 (from stock) in 4.5 mL LB+Kanamycin.

Thursday 12/07/18
Miniprep (NucleoSpin® Plasmid EasyPure)

Name	A260/A280	DNA conc.
pYB06k30	1.98	118 ng/uL

Amplification of the ccdB gblock. GC Enhancer and DMSO PCR.
Primers: ccdB_module_FW and ccdB_module_RV.
Anneal temperature: 64°C, Extension time: 30 secs, number of cycles: 25

Run electrophoresis gel (1%). PCR result:
ladder, PCR normal, negative control, PCR DMSO, negative control, PCR enhancer, negative control, ladder

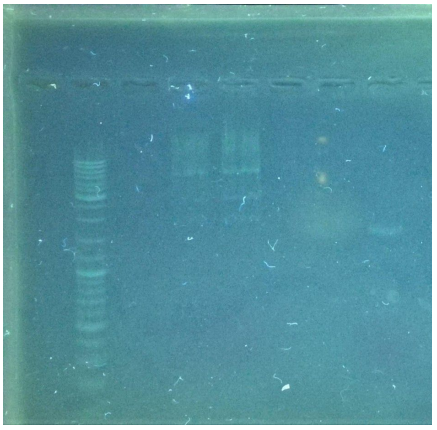


1 uL of this products are used as template for a new PCR with a bit less extension time.

Clean the PCR products (PCR clean-up protocol)

ccdB normal	ccdB DMSO
32,65 ng/uL	52,204 ng/uL

Digestion with E/P of ccdB amplicon and pYB06k30. Electrophoresis gel (1%)
1: ladder, 3-4: digestion of pYB06k30, 7: ccdB



Fragment size of the ccdB is 1.6 kb: it is correct.

Band purification of the pYB06k30 backbone (second band). Fragment size: 2.2 kb.
DNA band purification protocol: 16. 44 ng/uL.

Ligation reaction. 3:1 ratio and 5:1 ratio (insert:vector)

Transformation of electrocompetent and Mix&Go DB3.1 cells with the ligation products. Positive control of the ccdB lethal gene transforming electrocompetent DB3.1 with a control plasmid with ccdB. Thus, we can know if the DB3.1 strain is resistant to the ccdB or not. Plating in Km+Chromomax.

Monday 16/07/18
Transformation results:
Positive control of DB3.1 resistance to the ccdB: many blue colonies, so DB3.1 cells are resistant to the lethal gene.
Plates for the pGrey destination vector assembly: there are colonies. We pick 10 colonies to do colony PCR. Run electrophoresis gel (1%).
Colonies 1, 2 and 5 are chosen to do Miniprep. Inoculate in 5 mL LB+Km medium and incubate for 16 h at 37°C.

Tuesday 17/06/18
Miniprep (NucleoSpin® Plasmid EasyPure)

Name	A260/280	DNA conc. (ng/uL)
pGrey from Colony 1	1,93	419,387
pGrey from Colony 3	1,962	405,817
pGrey from colony 5	1,924	434,493

Restriction analysis with EcoRI/PstI. Electrophoresis gel (1%).
Result: Bands sizes are not the ones expected for the pGrey vector.

Tuesday 24/07/18
- Test of efficiency of the DB3.1 electrocompetent cells:
  Plasmid with ccdB AmpR (pccdB)
  Control: plasmid with AmpR (GB1205)

Electroporation of DB3.1 cells with 200 ng DNA. Transformation with 5 uL plasmid with ccdB (co=40.3 ng/uL) and 0.8 uL GB1205 (co=267 ng/uL). 1000 uL SOC. Plate 5, 10, 50, 100 uL of each transformation product in ampicillin plates. Incubate overnight at 37°C.

Wednesday 25/07/18
DB3.1 electrocompetent cells competence: DB3.1 cells have a competence of 10^7 CFU/ uL without ccdB and 10^6 CFU/uL with ccdB.
As the previous GB reactions didn't work we are doing TA cloning (Thermo protocol) of the ccdB linear module to enhance the efficiency of the GB reaction.

