EutS + EutMN - RD, Ligation, Transformation

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-07-19 to 2017-07-21

WEDNESDAY, 19/7/17

From G Block

1. eutS and eutMN
2. add 20uL of EB buffer (in miniprep kit) = 50ng/uL stock

Restriction Digest

1. Protocol attached

pSB1C3 (from iGEM) digest with EcoRI + PstI

   a. 6.7uL linearised vector
   b. 0.5uL EcoRI, PstI, DpnI, rSAP

EutS digest for pSB1C3

   a. 3.8uL eutS
   b. 0.5uL EcoRI, PstI

EutMN digest for pSB1C3

   a. 4.7uL eutMN
   b. 0.5uL EcoRI, PstI

Ligation

1. Protocol attached

Transformation

1. Protocol attached

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 20uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min at 80C

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: 50 ng (0.020 pmol)	1
4	Insert DNA: 37.5 ng (0.060 pmol)	4
5	Nuclease-free water	12
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.

- ✔ 7. Incubate at room temperature for 30 minutes
- ✔ 8. Heat inactivate at 65C for 10 minutes.
- ✔ 9. Chill on ice and transform

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins

13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.

14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before use

15. Spun down into a pellet at 5000rpm for 2 minutes

16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC

17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously

18. Incubate overnight at 37C

EutS + EutMN - RD, Ligation, Transformation

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-07-19 to 2017-07-21

THURSDAY, 20/7/17

Results: no colonies - used the wrong cells, saved digest mix from yesterday in fridge, so redo ligation, transformation and plate

EutS + EutMN - RD, Ligation, Transformation

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-07-19 to 2017-07-21

FRIDAY, 21/7/17

Results: colonies on EutS + EutMN, none on -ve control

- eutS + eutMN transformation successful - put in fridge to inoculate on monday and miniprep on tuesday

EutS + EutMN - overnight cultures

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Date: 2017-07-24

MONDAY, 24/7/17

Growing liquid overnight culture

- 10ml LB broth
- 1 colony
- 10µl chloramphenicol

Added to a 50ml falcon tube for incubation overnight at 37°C in shaker.

EutS + EutMN - miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-07-25 to 2017-07-26

TUESDAY, 25/7/17

Miniprep

1. Protocol attached

nanodrop results:

IMG_0023.JPG

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
1	25/07jessblank	Takano_Lab_2	25/07/2017 12:47:49	0.9	ng/µl	0.019	0.011	1.70	0.78	DNA
2	pSB1C3_eutSA	Takano_Lab_2	25/07/2017 12:49:15	161.0	ng/µl	3.219	1.842	1.75	1.07	DNA
3	pSB1C3_eutSB	Takano_Lab_2	25/07/2017 12:50:25	139.5	ng/µl	2.791	1.547	1.80	1.26	DNA
4	pSB1C3_eutSB	Takano_Lab_2	25/07/2017 12:51:07	37.8	ng/µl	0.755	0.539	1.40	0.41	DNA
5	pSB1C3_eutSC	Takano_Lab_2	25/07/2017 12:51:18	35.4	ng/µl	0.708	0.498	1.42	0.41	DNA
6	pSB1C3_eutSD	Takano_Lab_2	25/07/2017 12:52:21	115.1	ng/µl	2.302	1.329	1.73	0.97	DNA
7	pSB1C3_eutSE	Takano_Lab_2	25/07/2017 12:53:33	169.2	ng/µl	3.383	1.976	1.71	0.97	DNA
8	pSB1C3_eutSF	Takano_Lab_2	25/07/2017 12:54:21	109.3	ng/µl	2.185	1.347	1.62	0.93	DNA
9	pSB1C3_eutSG	Takano_Lab_2	25/07/2017 12:55:06	81.5	ng/µl	1.629	0.969	1.68	0.81	DNA
10	pSB1C3_eutSH	Takano_Lab_2	25/07/2017 12:56:19	101.9	ng/µl	2.038	1.112	1.83	1.20	DNA
11	25/07jessblank	Takano_Lab_2	25/07/2017 12:57:55	-0.8	ng/µl	-0.016	-0.019	0.84	0.16	DNA
12	25/07jessblank	Takano_Lab_2	25/07/2017 12:58:29	-1.5	ng/µl	-0.030	-0.030	1.01	0.32	DNA
13	25/07jessblank	Takano_Lab_2	25/07/2017 12:59:09	-0.6	ng/µl	-0.011	-0.020	0.58	0.10	DNA
14	pSB1C3_eutMNA	Takano_Lab_2	25/07/2017 13:00:00	86.4	ng/µl	1.728	0.941	1.84	1.07	DNA
15	pSB1C3_eutMNB	Takano_Lab_2	25/07/2017 13:01:33	54.5	ng/µl	1.091	0.608	1.79	1.20	DNA
16	pSB1C3_eutMNC	Takano_Lab_2	25/07/2017 13:02:20	113.6	ng/µl	2.271	1.649	1.38	0.43	DNA
17	pSB1C3_eutMNCredo	Takano_Lab_2	25/07/2017 13:03:05	13.8	ng/µl	0.276	0.243	1.13	0.29	DNA
18	pSB1C3_eutMND	Takano_Lab_2	25/07/2017 13:04:10	49.8	ng/µl	0.995	0.610	1.63	0.96	DNA
19	pSB1C3_eutMNE	Takano_Lab_2	25/07/2017 13:04:50	49.1	ng/µl	0.982	0.525	1.87	1.16	DNA
20	pSB1C3_eutMNE	Takano_Lab_2	25/07/2017 13:05:23	24.3	ng/µl	0.487	0.343	1.42	0.39	DNA
21	pSB1C3_eutMNF	Takano_Lab_2	25/07/2017 13:05:32	23.3	ng/µl	0.465	0.322	1.45	0.39	DNA
22	pSB1C3_eutMNG	Takano_Lab_2	25/07/2017 13:06:07	29.3	ng/µl	0.585	0.349	1.68	0.89	DNA
23	pSB1C3_eutMNH	Takano_Lab_2	25/07/2017 13:06:46	135.4	ng/µl	2.707	1.531	1.77	1.17	DNA

Restriction Digest

1. Protocol attached

Agarose gel

1. Protocol attached

Calc for gel - running 250ng			
	Construct	Concentration (ng/uL)	Vol for 250ng
1	EutS A	161	1.6uL
2	EutS B	139.5	1.8uL
3	EutS C	37.8	6.6uL
4	EutS D	115.1	2.2uL
5	EutS E	169.2	1.5uL
6	EutS F	109.3	2.3uL
7	EutS G	81.5	3.1uL
8	EutS H	101.9	2.5uL
9	EutMN A	86.4	2.9uL
10	EutMN B	54.5	4.6uL
11	EutMN C	113.6	2.2uL
12	EutMN D	49.8	5uL
13	EutMN E	24.3	10.3uL
14	EutMN F	23.3	10.7uL
15	EutMN G	29.3	8.5uL
16	EutMN H	135.4	1.8uL

Have 250ng in the 50uL RD mix - load as much as possible
leave out SC, MNE, MNF, MNG
order: ladder-SA-SB-SD-SE-SF-SG-SH-MNA-MNB-MNC-MND-MNH

gel result: too faint to determine so redo with 1000ng DNA

UVP06607July252017 2.jpg

QIAprep® Spin Miniprep Kit

Introduction

The QIAprep Spin Miniprep Kit (cat. nos. 27104 and 27106) can be stored at room temperature (15–25°C) for up to 12 months.

Materials

- Qiagen Kit

Procedure

Notes before starting

1. Optional: Add LyseBlue reagent to Buffer P1 at a ratio of 1 to 1000.
2. Add the provided RNase A solution to Buffer P1, mix and store at 2–8°C.
3. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

Protocol

4. Spin down the overnight cultures at 10000rpm for 10min. Discard supernatant into virkofn.
5. Resuspend the pelleted bacteria with 250ul of Buffer P1 (stored in fridge), and transfer the resuspended bacteria into a fresh 2mL eppendorf
6. Add 250ul of Buffer P2 to the 2mL Eppendorf with bacteria and mix gently (invert 4-6 times) Sample should turn blue if LyseBlue added to P1 (indicates cells have lysed). Incubate for 5min at room temperature (do not exceed 5min or plasmid will begin to degrade).
7. Add 350ul of Buffer N3 and mix gently (invert 4-6 times). Sample should be colourless and contain a white precipitant (is cell debris and genomic DNA? - so gently mix as to not dislodge). Centrifuge samples at 14,000rpm for 10min using a table top centrifuge
8. Transfer 750ul of the supernatant to a column placed on a 1.5ml Eppendorf tube (discard white precipitate). Centrifuge at 11,000rpm for 1 min using a table top centrifuge.
9. **If using endA+ strains or other bacteria strains with high nuclease activity or carbohydrate content:** Wash by adding 500ul Buffer PB. Centrifuge for 30–60 s at 13,000rpm and discard the flow-through,
10. Discard the flow-through. Place column onto new eppendorf tube and add 750ul PE buffer (with added ethanol to the stock buffer if not already done so). Incubate at room temperature for 5min. Centrifuge at 13,000rpm for 30secs.
11. Transfer column to fresh eppendorf. Centrifuge at 13,000rpm for 2min (dry out).
12. Transfer column into fresh eppendorf. Add 30ul of sterile dH20 (MiliQ) *(add directly onto column to ensure water pushes DNA through)* and incubate for 5 min at room temp. Centrifuge at 11,000rpm for 1min . **DO NOT DISCARD THIS FLOW-THROUGH. THIS CONTAINS THE EXTRACTED PLASMID.**

Restriction Digest

Introduction

By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
Tips:
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

Table1

	Component	Vol
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 50uL
4	DNA	Conc needed varies depending on purpose

Incubation

1. 1hr 37C, 20min 80C to inactivate

Agarose Gel

Introduction

- If plan to gel extract: use lower % gel, and run at a lower temp for longer
- 100mL = for larger casting tray

Materials

- 1% TAE buffer
- agarose
- SYBR Safe
- loading dye
- DNA ladder

Procedure

Make gel

1. Prepare 1% TAE agarose gel: dissolve 1g of agarose into 100mL of TAE buffer in a conical flask
2. Warm in microwave for 1min at max power
3. Remove flask from microwave with care, swirl gently and cool under running tap
4. Add 5ul of SYBR Safe
5. Prepare a casting tray with suitable comb
6. Pour to cool mixture into the casting tray and wait 15min until it solidifies

Run gel

7. Add 5ul of DNA and 1ul 6x loading dye
8. Load 6ul of DNA ladder (with added dye) alongside and all samples - NEB 1kb ladder (source)
9. Run gel at 100V for 45min ('run to red')

Visualise gel

10. Visualise on a transilluminator (SYBR Safe binds DNA and fluoresces under UV light)

EutS + EutMN - miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-07-25 to 2017-07-26

WEDNESDAY, 26/7/17

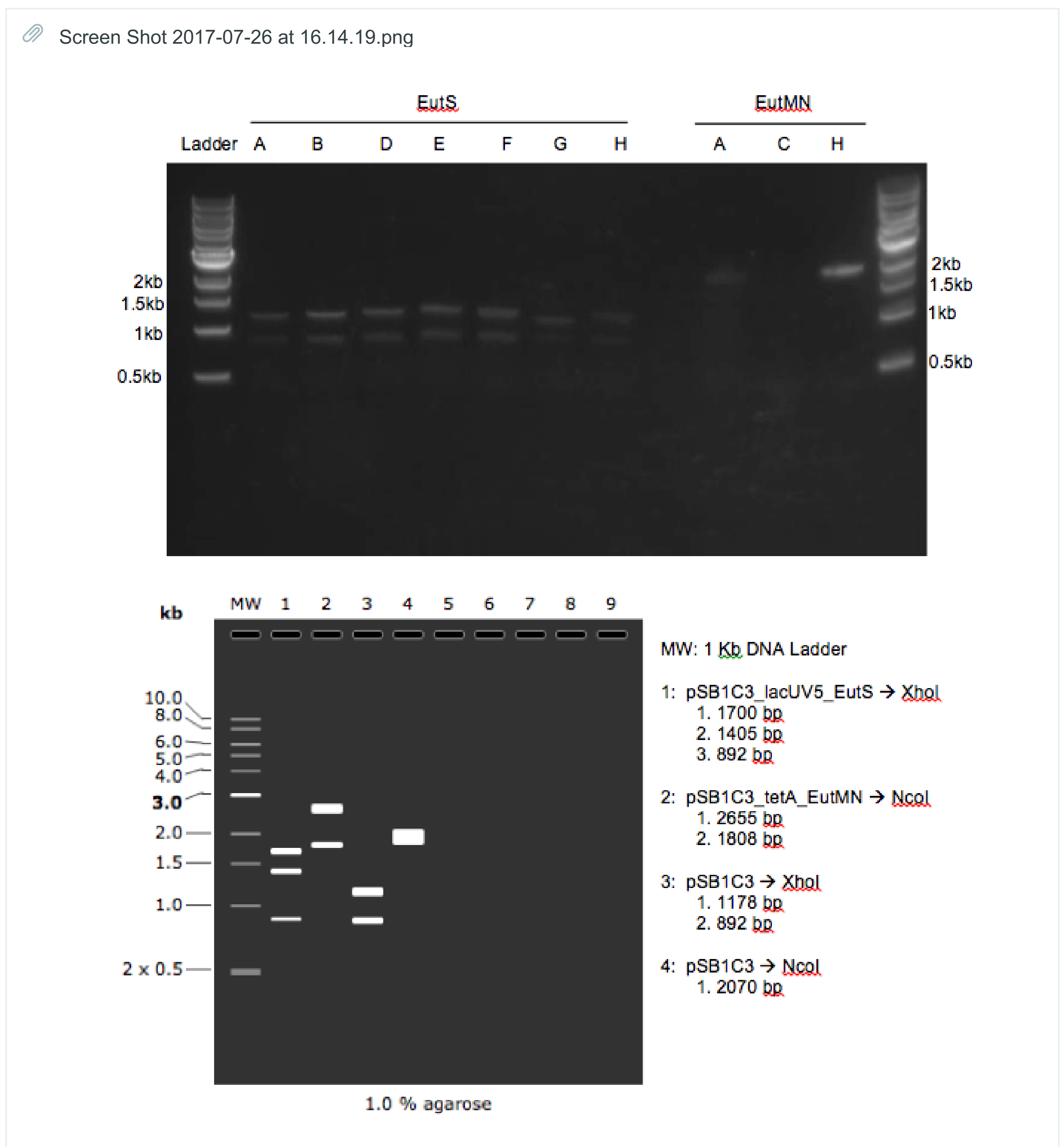
Task: redo RD and gel... use 1000ng of DNA - leave out SC, MNB, MND, MNE, MNF, MNG

Restriction Digest

1. Protocol attached

Gel calcs - running 1000ng

	Construct	Concentration (ng/uL)	Vol for 1000ng	Vol water to use
1	EutS A	161	6.2uL	11.3
2	EutS B	139.5	7.2uL	10.3
3	EutS C	37.8	26.5uL	
4	EutS D	115.1	8.7uL	8.8
5	EutS E	169.2	5.9uL	11.6
6	EutS F	109.3	9.1uL	8.4
7	EutS G	81.5	12.3uL	5.2
8	EutS H	101.9	9.8uL	7.7
9	EutMN A	86.4	11.6uL	5.9
10	EutMN B	54.5	18.3uL	
11	EutMN C	113.6	8.8uL	8.7
12	EutMN D	49.8	20.1uL	
13	EutMN E	24.3	41.2uL	
14	EutMN F	23.3	42.9uL	
15	EutMN G	29.3	34.1uL	
16	EutMN H	135.4	7.4uL	10.1



result: looks as though it didn't work, but the fact we got colonies on both and a clear negative suggests otherwise...

- so try a colony PCR of 20 from each plate (EutS + EutMN)

EutMN - RD, ligation, transformation

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-07-27

THURSDAY, 27/7/17

Restriction Digest:

pSB1C3 (from will) digest with EcoRI + PstI

- a. 6.7uL linearised vector
- b. 0.5uL EcoRI, PstI, DpnI, rSAP

EutMN digest for pSB1C3

- a. 4.7uL eutMN
- b. 0.5uL EcoRI, PstI

1. Protocol attached

Ligation

1. Protocol attached

Incubated at room temperature on the benchtop for about 3 hours

Transformation

1. Protocol attached

results: colonies seen, clean negative control

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

› 10X NEB CutSmart Buffer

› Restriction enzymes

› MilliQ water

› DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 50uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min 80C

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: pSB1C3	1
4	Insert DNA: EutMN	4
5	Nuclease-free water	12
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.

- ✔ 7. Incubate at room temperature for 30 minutes
- ✔ 8. Heat inactivate at 65C for 10 minutes.
- ✔ 9. Chill on ice and transform

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 µL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25µg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400µl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins
13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.
14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before use

15. Spun down into a pellet at 5000rpm for 2 minutes
16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC
17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously
18. Incubate overnight at 37C

EutS + EutMN - Colony PCR

Project: Manchester iGEM 2017 Shared Project
Authors: Ong Jun Yang
Date: 2017-07-27

THURSDAY, 27/7/17

Digests have all come back as backbone only..

Therefore will do a colony PCR to screen a large number of colonies.

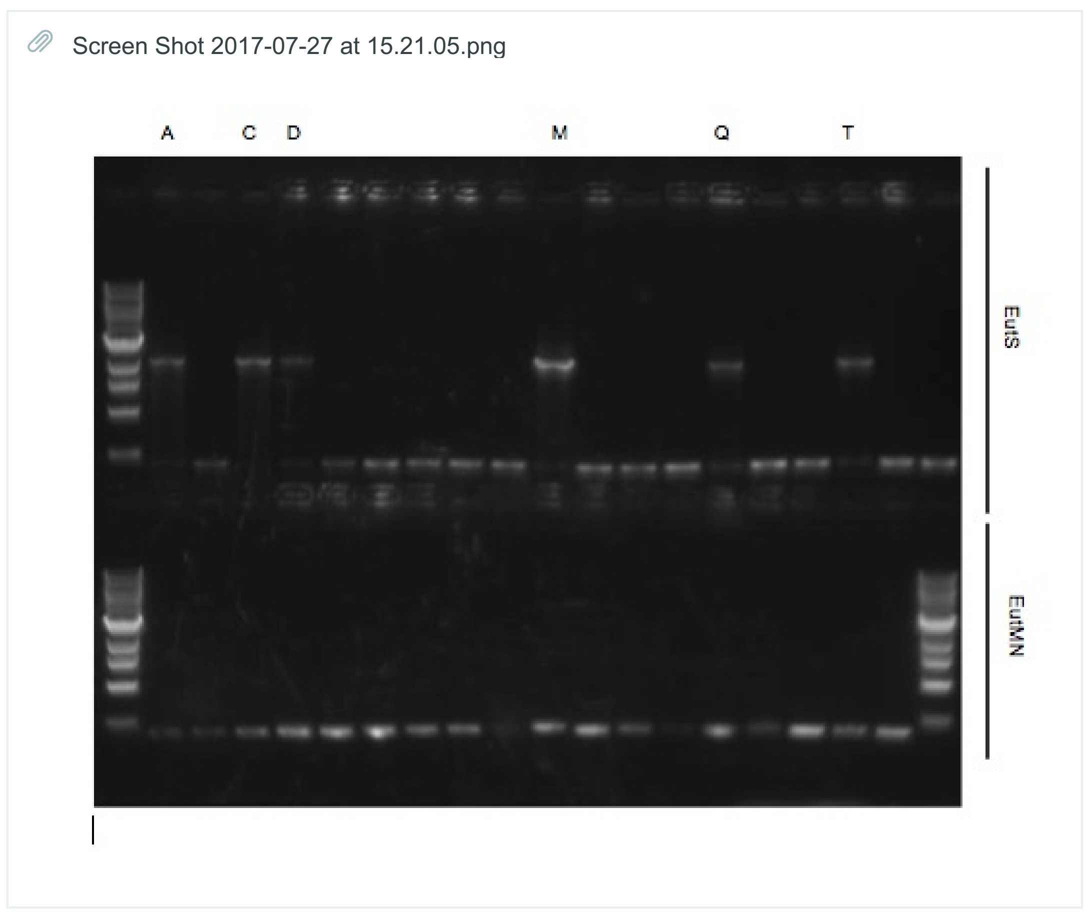
Use VF2 and VR primers

Follow OneTaq PCR protocol (attached to this entry)
Run 1% agarose gel looking for:
EutS = 2.2kb
EutMN = 2.7kb

Results:
EutS-A, EutS-C, EutS-D, EutS-M, EutS-Q, EutS-T all have EutS in pSB1C3

- incubate index plate overnight, then do overnight liquid cultures for those succesful and miniprep
- need to fully redo EutMN

Screen Shot 2017-07-27 at 15.21.05.png



OneTaq Mastermix PCR

Introduction

Copy of protocol from OneTaq NEB

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification. Taq DNA Polymerase is an enzyme widely used in PCR. The following guidelines are provided to ensure successful PCR using New England Biolabs' OneTaq Quick-Load 2X Master Mix with Standard Buffer. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure or low template concentrations may require further optimization.

Materials

- OneTaq Mix
- Primers
- Template
- ddH20

Procedure

Reaction set up:

Reaction

	A	B	C
1	**Reaction vol:**	**10**	
2	**Number Reactions:**	1	**42**
3	10 uM Fwd Primer	0.2	8.4
4	10 uM Rev Primer	0.2	8.4
5	Template	0	0
6	2x OneTaq Mix	5	210
7	ddH20	4.6	193.2
8			

Thermocycling conditions for a routine PCR:

Thermocycling

	A	B	C
1		**Temp**	**Time**
2	**Initial Denaturating**	95 oC	30 s
3	**30 cycles:**	95 oC	15-30s
4		~ 55 oC	15-60 s
5		68 oC	1 min/kb
6	**Final Extension**	68 oC	5 mins
7	**Hold**	4-10 oC	Inf

EutLK - RD, Ligation, Transformation

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-07-28

FRIDAY, 28/7/17

/From G Block

1. add 20uL EB buffer to EutLK = 50ng/uL stock

Restriction Digest

pSB1C3 (PCR product from will) digest with EcoRI + PstI

  a. 6.7uL linearised vector
  b. 0.5uL EcoRI, PstI, dpn1, rSAP

EutLK digest for pSB1C3

  a. 5.3uL eutMN
  b. 0.5uL EcoRI, PstI

1. Protocol attached

Ligation

1. Protocol attached

Transformation

1. Protocol attached

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

› 10X NEB CutSmart Buffer

› Restriction enzymes

› MilliQ water

› DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 50uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 2hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA	1
4	Insert DNA	4
5	Nuclease-free water	12
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.

7. Incubate room temperature for 30 minutes.

8. Heat inactivate at 65C for 10 minutes.

9. Chill on ice and transform

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins

13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.

14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before use

15. Spun down into a pellet at 5000rpm for 2 minutes

16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC

17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously

18. Incubate overnight at 37C

EutMN + EutLK - Colony PCR

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-07-31 to 2017-08-01

MONDAY, 31/7/17

To screen many colonies:

Follow OneTaq PCR protocol (attached to this entry)

- use VR and VF2 primers
- make index plate at same time - if positive result, can sample colony straight from here and do overnight cultures *(dont need to wait for plate to grow)*

Run 1% agarose gel (*protocol attached),* looking for:
EutMN = 2.7kb

result: EutMN-13 is the only one that worked - so overnight and miniprep

UVP06620July312017.jpg

OneTaq PCR

Introduction

Copy of protocol from OneTaq NEB

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification. Taq DNA Polymerase is an enzyme widely used in PCR. The following guidelines are provided to ensure successful PCR using New England Biolabs' OneTaq Quick-Load 2X Master Mix with Standard Buffer. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure or low template concentrations may require further optimization.

