iGEM2013 – Microbiology – BMB – SDU	
Project type: USER cloning **Project title:** USER cloning of pSB1C3, lac promoter and DXS (B. sub) **Sub project:**	**Creation date:** 13.08.03 **Written by: PRA** **Performed by:** PRA

1. SOPs in use

SOP0007_v01 Excision and ligation of PCR product in USER cloning

SOP0009_v01 TSB transformation

SOP0019_v01_plasmid Miniprep

iGEM2013_SOP010_v01_Restriction digest

iGEM2013_SOP015_v01_ ligation

iGEM2013_SOP0014_v01_ Gel purification

iGEM2013_SOP0017_v01 Fast digest

2. Purpose

3. Overview

Day	SOPs	Persons	Experiments
1	SOP0007 SOP0009	PRA PRA	USER reaction for pSB1C3, Lac promoter and Dxs (B. sub) TSB transformation of USER reaction
2	SOP0021 SOP0021	SIS, SF SIS	Colony PCR with Mytaq and primer VR and VF in order to check for the right inset size. Colony PCR with Mytaq and primer VR and VF in order to check for the right inset size.

3	SOP0019 SOP0003	SIS	Plasmid Miniprep on ONC of colony 1. Bacterial freezing stock
4	SOP0010 SOP0014	PRA SF	10 uL blue 105 digested with EcoRI and XbaI for 30 min at 37 deg in a green fast digest buffer and runned on a gel. Gelpurification
5	SOP0015	SF	Ligation of LacI into the plasmid.
6	SOP0009	SIS, SF	TSB Transformation of ligation with LacI
7	 SOP0009	MH SF	ONC of colony 1 for miniprep (plasmid to be used for sequencing) TSB transforamtion of rest of ligation with LacI
8	SOP0021 SOP0021	PRA PRA	Colony PCR Colony PCR
9	SOP0021 SOP0012 SOP0014 SOP0015 SOP0009	PRA AK AK PRA PRA, AK	Colony PCR with lacI fwd primer Digest of pSB1C3-araC (blue 154) with EcoRI and Xba Gel purification of digest (red 159) Ligation of gel purification with digest lacI (red 156) TSB transformation of ligation
10	SOP0019	SIS	Plasmid Miniprep of ONC in order to digest with EcoRI and PstI (to check for the right insert size) and EcoRI alone (to linearize the plamid).
11		MH	Colony PCR of transformed cells from day 9 with expected sequence: pSB1C3-lacI-lac-Dxs(sub)
12		MH	Preparation of sequencing mixture for expected sequence: pSB1C3-lac-Dxs (sub)
13	SOP0017	MHK	Fast digest of blue 166 (colony of colony PCR with weird length) with EcoRI and PstI and EcoRI alone
14	SOP0010	PRA	Restriction digest of blue 166 (colony of colony PCR with weird length) with EcoRI and PstI and EcoRI alone
15		MH	Preparation of sequencing mixture for expected sequence: lacI-lac-dxs(sub)? with the knowledge that the colony PCR results showed an unexpected length of insert.
16	SOP0017	PRA, MHK	Test digest of Blue 167 (pSB1C3-LacI-Lac-dxs(B. sub)) with EcoRI+PstI and with EcoRI alone
17	SOP0014	MH, SIS	TSB transformation of blue 154 (pSB1C3-Plac-dxs(sub)) into KG22
18	SOP0015 SOP0009	HWJ	Ligation of pSB1C3-Plac-Dxs(B.sub) with natural LacI without LVA-tag. Transformation of ligation into MG1655
19	SOP0021	SF	Colony PCR
20	SOP0019 SOP0017	SF	Plasmid miniprep Test Digest

21	SOP0021 SOP0017 SOP0014 SOP0015 SOP0019	HWJ HWJ SF SF	Miniprep Fast digest Gelpurification Ligation TSB transformation
22	SOP0021	MHK	Colony PCR Colony PCR of colony 7 from previous colony PCR
23	SOP0019	MHK	Miniprep of pSB1C3-LacI(N)-Plac-DXS(B) from colony #7