Adenine adding to the ccdb gblock and amplicon
Final volume=10 uL

2uL buffer
0,2 uL MyTaq
5 uL ccdB
2,8 uL H20
Thermocycler: 72ºC 10-15 min

TA cloning
4 uL extension product
1 uL NaCl
1 uL TOPO vector
5 min at RT
Dyalization of the TA cloning product, as the high salt content can lower electroporation efficiency.
Transformation of electrocompetent DB3.1 cells with 1 uL TA cloning product. Plating in kanamycin, IPTG+X-gal agar plates.

Thursday 26/07/18
TA cloning transformation result: all colonies blue, so they are the TOPO vector without the ccdB insert.
Primers design to amplificate the pGreen vector backbone with EcoRI and PstI sites: RB_pGrey_FOR and LB_pGrey_RV.

Monday 30/07/18
As the TA cloning didn't work, we are repeating the GB assembly reaction with the linearized gBlock and ccdB amplicon.
Transformation in DB3.1 electrocompetent cells and 700 uL SOC. Plating 30 and 500 uL in kanamycin selection antibiotic. Incubate overnight 37ºC.

Tuesday 31/07/2018
Inoculate one colony in LB medium with kanamycin and incubate for 16 h at 37ºC.

Wednesday 1/08/2018
Miniprep of the supposed pGrey alpha 1 vector (NucleoSpin® Plasmid EasyPure kit)

Name	260/280	ng/µL
pGrey alpha1	1,928	172,476

Restriction assay of the pGrey alpha1 (digestion with EcoRI/PstI).
Run electrophoresis gel 1%. Result: there was only one band.
The plasmid is not the pGrey alpha1 vector.

As the GB reaction didn't work, we will try to assemble the pGrey vector with a 3rd approach: amplification of the pGreen alpha1 backbone adding E/P sites and ligation of this amplicon with the ccdB module digested with E/P.

PCR of pGreen backbone with normal conditions, DMSO and GC enhancer:
Primers RB_pGrey_FOR and LB_pGrey_RV.
25 cycles, annealing T^a = 67ºC, ext time: 1 min 18 sec.
Run electrophoresis gel (1%): there is no band. Repeat PCR.

Thursday 2/08/2018

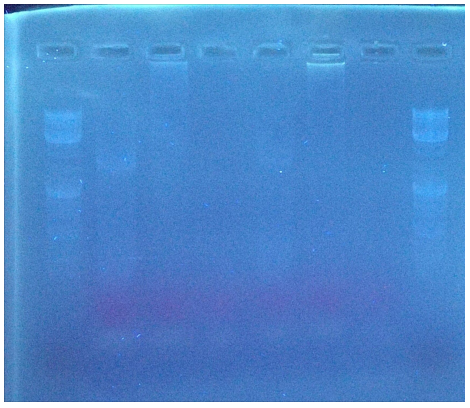
Again: PCR of pGreen backbone adding E/P sites. Extension time of 2min 30 sec.
Run electrophoresis gel 1% and purificate the backbone band (2.6 kb)
Band purification:

Name	260/280	ng/µL
DMSO	1,649	23,286
GC Enhacer	1,79	46,669

Digestion of the pGreen backbone and ccdB module with E/P.
Ligation reaction. Ratio 3:1 (ccdB gblock:backbone)
Transformation in 10G electrocompetent cells. Plating in Km+Chromomax.