Materials

- OneTaq Mix
- Primers
- Template
- ddH20 (MilliQ)

Procedure

Reaction set up:

Reaction

	A	B	C
1	**Reaction vol:**	**10**	
2	**Number Reactions:**	1	**65**
3	10 uM Fwd Primer	0.2	13
4	10 uM Rev Primer	0.2	13
5	Template	0	0
6	2x OneTaq Mix	5	325
7	ddH20	4.6	299
8			

Thermocycling conditions for a routine PCR:

Thermocycling

	A	B	C
1		**Temp**	**Time**
2	**Initial Denaturating**	95 oC	30 s
3	**30 cycles:**	95 oC	15-30s
4		~ 55 oC	15-60 s
5		68 oC	1 min/kb
6	**Final Extension**	68 oC	5 mins
7	**Hold**	4-10 oC	Inf

Agarose Gel

Introduction

- If plan to gel extract: use lower % gel, and run at a lower temp for longer
- 100mL = for larger casting tray

Materials

- 1% TAE buffer
- agarose
- SYBR Safe
- loading dye
- DNA ladder

Procedure

Make gel

1. Prepare 1% TAE agarose gel: dissolve 1g of agarose into 100mL of TAE buffer in a conical flask
2. Warm in microwave for 1min at max power
3. Remove flask from microwave with care, swirl gently and cool under running tap
4. Add 5ul of SYBR Safe
5. Prepare a casting tray with suitable comb
6. Pour to cool mixture into the casting tray and wait 15min until it solidifies

Run gel

7. Add 5ul of DNA and 1ul 6x loading dye
8. Load 6ul of DNA ladder (with added dye) alongside and all samples - NEB 1kb ladder (source)
9. Run gel at 100V for 45min ('run to red')

Visualise gel

10. Visualise on a transilluminator (SYBR Safe binds DNA and fluoresces under UV light)

EutS - Miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-07-31 to 2017-08-01

MONDAY, 31/7/17

Inoculate EutS A, C, D, M, Q, T - overnight cultures

Agarose Gel

Introduction

- If plan to gel extract: use lower % gel, and run at a lower temp for longer
- 100mL = for larger casting tray

Materials

- 1% TAE buffer
- agarose
- SYBR Safe
- loading dye
- DNA ladder

Procedure

Make gel

1. Prepare 1% TAE agarose gel: dissolve 1g of agarose into 100mL of TAE buffer in a conical flask
2. Warm in microwave for 1min at max power
3. Remove flask from microwave with care, swirl gently and cool under running tap
4. Add 5ul of SYBR Safe
5. Prepare a casting tray with suitable comb
6. Pour to cool mixture into the casting tray and wait 15min until it solidifies

Run gel

7. Add 5ul of DNA and 1ul 6x loading dye
8. Load 6ul of DNA ladder (with added dye) alongside and all samples - NEB 1kb ladder (source)
9. Run gel at 100V for 45min ('run to red')

Visualise gel

10. Visualise on a transilluminator (SYBR Safe binds DNA and fluoresces under UV light)

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 20uL
4	DNA	1000ng

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

QIAprep® Spin Miniprep Kit

Introduction

The QIAprep Spin Miniprep Kit (cat. nos. 27104 and 27106) can be stored at room temperature (15–25°C) for up to 12 months.

Materials

- Qiagen Kit

Procedure

Notes before starting

1. Optional: Add LyseBlue reagent to Buffer P1 at a ratio of 1 to 1000.
2. Add the provided RNase A solution to Buffer P1, mix and store at 2–8°C.
3. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

Protocol

4. Spin down the overnight cultures at 10000rpm for 10min. Discard supernatant into virkofn.
5. Resuspend the pelleted bacteria with 250ul of Buffer P1 (stored in fridge), and transfer the resuspended bacteria into a fresh 2mL eppendorf
6. Add 250ul of Buffer P2 to the 2mL Eppendorf with bacteria and mix gently (invert 4-6 times) Sample should turn blue if LyseBlue added to P1 (indicates cells have lysed). Incubate for 5min at room temperature (do not exceed 5min or plasmid will begin to degrade).
7. Add 350ul of Buffer N3 and mix gently (invert 4-6 times). Sample should be colourless and contain a white precipitant (is cell debris and genomic DNA? - so gently mix as to not dislodge). Centrifuge samples at 14,000rpm for 10min using a table top centrifuge
8. Transfer 750ul of the supernatant to a column placed on a 1.5ml Eppendorf tube (discard white precipitate). Centrifuge at 11,000rpm for 1 min using a table top centrifuge.
9. **If using endA+ strains or other bacteria strains with high nuclease activity or carbohydrate content:** Wash by adding 500ul Buffer PB. Centrifuge for 30–60 s at 13,000rpm and discard the flow-through,
10. Discard the flow-through. Place column onto new eppendorf tube and add 750ul PE buffer (with added ethanol to the stock buffer if not already done so). Incubate at room temperature for 5min. Centrifuge at 13,000rpm for 30secs.
11. Transfer column to fresh eppendorf. Centrifuge at 13,000rpm for 2min (dry out).
12. Transfer column into fresh eppendorf. Add 30ul of sterile dH20 (MiliQ) or EB Buffer *(add directly onto column to ensure water pushes DNA through)* and incubate for 5 min at room temp. Centrifuge at 11,000rpm for 1min . **DO NOT DISCARD THIS FLOW-THROUGH. THIS CONTAINS THE EXTRACTED PLASMID.**

Nanodrop: *calculate DNA concentration in sample*

13. Load 1uL of MiliQ water (or EB if used at step 8) to the Nanodrop and blank. Clean and load another 1ul of MiliQ water/EB, measure and proceed only if clean (absorbance is zero at all measured wavelengths).

14. Load 1uL of sample above (miniprep plasmid) and measure DNA concentration.

15. Check A260/280 ~ 1.8 (DNA:protein ratio) and A260/230 ~2.0 (DNA:contaminant ratio)

EutLK - Restriction Digests

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-01 to 2017-08-02

TUESDAY, 1/8/17

Restriction Digests (1)

pSB1C3 (PCR product from will) digest 1

pSB1C3 PCR product RD 1

	A	B
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each (ecorI + pstI)
3	MilliQ water	up to 50uL
4	DNA (pSB1C3)	6.7uL

EutLK digest 1 for pSB1C3

EutLK RD 1

	A	B
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each (ecorI + pstI)
3	MilliQ water	up to 50uL
4	DNA (eutLK)	5.3uL

1. Protocol attached
2. 37C incubation overnight

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

› 10X NEB CutSmart Buffer

› Restriction enzymes

› MilliQ water

› DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 20uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

Note: The table shows a ligation using a molar ratio of 1:3 vector to insert for vector of 4kb and insert of 1kb. *Use NEB calculator to calculate molar ratios.*

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: 50 ng (0.020 pmol)	
4	Insert DNA: 37.5 ng (0.060 pmol)	
5	Nuclease-free water	17
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.

7. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 30 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.

8. Heat inactivate at 65C for 10 minutes.

9. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

   Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins

13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.

14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before use

15. Spun down into a pellet at 5000rpm for 2 minutes

16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC

17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously

18. Incubate overnight at 37C

EutLK - miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-01 to 2017-08-02

TUESDAY, 1/8/17

Inoculate 1, 2, 3, 4, 5, 6 - overnight culture

these are the ones which did not show a band on the gel - worth a shot

QIAprep® Spin Miniprep Kit

Introduction

The QIAprep Spin Miniprep Kit (cat. nos. 27104 and 27106) can be stored at room temperature (15–25°C) for up to 12 months.

Materials

- Qiagen Kit

Procedure

Notes before starting

1. Optional: Add LyseBlue reagent to Buffer P1 at a ratio of 1 to 1000.
2. Add the provided RNase A solution to Buffer P1, mix and store at 2–8°C.
3. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

Protocol

4. Spin down the overnight cultures at 10000rpm for 10min. Discard supernatant into virkofn.
5. Resuspend the pelleted bacteria with 250ul of Buffer P1 (stored in fridge), and transfer the resuspended bacteria into a fresh 2mL eppendorf
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8. Transfer 750ul of the supernatant to a column placed on a 1.5ml Eppendorf tube (discard white precipitate). Centrifuge at 11,000rpm for 1 min using a table top centrifuge.
9. **If using endA+ strains or other bacteria strains with high nuclease activity or carbohydrate content:** Wash by adding 500ul Buffer PB. Centrifuge for 30–60 s at 13,000rpm and discard the flow-through,
10. Discard the flow-through. Place column onto new eppendorf tube and add 750ul PE buffer (with added ethanol to the stock buffer if not already done so). Incubate at room temperature for 5min. Centrifuge at 13,000rpm for 30secs.
11. Transfer column to fresh eppendorf. Centrifuge at 13,000rpm for 2min (dry out).
12. Transfer column into fresh eppendorf. Add 30ul of sterile dH20 (MiliQ) or EB Buffer *(add directly onto column to ensure water pushes DNA through)* and incubate for 5 min at room temp. Centrifuge at 11,000rpm for 1min . **DO NOT DISCARD THIS FLOW-THROUGH. THIS CONTAINS THE EXTRACTED PLASMID.**

Nanodrop: *calculate DNA concentration in sample*

- ✔ 13. Load 1uL of MiliQ water (or EB if used at step 8) to the Nanodrop and blank. Clean and load another 1ul of MiliQ water/EB, measure and proceed only if clean (absorbance is zero at all measured wavelengths).
- ✔ 14. Load 1uL of sample above (miniprep plasmid) and measure DNA concentration.
- ✔ 15. Check A260/280 ~ 1.8 (DNA:protein ratio) and A260/230 ~2.0 (DNA:contaminant ratio)

Single Temperature Restriction Digest

Introduction

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- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 50uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

Agarose Gel

Introduction

- If plan to gel extract: use lower % gel, and run at a lower temp for longer
- 100mL = for larger casting tray

Materials

- 1% TAE buffer
- agarose
- SYBR Safe
- loading dye
- DNA ladder

Procedure

Make gel

1. Prepare 1% TAE agarose gel: dissolve 1g of agarose into 100mL of TAE buffer in a conical flask
2. Warm in microwave for 1min at max power
3. Remove flask from microwave with care, swirl gently and cool under running tap
4. Add 5ul of SYBR Safe
5. Prepare a casting tray with suitable comb
6. Pour to cool mixture into the casting tray and wait 15min until it solidifies

Run gel

7. Add 5ul of DNA and 1ul 6x loading dye
8. Load 6ul of DNA ladder (with added dye) alongside and all samples - NEB 1kb ladder (source)
9. Run gel at 100V for 45min ('run to red')

Visualise gel

10. Visualise on a transilluminator (SYBR Safe binds DNA and fluoresces under UV light)

EutMN + EutLK - Colony PCR

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-07-31 to 2017-08-01

TUESDAY, 1/8/17

Run 1% agarose gel (*protocol attached),* looking for:
EutLK = 3kb

result:
possible that the vector self ligated = 500bp or with RFP = 1500bp
pick those which dont have bands (EutLK - 1,2,3,4,5,6), and miniprep - may be that the pcr did not work, it is a large fragment

UVP06623Aug12017.jpg

1. Inoculate EutLK 1, 2, 3, 4, 5, 6 - to miniprep tomorrow
2. Also retry the restriction digest of eutLK and pSB1C3 overnight

EutMN - Miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-01

TUESDAY, 1/8/17

Miniprep
nanodrop results:

IMG_0046 copy.JPG

10	pSB1C3_EutMN13.A	Takano_Lab_2	01/08/2017 13:03:53	146.4	ng/µl	2.928	1.548	1.89	1.64	DNA
11	pSB1C3_EutMN13.B	Takano_Lab_2	01/08/2017 13:04:30	369.8	ng/µl	7.395	6.355	1.16	1.16	DNA
12	pSB1C3_EutMN13.Bredo	Takano_Lab_2	01/08/2017 13:05:06	169.7	ng/µl	3.394	1.761	1.93	1.95	DNA
13	pSB1C3_EutMN13.C	Takano_Lab_2	01/08/2017 13:06:50	131.1	ng/µl	2.622	1.357	1.93	1.80	DNA

Restriction Digest
1000ng DNA
with NcoI

1% agarose gel
expected bands at: 2655 + 1808
results:

UVP06625Aug12017.jpg

IT WORKED!

- now send pSB1C3_eutMN for sequencing

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 50uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

QIAprep® Spin Miniprep Kit

Introduction

The QIAprep Spin Miniprep Kit (cat. nos. 27104 and 27106) can be stored at room temperature (15–25°C) for up to 12 months.

Materials

- Qiagen Kit

Procedure

Notes before starting

1. Optional: Add LyseBlue reagent to Buffer P1 at a ratio of 1 to 1000.
2. Add the provided RNase A solution to Buffer P1, mix and store at 2–8°C.
3. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

Protocol

4. Spin down the overnight cultures at 10000rpm for 10min. Discard supernatant into virkofn.
5. Resuspend the pelleted bacteria with 250ul of Buffer P1 (stored in fridge), and transfer the resuspended bacteria into a fresh 2mL eppendorf
6. Add 250ul of Buffer P2 to the 2mL Eppendorf with bacteria and mix gently (invert 4-6 times) Sample should turn blue if LyseBlue added to P1 (indicates cells have lysed). Incubate for 5min at room temperature (do not exceed 5min or plasmid will begin to degrade).
7. Add 350ul of Buffer N3 and mix gently (invert 4-6 times). Sample should be colourless and contain a white precipitant (is cell debris and genomic DNA? - so gently mix as to not dislodge). Centrifuge samples at 14,000rpm for 10min using a table top centrifuge
8. Transfer 750ul of the supernatant to a column placed on a 1.5ml Eppendorf tube (discard white precipitate). Centrifuge at 11,000rpm for 1 min using a table top centrifuge.
9. **If using endA+ strains or other bacteria strains with high nuclease activity or carbohydrate content:** Wash by adding 500ul Buffer PB. Centrifuge for 30–60 s at 13,000rpm and discard the flow-through,
10. Discard the flow-through. Place column onto new eppendorf tube and add 750ul PE buffer (with added ethanol to the stock buffer if not already done so). Incubate at room temperature for 5min. Centrifuge at 13,000rpm for 30secs.
11. Transfer column to fresh eppendorf. Centrifuge at 13,000rpm for 2min (dry out).
12. Transfer column into fresh eppendorf. Add 30ul of sterile dH20 (MiliQ) or EB Buffer *(add directly onto column to ensure water pushes DNA through)* and incubate for 5 min at room temp. Centrifuge at 11,000rpm for 1min . **DO NOT DISCARD THIS FLOW-THROUGH. THIS CONTAINS THE EXTRACTED PLASMID.**

Nanodrop: *calculate DNA concentration in sample*

- ✔ 13. Load 1uL of MiliQ water (or EB if used at step 8) to the Nanodrop and blank. Clean and load another 1ul of MiliQ water/EB, measure and proceed only if clean (absorbance is zero at all measured wavelengths).

- ✔ 14. Load 1uL of sample above (miniprep plasmid) and measure DNA concentration.

- ✔ 15. Check A260/280 ~ 1.8 (DNA:protein ratio) and A260/230 ~2.0 (DNA:contaminant ratio)

Agarose Gel

Introduction

- If plan to gel extract: use lower % gel, and run at a lower temp for longer
- 100mL = for larger casting tray

Materials

- 1% TAE buffer
- agarose
- SYBR Safe
- loading dye
- DNA ladder

Procedure

Make gel

1. Prepare 1% TAE agarose gel: dissolve 1g of agarose into 100mL of TAE buffer in a conical flask
2. Warm in microwave for 1min at max power
3. Remove flask from microwave with care, swirl gently and cool under running tap
4. Add 5ul of SYBR Safe
5. Prepare a casting tray with suitable comb
6. Pour to cool mixture into the casting tray and wait 15min until it solidifies

Run gel

7. Add 5ul of DNA and 1ul 6x loading dye
8. Load 6ul of DNA ladder (with added dye) alongside and all samples - NEB 1kb ladder (source)
9. Run gel at 100V for 45min ('run to red')

Visualise gel

10. Visualise on a transilluminator (SYBR Safe binds DNA and fluoresces under UV light)

EutS - Miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

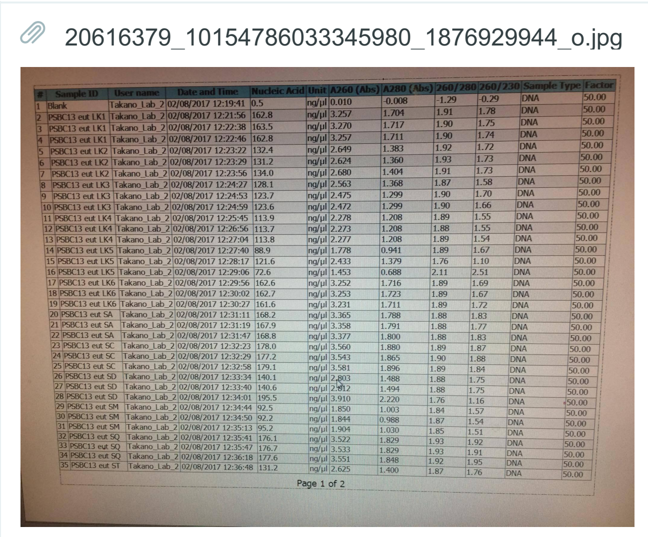
Dates: 2017-07-31 to 2017-08-01

TUESDAY, 1/8/17

Miniprep

nanodrop results:

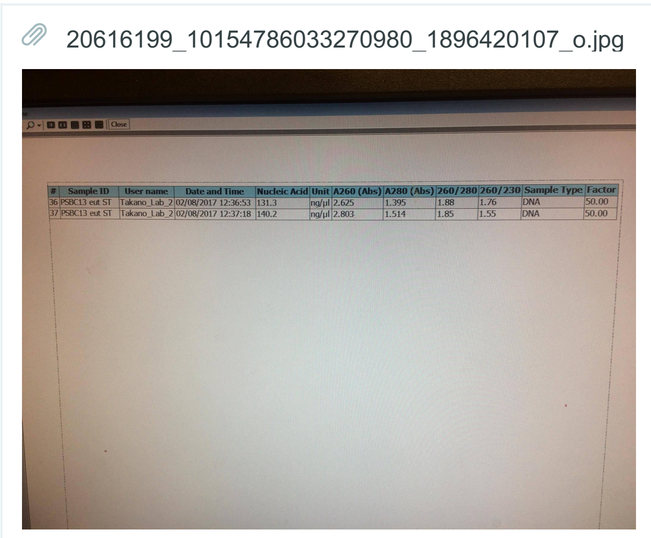
20616379_10154786033345980_1876929944_o.jpg



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	Blank	Takano_Lab_2	02/08/2017 12:19:41	0.5	ng/µl	0.010	-0.008	-1.29	-0.29	DNA	50.00
2	PSBC13 eut LK1	Takano_Lab_2	02/08/2017 12:21:56	162.8	ng/µl	3.257	1.704	1.91	1.78	DNA	50.00
3	PSBC13 eut LK1	Takano_Lab_2	02/08/2017 12:22:38	163.5	ng/µl	3.270	1.717	1.90	1.75	DNA	50.00
4	PSBC13 eut LK1	Takano_Lab_2	02/08/2017 12:22:46	162.8	ng/µl	3.257	1.711	1.90	1.74	DNA	50.00
5	PSBC13 eut LK2	Takano_Lab_2	02/08/2017 12:23:22	132.4	ng/µl	2.649	1.383	1.92	1.72	DNA	50.00
6	PSBC13 eut LK2	Takano_Lab_2	02/08/2017 12:23:29	131.2	ng/µl	2.624	1.360	1.93	1.73	DNA	50.00
7	PSBC13 eut LK2	Takano_Lab_2	02/08/2017 12:23:56	134.0	ng/µl	2.680	1.404	1.91	1.73	DNA	50.00
8	PSBC13 eut LK3	Takano_Lab_2	02/08/2017 12:24:27	128.1	ng/µl	2.563	1.368	1.87	1.58	DNA	50.00
9	PSBC13 eut LK3	Takano_Lab_2	02/08/2017 12:24:53	123.7	ng/µl	2.475	1.299	1.90	1.70	DNA	50.00
10	PSBC13 eut LK3	Takano_Lab_2	02/08/2017 12:24:59	123.6	ng/µl	2.472	1.299	1.90	1.66	DNA	50.00
11	PSBC13 eut LK4	Takano_Lab_2	02/08/2017 12:25:45	113.9	ng/µl	2.278	1.208	1.89	1.55	DNA	50.00
12	PSBC13 eut LK4	Takano_Lab_2	02/08/2017 12:26:56	113.7	ng/µl	2.273	1.208	1.88	1.55	DNA	50.00
13	PSBC13 eut LK4	Takano_Lab_2	02/08/2017 12:27:04	113.8	ng/µl	2.277	1.208	1.89	1.54	DNA	50.00
14	PSBC13 eut LK5	Takano_Lab_2	02/08/2017 12:27:40	88.9	ng/µl	1.778	0.941	1.89	1.67	DNA	50.00
15	PSBC13 eut LK5	Takano_Lab_2	02/08/2017 12:28:17	121.6	ng/µl	2.433	1.379	1.76	1.10	DNA	50.00
16	PSBC13 eut LK5	Takano_Lab_2	02/08/2017 12:29:06	72.6	ng/µl	1.453	0.688	2.11	2.51	DNA	50.00
17	PSBC13 eut LK6	Takano_Lab_2	02/08/2017 12:29:56	162.6	ng/µl	3.252	1.716	1.89	1.69	DNA	50.00
18	PSBC13 eut LK6	Takano_Lab_2	02/08/2017 12:30:02	162.7	ng/µl	3.253	1.723	1.89	1.67	DNA	50.00
19	PSBC13 eut LK6	Takano_Lab_2	02/08/2017 12:30:27	161.6	ng/µl	3.231	1.711	1.89	1.72	DNA	50.00
20	PSBC13 eut SA	Takano_Lab_2	02/08/2017 12:31:11	168.2	ng/µl	3.365	1.788	1.88	1.83	DNA	50.00
21	PSBC13 eut SA	Takano_Lab_2	02/08/2017 12:31:19	167.9	ng/µl	3.358	1.791	1.88	1.77	DNA	50.00
22	PSBC13 eut SA	Takano_Lab_2	02/08/2017 12:31:47	168.8	ng/µl	3.377	1.800	1.88	1.83	DNA	50.00
23	PSBC13 eut SC	Takano_Lab_2	02/08/2017 12:32:23	178.0	ng/µl	3.560	1.880	1.89	1.87	DNA	50.00
24	PSBC13 eut SC	Takano_Lab_2	02/08/2017 12:32:29	177.2	ng/µl	3.543	1.865	1.90	1.88	DNA	50.00
25	PSBC13 eut SC	Takano_Lab_2	02/08/2017 12:32:58	179.1	ng/µl	3.581	1.896	1.89	1.84	DNA	50.00
26	PSBC13 eut SD	Takano_Lab_2	02/08/2017 12:33:34	140.1	ng/µl	2.803	1.488	1.88	1.75	DNA	50.00
27	PSBC13 eut SD	Takano_Lab_2	02/08/2017 12:33:40	140.6	ng/µl	2.812	1.494	1.88	1.75	DNA	50.00
28	PSBC13 eut SD	Takano_Lab_2	02/08/2017 12:34:01	195.5	ng/µl	3.910	2.220	1.76	1.16	DNA	50.00
29	PSBC13 eut SM	Takano_Lab_2	02/08/2017 12:34:44	92.5	ng/µl	1.850	1.003	1.84	1.57	DNA	50.00
30	PSBC13 eut SM	Takano_Lab_2	02/08/2017 12:34:50	92.2	ng/µl	1.844	0.988	1.87	1.54	DNA	50.00
31	PSBC13 eut SM	Takano_Lab_2	02/08/2017 12:35:13	95.2	ng/µl	1.904	1.030	1.85	1.51	DNA	50.00
32	PSBC13 eut SQ	Takano_Lab_2	02/08/2017 12:35:41	176.1	ng/µl	3.522	1.829	1.93	1.92	DNA	50.00
33	PSBC13 eut SQ	Takano_Lab_2	02/08/2017 12:35:47	176.7	ng/µl	3.533	1.829	1.93	1.91	DNA	50.00
34	PSBC13 eut SQ	Takano_Lab_2	02/08/2017 12:36:18	177.6	ng/µl	3.551	1.848	1.92	1.95	DNA	50.00
35	PSBC13 eut ST	Takano_Lab_2	02/08/2017 12:36:48	131.2	ng/µl	2.625	1.400	1.87	1.76	DNA	50.00

Page 1 of 2

20616199_10154786033270980_1896420107_o.jpg



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
36	PSBC13 eut ST	Takano_Lab_2	02/08/2017 12:36:53	131.3	ng/µl	2.625	1.395	1.88	1.76	DNA	50.00
37	PSBC13 eut ST	Takano_Lab_2	02/08/2017 12:37:18	140.2	ng/µl	2.803	1.514	1.85	1.55	DNA	50.00

Restriction Digest

Calcs to run 1000ng				
	Construct	Conc (ng/uL)	Vol for 1000ng (uL)	Vol of water
1	EutS - A	168	6	11
2	EutS - C	178	5.6	11.4
3	EutS - D	150	6.7	10.3
4	EutS - M	93	10.8	6.2
5	EutS - Q	176	5.7	11.3
6	EutS - T	134	7.5	9.5

1. 1000ng DNA (table above), water (table above), 1uL XhoI, 2uL CutSmart Buffer

2. 37C for 1.5hr, 65C for 20mins

results:
expected: 1700 + 1405 + 892

UVP06636Aug22017 2.jpg

all confirmed - can send for sequencing

EutLK - Restriction Digests

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-01 to 2017-08-02

WEDNESDAY, 2/8/17

Restriction Digests (2)

- not using HF enzymes (cannot be heat inactivated)

EutLK digest 2 for pSB1C3

- use 2.3ul DNA (half of what should be used for 20ul) - dont want to waste G block, so do 10uL reaction

EutLK RD 2

	A	B
1	10X CutSmart Buffer	2.5uL
2	Restriction enzymes	0.5uL each (ecorI + pstI)
3	MilliQ water	up to 10uL
4	DNA (eutMN)	2.3uL

- add 5ul extra water (too much cutsmart - added enough for 25ul reaction, wanted 10ul)

pSB1C3 PCR product digest 2

- use 3.3uL (half 6.7) in 10ul reaction

pSB1C3 PCR product RD 2

	A	B
1	10X CutSmart Buffer	2.5uL
2	Restriction enzymes	0.5uL each (PstI EcoRI rSAP Dpn1)
3	MilliQ water	up to 10uL
4	DNA (pSB1C3)	3.3uL

- add 5ul extra water (too much cutsmart - added enough for 25ul reaction, wanted 10ul)