4. Materials required

Materials in use

Name	Components (Concentrations)	Manufacturer / Cat. #	Room	Safety considerations
Green 91	rbs-DXS (B. sub) PCR			
Green 94	Lac promoter PCR			
Green 97	pSB1C3 PCR			

5. Other comments

6. Experiment history

Date (YY.MM.DD)	SOPs	Alterations to SOPs and remarks to experiments

File name: iGEM2013_0026_Protocol_USER_Cloning_Lac promoter_RBS-DXS(B-Sub)

13.08.03	USER reaction TSB transformation	20 µL total reaction with pfu 10x buffer 10 fmol pSB1C3 to each reaction: 1:0:0 control: no insert 1:2:2: 20 fmol DXS, 20 fmol Lac promoter 1:5:5: 50 fmol DXS, 50 fmol Lac promoter 30 min at 37 degrees 30 min at RT Performed according to protocol, with 1 h phenotypical expression
13.08.05 13.08.05	Colony PCR Colony PCR	Colony PCR with Mytaq and primer 004 and 005 in order to check for the right insert size (ap. 2500 bp). 10 µL total reaction. 6 colony PCRs were performed: 1) Transformation 1:2:2 big colony 2)Transformation 1:2:2 small colony 3)Transformation 1:5:5 big colony 4)Transformation 1:5:5 small colony 5)Negative control small colony 6)Negative control big colony. Colony PCR was also performed on colonies from the negative control plate, in order to check if the plasmid is religated and this is the reason for so many colonies on the negative control. Colony PCR with Mytaq and primer 004 and 005 in order to check for the right insert size (ap. 2500 bp). 10 µL total reaction. 1)-4)Transformation 1:2:2 5)-8) Transformation 1:5:5 9)-12) Negative control
13.08.06	Plasmid miniprep Bacterial freezing stock	Plasmid miniprep was performed on a ONC of colony 1 from transformation 13.08.03. It was eluted in 50 µL MQ water.

13.08.12	Fast digest Gel purification Ligation	10 uL blue 125 EcoRI and XbaI for 30 min at 37 deg in a green fast digest buffer and runned on a gel. The spins were performed at 14.500 g, and because of multiple samples the 60 sec wait were longer. Nanodrop was performed. The samples were: pSB1C3-Lac:DXS:LacI 1:2 1:5 1:0 always 10 fmol “Plasmid”. Water until at total volume of 20 µL
13.08.13	Ligation	The SOP was followed
13.08.15	Transformation	The SOP was followed
13.08.16	ONC TSB transformation	An overnight culture containing 10 ml of LB and colony 1 was set in the heating cabinet. The plasmid is to be used for sequencing using primers 4, 5, and 19. SOP followed with 1h phenotypical expression.
13.08.17	Colony PCR Colony PCR	Colony PCR with MyTaq and verification primers 1uL of each primer 3uL H2O 5uL MyTaq Colony 11-14 and 18-21 Colony 10 and 16 as control Colony PCR with MyTaq and verification primers 1uL of each primer 3uL H2O 5uL MyTaq Colony 11-14 Colony 10 as control

File name: iGEM2013_0026_Protocol_USER_Cloning_Lac promoter_RBS-DXS(B-Sub)

13.08.18	Colony PCR	Colony PCR with MyTaq and primer 60 and 5 3uL H2O 5uL MyTaq Colony 19
	Restriction digest	1ug blue 154 was digested with EcoRI and XbaI
	Gel purification	Gel purification SOP followed. Eluted in 30uL
	Ligation	Red 159 was used as backbone and Red 156 was insert. 10 fmol bacbone in each reaction. The following relation was used 1:0 negative control 1:1 10 fmol red 156 1:2 20 fmol red 156
	TSB transformation	SOP followed. 15uL ligation reaction used phenotypical ecpression for 45 min