Friday 3/08/2018
Results of the assembly of pGrey: negative
Again: PCR of pGreen backbone adding E/P sites. Run electrophoresis gel 1%.
The size of the pGreen backbone band is correct

Monday 6/08/18
PCR of ccdB from the gblock and the previous PCR. Anneal temperature: 64ºC, extension time: 40 secs, number of cycles: 25.
ladder; Normal PCR (gblock), normal PCR (previous amplificated), c-, DMSO PCR (gblock), DMSO (previous amplificated), C-, ladder



ccdB is degraded (ccdB band in well 2 and well 5). We repeat it changing some PCR conditions: ext. time = 30 secs, number of cycles: 30. Tª gradient: 63ºC Td: 3.5 (PCR reactions at 62.9 and 63.8).
Run electrophoresis gel: PCR didn't work

Tuesday 7/08/18
We are repeating the ccdB module PCR: Anneal temperature: 64ºC, Extension time: 28 secs, number of cycles: 25
Result of the electrophoresis gel: PCR didn't work.

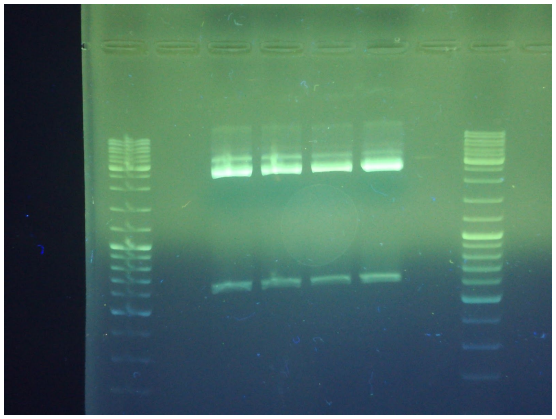
Wednesday 8/08/18
We are repeating ccdB PCR with new aliquote of both primers and new dilution of the gblock.
PCR conditions: anneal temperature: 64ºC, Ext. time: 30 sec, 25 cycles
Result of the electrophoresis gel: PCR didn't work

We are repeating the ccdB PCR with ext. time 17 secs x30 cycles.
Result of the electrophoresis gel: PCR didn't work

Monday 13/08/18
We have measured the concentration of the gBlock and it was 7.9 ng/uL instead of 10 ng/ul.
Repeat the PCR amplification diluting the template until. 0.5 ng/uL
PCR conditions: Anneal temperature: 64°C, extension time: 30 secs x 30 cycles
Result of the electrophoresis gel: PCR didn't work

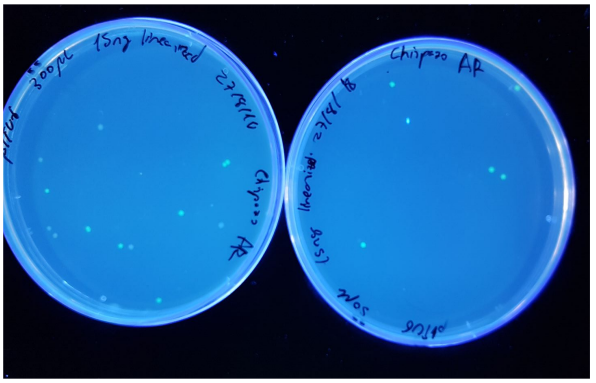
Monday 20/08/18
As we are troubleshooting with the ccdB module, we have decided that the pGreen alpha1 destination plasmid will be used as a linearized vector for the TU's assemblies.

Digestion reaction of pGreen alpha 1 vector with BsaI. 1h 37°C.
Run electrophoresis gel 1%. Cut the 2nd band.
ladder, pGreen alpha1, ladder



Tuesday 21/08/18
DNA band purification (NucleoSpin® Gel and PCR Clean-up kit)

Name	260/280 ratio	DNA conc. (ng/uL)
pGreen alpha 1 linearized	1.906	26.5
pGreen alpha 1 linearized (2nd. elution)	1.702	11.82

Monday 27/08/18
Assembly of pITU6 with different amounts of linearized destination plasmid (from 15 to 40 ng; 5 ng of step). Transformation of 10G electrocompetent cells and plating in Km solid medium. Incubate overnight.

Tuesday 28/08/18
Transformation result: 15-20 ng are the conc. with less colony background (only 1 of the colonies is not expressing the sfGFP).