EutLK - miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

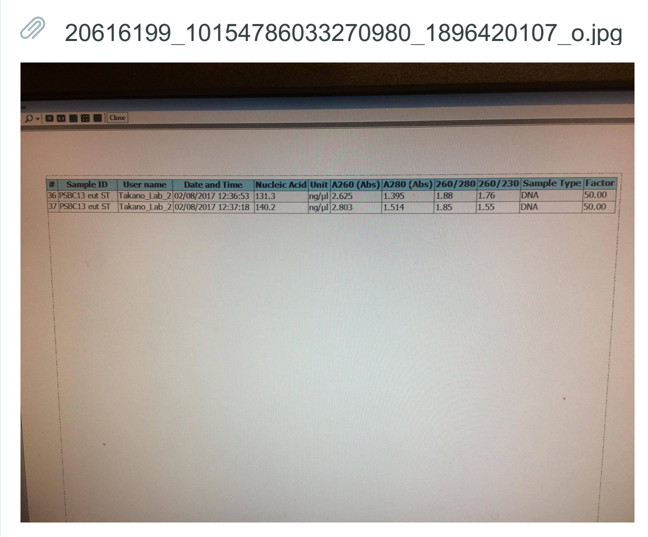
Dates: 2017-08-01 to 2017-08-02

WEDNESDAY, 2/8/17

Miniprep

nanodrop results:

20616199_10154786033270980_1896420107_o.jpg



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
36	PSBC13 eut ST	Takano_Lab_2	02/08/2017 12:36:53	131.3	ng/µl	2.625	1.395	1.88	1.76	DNA	50.00
37	PSBC13 eut ST	Takano_Lab_2	02/08/2017 12:37:18	140.2	ng/µl	2.803	1.514	1.85	1.55	DNA	50.00

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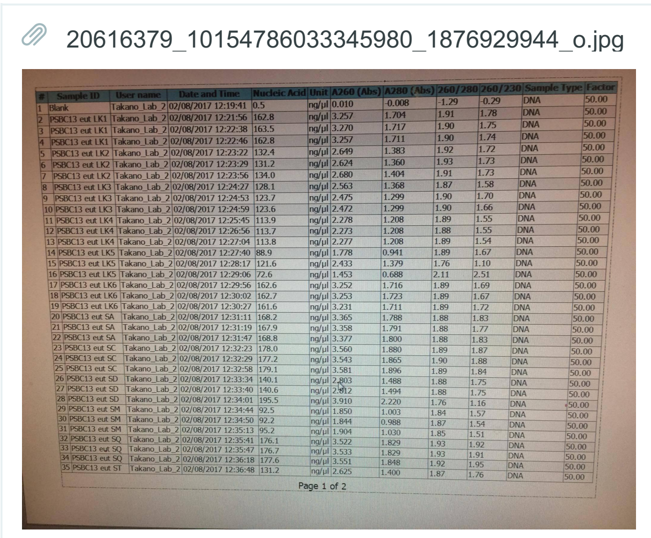

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3	PSBC13 eut LK1	Takano_Lab_2	02/08/2017 12:22:38	163.5	ng/µl	3.270	1.717	1.90	1.75	DNA	50.00
4	PSBC13 eut LK1	Takano_Lab_2	02/08/2017 12:22:46	162.8	ng/µl	3.257	1.711	1.90	1.74	DNA	50.00
5	PSBC13 eut LK2	Takano_Lab_2	02/08/2017 12:23:22	132.4	ng/µl	2.649	1.383	1.92	1.72	DNA	50.00
6	PSBC13 eut LK2	Takano_Lab_2	02/08/2017 12:23:29	131.2	ng/µl	2.624	1.360	1.93	1.73	DNA	50.00
7	PSBC13 eut LK2	Takano_Lab_2	02/08/2017 12:23:56	134.0	ng/µl	2.680	1.404	1.91	1.73	DNA	50.00
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11	PSBC13 eut LK4	Takano_Lab_2	02/08/2017 12:25:45	113.9	ng/µl	2.278	1.208	1.89	1.55	DNA	50.00
12	PSBC13 eut LK4	Takano_Lab_2	02/08/2017 12:26:56	113.7	ng/µl	2.273	1.208	1.88	1.55	DNA	50.00
13	PSBC13 eut LK4	Takano_Lab_2	02/08/2017 12:27:04	113.8	ng/µl	2.277	1.208	1.89	1.54	DNA	50.00
14	PSBC13 eut LK5	Takano_Lab_2	02/08/2017 12:27:40	88.9	ng/µl	1.778	0.941	1.89	1.67	DNA	50.00
15	PSBC13 eut LK5	Takano_Lab_2	02/08/2017 12:28:17	121.6	ng/µl	2.433	1.379	1.76	1.10	DNA	50.00
16	PSBC13 eut LK5	Takano_Lab_2	02/08/2017 12:29:06	72.6	ng/µl	1.453	0.688	2.11	2.51	DNA	50.00
17	PSBC13 eut LK6	Takano_Lab_2	02/08/2017 12:29:56	162.6	ng/µl	3.252	1.716	1.89	1.69	DNA	50.00
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19	PSBC13 eut LK6	Takano_Lab_2	02/08/2017 12:30:27	161.6	ng/µl	3.231	1.711	1.89	1.72	DNA	50.00
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22	PSBC13 eut SA	Takano_Lab_2	02/08/2017 12:31:47	168.8	ng/µl	3.377	1.800	1.88	1.83	DNA	50.00
23	PSBC13 eut SC	Takano_Lab_2	02/08/2017 12:32:23	178.0	ng/µl	3.560	1.880	1.89	1.87	DNA	50.00
24	PSBC13 eut SC	Takano_Lab_2	02/08/2017 12:32:29	177.2	ng/µl	3.543	1.865	1.90	1.88	DNA	50.00
25	PSBC13 eut SC	Takano_Lab_2	02/08/2017 12:32:58	179.1	ng/µl	3.581	1.896	1.89	1.84	DNA	50.00
26	PSBC13 eut SD	Takano_Lab_2	02/08/2017 12:33:34	140.1	ng/µl	2.803	1.488	1.88	1.75	DNA	50.00
27	PSBC13 eut SD	Takano_Lab_2	02/08/2017 12:33:40	140.6	ng/µl	2.812	1.494	1.88	1.75	DNA	50.00
28	PSBC13 eut SD	Takano_Lab_2	02/08/2017 12:34:01	195.5	ng/µl	3.910	2.220	1.76	1.16	DNA	50.00
29	PSBC13 eut SM	Takano_Lab_2	02/08/2017 12:34:44	92.5	ng/µl	1.850	1.003	1.84	1.57	DNA	50.00
30	PSBC13 eut SM	Takano_Lab_2	02/08/2017 12:34:50	92.2	ng/µl	1.844	0.988	1.87	1.54	DNA	50.00
31	PSBC13 eut SM	Takano_Lab_2	02/08/2017 12:35:13	95.2	ng/µl	1.904	1.030	1.85	1.51	DNA	50.00
32	PSBC13 eut SQ	Takano_Lab_2	02/08/2017 12:35:41	176.1	ng/µl	3.522	1.829	1.93	1.92	DNA	50.00
33	PSBC13 eut SQ	Takano_Lab_2	02/08/2017 12:35:47	176.7	ng/µl	3.533	1.829	1.93	1.91	DNA	50.00
34	PSBC13 eut SQ	Takano_Lab_2	02/08/2017 12:36:18	177.6	ng/µl	3.551	1.848	1.92	1.95	DNA	50.00
35	PSBC13 eut ST	Takano_Lab_2	02/08/2017 12:36:48	131.2	ng/µl	2.625	1.400	1.87	1.76	DNA	50.00

Page 1 of 2

Restriction Digest

Calcs to run 1000ng				
	Construct	Conc (ng/uL)	Vol for 1000ng	Vol of water
1	EutLK - 1	163	6.1	10.9
2	EutLK - 2	132	7.6	9.4
3	EutLK - 3	124	8.1	8.9
4	EutLK - 4	113	8.8	8.2
5	EutLK - 5	94	10.6	6.4
6	EutLK - 6	162	6.2	10.8

1. 1000ng DNA (table above), water (table above), 1uL BamHI, 2uL CutSmart Buffer
2. 37C for 1.5hr, Is not for a ligation, just a gel - so no need to inactivate

1% agarose gel

expected bands at 3830 + 925

if just backbone, expect band at 2070 (pSB1C3 w/o stuffer gene - check with will this is the case)

results: none worked:

Screen Shot 2017-08-02 at 18.02.58.png

pSB1C3 - RD, gel

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-02 to 2017-08-03

WEDNESDAY, 2/8/17

Restriction Digest

digest pSB1C3 plasmid

- nanodrop = 160ng/ul
- so for 1000ng use 6.25uL
- do 20ul reaction

Table1

	A	B
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	0.5uL each (pstI EcoRI rSAP)
3	MilliQ water	7uL
4	DNA (pSB1C3)	1000ng = 6.25ul

run gel - looks weird. do not trust the pSB1C3 in iGEM stock
result:

UVP06628Aug22017.jpg

digest pSB1C3 from will

- nanodrop = 145ng/ul
- so for 1000ng use 6.9uL
- 9ul water .

run 1% gel of digested pSB1C3 plasmid (1000ng - load 20uL) - expected bands at 2029bp + 1110bp

UVP06636Aug22017.jpg

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	0.5uL each
3	MilliQ water	up to 20uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min at 80 to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

Gel Extraction (Qiagen)

Introduction

Materials

› Qiagen Kit

Procedure

Gel extraction

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube = **XXXmg**. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.
4. Add 1 gel volume isopropanol to the sample and mix by inverting.
5. Transfer 750µl of supernatant to a column placed on a 1.5mp eppendorf. Centrifuge at 11,000rpm for 1 min.
6. Discard flow through. Place column in a new Eppendorf tube. Add 500µl of PB buffer and centrifuge column at 13,000 rpm for 30 seconds.
7. Discard flow through. Place column in a new eppendorf. Add 750µl of PE buffer (check ethanol has been added). Incubate at room temp for 5 mins, then centrifuge at 13,000 rpm for 30 seconds.
8. Transfer column into fresh Eppendorf. Centrifuge at 13,000 rpm for 2 mins.
9. Transfer column to new eppendorf. Add 30µl of MiliQ water and incubate for 5 mins at room temp
10. Final centrifuge at 11,000 rpm for 1 minute.
11. Test sample using Nanodrop =
12. (graph for gel extraction does not look like one for purification)
13.

EutLK - Ligation, Transformation

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-03 to 2017-08-04

THURSDAY, 3/8/17

Ligation

- add in 1uL of ATP to each reaction

L1 - for RD 1 (use HF enzyme..

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA	1
4	Insert DNA	4
5	Nuclease-free water	12
6	T4 DNA Ligase	1
7	Total	20

L2 - For RD 2 (non HF enzymes - can be heat inactivated

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA:	1
4	Insert DNA:	4
5	Nuclease-free water	12
6	T4 DNA Ligase	1
7	Total	20

use 37ng vector (2.6uL)
1:1 = 50ng insert (1uL)
2:1 = 100ng insert (2uL)

L3 - using the gel extracted pSB1C3 plasmid + RD2 EutLK (1:1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	EutLK	1
4	pSB1C3 extracted	2.6
5	Nuclease-free water	13.4
6	T4 DNA Ligase	1
7	Total	20

L4 - using gel extracted pSB1C3 plasmid + RD 2 eutLK (2…

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	EutLK	2
4	pSB1C3 extracted	2.6
5	Nuclease-free water	12.4
6	T4 DNA Ligase	1
7	Total	20

1. room temperature for 1hr, 65C for 10mins
2. Chill on ice

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

› 10X NEB CutSmart Buffer

› Restriction enzymes

› MilliQ water

› DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2		
	Component	Vol
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 50uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

Note: The table shows a ligation using a molar ratio of 1:3 vector to insert for vector of 4kb and insert of 1kb. *Use NEB calculator to calculate molar ratios.*

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: 50 ng (0.020 pmol)	
4	Insert DNA: 37.5 ng (0.060 pmol)	
5	Nuclease-free water	17
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.
7. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 30 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.
8. Heat inactivate at 65C for 10 minutes.
9. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

    Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

- ✔ 12. Add 1-5µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins
- ✔ 13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.
- ✔ 14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

  SOC should be stored at 4°C, but can be warmed to room temperature before use
- ✔ 15. Spun down into a pellet at 5000rpm for 2 minutes
- ✔ 16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC
- ✔ 17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously
- ✔ 18. Incubate overnight at 37C

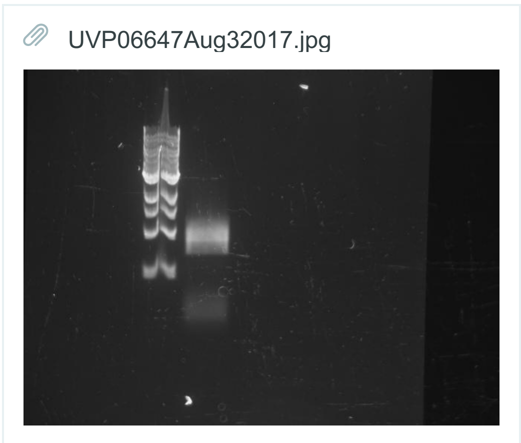
pSB1C3 - RD, gel

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-02 to 2017-08-03

THURSDAY, 3/8/17

redo:

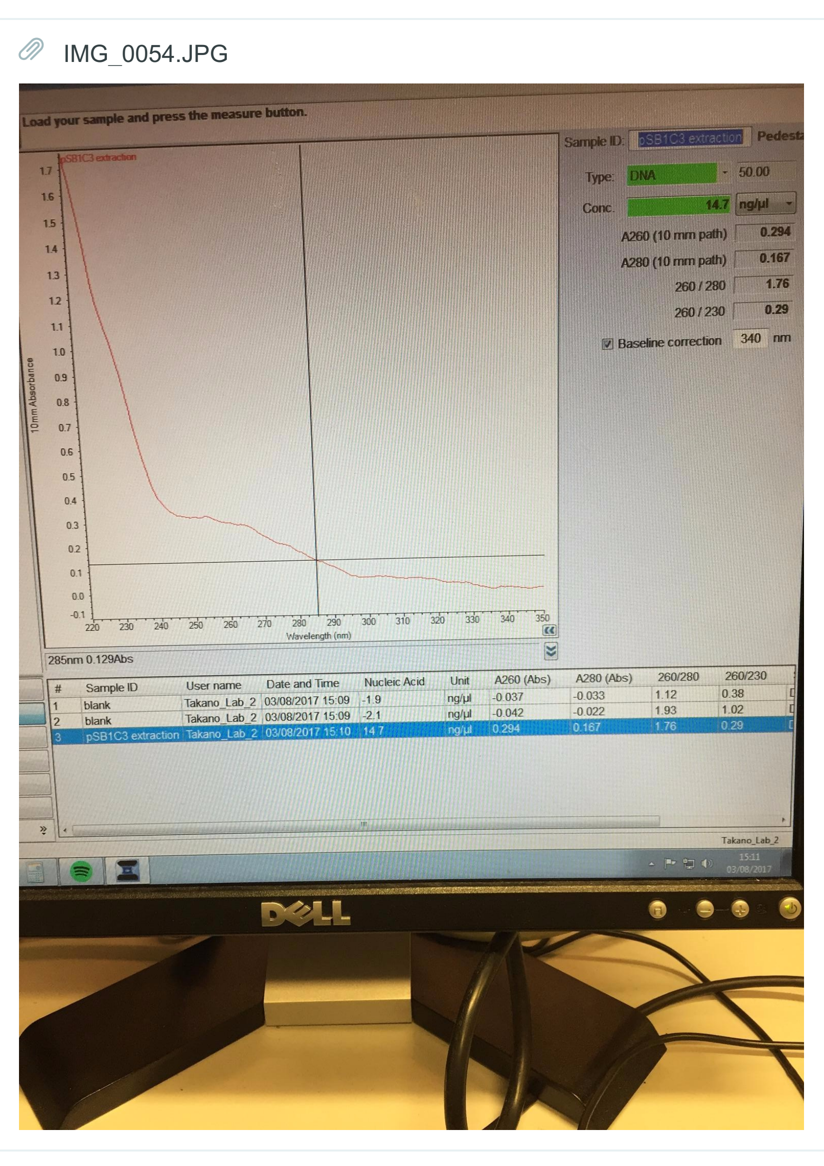
UVP06647Aug32017.jpg



Gel Extraction

gel weight = 100mg
nanodrop conc = 14ng/ul

IMG_0054.JPG



EutLK - Ligation, Transformation

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-03 to 2017-08-04

FRIDAY, 4/8/17

Transformation

Transform 5uL into 50uL cells (use one 200uL tube)

- protocol attached

EutS + EutMN - Assembly

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-07 to 2017-08-10

MONDAY, 7/8/17

- Only use those which have been sent for sequencing (EutS-C, EutS-Q, EutMN-13)
- From index plate - 3x EutS-C, 3x EutS-Q, 2x EutMN-13 (ovenight cultures) - need more

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	5uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 50uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

Gel Extraction (Qiagen)

Introduction

Materials

› Qiagen Kit

Procedure

Gel extraction

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube = **XXXmg**. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.
4. Add 1 gel volume isopropanol to the sample and mix by inverting.
5. Transfer 750µl of supernatant to a column placed on a 1.5mp eppendorf. Centrifuge at 11,000rpm for 1 min.
6. Discard flow through. Place column in a new Eppendorf tube. Add 500µl of PB buffer and centrifuge column at 13,000 rpm for 30 seconds.
7. Discard flow through. Place column in a new eppendorf. Add 750µl of PE buffer (check ethanol has been added). Incubate at room temp for 5 mins, then centrifuge at 13,000 rpm for 30 seconds.
8. Transfer column into fresh Eppendorf. Centrifuge at 13,000 rpm for 2 mins.
9. Transfer column to new eppendorf. Add 30µl of MiliQ water and incubate for 5 mins at room temp
10. Final centrifuge at 11,000 rpm for 1 minute.
11. Test sample using Nanodrop =
12. (graph for gel extraction does not look like one for purification)
13.

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

Note: The table shows a ligation using a molar ratio of 1:3 vector to insert for vector of 4kb and insert of 1kb. *Use NEB calculator to calculate molar ratios.*

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA:	2.4
4	Insert DNA:	13.7
5	Nuclease-free water	0.9
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.

7. For cohesive (sticky) ends, incubate at 16°C (on bench) overnight or room temperature for 30 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.

8. Heat inactivate at 65C for 10 minutes.

9. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

   Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins

13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.

14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before use

15. Spun down into a pellet at 5000rpm for 2 minutes

16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC

17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously

18. Incubate overnight at 37C

EutS + MN - sequencing

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-07

MONDAY, 7/8/17

need to submit:

- 15uL DNA at 50ng/uL, and 10uL extra for each additional read
- 10uL primer at 10uM, and 5uL extra for each additional read

plan:

pSB1C3_EutS (C + Q)

- use VR, VF2, EutS reverse, LacI forward

pSB1C3_EutMN (13)

- use VR, VF2, EutM reverse, TetR forward

so need:

- VR for 3 = 20uL
- VF2 for 3 = 20uL
- EutS-R for 2 = 15uL
- LacI-F for 2 = 15uL
- EutMN-R for 1 = 10uL
- TetR-F for 1 = 10uL
- pSB1C3_EutS C for 4 reads = 45uL
- pSB1C3_EutS C Q for 4 reads = 45uL
- pSB1C3_EutMN 13 for 4 reads = 45uL

Table1

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	Water vol needed (uL)
1	pSB1C3_ EutS C	233.3	9.6442348907	35.3557651093
2	pSB1C3_ EutS Q	181.6	12.3898678414	32.6101321586
3	pSB1C3_ Eut MN 13	142.7	15.7673440785	29.2326559215

Results

pSB1C3-EutS - C - large insertion at 2711 (3 primers showing the same)

pSB1C3-EutS - Q - mutated spot at 3671 (only 1 primer covers this so check)

pSB1C3-EutMN - the VF2 primer did not work

- resend MN with VF2
- design alternative primers to VF2 for eutMN region missing
- resend S-Q with VR

EutS + EutMN - Assembly

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-07 to 2017-08-10

TUESDAY, 8/8/17

- Miniprep and pool all Eut constructs
- Which EutS should we use? have sent both for sequencing, wont have results yet - just try with both (cells are in shots of 4)

EutLK - Colony PCR

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-09 to 2017-08-10

WEDNESDAY, 9/8/17

Table1

	A	B	C
1	**Reaction vol:**	**10**	
2	**Number Reactions:**	1	**60**
3	10 uM Fwd Primer	0.2	12
4	10 uM Rev Primer	0.2	12
5	Template	0	0
6	2x OneTaq Mix	5	300
7	ddH20	4.6	276
8			

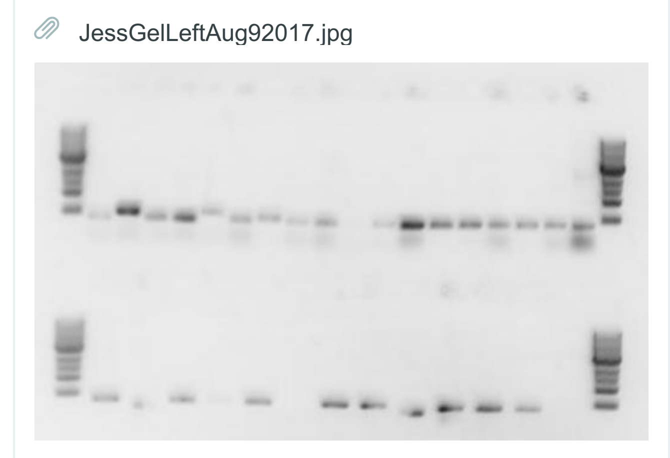
Table2

	A	B	C
1		**Temp**	**Time**
2	**Initial Denaturating**	95 oC	30 s
3	**30 cycles:**	95 oC	15-30s
4		~ 55 oC	15-60 s
5		68 oC	3 mins
6	**Final Extension**	68 oC	5 mins
7	**Hold**	4-10 oC	Inf

Run gel

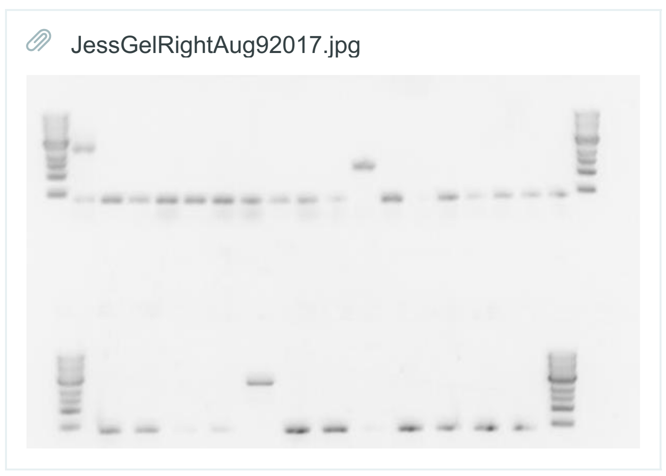
- looking for 3kb band

JessGelLeftAug92017.jpg



L2 samples - all negative

JessGelRightAug92017.jpg



L3. 1-20 and L4. 1-10 samples (L3.1, L4.3 positive) (L3.11 RFP = digest failed)

- L3.1, L4.3 were positive - L3.11 band is lower = rfp

EutS + EutMN - Assembly

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-07 to 2017-08-10

WEDNESDAY, 9/8/17

Restriction digest

1000ng pSB1C3_EutS with SpeI + PstI

- nanodrop C = 183.7ng/ul
- nanodrop Q = 185.7ng/uL
    a. 5.5uL DNA, 11uL water, 0.5uL Spe, pst, rsap, 2uL cutsmart

1000ng pSB1C3_EutMN with XbaI + PstI

- nanodrop = 166.3ng/uL
    a. 6uL DNA, 10.5uL water, 0.5uL xba, pst, 2uL cutsmart

1. 37C 1hr, 80C 20min

Gel extract

1. run gel and extract pSB1C3-EutS bands from ~4000bp, and EutMN band from ~ 2400bp

- S-C gel weight = 0.08g
- S-Q gel weight = 0.07g
- MN-13 gel weight = 0.11g

nanodrop results:

- EutS-C = 13.3ng/ul
- EutS-Q = 12.1ng/ul
- EutMN = 4.9ng/ul

Ligation

- Try both EutS vectors with EutMN at 3:1 ratio
- L1 = EutS-C; L2 = EutS-Q
- do overnight

EutS + EutMN - re-sequencing

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-09

WEDNESDAY, 9/8/17

Results (from before)

pSB1C3-EutS - C - large insertion at 2711 (3 primers showing the same)
pSB1C3-EutS - Q - mutated spot at 3671 (only 1 primer covers this so check)
pSB1C3-EutMN - the VF2 primer did not work

- resend MN with VF2
- design alternative primers to VF2 for eutMN region missing
- resend S-Q with VR

need :
15uL eutMN with 10uL 10uM VF2
15uL eutS with 10uL 10uM VR

Table1

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	Water vol needed (uL)
1	pSB1C3_ EutS Q	181.6	4.1299559471	10.8700440529
2	pSB1C3_ Eut MN 13	142.7	5.2557813595	9.7442186405