13.08.19	Plasmid miniprep	Plasmid Miniprep of ONC were performed in order to digest with EcoRI and PstI (to check for the right insert size) and EcoRI alone (to linearize the plamid). Two minipreps were performed (both eluated in 50 uL water), but they gave around the same nanodrop so they were pooled.
	Colony PCR	A colony PCR was performed with the following colonies as templates: 1:1 9 thru 12 1:2 13 thru 16 1:0 Control on 39 Primers: 4 and 5 Annealing temperature: Elongation time: 15 sec
	Sequencing mixture	3 mixtures were prepared of 17 ul volume with 2 ul of the following primers: 4, 5, and 19 given the corresponding numbers: 22, 23, 24
	Fast digest	1000ng blue 166 was digested with EcoRI and PstI and EcoRI alone. The 10x FastDigest green buffer was used, and the products were loaded directly on a gel.
13.08.20	Restriction digest	1000ng blue 164 was digested with EcoRI and PstI and EcoRI alone.
13.08.20	Sequencing mixture	3 mixtures from blue 166 were prepared of 17 ul volume with 2 ul of the following primers: 4, 5, and 15 given the corresponding numbers: 47, 48, and 49
13.08.20	miniprep	Plasmid Miniprep of ONC was performed in order to Two minipreps were performed (both eluted in 100 uL water)samples blå167 and blå 168.

13.08.21	Fast digest	Fast digest of 1000ng Blue 167 (pSB1C3-LacI-Lac-dxs(B.sub)) with EcoRI+PstI and with EcoRI alone. 10x FastDigest green buffer was used. 20µL total volume for reactions. The mix was given 25 min at 37 deg C to digest.
13.08.27	TSB transformation	TSB transformation of blue 154 into KG22 cells. Expression time: 1 hour. A very small volume was present in the eppendorf. However, the transformation was attempted anyway in the eppendorf that the sample was stored in.
13.09.18	Ligation Transformation	Ligation of pSB1C3-Plac-Dxs(B.sub) with natural LacI without LVA-tag. Transformation of ligation into MG1655
13.09.19	Colony PCR with myTaq	Colony PCR with pSB1C3-LacI(n)-pLac-dxs(sub) in MG1655 with ligationconcentrations 1:0, 1:1 and 1:2.
13.09.20	Miniprep of ONC Test digest	Miniprep and nanodrop Test digest to see if insert is correct. Digested with E and E,P first digest was wierd so testdigest was performed again.
13.09.21	Gelpurification Ligation TSB transformation	The SOP was followed. Nanodropmeasurements was made Ligation of pSBB1C3-pLac-DXS(Sub) (Red259) pcon LacI (Red239). The ligations were made with concentrations 10fmol:o,20 and 50 fmol. 3 hours at roomtemperature.
13.09.21	Miniprep Digest Ligation Transformation	miniprep yielded 30,2 ng/µl Digested with E, X and fast AP

13.09.22	ColonyPCR	Colony PCR of transformation of pSb1C3-LacI(N)-Plac-DXS(B) Colony 1+2 pSB1C3 Colony 3-8 1:2 pSb1C3:LacI(N)-Plac-DXS(B) Colony 9-16 1:5 pSb1C3:LacI(N)-Plac-DXS(B) VF2 og VR (primer 4 og 5)
	ColonyPCR	Colony 7 from the previous transformation was used for three times colony PCR again. VF2 and VR (primer 4 og primer 5)
13.09.27	Miniprep	Miniprep of colony #7 containing pSB1C3-LacI(N)-Plac-DXS(B)

7. Sample specification

Sample name	Sample content	Concentration	Used for / Saved where
White 13	Transformed MG1655 E.coli with pSB1C3-lac-rbs-dxs(B.sub).		-80 deg freezer.
Blue 105	Plasmid purification of pSB1C3-lac-rbs-dxs(B.sub)	35,4 ng/µL	Green box in the iGEM fridge.
Red 131	pSB1C3-lac-rbs-dxs(B.sub) digested with XbaI and EcoRI	5.0 ng/µL	Green box in the iGEM fridge.
Blue 166	Miniprep of pSB1C3-LacI-Lac-dxs(B.sub)	441,1 ng/uL	To be digested with EcoRI and PstI and EcoRI. Stored in the green box in the iGEM fridge.
Blå 167	pSB1C3-LacI-Lac-dxs(B.sub)	220,9	iGEM fridge.
Blå 168	pSB1C3-LacI-Lac-dxs(B.sub)	145,3	iGEM fridge.
Blå 256	pSB1C3-LacI-Lac-dxs(B.sub)	30,2	iGEM fridge.
Blå 2XX	pSB1C3-LacI(N)-Plac-DXS(B)	16,0	iGEM fridge

8. Remarks on setup

9. Results and conclusions

05.08.13

Colonies appeared on transformation plate 1:2:2 and 1:5:5, but also on the negative control (more than 50) and no colonies appeared on the pSB1C3-GFP plate.

Result for the colony PCR:

10 µL was loaded in each well on a 1% agarose gel. ladder: red.

None of the bands had the right length. The bands seen are from the negative control plate.

Results for the new colony PCR:

10 µL was loaded in each well on a 1% agarose gel. ladder: red.

Colony 1, 2, 3, 5 and 7 had the appropriate length around 2400bp. An ON culture of colony 1 was put in the incubator at 37 deg.

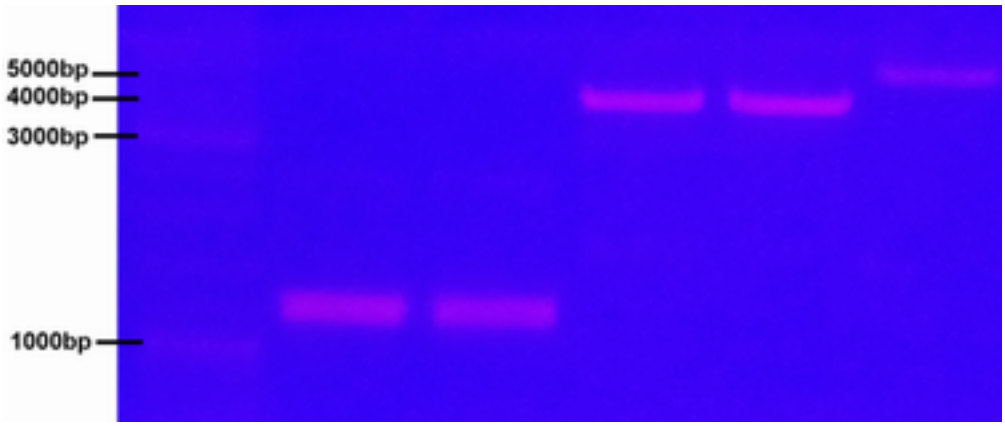
06.08.13

The concentration of the purified plasmid (blue 105): 35,4 ng/µL.

13.08.12

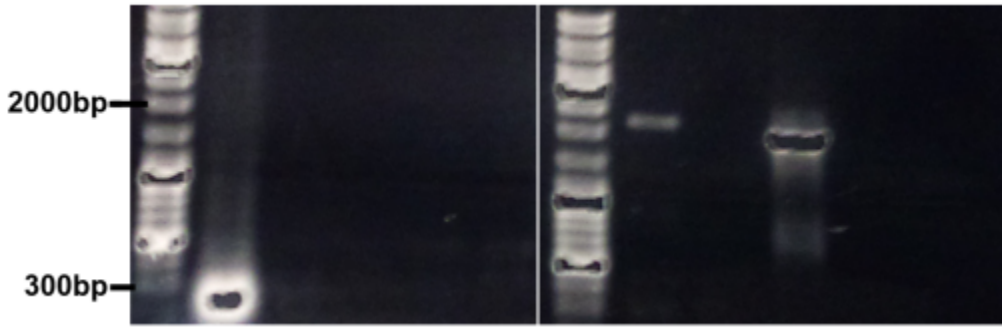
Digest of pSB1C3-lac-dxs (lane 4):

File name: iGEM2013_0026_Protocol_USER_Cloning_Lac promoter_RBS-DXS(B-Sub)



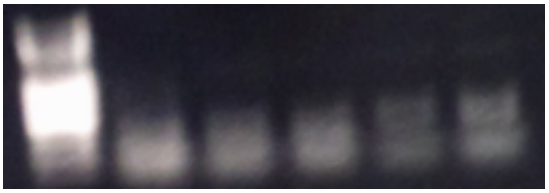
The band in lane 4 had the right lenght (4172bp) around 4000bp and were cut out and purified.