Results

pSB1C3-EutS has the same mutation - so colony Q is def mutated and unusable - so send remaining colonies for sequencing and deisng PCr primers to remove this mutation
pSB1C3-EutMN now successfully sequencing and correct - can submit and use

EutLK - Colony PCR

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-09 to 2017-08-10

THURSDAY, 10/8/17

- overnight cultures from index plate

EutS + EutMN - Assembly

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-07 to 2017-08-10

THURSDAY, 10/8/17

- heat inactivate ligation reaction for 10min at 65C

Transformation

- unsucessful - no colonies

(but the eutS used had a mutation anyway)

EutS - sequencing (other colonies)

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-10

THURSDAY, 10/8/17

for 4 constructs = 25ul at 10uM

- VR
- VF2
- laci forward
- euts reverse

for 4 reads = 45ul

- euts - a
- euts - d
- euts - m
- euts - t

Table1

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	Water vol needed (uL)
1	pSB1C3_ EutS a	154.9	14.5255003228	30.4744996772
2	pSB1C3_ EutS d	136	16.5441176471	28.4558823529
3	pSB1C3_ Eut S m	95.2	23.6344537815	21.3655462185
4	pSB1C3_ Eut S t	120.5	18.6721991701	26.3278008299

Results:
all correct

EutLK - Miniprep, RD, gel - Alice

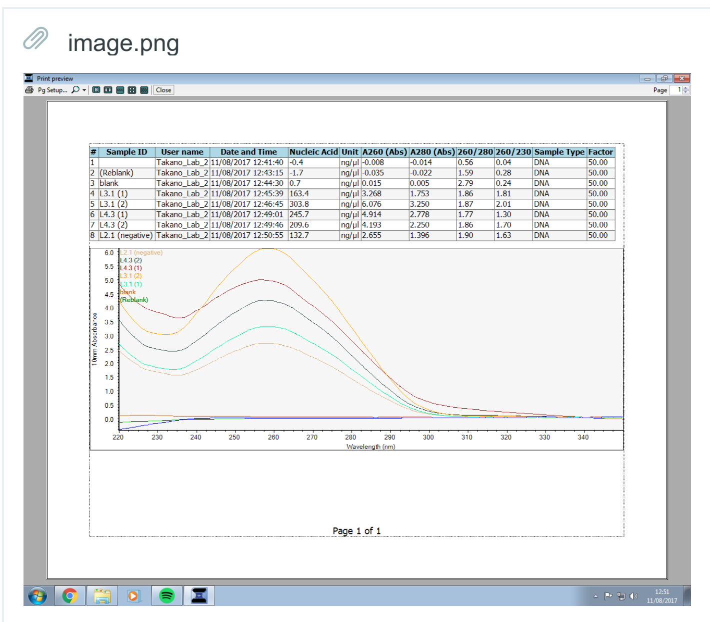
Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-11

FRIDAY, 11/8/17

Miniprep of 2 samples L3.1 and L4.3 (only positive results from previous colony PCR). 2 overnights made from each sample, so in total 2x L3.1 and 2x L4.3 samples. Samples are also on index plate. Miniprep has also been done on a negative sample (from colony PCR), so it can be sent for sequencing

Nanodrop results

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1		Takano_Lab_2	11/08/2017 12:41:40	-0.4	ng/µl	-0.008	-0.014	0.56	0.04	DNA	50.00
2	(Reblank)	Takano_Lab_2	11/08/2017 12:43:15	-1.7	ng/µl	-0.035	-0.022	1.59	0.28	DNA	50.00
3	blank	Takano_Lab_2	11/08/2017 12:44:30	0.7	ng/µl	0.015	0.005	2.79	0.24	DNA	50.00
4	L3.1 (1)	Takano_Lab_2	11/08/2017 12:45:39	163.4	ng/µl	3.268	1.753	1.86	1.81	DNA	50.00
5	L3.1 (2)	Takano_Lab_2	11/08/2017 12:46:45	303.8	ng/µl	6.076	3.250	1.87	2.01	DNA	50.00
6	L4.3 (1)	Takano_Lab_2	11/08/2017 12:49:01	245.7	ng/µl	4.914	2.778	1.77	1.30	DNA	50.00
7	L4.3 (2)	Takano_Lab_2	11/08/2017 12:49:46	209.6	ng/µl	4.193	2.250	1.86	1.70	DNA	50.00
8	L2.1 (negative)	Takano_Lab_2	11/08/2017 12:50:55	132.7	ng/µl	2.655	1.396	1.90	1.63	DNA	50.00

Restriction Digest

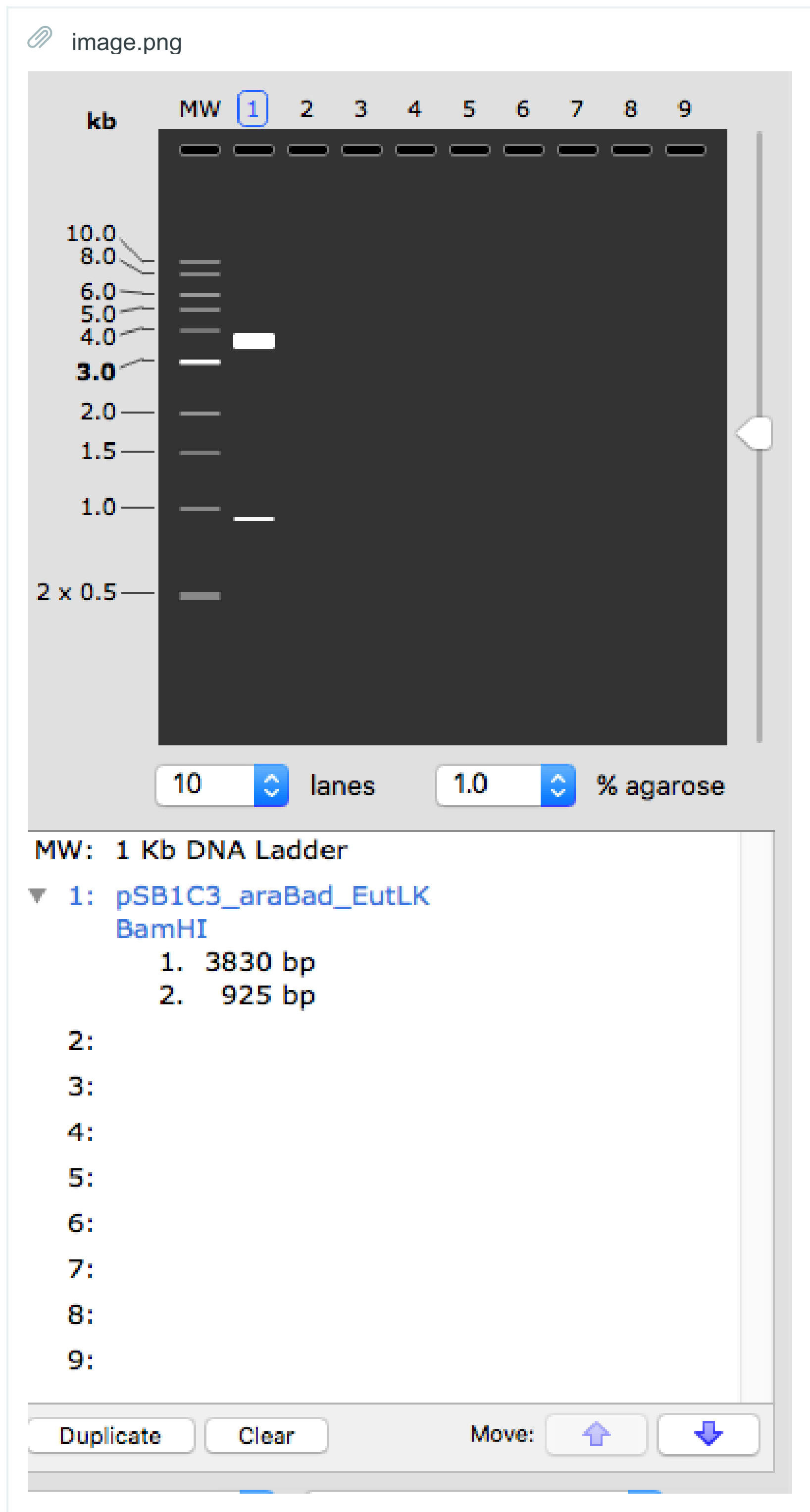
To make up to 20ul:
* To get 1000ng of DNA for PCR
1000/DNA in ng/ul (the nanodrop sample value)

Table1

	A	B	C	D
1	Sample I.D.	DNA ng/ul	DNA in ul for 100ng	H2O
2	L3.1 (1)	163.4	6.1	11.9
3	L3.1 (2)	303.8	3.3	13.7
4	L4.3 (1)	245.7	4	13
5	L4.3 (2)	209.6	4.8	12.2

+ add 2ul cutsmart and 1ul BamHI, run for 90 mins at 37°C (BamHI is not sensitive to heat inactivation).

Predicted gel



Gel Results

looks like digestion didnt work - not sure why. - redo

EutS - Miniprep - Owen

Project: Manchester iGEM 2017 Shared Project

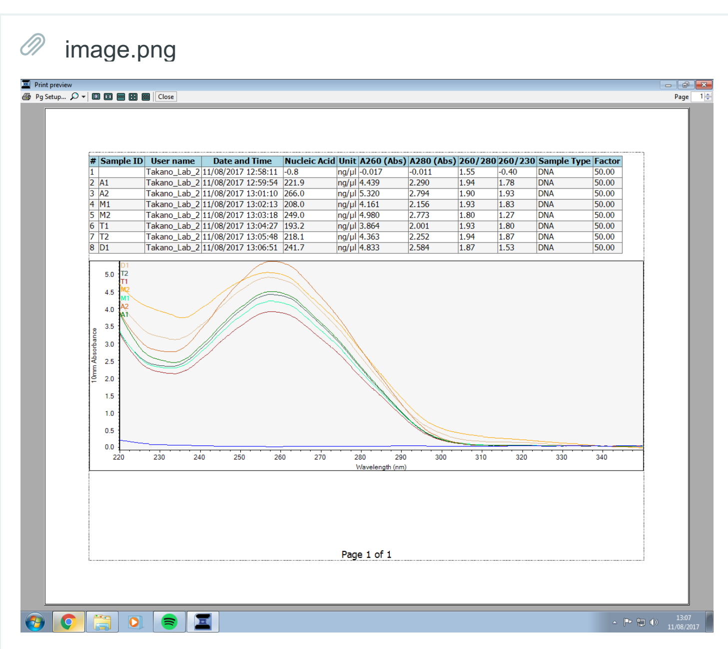
Authors: Jessica Burns

Date: 2017-08-11

FRIDAY, 11/8/17

- miniprep EutS A/D/M/T from overnights
- pool to ones from the same colony (ie. put EutS A1 and A2 together. etc.) then nanodrop

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1		Takano_Lab_2	11/08/2017 12:58:11	-0.8	ng/µl	-0.017	-0.011	1.55	-0.40	DNA	50.00
2	A1	Takano_Lab_2	11/08/2017 12:59:54	221.9	ng/µl	4.439	2.290	1.94	1.78	DNA	50.00
3	A2	Takano_Lab_2	11/08/2017 13:01:10	266.0	ng/µl	5.320	2.794	1.90	1.93	DNA	50.00
4	M1	Takano_Lab_2	11/08/2017 13:02:13	208.0	ng/µl	4.161	2.156	1.93	1.83	DNA	50.00
5	M2	Takano_Lab_2	11/08/2017 13:03:18	249.0	ng/µl	4.980	2.773	1.80	1.27	DNA	50.00
6	T1	Takano_Lab_2	11/08/2017 13:04:27	193.2	ng/µl	3.864	2.001	1.93	1.80	DNA	50.00
7	T2	Takano_Lab_2	11/08/2017 13:05:48	218.1	ng/µl	4.363	2.252	1.94	1.87	DNA	50.00
8	D1	Takano_Lab_2	11/08/2017 13:06:51	241.7	ng/µl	4.833	2.584	1.87	1.53	DNA	50.00

EutLK - RD, gel (repeat)

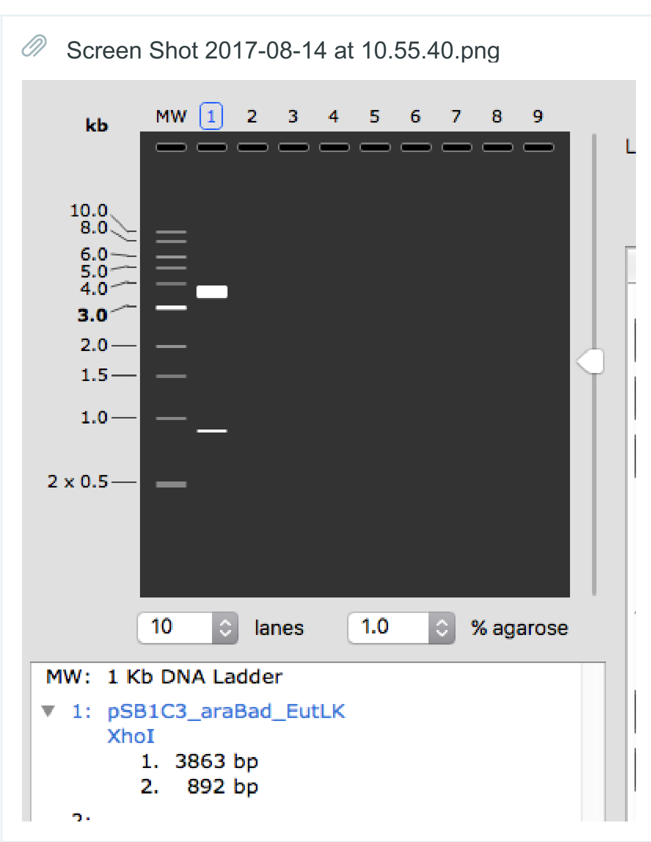
Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-14

MONDAY, 14/8/17

used bamhIII before to digest - problem is likely not the enzyme but just to be safe and not waste the DNa, going to use xhoI (which we have used before and know works)
digest 1000ng with xhoI

- L3.1 = 240ng/uL - so use 4uL
- L4.3 = 204ng/uL - so use 5uL

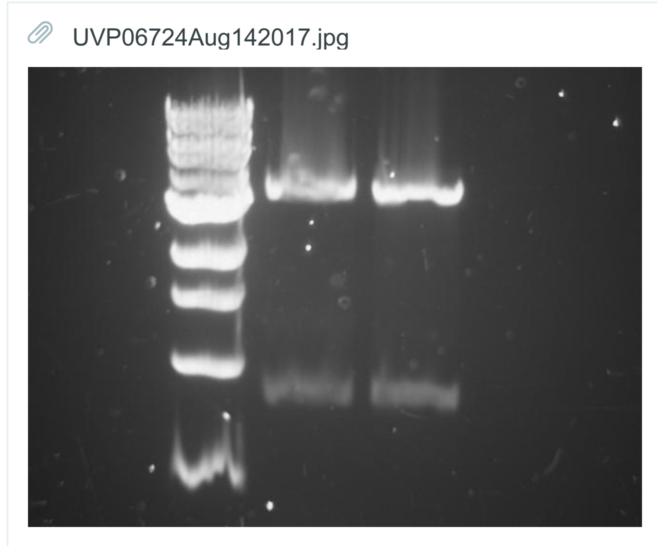
predicted:



Screen Shot 2017-08-14 at 10.55.40.png

result:

UVP06724Aug142017.jpg



- send for sequencing

EutSMN biobrick assembly

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-14 to 2017-08-16

MONDAY, 14/8/17

Restriction digest

1000ng pSB1C3_EutS with SpeI + PstI

- nanodrop EutS D = 136ng/uL
    a. 7.5uL DNA, 9L water, 0.5uL Spe, pst, rsap, 2uL cutsmart

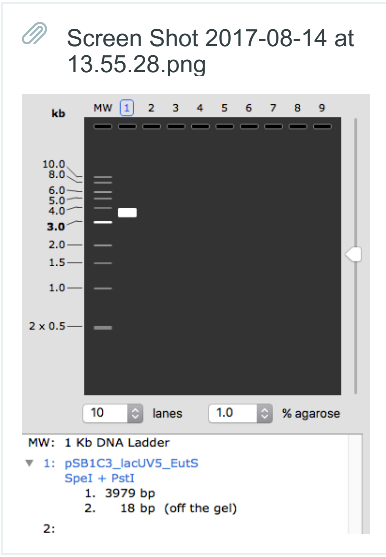
(3 lots - last time got only a tiny bit) 1000ng pSB1C3_EutMN with XbaI + PstI

- nanodrop Eut MN 13 = 166.3ng/uL
    a. 6uL DNA, 11uL water, 0.5uL xba, pst, 2uL cutsmart

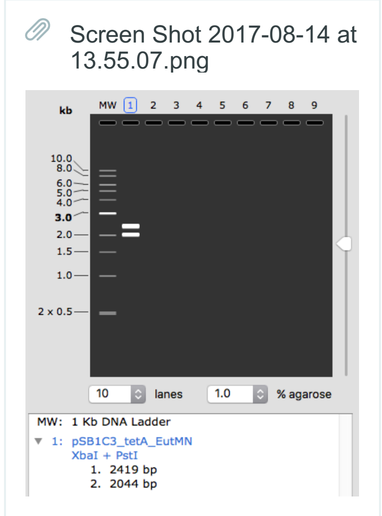
1. 37C 1hr, 80C 20min

Gel extract

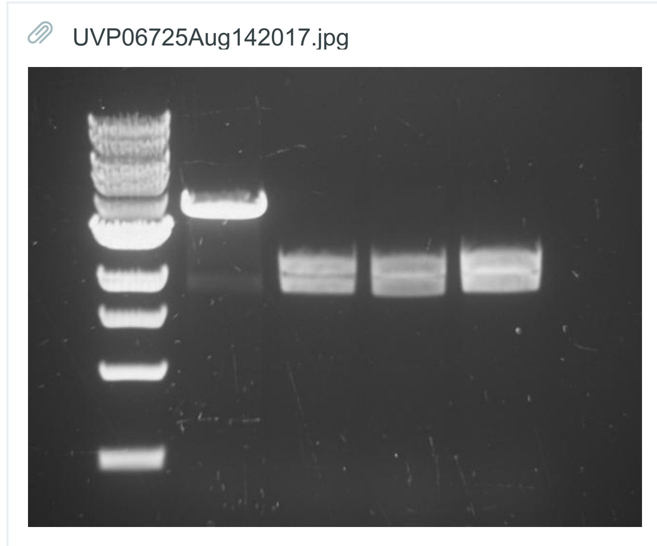
Screen Shot 2017-08-14 at 13.55.28.png



Screen Shot 2017-08-14 at 13.55.07.png



UVP06725Aug142017.jpg

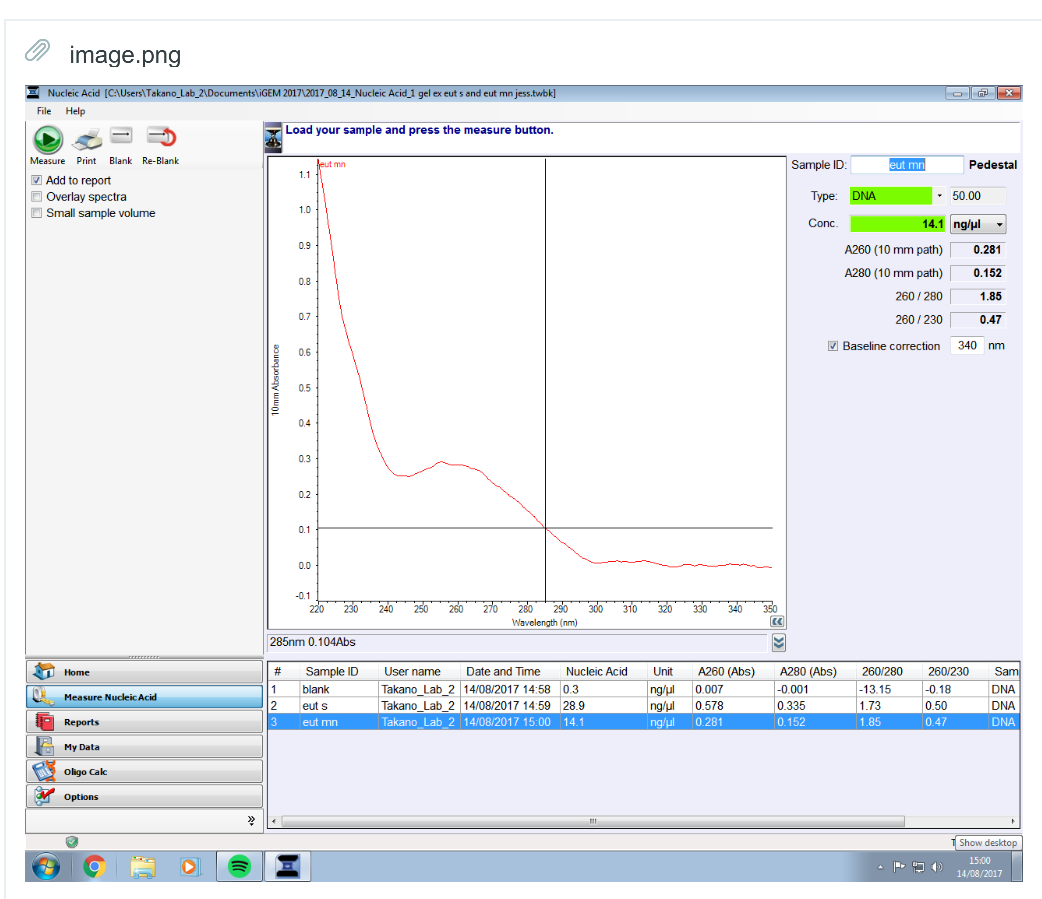


1. run gel and extract pSB1C3-EutS bands from ~4000bp, and EutMN band from ~ 2400bp

gel weights = all 100mg

nanodrop results:

image.png



Ligation

- **3:1 =** 40ng vector (1.4uL) + 72.95ng insert (5.2uL)

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA:	1.4
4	Insert DNA:	5.2
5	Nuclease-free water	10.4
6	T4 DNA Ligase	1
7	Total	20

do 3 lots of 3:1 + 1 negative
room temp overnight

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

Note: The table shows a ligation using a molar ratio of 1:3 vector to insert for vector of 4kb and insert of 1kb. *Use NEB calculator to calculate molar ratios.*

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: 50 ng (0.020 pmol)	
4	Insert DNA: 37.5 ng (0.060 pmol)	
5	Nuclease-free water	17
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.

7. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 30 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.