Colony PCR 1:



No bands appeared with colony 10-14 and only one appeared with colony 19. Colony 19 has a length differing from the re-ligation.

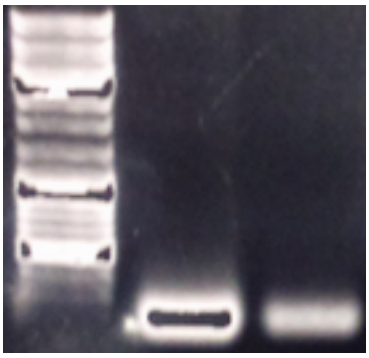
Colony PCR 2:



No bands appeared.

13.08.18

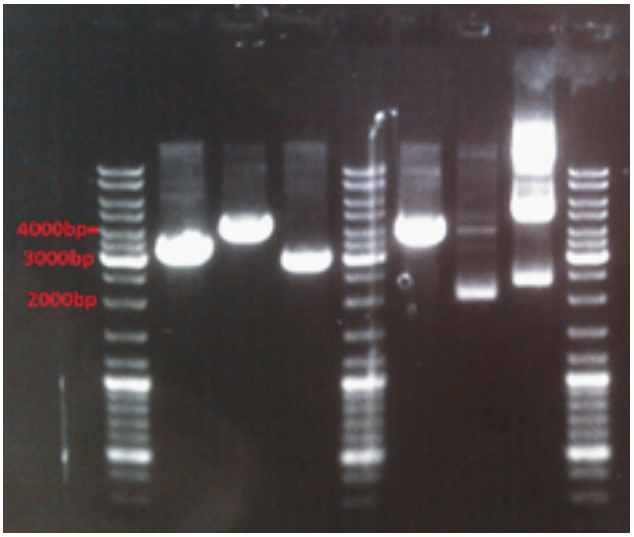
Colony PCR with lacI forward:



No bands appeared.
Another round of cloning was commenced.

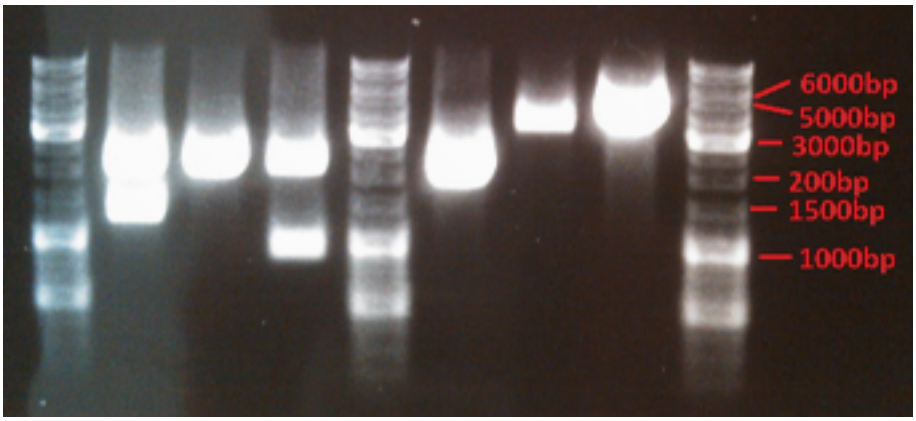
13.08.19
Nanodrop measurements of plasmid miniprep: 441,1 ng/uL.

Below: Fast digest of Blue 166 with EcoRI loaded on a 1% agarose gel. Red ladder is used.
Blue 166 is loaded in well number 8. Many bands appeared with several above 10000bp. A clear band appeared around 2500bp and a wide band around 5000bp. The expected length was ~5400bp (pSB1C3 ~2000bp, LacI ~1200bp, lac ~200, DXS (B. sub.) ~2000).