8. Heat inactivate at 65C for 10 minutes.

9. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

   Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1-5µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins
13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.
14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before usem

15. Spun down into a pellet at 5000rpm for 2 minutes
16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC
17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously
18. Incubate overnight at 37C

eutLK - sequencing

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-14

MONDAY, 14/8/17

need 6 reads = 65uL at 50ng/uL
need 2 lots of primers (VR, VF2, araC f, araC r, eutL f, eutL r) = 15uL at 10uM

Table1

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	Water vol needed (uL)
1	pSB1C3_ EutLK 3.1	240	13.5416666667	51.4583333333
2	pSB1C3_ EutLK 4.3	204	15.931372549	49.068627451

EutLK - colony PCR (repeat)

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-15 to 2017-08-16

TUESDAY, 15/8/17

set up:

Table1

	A	B	C
1	**Reaction vol:**	**10**	
2	**Number Reactions:**	1	**25**
3	10 uM Fwd Primer	0.2	5
4	10 uM Rev Primer	0.2	5
5	Template	0	0
6	2x OneTaq Mix	5	125
7	ddH20	4.6	115
8			

Table2

	A	B	C
1		**Temp**	**Time**
2	**Initial Denaturating**	95 oC	30 s
3	**30 cycles:**	95 oC	15-30s
4		~ 55 oC	15-60 s
5		68 oC	1 mins
6	**Final Extension**	68 oC	5 mins
7	**Hold**	4-10 oC	Inf

EutSMN biobrick assembly

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-14 to 2017-08-16

TUESDAY, 15/8/17

heat inactivate 65C for 10mins

- do transformation

EutLK - colony PCR (repeat)

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-15 to 2017-08-16

WEDNESDAY, 16/8/17

Run gel looking for: 1kb band

- all negative

EutSMN biobrick assembly

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-14 to 2017-08-16

WEDNESDAY, 16/8/17

8 colonies grew over 3 identical plates - negative was all clear

- overnight cultures of all those colonies

EutSMN - Miniprep + sequencing

Project: Manchester iGEM 2017 Shared Project

Authors: Ong Jun Yang

Dates: 2017-08-17 to 2017-08-21

THURSDAY, 17/8/17

overnight cultures of EutSMN 5, 6, 7

EutSMN - miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

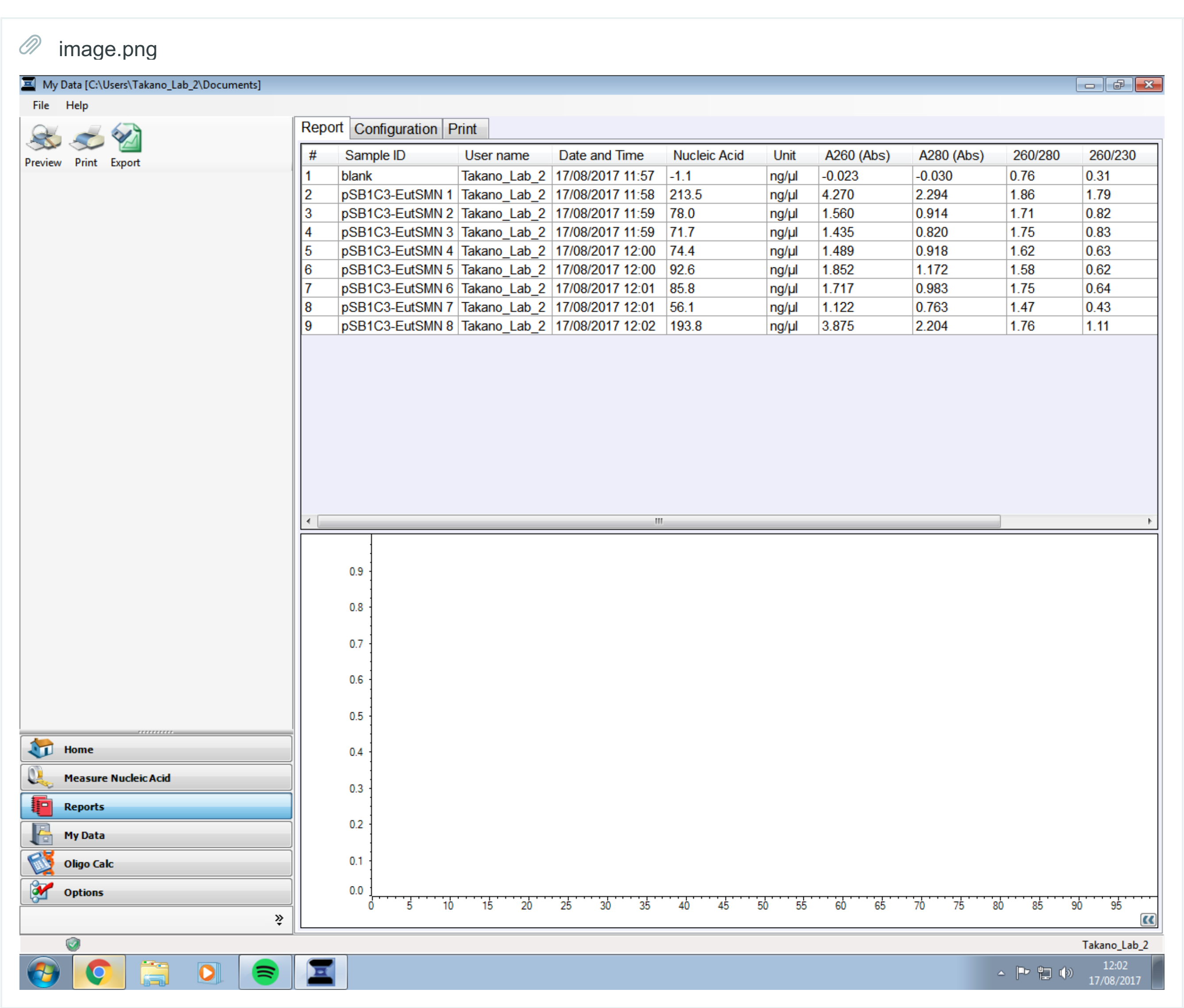
Date: 2017-08-17

THURSDAY, 17/8/17

Miniprep

nanodrop results:

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	blank	Takano_Lab_2	17/08/2017 11:57	-1.1	ng/µl	-0.023	-0.030	0.76	0.31
2	pSB1C3-EutSMN 1	Takano_Lab_2	17/08/2017 11:58	213.5	ng/µl	4.270	2.294	1.86	1.79
3	pSB1C3-EutSMN 2	Takano_Lab_2	17/08/2017 11:59	78.0	ng/µl	1.560	0.914	1.71	0.82
4	pSB1C3-EutSMN 3	Takano_Lab_2	17/08/2017 11:59	71.7	ng/µl	1.435	0.820	1.75	0.83
5	pSB1C3-EutSMN 4	Takano_Lab_2	17/08/2017 12:00	74.4	ng/µl	1.489	0.918	1.62	0.63
6	pSB1C3-EutSMN 5	Takano_Lab_2	17/08/2017 12:00	92.6	ng/µl	1.852	1.172	1.58	0.62
7	pSB1C3-EutSMN 6	Takano_Lab_2	17/08/2017 12:01	85.8	ng/µl	1.717	0.983	1.75	0.64
8	pSB1C3-EutSMN 7	Takano_Lab_2	17/08/2017 12:01	56.1	ng/µl	1.122	0.763	1.47	0.43
9	pSB1C3-EutSMN 8	Takano_Lab_2	17/08/2017 12:02	193.8	ng/µl	3.875	2.204	1.76	1.11

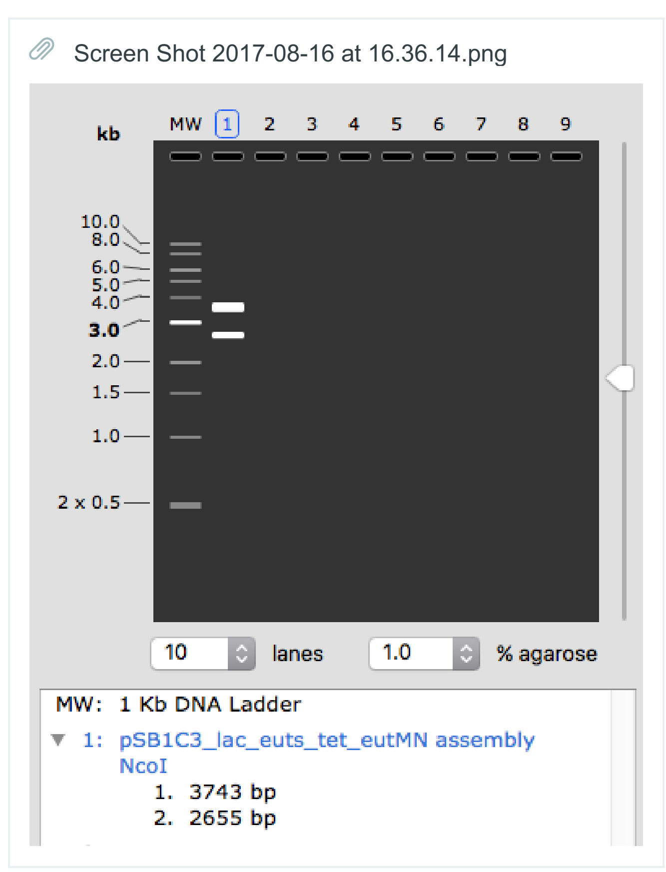
Restriction Digest

1000ng with NcoI

Gel calcs - running 1000ng				
	Construct	Concentration (ng/uL)	Vol for 1000ng	Vol water to use
1	Eut SMN 1	213	4.6948356808	12.8051643192
2	Eut SMN 2	78	12.8205128205	4.6794871795
3	Eut SMN 3	71	14.0845070423	3.4154929577
4	Eut SMN 4	74	13.5135135135	3.9864864865
5	Eut SMN 5	93	10.752688172	6.747311828
6	Eut SMN 6	86	11.6279069767	5.8720930233
7	Eut SMN 7	56	17.8571428571	-0.3571428571
8	Eut SMN 8	192	5.2083333333	12.2916666667

Run gel

expected:



results: (1-4 top row, 5-8 bottom row)

if pSB1C3-eutS religated = one band at 3997

if pSB1C3-eutS-eutMN digest didnt work = one band at 6398

UVP06765Aug172017.jpg

top ladder is wonky - is possible that 1 and 3 are correct
5 and 7 are correct
6 is probably correct - incomplete digestion causing top band
- so send 5, 6, 7 for sequencing

Eut LK PCR + Gibson assembly

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-18

FRIDAY, 18/8/17

- cPrimers resuspended andspun down final conc = 100mM
- Working stock made of 10mM , the rest put in freezer

3 x reactions

- LK
- LK gibson
- BB gibson

For LK and LK gibson template is Eut Gblock
For BB gibson template is vector PSB1C3
Master mix= CloneAmp HiFi PCR premix
See CloneAmp HiFi PCR premix protocol but final volume is 50µl not 25µl.

CloneAmp HiFi PCR Premix Protocol-At-A-Glance_092612.pdf

Table1

	A	ul
1	Forward Primer	1.25
2	Reverse Primer	1.25
3	Template	0.2
4	2x master mix	25
5	ddH2O	22.3
6		Total = 50ul

PCR conditions
Steps 2,3 and 4 are repeated 35x
Step 4 is dependent on template (5 seconds per KB). G Block is 3kb so 15seconds. Vector is 2Kb.

Table2			
	Step	Temp C	Time
1		98	2 mins
2	repeated x35	98	10secs
3	repeated x35	55	5secs
4	repeated x35	72	15secs
5		72	1 mins
6		10	pause

Run on Gel (Gel set up = LK, LK gibson, BB Gibson)
Results, one correct band for LK gibson only.

PCR repeated (exactly the same protocol as above)
Gel ran + rest of LKgibson PCR product also ran for gel extraction .

Lane map

Table3							
	A	B	C	D	E	F	G
1	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
2	Ladder	PCR 2 LK	PCR 1 LK gibson	PCR 1 BB gibson	Empty	PCR 1 LK gibson	PCR 1 LK Gibson

UVP06780Aug182017.jpg

UVP06781Aug182017.jpg

- gel extracted LK gibsons - but the pSB1C3 gibson did not work, so ordered new primers
- Do PCR purification of lane 2 (LK PCR), then digest it
- Also digest and run pSB1C3, then gel extract it
- Then ligate

EutSMN - Miniprep + sequencing

Project: Manchester iGEM 2017 Shared Project

Authors: Ong Jun Yang

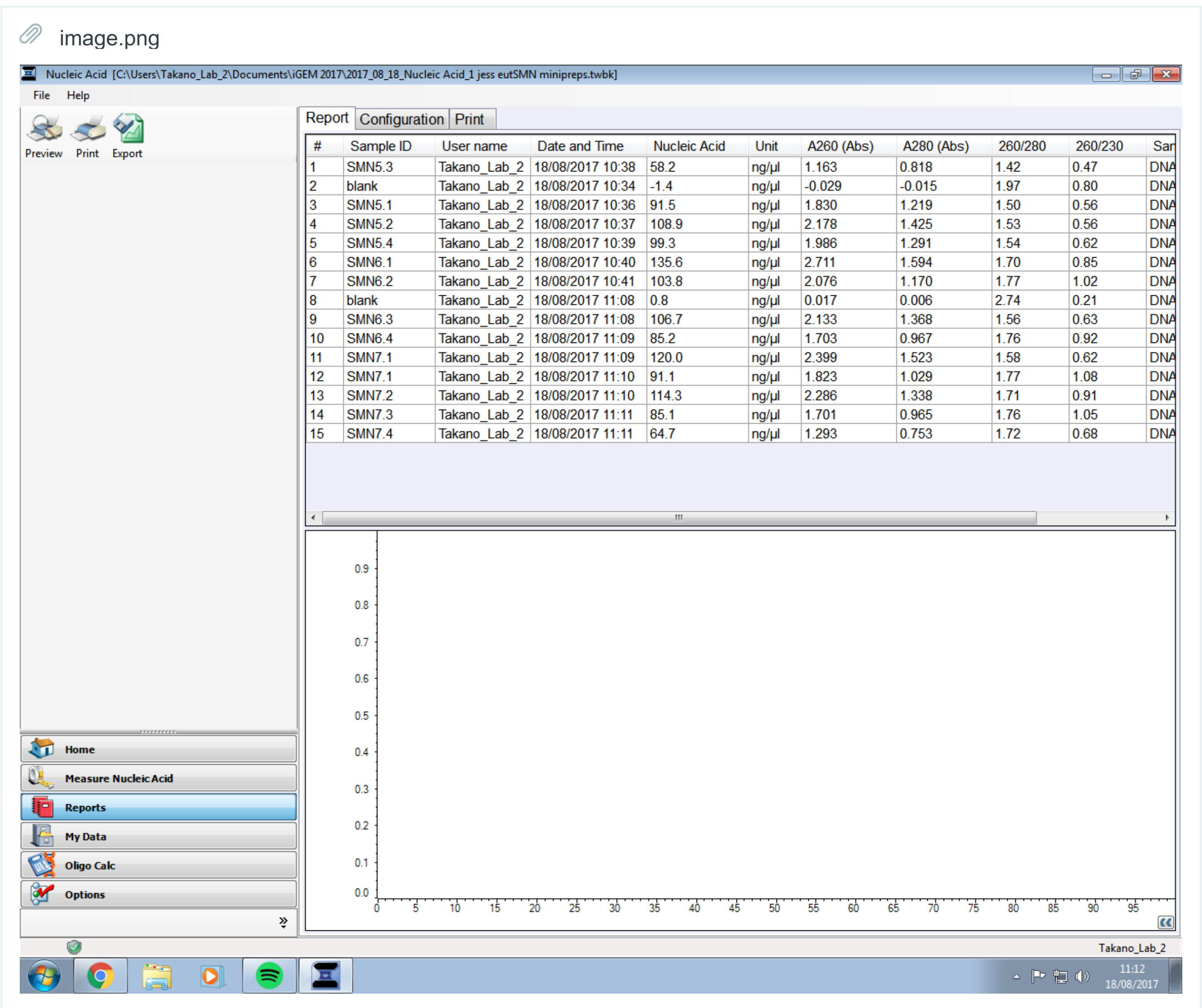
Dates: 2017-08-17 to 2017-08-21

FRIDAY, 18/8/17

Miniprep

nanodrop results: very low 260/230 values - redo

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sar
1	SMN5.3	Takano_Lab_2	18/08/2017 10:38	58.2	ng/µl	1.163	0.818	1.42	0.47	DNA
2	blank	Takano_Lab_2	18/08/2017 10:34	-1.4	ng/µl	-0.029	-0.015	1.97	0.80	DNA
3	SMN5.1	Takano_Lab_2	18/08/2017 10:36	91.5	ng/µl	1.830	1.219	1.50	0.56	DNA
4	SMN5.2	Takano_Lab_2	18/08/2017 10:37	108.9	ng/µl	2.178	1.425	1.53	0.56	DNA
5	SMN5.4	Takano_Lab_2	18/08/2017 10:39	99.3	ng/µl	1.986	1.291	1.54	0.62	DNA
6	SMN6.1	Takano_Lab_2	18/08/2017 10:40	135.6	ng/µl	2.711	1.594	1.70	0.85	DNA
7	SMN6.2	Takano_Lab_2	18/08/2017 10:41	103.8	ng/µl	2.076	1.170	1.77	1.02	DNA
8	blank	Takano_Lab_2	18/08/2017 11:08	0.8	ng/µl	0.017	0.006	2.74	0.21	DNA
9	SMN6.3	Takano_Lab_2	18/08/2017 11:08	106.7	ng/µl	2.133	1.368	1.56	0.63	DNA
10	SMN6.4	Takano_Lab_2	18/08/2017 11:09	85.2	ng/µl	1.703	0.967	1.76	0.92	DNA
11	SMN7.1	Takano_Lab_2	18/08/2017 11:09	120.0	ng/µl	2.399	1.523	1.58	0.62	DNA
12	SMN7.1	Takano_Lab_2	18/08/2017 11:10	91.1	ng/µl	1.823	1.029	1.77	1.08	DNA
13	SMN7.2	Takano_Lab_2	18/08/2017 11:10	114.3	ng/µl	2.286	1.338	1.71	0.91	DNA
14	SMN7.3	Takano_Lab_2	18/08/2017 11:11	85.1	ng/µl	1.701	0.965	1.76	1.05	DNA
15	SMN7.4	Takano_Lab_2	18/08/2017 11:11	64.7	ng/µl	1.293	0.753	1.72	0.68	DNA

overnight cultures

stored in cold room

EutLK - Biobrick assembly

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-21 to 2017-08-22

MONDAY, 21/8/17

1. Digest pSB1C3 with , run gel and extract
2. PCR purification of LK PCR product, then digest
3. Ligation of LK to pSB1C3 and transformation

Restriction Digest

of pSB1C3 plasmid

- nanodrop = 153.2ng/ul
- so for 1000ng use 6.2uL
- do 20ul reaction

Table1

	A	Volume (uL)
1	10X CutSmart Buffer	2
2	Restriction enzymes	0.5 each (pstI EcoRI rSAP)
3	MilliQ water	10.3
4	DNA (pSB1C3)	6.2

- 1hr at 37C, 20min at 80C

Run gel

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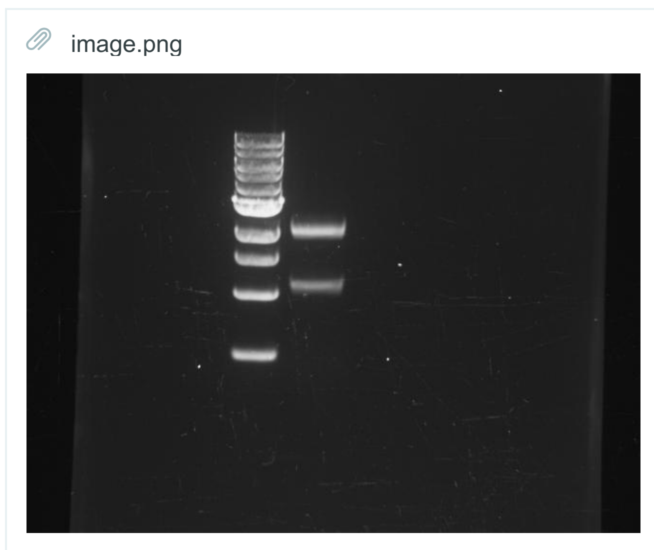
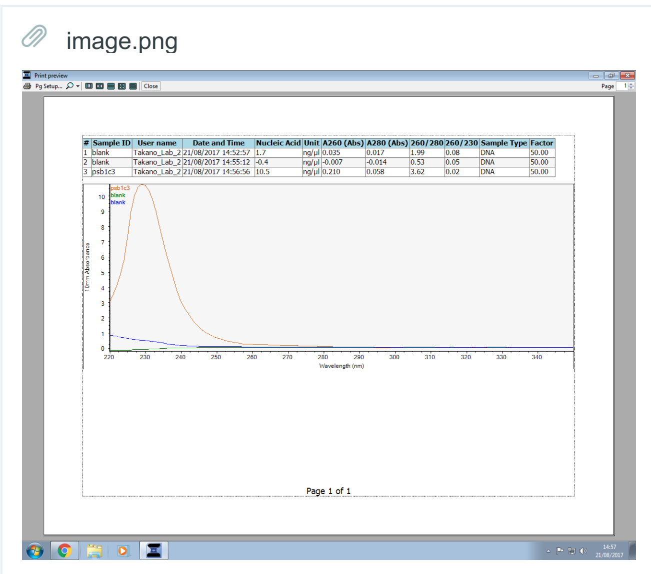


2 bands = 2029bp + 1100bp

Gel Extraction

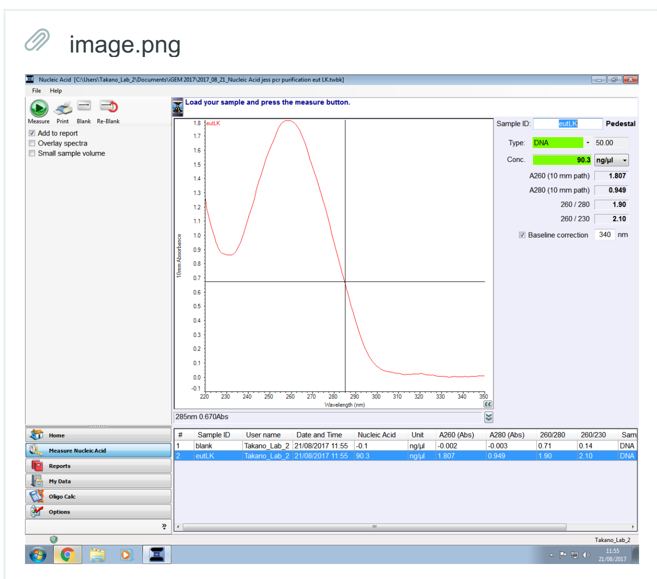
Gel extraction of top band 2029bp
gel weight = 300mg
nanodrop conc = 10.5ng/µl



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	blank	Takano_Lab_2	21/08/2017 14:52:57	1.7	ng/µl	0.035	0.017	1.99	0.08	DNA	50.00
2	blank	Takano_Lab_2	21/08/2017 14:55:12	-0.4	ng/µl	-0.007	-0.014	0.53	0.05	DNA	50.00
3	psb1c3	Takano_Lab_2	21/08/2017 14:56:56	10.5	ng/µl	0.210	0.058	3.62	0.02	DNA	50.00

PCR Purification

of eutLK



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sam
1	blank	Takano_Lab_2	21/08/2017 11:55	-0.1	ng/µl	-0.002	-0.003	0.71	0.14	DNA
2	eutLK	Takano_Lab_2	21/08/2017 11:55	90.3	ng/µl	1.807	0.949	1.90	2.10	DNA

Restriction Digest

of eutLK

- nanodrop = 90.3ng/ul
- so for 500ng use 5.6uL
- do 20ul reaction
- so 25ng in 1ul

Table2

	A	Volume (uL)
1	10X CutSmart Buffer	2
2	Restriction enzymes	0.5 each (pstI EcoRI)
3	MilliQ water	11.4
4	DNA (LK)	5.6

Ligation

of pSB1C3 and eutLK

2 lots of 3:1 = 40ng vector + 155.6ng insert

Table3

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: pSB1C3	4
4	Insert DNA: eutLK	6
5	Nuclease-free water	7
6	T4 DNA Ligase	1
7	Total	20

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 20uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

Note: The table shows a ligation using a molar ratio of 1:3 vector to insert for vector of 4kb and insert of 1kb. *Use NEB calculator to calculate molar ratios.*

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: 50 ng (0.020 pmol)	
4	Insert DNA: 37.5 ng (0.060 pmol)	
5	Nuclease-free water	17
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.

7. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 30 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.

8. Heat inactivate at 65C for 10 minutes.

9. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

    Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

EutS/MN/SMN - IPTG/tet induction

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-21 to 2017-08-22

MONDAY, 21/8/17

- overnights of eutS, eutMN, eutSMN

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins
13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.
14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before use
15. Spun down into a pellet at 5000rpm for 2 minutes
16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC
17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously
18. Incubate overnight at 37C

EutSMN - Miniprep + sequencing

Project: Manchester iGEM 2017 Shared Project

Authors: Ong Jun Yang

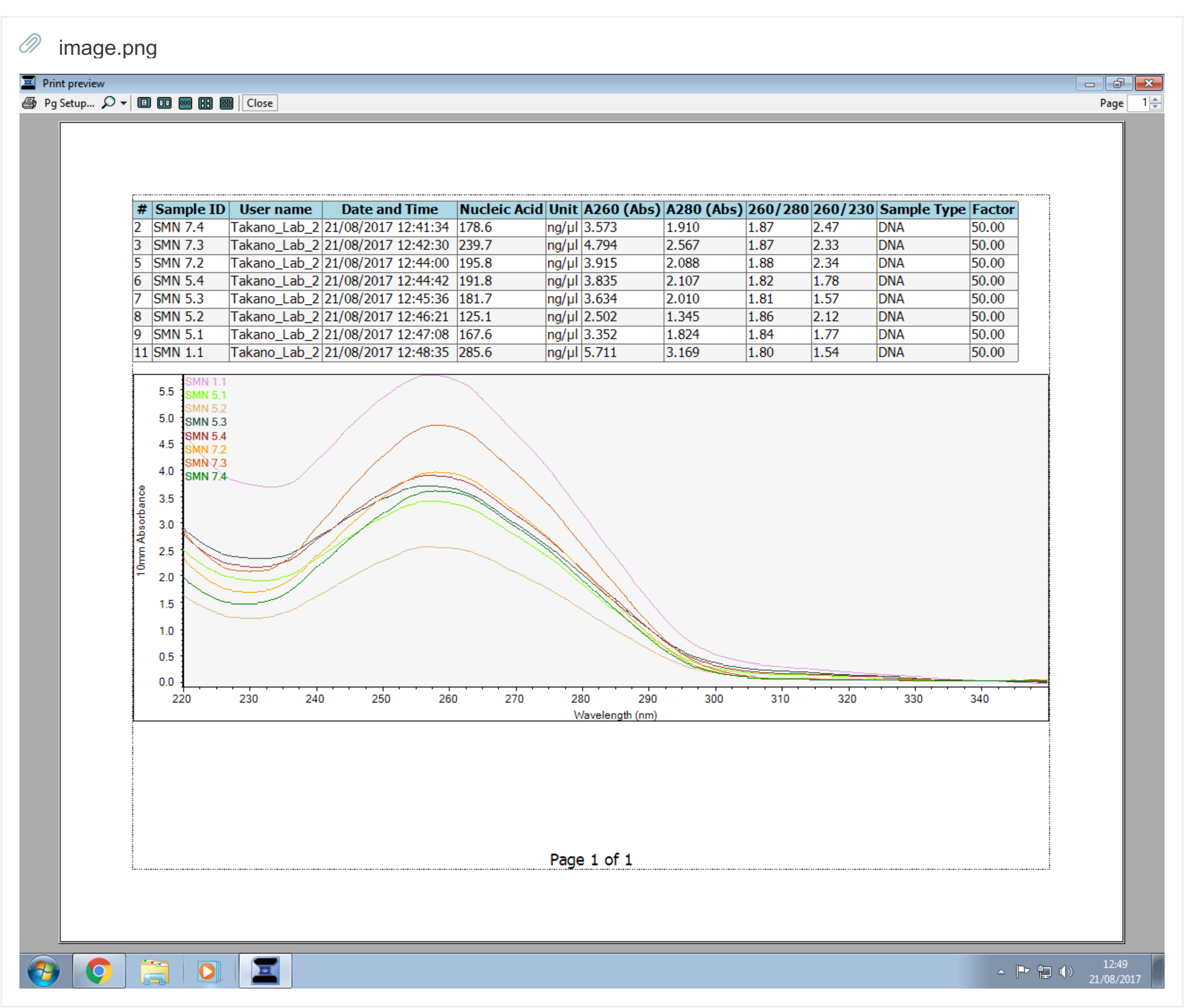
Dates: 2017-08-17 to 2017-08-21

MONDAY, 21/8/17

Miniprep

nanodrop results:

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
2	SMN 7.4	Takano_Lab_2	21/08/2017 12:41:34	178.6	ng/µl	3.573	1.910	1.87	2.47	DNA	50.00
3	SMN 7.3	Takano_Lab_2	21/08/2017 12:42:30	239.7	ng/µl	4.794	2.567	1.87	2.33	DNA	50.00
5	SMN 7.2	Takano_Lab_2	21/08/2017 12:44:00	195.8	ng/µl	3.915	2.088	1.88	2.34	DNA	50.00
6	SMN 5.4	Takano_Lab_2	21/08/2017 12:44:42	191.8	ng/µl	3.835	2.107	1.82	1.78	DNA	50.00
7	SMN 5.3	Takano_Lab_2	21/08/2017 12:45:36	181.7	ng/µl	3.634	2.010	1.81	1.57	DNA	50.00
8	SMN 5.2	Takano_Lab_2	21/08/2017 12:46:21	125.1	ng/µl	2.502	1.345	1.86	2.12	DNA	50.00
9	SMN 5.1	Takano_Lab_2	21/08/2017 12:47:08	167.6	ng/µl	3.352	1.824	1.84	1.77	DNA	50.00
11	SMN 1.1	Takano_Lab_2	21/08/2017 12:48:35	285.6	ng/µl	5.711	3.169	1.80	1.54	DNA	50.00

Sequencing

Send EutSMN 5, (for 8 reads) = 85uL at 50ng/uL

also send another eutSMN 5 (x) - will only do 8 per tube - ask you to upgrade to power read

Primers VR, VF2, LacI-F, EutS-F, EutS-R, TetR-F, TetR-R, EutM-F, EutM-R (for 1 construct) = 10uL at 10uM

Table2

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	Water vol needed (uL)
1	pSB1C3_ EutSMN 5	155.7	30.5073859987	64.4926140013
2	pSB1C3_ EutSMN 5 x	155.7	4.816955684	10.183044316

EutLK - Biobrick assembly

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-21 to 2017-08-22

TUESDAY, 22/8/17

room tempovernight, 65C for 10min

Transformation

use cells from will

Ligation

Repeat of ligation using new backbone from gel extraction .

Nanodrop of pSB1C3= 20.7

Ligation calculator (NEB website)

Insert DNA length (EutLk) = 2627bp

Vector DNAlength (pSBC13) =2029bp

Vector DNA mass = 40 (40 -50 optimum for vector)

Because concentration of eutLK =90.3 ng/ul

Want 500ng to use (1000 would be better if we had enough)

so 500/90.3 = 5.6

so there is 500ng of DNA in 5.6uL

so by adding 5.6 uL of DNA stock within a 20uL reaction there is now 500ng of DNA within 20ul

so in 1uL there is 25ng of DNA

We want a ratio of 3:1 (insert:vector) so use NEBLigation calculator to determine ng of pSBC13 DNA to use.

NEB calculator indicates that required DNA mass for insert is 155.4 ng

so 155.4/25 =16.2uL (from amount of EutLK DNA in 1uL1)

we want 40 ng of vector (concentration of vector pSB1C3 = 20.7)

so 40/20.7 = 1.93 (rounded to 2)

1.72uL (EutLK DNA) = 5.

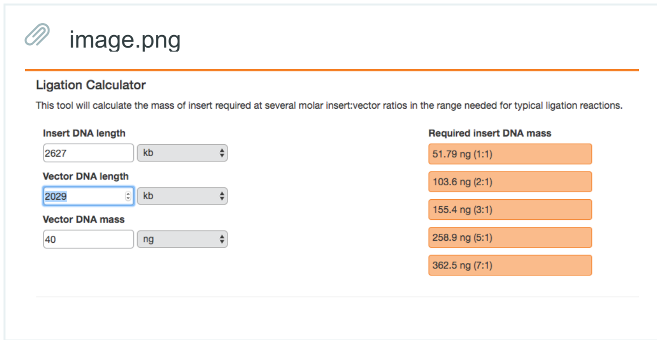


Table4	A	B
1	ATP (20mM)	1uL
2	T4 DNA Ligase	1uL
3	T4 DNA ligase buffer 10x	2uL
4	pSBC13 DNA (from gel extract)	2uL
5	Eut LK DNA	6.2uL
6	MilliQ H2O	7.8uL
7	Total	20uL

Ligation to run overnight.

EutS/MN/SMN - IPTG/tet induction

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-21 to 2017-08-22

TUESDAY, 22/8/17

- Inoculate flask of 50mL LB (+chl) with 200uL of the overnight
- Grow at 37C until reaches OD 0.5

Table3

	Time (mins)	OD - S	OD - S+	OD - MN	OD - MN+	OD - SMN	OD - SM
1	60	0.3	0.3	0.3	0.3	0.3	
2	90	0.48	0.47	0.48	0.5	0.57	

- Induce: add 250uM IPTG to eutS, 100nM tet to eutMN, and both to eutSMN
  - *induced at 13:30*

Table2

	Inducer	Stock conc (mM)	Desired conc (uM)	Vol to add to 50ml (uL)
1	IPTG	100	250	125
2	Tetracycline	0.214	0.1	23.364485…

Table1

	Flask No	Construct	Inducer + conc
1	1	eutS	none
2	2	eutS	250uM IPTG
3	3	eutMN	none
4	4	eutMN	100nM Tet
5	5	eutSMN	none
6	6	eutSMN	250uM IPTG + 100nM Tet

- Allow to express for 4hr *(17:30)* and 20hr *(09:30)* at 20C

Table4			
		OD when harvest at 4hr	OD when harvest at 20hr
1	S	0.65	0.78
2	S+	0.66	0.81
3	MN	0.70	0.85
4	MN+	0.67	0.79
5	SMN	0.70	0.82
6	SMN+	0.71	0.84

- Harvest cells: take 2 x 1ml from flask, centrifuge, freeze the pellet until ready to use

eutLK - colony PCR

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-23

WEDNESDAY, 23/8/17

Table1

	A	B	C
1	**Reaction vol:**	**10**	
2	**Number Reactions:**	1	**20**
3	10 uM araC Fwd Primer	0.2	4
4	10 uM eutL Rev Primer	0.2	4
5	Template	0	0
6	2x OneTaq Mix	5	100
7	ddH20	4.6	92
8			

Table2

	A	B	C
1		**Temp**	**Time**
2	**Initial Denaturating**	95 oC	30 s
3	**30 cycles:**	95 oC	15-30s
4		~ 55 oC	15-60 s
5		68 oC	1 mins
6	**Final Extension**	68 oC	5 mins
7	**Hold**	4-10 oC	Inf

Run gel
looking for ~1kb band

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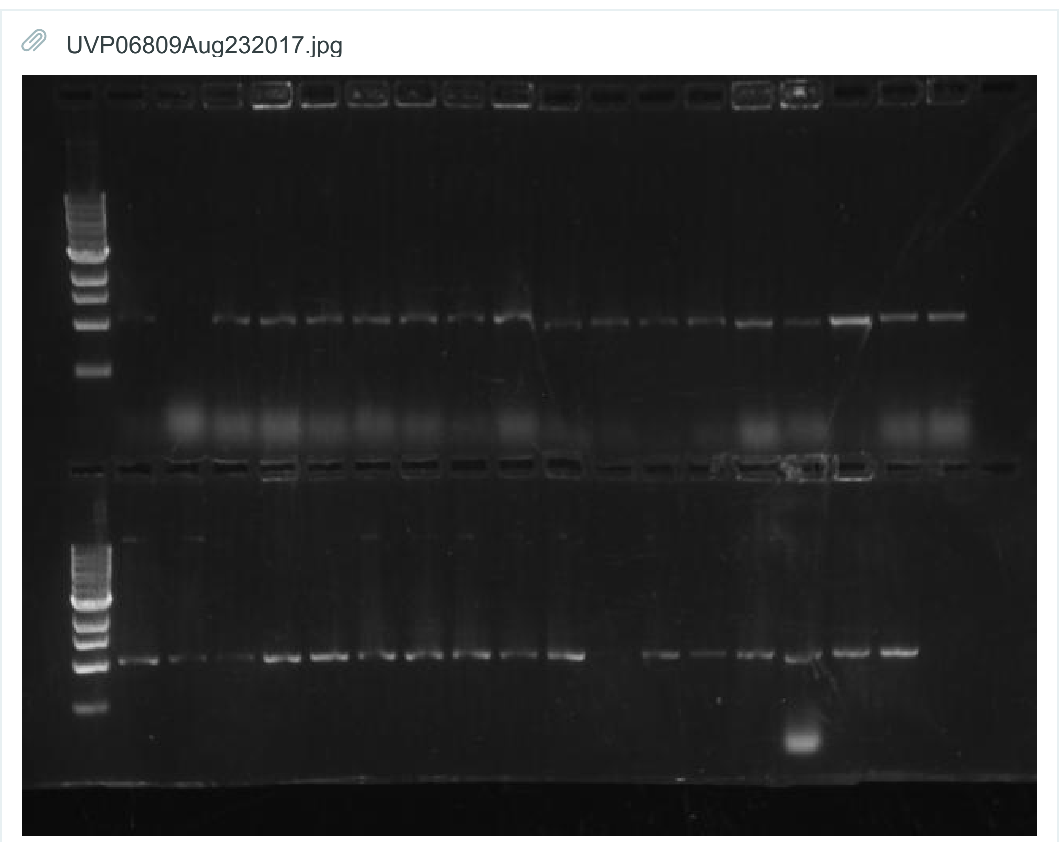

eutS, MN, SMN - SDS PAGE

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-23 to 2017-08-24

WEDNESDAY, 23/8/17

50mM TrisHCL (ph7.5)

2uM EDTA

0.1% Triton 100

5uM MgCl

0.1mg/ml Lysozyme

DNAase 1:1000

1. freeze-thaw repeat (use -80C)
2. add DNase + MgCl (1ul each) after freeze-thawing

solution shouldn't be viscous after DNAase addition

1. centrifuge 10min on full
2. pipette supernatant into new eppendorf = soluble fraction
3. resuspend pellet in buffer (above) = insoluble fraction
4. add equal amounts sample and SDS loading buffer (10uL + 10uL)
5. boil in PCR machine (95C 10 min)

Eut SMN - re-sequencing

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Date: 2017-08-24

THURSDAY, 24/8/17

Nanodrop concentrations

pSBC13 EutSMN 7 = 214.6

Table1

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	Water vol needed (uL)
1	pSB1C3_ EutSMN 7	214.6	22.1342031687	72.8657968313
2	pSB1C3_ EutSMN 7 x	214.6	3.4948741845	11.5051258155

EutLK - colony PCR (retry)

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Date: 2017-08-24

THURSDAY, 24/8/17

retry with VR and eutL Fwd

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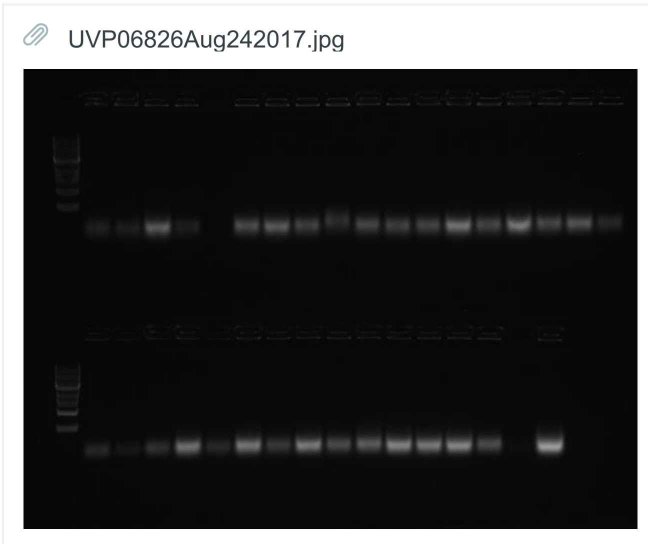


all negative results

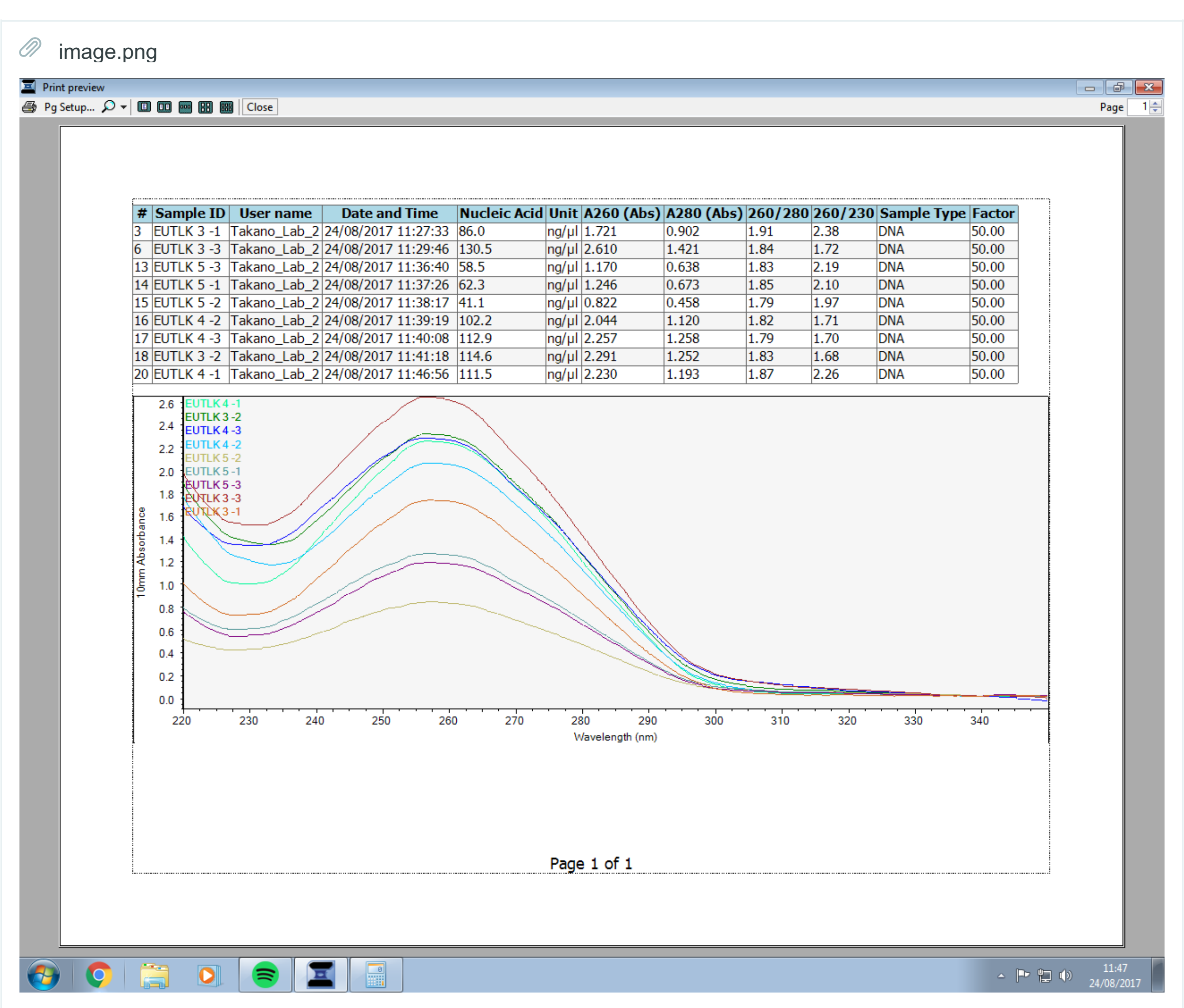
EutLK - miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-24

THURSDAY, 24/8/17

Miniprep

nanodrop results:



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
3	EUTLK 3 -1	Takano_Lab_2	24/08/2017 11:27:33	86.0	ng/µl	1.721	0.902	1.91	2.38	DNA	50.00
6	EUTLK 3 -3	Takano_Lab_2	24/08/2017 11:29:46	130.5	ng/µl	2.610	1.421	1.84	1.72	DNA	50.00
13	EUTLK 5 -3	Takano_Lab_2	24/08/2017 11:36:40	58.5	ng/µl	1.170	0.638	1.83	2.19	DNA	50.00
14	EUTLK 5 -1	Takano_Lab_2	24/08/2017 11:37:26	62.3	ng/µl	1.246	0.673	1.85	2.10	DNA	50.00
15	EUTLK 5 -2	Takano_Lab_2	24/08/2017 11:38:17	41.1	ng/µl	0.822	0.458	1.79	1.97	DNA	50.00
16	EUTLK 4 -2	Takano_Lab_2	24/08/2017 11:39:19	102.2	ng/µl	2.044	1.120	1.82	1.71	DNA	50.00
17	EUTLK 4 -3	Takano_Lab_2	24/08/2017 11:40:08	112.9	ng/µl	2.257	1.258	1.79	1.70	DNA	50.00
18	EUTLK 3 -2	Takano_Lab_2	24/08/2017 11:41:18	114.6	ng/µl	2.291	1.252	1.83	1.68	DNA	50.00
20	EUTLK 4 -1	Takano_Lab_2	24/08/2017 11:46:56	111.5	ng/µl	2.230	1.193	1.87	2.26	DNA	50.00

Restriction Digest

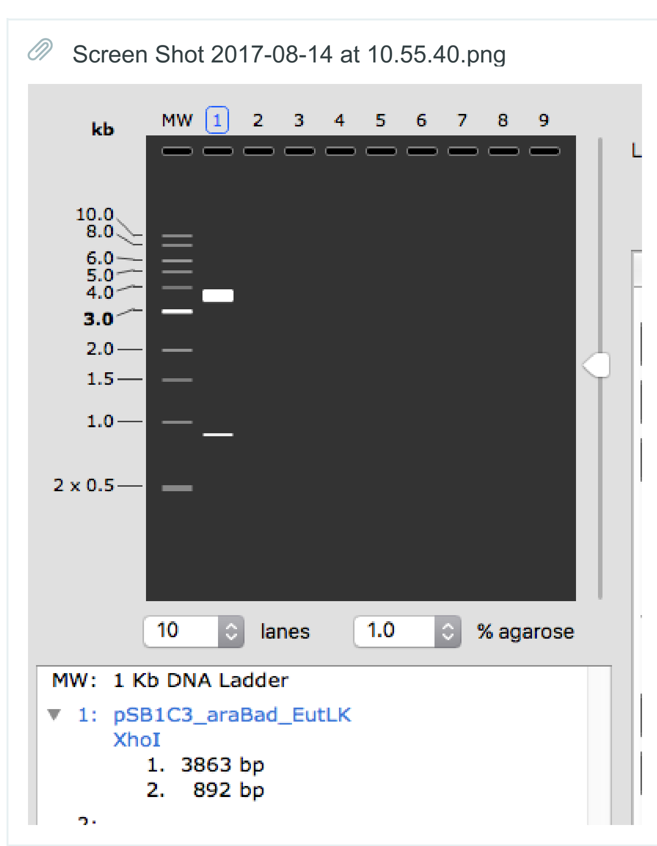
- digest 1000ng with 1uL **xhol,** 2uL cutsmart

Table1				
	Construct	Stock conc (ng/ul)	Vol of DNA needed for 1000ng (uL)	Vol of water needed to make 20uL (uL)
1	pSB1C3-EutLK 3	110	9.0909090909	8.4090909091
2	pSB1C3-EutLK 4	109	9.1743119266	8.3256880734
3	pSB1C3-EutLK 5	54	18.5185185185	-1.0185185185

- 1hr 37C

Run gel
predicted:

Screen Shot 2017-08-14 at 10.55.40.png



result:

UVP06823Aug242017.jpg

- shows wrong bands - shows what would happen if we just had the backbone with no stuffer gene - even though the colony PCR showed positive results :(
- retry the colony PCR with different primers..

EutLK - sequencing

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-24

THURSDAY, 24/8/17

need to submit:

- 15uL DNA at 50ng/uL, and 10uL extra for each additional read
- 10uL primer at 10uM, and 5uL extra for each additional read

EutLK 3, 4, 5 for 6 reads each = **65uL at 50ng/uL**

Table1

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	water vol needed (uL)
1	pSB1C3-EutLK 3	110	29.5454545455	35.4545454545
2	pSB1C3-EutLK 4	109	29.8165137615	35.1834862385
3	pSB1C3-EutLK 5	54	60.1851851852	4.8148148148

using primers:
VR, VF2, eutL fwd, eutL rev, araC fwd, araC rev (3 times) = **20uL at 10uM**

EutS/MN/SMN - IPTG/tet induction - repeat Alice

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-24

THURSDAY, 24/8/17

- cultures of S, MN, grown overnight used
- Inoculate flask of 50mL LB (+chl) with 200uL of the overnight
- Grow at 37C until reaches OD 0.5
- No Samples used for EutSMN as sample 5 was used. Sequencing has shown that EutSMN sample 5 lacks insert.
- Sample 7 of EutSMN needed to test combination of IPTG and tetracycline.

Table3

	Time (mins)	OD - S	OD - S+	OD - MN	OD - MN+
1	60	0.0087	0.034	0.037	0.037
2	140	0.137	0.274	0.336	0.320
3	180	0.200	0.562	0.796	0.780

- Induce: 250uM IPTG for EutS, 100nm tetracycline to EutMN

Table2

	Inducer	Stock conc (mM)	Desired conc (uM)	Vol to add to 50ml (uL)
1	IPTG	100	250	125
2	Tetracycline	0.214	0.1	23.364485…

- Induced at 3pm (Harvest at 11am next day, take OD reading aswell). Expressed for 10 hours at 20 °C.

Table4

	Flask No	Construct	Inducer + conc
1	1	eutS	none
2	2	eutS	250uM IPTG
3	3	eutMN	none
4	4	eutMN	100nM Tet

Table5			
		OD when harvest at 20hr (1 in 10 dilution)	OD
1	S	0.285	2.85
2	S+	0.18	1.8
3	MN	0.195	1.95
4	MN+	0.166	1.66

these values would be expected (last try werent)

Harvest cells:

- split culture into 2 falcon tubes (~25ml in each)
- centrifuge for 10min at 8000rpm
- pour off supernatant
- resuspend pellet in 1.25ml buffer (on ice)
- pool samples (on ice)
- sonicate each sample at 6mins, 30%, 50% power
- split into two 2ml eppendorf tubes (= 2 samples)
- centrifuge on max at 10min
- sepearate sol and insol fractions
- resuspend insol in same vol water (same vol as sol sample)
- add equal amounts sample and SDS loading buffer (30uL + 30uL)
- boil in PCR machine (95C 10 min)
- load 5uL and run (2 gels - one for WB)

order on gel = Ladder, S-, S+, S-(insol), S+(insol), MN-, MN+, MN-(insol), MN+(insol)

order on western = S-, Ladder, S+, S-(insol), S+(insol), MN-, MN+, MN-(insol), MN+(insol)

eutS, MN, SMN - SDS PAGE

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-08-23 to 2017-08-24

THURSDAY, 24/8/17

1. load and run

eutSMN - RD gel

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-24

THURSDAY, 24/8/17

Gel calcs - running 1000ng				
	Construct	Concentration (ng/uL)	Vol for 1000ng	Vol water to use
1	Eut SMN 5	155.7	6.4226075787	11.0773924213
2	Eut SMN 7	201	4.9751243781	12.5248756219

1 - with NcoI
2 - with EcoRI + PstI

UVP06820Aug242017.jpg

2 = SMN 5 + NcoI, 3 = SMN 5 + EcoRi/PstI, 4 = 7 + N, 5 = 7 + EP ?check order?