Below:Fast digest of Blue 166 with EcoRI and PstI loaded on a 1% agarose gel. Red ladder is used.
Blue 166 is loaded in well number 8. A wide band appeared around 5000bp, which was not expected (expected: a band at 2000bp and a band at approximately 3400bp).

File name: iGEM2013_0026_Protocol_USER_Cloning_Lac promoter_RBS-DXS(B-Sub)



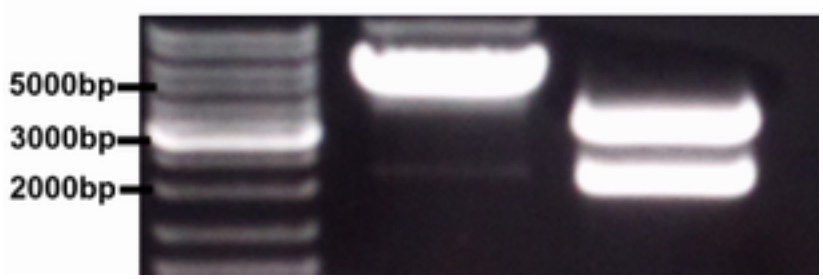
Colony PCR for new round of cloning:

13.08.20

miniprep: blå167 = 220,9 ng/µl and blå168 = 145,3 ng/µl

13.08.21

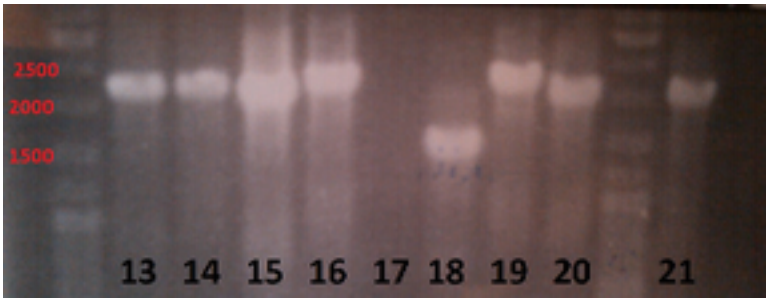
Test fast digest:



The linearized plasmid has a length around 5500bp, and the EcoRI and PstI cut plasmid has lengths around 2000 and 3500bp, which are all the correct lengths for pSB1C3-lacI-Plac-dxs(B.sub).

13.09.19

Resultat fra colonyPCR

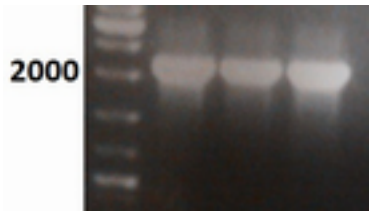


File name: iGEM2013_0026_Protocol_USER_Cloning_Lac promoter_RBS-DXS(B-Sub)

Well load: Red ladder, colony 13-16 (psB1C3-LacI without LVA-DXS(B) 1:1), colony 17-20 (pSB1C3-LacI without LVA-DXS(B) 1:2), red ladder, colony 21 (pSB1C3-LacI without LVA-DXS(B) 1:0).
All bands except colony 18 had the same length as the religation (a bit above 2000bp). No band appeared for colony 17. Colony 18 had a length of approximately 1600bp. This is not the expected length.
The gel contained no Ethidium Bromide and was therefore dyed after the gel had run. The gel was put in 180mL TAE buffer with three drops of EtBr and lightly shaken every five minutes for twenty minutes.

13.09.20
Nanodrop was performed. Blue 254 and 255 concentrations 34,7 and 41,3.

13.09.22
Colony PCR 1
Only one band of correct length appeared. This was a weak band for colony 7 above 3000bp.



Colony PCR 2

13.09.27
Miniprep produced sample Blue 2XX containing pSB1C3-LacI(N)-Plac-DXS(B) with a concentration of 16,0ng/µL.

10. Appendices

File name: iGEM2013_0026_Protocol_USER_Cloning_Lac promoter_RBS-DXS(B-Sub)