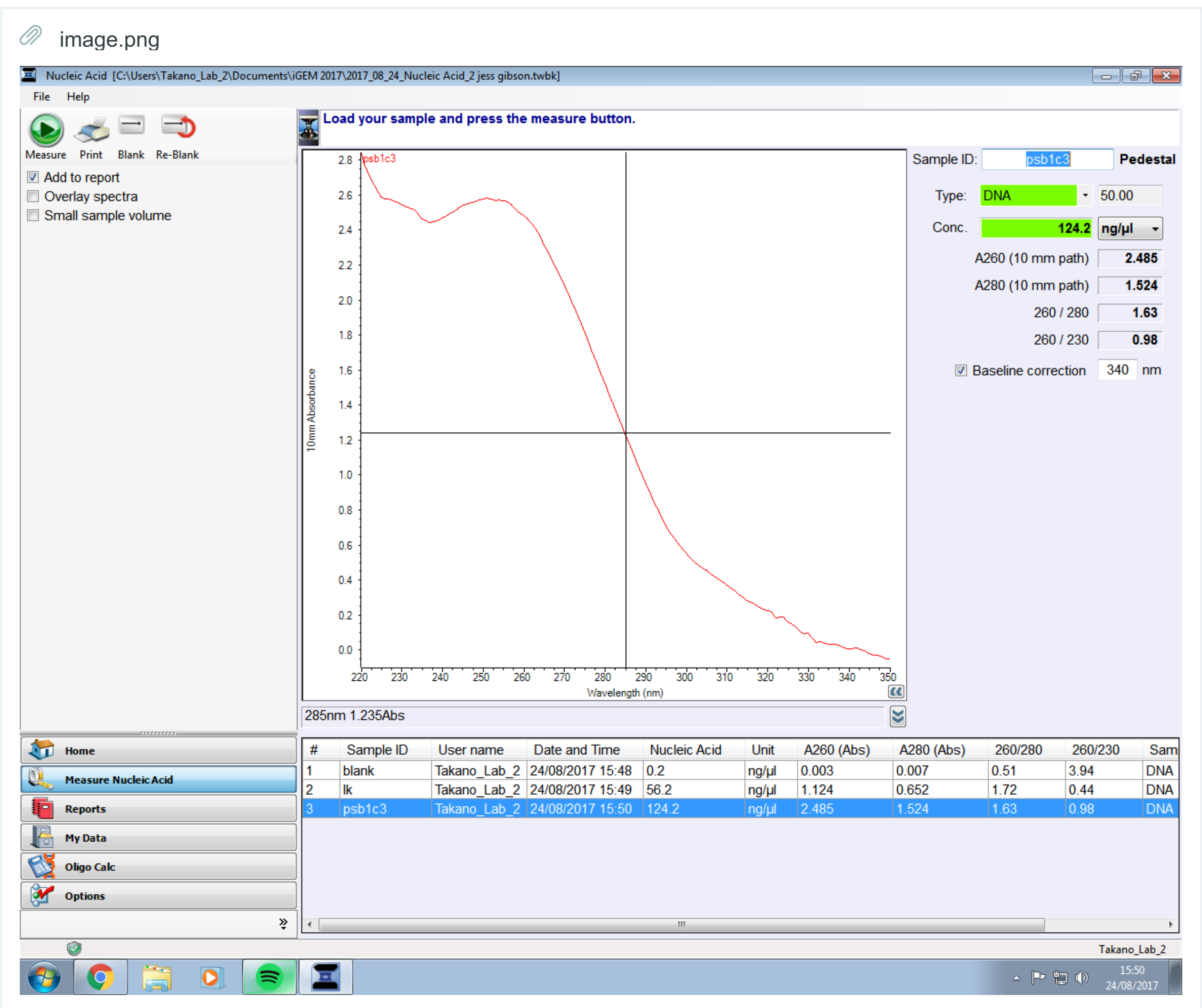
results = SMN 7 looks correct - send for seq

eutlk - gibson + transformation

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-24

THURSDAY, 24/8/17

image.png



3:1 = 198.4ng LK + 50ng pSB1C3 (0.12pmols + 0.04pmols)

(limited as only have a little bit of LK)

LK is at 56.2ng/ul

pSB1C3 is at 124.2ng/uL

Table1

	A	B
1	gibson mix (x2)	5
2	water	1.1
3	insert	3.5
4	vector	0.4

15min at 50C

transform

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1µl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins

13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.

14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before use

15. Spun down into a pellet at 5000rpm for 2 minutes

16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC

17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously

18. Incubate overnight at 37C

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
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Preparation of chemical competent cells:

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16. Removed 800µl of supernatant and resuspended in remaining 200µl of SOC
17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200µl of the cells into the agar plates made previously
18. Incubate overnight at 37C

eutlk - pcr amplification + gel ex

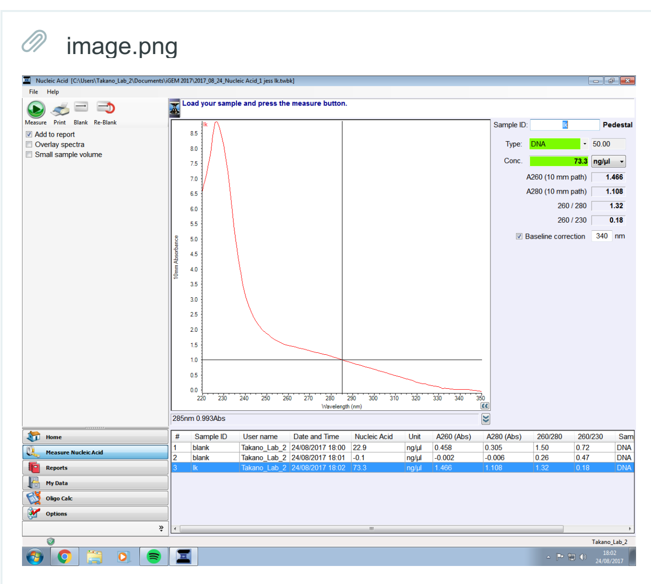
Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Date: 2017-08-24

THURSDAY, 24/8/17

image.png



EutLK (gibson) - colony PCR

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-08-25

FRIDAY, 25/8/17

Table1

	A	B	C
1	**Reaction vol:**	**10**	
2	**Number Reactions:**	1	**40**
3	10 uM VR Primer	0.2	8
4	10 uM eutL Fwd Primer	0.2	8
5	Template	0	0
6	2x OneTaq Mix	5	200
7	ddH20	4.6	184
8			

Table2

	A	B	C
1		**Temp**	**Time**
2	**Initial Denaturating**	95 oC	30 s
3	**30 cycles:**	95 oC	15-30s
4		~ 55 oC	15-60 s
5		68 oC	1 mins
6	**Final Extension**	68 oC	5 mins
7	**Hold**	4-10 oC	Inf

run gel

eutLK - miniprep, RD, gel

Project: Manchester iGEM 2017 Shared Project
Authors: Alice Fraser
Date: 2017-08-29

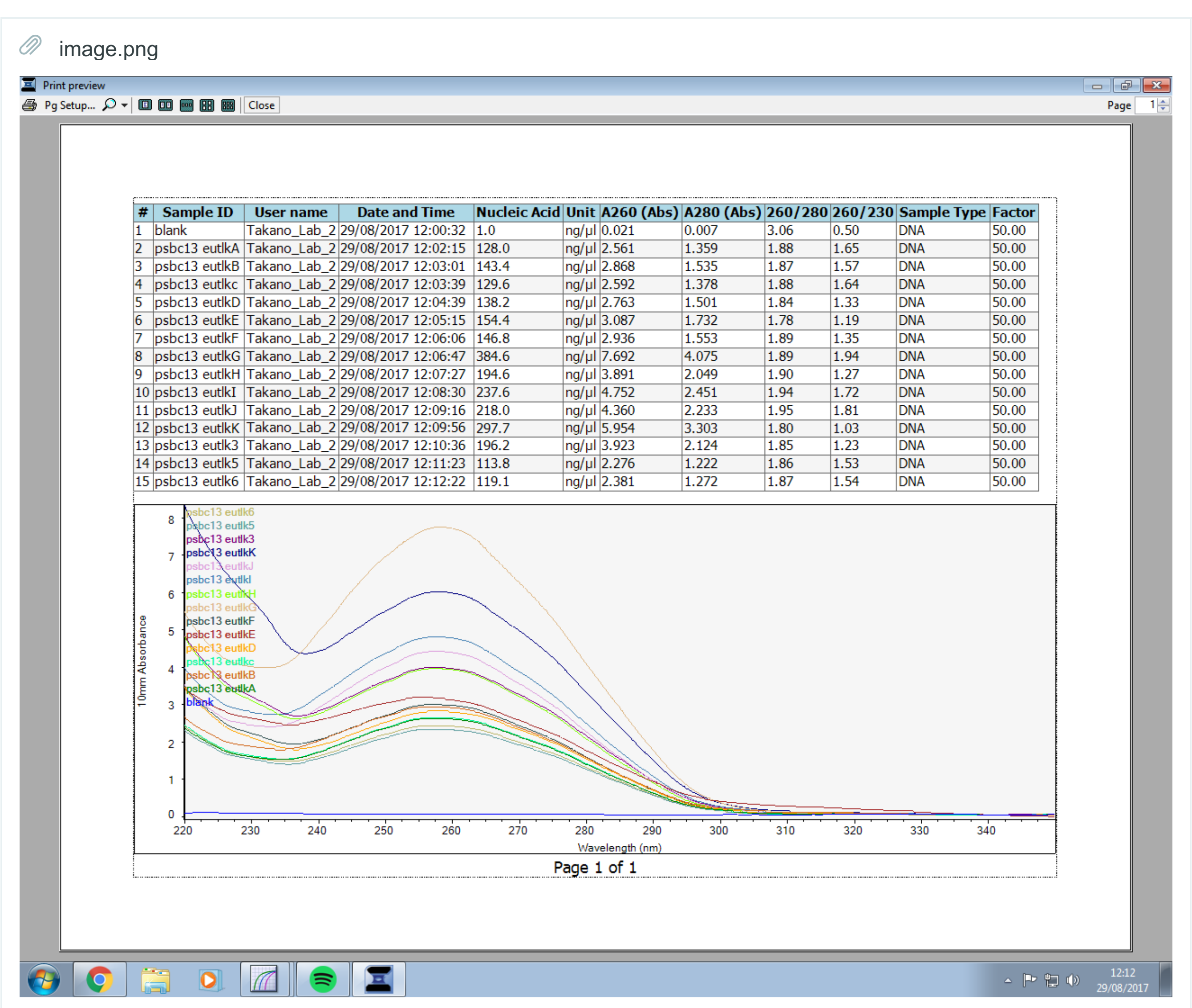
TUESDAY, 29/8/17

Miniprep

of overnights will has done

nanodrop results:

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	blank	Takano_Lab_2	29/08/2017 12:00:32	1.0	ng/µl	0.021	0.007	3.06	0.50	DNA	50.00
2	psbc13 eutlkA	Takano_Lab_2	29/08/2017 12:02:15	128.0	ng/µl	2.561	1.359	1.88	1.65	DNA	50.00
3	psbc13 eutlkB	Takano_Lab_2	29/08/2017 12:03:01	143.4	ng/µl	2.868	1.535	1.87	1.57	DNA	50.00
4	psbc13 eutlkc	Takano_Lab_2	29/08/2017 12:03:39	129.6	ng/µl	2.592	1.378	1.88	1.64	DNA	50.00
5	psbc13 eutlkD	Takano_Lab_2	29/08/2017 12:04:39	138.2	ng/µl	2.763	1.501	1.84	1.33	DNA	50.00
6	psbc13 eutlkE	Takano_Lab_2	29/08/2017 12:05:15	154.4	ng/µl	3.087	1.732	1.78	1.19	DNA	50.00
7	psbc13 eutlkF	Takano_Lab_2	29/08/2017 12:06:06	146.8	ng/µl	2.936	1.553	1.89	1.35	DNA	50.00
8	psbc13 eutlkG	Takano_Lab_2	29/08/2017 12:06:47	384.6	ng/µl	7.692	4.075	1.89	1.94	DNA	50.00
9	psbc13 eutlkH	Takano_Lab_2	29/08/2017 12:07:27	194.6	ng/µl	3.891	2.049	1.90	1.27	DNA	50.00
10	psbc13 eutlkI	Takano_Lab_2	29/08/2017 12:08:30	237.6	ng/µl	4.752	2.451	1.94	1.72	DNA	50.00
11	psbc13 eutlkJ	Takano_Lab_2	29/08/2017 12:09:16	218.0	ng/µl	4.360	2.233	1.95	1.81	DNA	50.00
12	psbc13 eutlkK	Takano_Lab_2	29/08/2017 12:09:56	297.7	ng/µl	5.954	3.303	1.80	1.03	DNA	50.00
13	psbc13 eutlk3	Takano_Lab_2	29/08/2017 12:10:36	196.2	ng/µl	3.923	2.124	1.85	1.23	DNA	50.00
14	psbc13 eutlk5	Takano_Lab_2	29/08/2017 12:11:23	113.8	ng/µl	2.276	1.222	1.86	1.53	DNA	50.00
15	psbc13 eutlk6	Takano_Lab_2	29/08/2017 12:12:22	119.1	ng/µl	2.381	1.272	1.87	1.54	DNA	50.00

Restriction digest

1000ng with BamHI

Table2

	Construct	Conc (ng/uL)	amount for 1000ng	water to add to reaction
1	pSB1C3-EutLK 3	196.2	5.0968399592	12.4031600408
2	pSB1C3-EutLK 5	113.8	8.7873462214	8.7126537786
3	pSB1C3-EutLK 6	119.1	8.3963056255	9.1036943745
4	pSB1C3-EutLK A	128.0	7.8125	9.6875
5	pSB1C3-EutLK B	143.4	6.9735006974	10.5264993026
6	pSB1C3-EutLK C	129.6	7.7160493827	9.7839506173
7	pSB1C3-EutLK D	138.2	7.2358900145	10.2641099855
8	pSB1C3-EutLK E	154.4	6.4766839378	11.0233160622
9	pSB1C3-EutLK F	146.8	6.8119891008	10.6880108992
10	pSB1C3-EutLK G	384.6	2.6001040042	14.8998959958
11	pSB1C3-EutLK H	194.6	5.1387461459	12.3612538541
12	pSB1C3-EutLK I	237.6	4.2087542088	13.2912457912
13	pSB1C3-EutLK J	218.0	4.5871559633	12.9128440367
14	pSB1C3-EutLK K	297.7	3.3590863285	14.1409136715

Table1

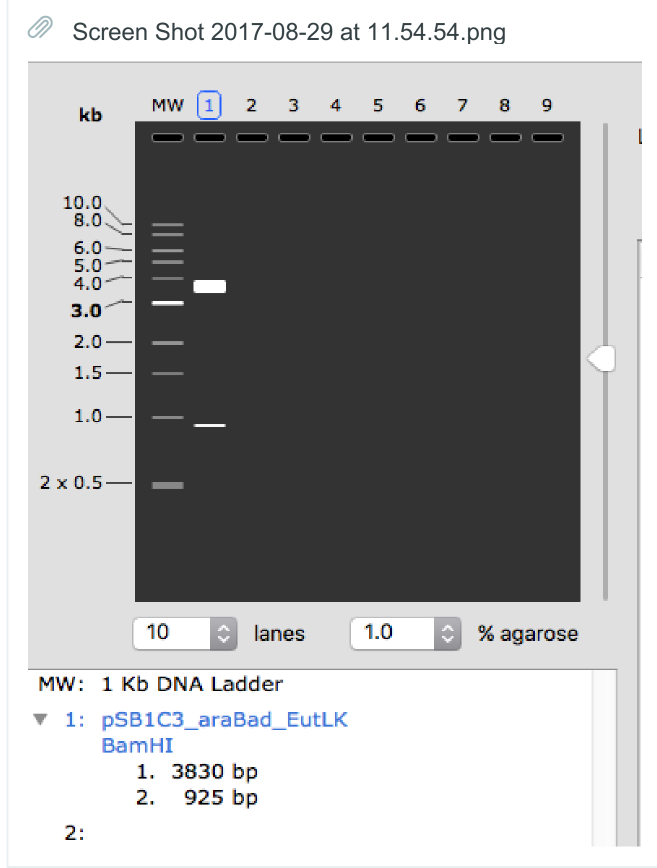
	A	B
1	BamHI	0.5
2	cutsmart	2
3	eutLK	table above
4	water	table above

- 37C for 1hr (just running on gel - no need to inactivate - BamHI cant be heat inactivated anyway)

Run gel

expected:

Screen Shot 2017-08-29 at 11.54.54.png



result:

Lanes Ladder, a,b,c,d,e,f,g,h,i,3,5,6,

UVP06838Aug292017.jpg

Eut LK miniprep, sequencing - G (Alice)

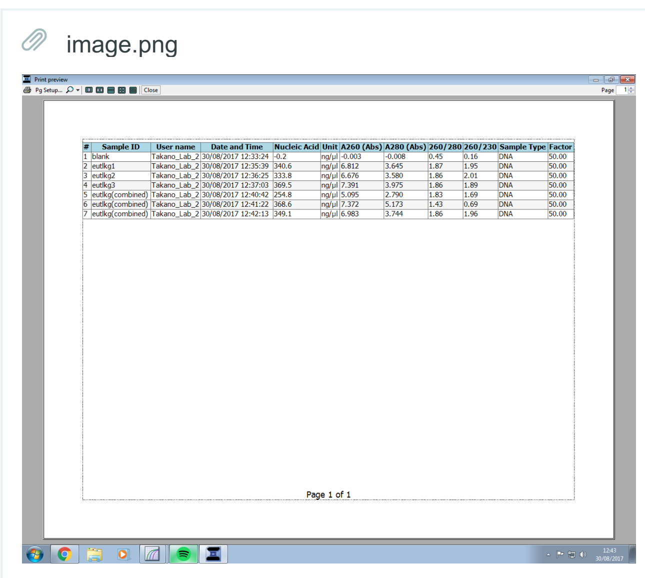
Project: Manchester iGEM 2017 Shared Project
Authors: Alice Fraser
Date: 2017-08-30

WEDNESDAY, 30/8/17

- Colonies grown overnight (G from index plate) as this was the only positive result shown on gel yesterday.

Nanodrop results

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	blank	Takano_Lab_2	30/08/2017 12:33:24	-0.2	ng/µl	-0.003	-0.008	0.45	0.16	DNA	50.00
2	eutlkg1	Takano_Lab_2	30/08/2017 12:35:39	340.6	ng/µl	6.812	3.645	1.87	1.95	DNA	50.00
3	eutlkg2	Takano_Lab_2	30/08/2017 12:36:25	333.8	ng/µl	6.676	3.580	1.86	2.01	DNA	50.00
4	eutlkg3	Takano_Lab_2	30/08/2017 12:37:03	369.5	ng/µl	7.391	3.975	1.86	1.89	DNA	50.00
5	eutlkg(combined)	Takano_Lab_2	30/08/2017 12:40:42	254.8	ng/µl	5.095	2.790	1.83	1.69	DNA	50.00
6	eutlkg(combined)	Takano_Lab_2	30/08/2017 12:41:22	368.6	ng/µl	7.372	5.173	1.43	0.69	DNA	50.00
7	eutlkg(combined)	Takano_Lab_2	30/08/2017 12:42:13	349.1	ng/µl	6.983	3.744	1.86	1.96	DNA	50.00

Page 1 of 1

need to submit:

- 15uL DNA at 50ng/uL, and 10uL extra for each additional read
- 10uL primer at 10uM, and 5uL extra for each additional read

6 reads in total using primers listed below.
DNA at conc of 50ng/µl , total of **65 µl**

Table1

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	water vol needed (uL)
1	pSB1C3-EutLK G	349.1	9.3096533944	55.6903466056

using primers:
VR, VF2, eutL fwd, eutL rev, araC fwd, araC rev (3 times) = **10uL at 10uM**

Eut S/SMN + Tags

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-08-31 to 2017-09-04

THURSDAY, 31/8/17

-Digest
-Gel extraction
-Ligation
-Transformation
-DIGEST-
-First a digest was performed to remove EutS/SMN from the pSB1C3 backbone and also to prepare the pSB4A5 backbone for insertion.
-Using Snapgene, restriction sites EcoRI and PstI in the biobrick prefix were identified. These sites also cut pSB4A5 favourably

-LIGATION-
-Once the relevant digest products were extracted from the gel they were combined with a ligation.
-Negative control of just pSB4A5 was used.
-Reagents were combined as shown in the table:

Table1

	in ul	-ve	Eut S	Eut SMN
1	Buffer	5	5	5
2	Vector	1.5	1.5	1.5
3	Insert	0	3.5	3.5
4	Ligase	0.5	0.5	0.5
5	Water	3.5	0	0
6	Total	10.5	10.5	10.5

-TRANSFORMATION-
-1.6ul of ligation products were added into 16ul of ultra competent cells (not good to go over 10% apparently)
-Tubes were left in the 37°C shake incubator for 1 hour
-Whole volume was then transferred under asceptic conditions to an ampicillin agar plate and a spread plate was prepared.

EutS/MN/SMN - IPTG/tet/ induction (Alice)

Project: Manchester iGEM 2017 Shared Project
Authors: Alice Fraser
Date: 2017-08-31

THURSDAY, 31/8/17

- cultures of S, MN, SMN , LK grown overnight used (SMN sequencing now shows it is correct)
- Still waiting in sequencing data for LK
- Inoculate flask of 50mL LB (+chl) with 200uL of the overnight
- Grow at 37C until reaches OD 0.5
- Sample 7 of EutSMN needed to test combination of IPTG and tetracycline.

Table3

	Time (mins)	OD S -	OD S +	OD - MN	OD - MN+	OD SMN -	OD SMN +
1	80 (1pm)	0.071	0.082	0.091	0.05	0.086	0.078
2	160 (2.20)	0.35	0.36	0.43	0.274	0.38	0.38
3	200 (3pm)	0.53	0.55	0.45	0.46	0.57	0.54

- Induce: 250uM IPTG for EutS, 100nm tetracycline to EutMN and both form Eut SMN

Table2

	Inducer	Stock conc (mM)	Desired conc (uM)	Vol to add to 50ml (uL)
1	IPTG	100	250	125
2	Tetracycline	0.214	0.1	23.364485…

- Induced at 3pm (Harvest at 11am next day, take OD reading aswell). Expressed for 20 hours at 20 °C.

Table4

	Flask Name	Construct	Inducer + conc	OD600 at 20hr harvest (1 in 10 dilution)	OD600 from plate reader	GFP
1	S-	eutS	none	0.39	v	d
2	S+	eutS	250uM IPTG	0.044		
3	MN-	eutMN	none	0.36		
4	MN+	eutMN	100nM Tet	0.39		
5	SMN-	eutSMN	none	0.0047		
6	SMN+	eutSMN	100nmTet and 250uM IPTG	0.076		

SDS page gel 10% and 12% + western blots

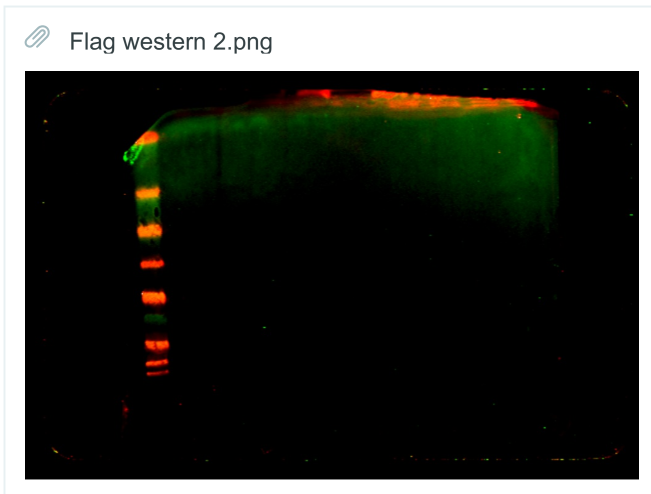
Project: Manchester iGEM 2017 Shared Project
Authors: Alice Fraser
Date: 2017-09-01

FRIDAY, 1/9/17

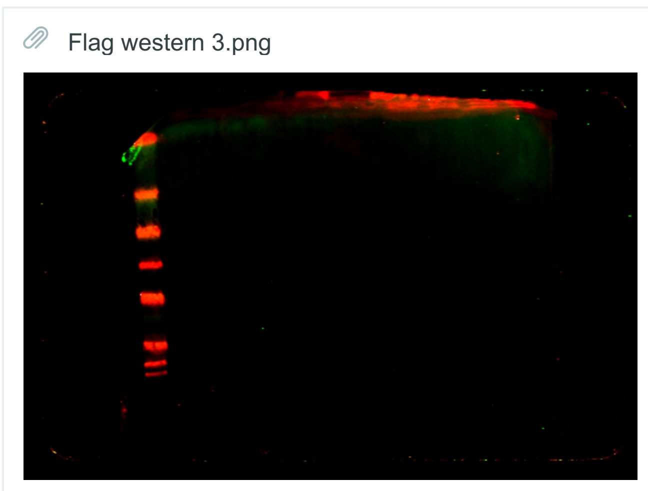
- Soluble and insoluble fractions from induction of Eut S and MN used ran on SDS page 12% gel (better for seeing smaller proteins)
- Western blot using anti flag and anti his antibodies.
- I-bind system used (protocol|: https://tools.thermofisher.com/content/sfs/manuals/ibind_qrc.pdf)

Western Blot results
Flag

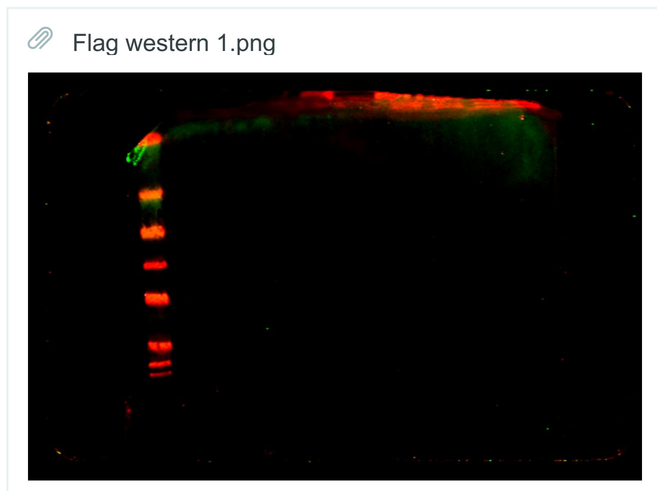
Flag western 2.png



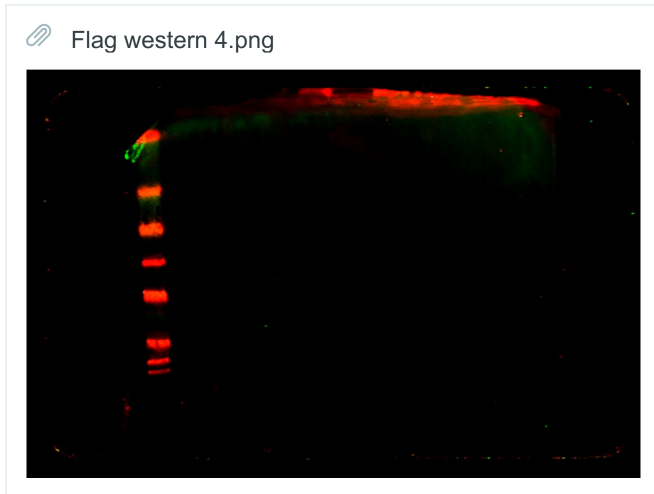
Flag western 3.png



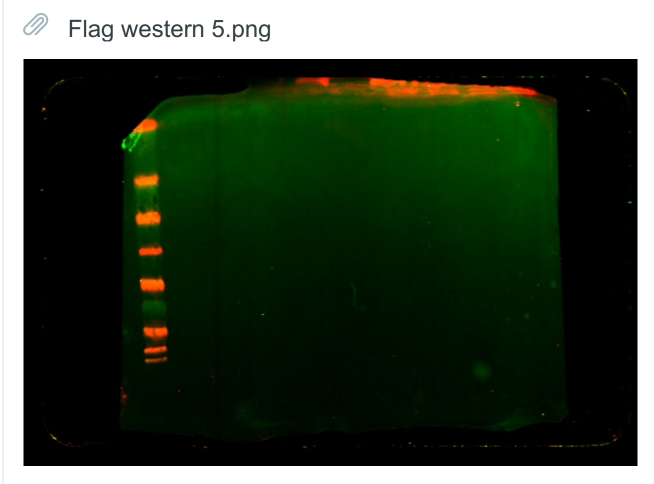
Flag western 1.png



Flag western 4.png

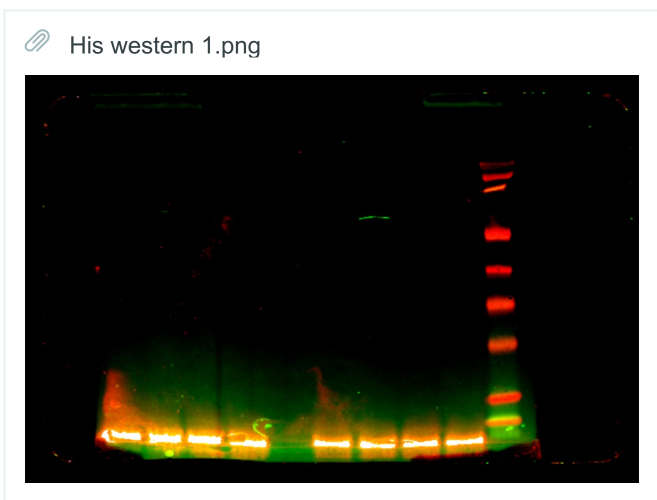


Flag western 5.png

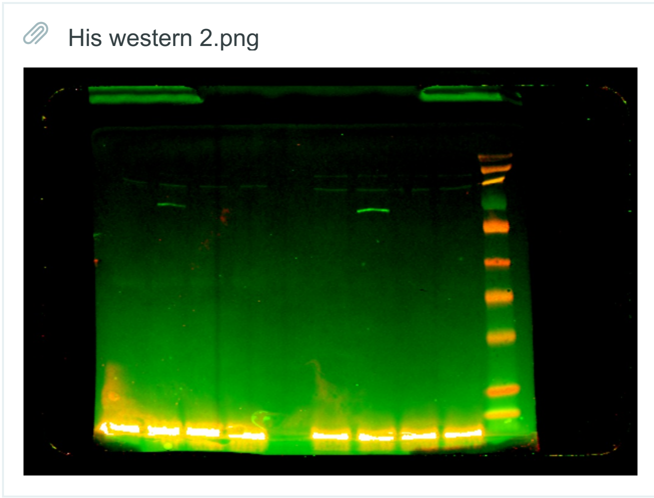


His

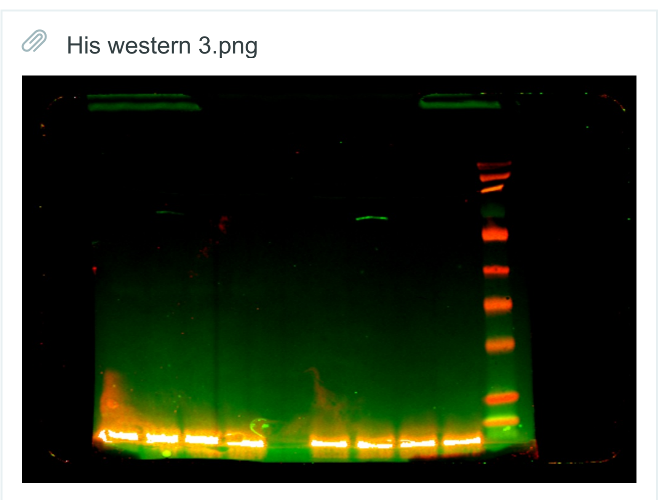
His western 1.png



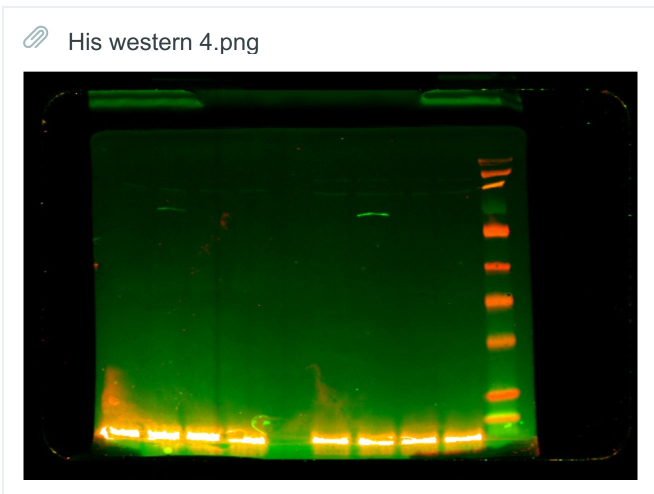
His western 2.png



His western 3.png



His western 4.png



Assemble LKSMN (in pSB1C3)

Project: Manchester iGEM 2017 Shared Project
Authors: Amber Hall
Dates: 2017-09-04 to 2017-09-20

MONDAY, 4/9/17

- Digest eutLK with **EcoRI + SpeI**
- Digest pSB1C3-EutSMN with **EcoRI + XbaI**
- LK at 367ng/uL
- SMN at 158ng/uL

pSB1C3-eutLK digest

	A	B
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	0.5uL each
3	MilliQ water	14.3
4	DNA	2.7

pSB1C3-eutSMN digst

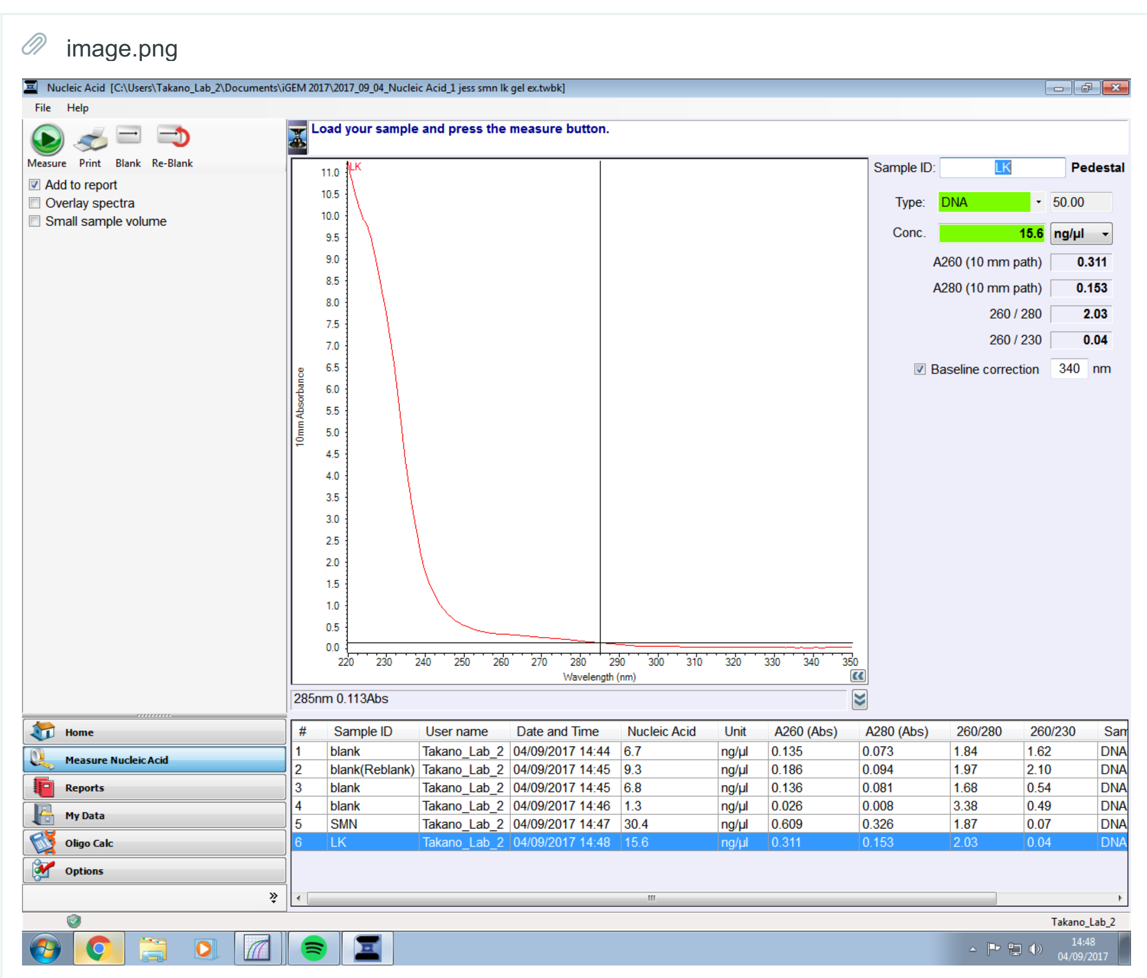
	A	B
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	0.5uL each
3	MilliQ water	10.7
4	DNA	6.3

- Run gel and extract LK at 2708bp and pSB1C3 at 6383bp

UVP06882Sept42017.jpg

nanodrop results:

image.png



- Ligation (with negative control)

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: SMN (50ng)	1.5
4	Insert DNA: LK (63.64ng)	4
5	Nuclease-free water	11.5
6	T4 DNA Ligase	1
7	Total	20

room temp for 1hr, 65C for 10min

- Transformation
- Plate

Single Temperature Restriction Digest

Introduction

More information from NEB can be found here.

Double Digests can be designed using NEB's Double Digest Finder.

See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

NEBcloner will help guide your reaction buffer selection when setting up double digests.

Tips:
- By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes, but often a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.
- Keep enzymes on ice, and add last to reaction mix
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- In some cases, incubation time can be decreased with an excess of enzyme, or increase (eg. to overnight) by using less enzyme
- Store most restriction enzymes a -20C. For a few enzymes, storage at -70°C is recommended for periods longer than 30 days
- 10X NEBuffers should also be stored at -20°C
- Dpn1: cut template vector but not PCR product, so will reduce background
- rSAP: heat inactivatible alkaline phosphatase - will remove 5' phosphate from vector so limit self ligation. *only add rSAP to either the vector or insert, NOT BOTH - you need one 5' P to ligate*

Control experiments:
- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing

Materials

- 10X NEB CutSmart Buffer
- Restriction enzymes
- MilliQ water
- DNA sample

Procedure

Reaction Mix

1. Set up the following

Table2

	Component	Vol
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	1uL each
3	MilliQ water	up to 20uL
4	DNA	Conc needed varies depending on purpose

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

Incubation

4. 1hr 37C, 20min to inactivate (temperature depends on enzyme used - See the NEBuffer Activity/Performance Chart with Restriction Enzymes)

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA
- Insert DNA
- Nuclease-free water

Procedure

Tips

1. T4 DNA Ligase should be added last
2. *Aliquote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.*
3. Negative control - no insert DNA, replace with water

Set up the T4 DNA Ligase Reaction

Note: The table shows a ligation using a molar ratio of 1:3 vector to insert for vector of 4kb and insert of 1kb. *Use NEB calculator to calculate molar ratios.*

4. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.
5. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: 50 ng (0.020 pmol)	
4	Insert DNA: 37.5 ng (0.060 pmol)	
5	Nuclease-free water	17
6	T4 DNA Ligase	1
7	Total	20

6. Gently mix the reaction by pipetting up and down and microfuge briefly.
7. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 30 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.
8. Heat inactivate at 65C for 10 minutes.
9. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

   Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

Chemical Transformation

Introduction

Materials

- LB medium (Luria Bertrani medium = rich media to grow bacteria)
- TSS buffer (to prepare chemically competent cells)
- S.O.C. medium (helps obtain the maximal transformation efficiency)
- LB agar (gel where bacteria can grow)
- Antibiotic stock: Chloramphenicol (CAL) at stock concentration 25mg/ml

Procedure

Preparation of chemical competent cells:

TSS Competent E. coli Preparation

1. Inoculate DH5α cells into 50mL LB and incubate at 37°C
2. Monitor growth every 30mins by measuring optical density at 600nm (OD600); until reach OD600 = 0.4-0.6
3. Once the proper optical density has been achieved, aliquot 50mL into PP centrifuge tubes and centrifuge under 4000RCF for 10 min at 4°C.
4. Resuspend each tube in 5mL of pre-chilled TSS buffer with gentle vortexing (≤50%).
5. Chill TSS suspended cells on ice for 15 min. Prepare/label 50x 1.5mL PP tubes (snap-cap) during this time.
6. Distribute 200 μL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
7. Cells can be used immediately, or stored at -80°C.

LB Agar plates preparation:

Prepare LB containing chloramphenicol (CAL) (at 25μg/ml)

8. Melt LB in micorwave (defrost setting for 15mins)
9. Cool LB by running cold water over
10. Stock of 25mg/ml CAL → so add 400μl CAL to 400ml LB = 25ug/ml CAL in LB
11. Pour plates (in fume hood) and allow to solidify

Chemical transformation:

Not fully understood how this works: heat shock triggers cells to open pores and so increase DNA uptake (promotes transformation)

12. Add 1μl of DNA to 50ul of competent cells, mix well and place on ice for at least 30mins
13. Heat shock cells at 42°C for 30secs, followed by 2min incubation on ice.
14. Add 1ml of SOC medium to the cells and incubate for 45min at 37°C at 180rpm (to allow (antiobiotic resistance) protein expression)

    SOC should be stored at 4°C, but can be warmed to room temperature before use
15. Spun down into a pellet at 5000rpm for 2 minutes
16. Removed 800μl of supernatant and resuspended in remaining 200μl of SOC
17. Plate and spread (glass spreader sterilised over a flame and in ethanol) 200μl of the cells into the agar plates made previously
18. Incubate overnight at 37C

Eut S/MN/SM/LK induction

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-09-04 to 2017-09-05

MONDAY, 4/9/17

New transformants used for overnights.

- cultures of S, MN, SMN , LK grown overnight used
- Still waiting in sequencing data for LK
- Inoculate flask of 50mL LB (+chl) with 200uL of the overnight
- Grow at 37C until reaches OD 0.5
- Sample 7 of EutSMN needed to test combination of IPTG and tetracycline.

Table3

	Time (mins)	OD S -	OD S +	OD - MN	OD - MN+	OD SMN -	OD SMN +	
1	90	0.0161	0.0160	0.0077	0.0075	0.139	0.0122	
2	150	0.067	0.60	0.024	0.024	0.059	0.049	
3	220	0.21	0.21	0.081	0.15	0.20	0.17	
4	280	0.43	0.42	0.18	0.23	0.45	0.44	
5	360 (MN only)	induced	induced	0.52	0.48	induced	induced	i

- Induce: 250uM IPTG for EutS, 100nm tetracycline to EutMN, both IPTG and tetracycline for Eut SMN and 1mM of arabinose for LK.

Table2

	Inducer	Stock conc (mM)	Desired conc (uM)	Vol to add to 50ml (uL)
1	IPTG	100	250	125
2	Tetracycline	0.214	0.1	23.364485…
3	Arabinose	100mM	1mM	500uL

- S, SMN and LK Induced at 4pm , LK induced at 5.20pm
- Expressed for 20 hours at 20 °C.
- Harvest S, SMN and LK at 12pm, take OD reading and GFP reading in triplicate. Havest MN at 1.20pm. See layout below
- 1 in 10 dilution will be needed to take OD reading

Plate reader layout

*** Will need to get final mn readings seperately later (can use same plate reader just use clean wells)**

Well1												
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	put 200ul in											
D												
E	s-	s+	smn-	smn+	lk-	lk+	blank (LB)	**mn-**	**mn+**			
F	s-	s+	smn-	smn+	lk-	lk+	blank (LB)	**mn-**	**mn+**			
G	s-	s+	smn-	smn+	lk-	lk+	blank (LB)	**mn-**	**mn+**			
H												

OD600 and GFP readings after 20hours

Table4					
	Flask Name	Construct	Inducer + conc	OD600 from plate reader	GFP
1	S-	eutS	none	1.428	210
2	S+	eutS	250uM IPTG	1.378	208
3	MN-	eutMN	none	1.43	211
4	MN+	eutMN	100nM Tet	1.429	208
5	SMN-	eutSMN	none	1.43	215
6	SMN+	eutSMN	100nmTet and 250uM IPTG	0.353	312
7	LK-	eutLK	none	1.319	218
8	LK+	eutLK	500uL arabinose	0.312	306

Harvest cells:

- split culture into 2 falcon tubes (~25ml in each)
- centrifuge for 10min at 8000rpm
- pour off supernatant (can be frozen at this point if necessary)
- resuspend pellet in 1.25ml buffer (on ice)
- pool samples (on ice)
- sonicate each sample at 2mins, 30%, 40% power
- split into two 2ml eppendorf tubes (= 2 samples) - one lot were frozen at this point
- centrifuge on max at 10min
- sepearate sol and insol fractions
- resuspend insol in same vol water (same vol as sol sample)
- add equal amounts sample and SDS loading buffer (30uL + 30uL)

Eut S/SMN + Tags

Project: Manchester iGEM 2017 Shared Project

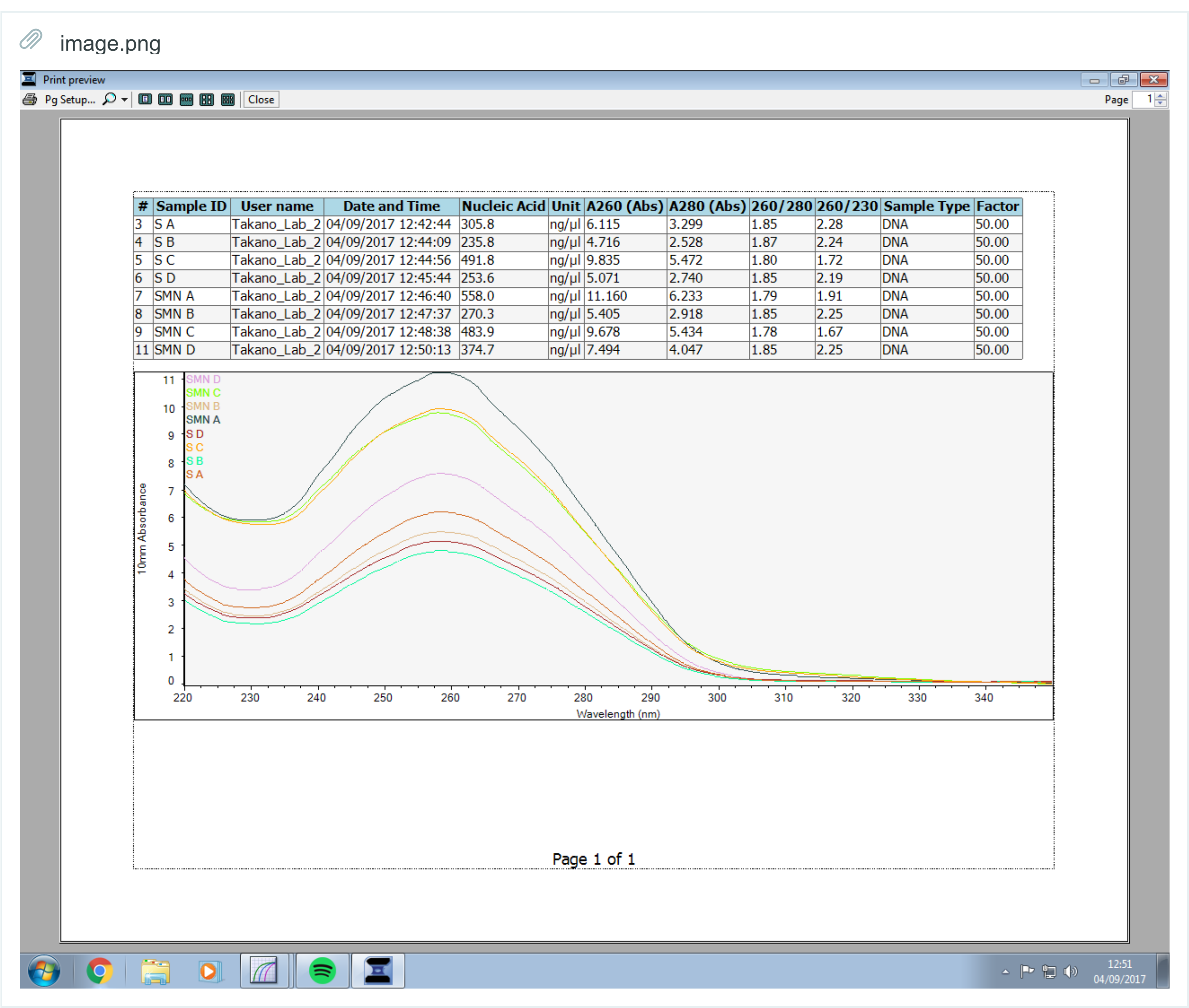
Authors: Jessica Burns

Dates: 2017-08-31 to 2017-09-04

MONDAY, 4/9/17

-From overnights, 4 colonies from EutS and 4 from EutSMN were minipreped:

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
3	S A	Takano_Lab_2	04/09/2017 12:42:44	305.8	ng/µl	6.115	3.299	1.85	2.28	DNA	50.00
4	S B	Takano_Lab_2	04/09/2017 12:44:09	235.8	ng/µl	4.716	2.528	1.87	2.24	DNA	50.00
5	S C	Takano_Lab_2	04/09/2017 12:44:56	491.8	ng/µl	9.835	5.472	1.80	1.72	DNA	50.00
6	S D	Takano_Lab_2	04/09/2017 12:45:44	253.6	ng/µl	5.071	2.740	1.85	2.19	DNA	50.00
7	SMN A	Takano_Lab_2	04/09/2017 12:46:40	558.0	ng/µl	11.160	6.233	1.79	1.91	DNA	50.00
8	SMN B	Takano_Lab_2	04/09/2017 12:47:37	270.3	ng/µl	5.405	2.918	1.85	2.25	DNA	50.00
9	SMN C	Takano_Lab_2	04/09/2017 12:48:38	483.9	ng/µl	9.678	5.434	1.78	1.67	DNA	50.00
11	SMN D	Takano_Lab_2	04/09/2017 12:50:13	374.7	ng/µl	7.494	4.047	1.85	2.25	DNA	50.00

Table2

	Construct]	conc (ng/uL)	amount for 1000ng DNA (uL)	amount of water for 20uL reaction (uL)
1	S A	305	3.2786885246	13.7213114754
2	S B	235	4.2553191489	12.7446808511
3	S C	491	2.0366598778	14.9633401222
4	S D	253	3.95256917	13.04743083
5	SMN A	558	1.7921146953	15.2078853047
6	SMN B	270	3.7037037037	13.2962962963
7	SMN C	483	2.0703933747	14.9296066253
8	SMN D	374	2.6737967914	14.3262032086

digets

	A	B
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	0.5uL each
3	MilliQ water	Above
4	DNA	Above

Assemble LKSMN (in pSB1C3)

Project: Manchester iGEM 2017 Shared Project

Authors: Amber Hall

Dates: 2017-09-04 to 2017-09-20

TUESDAY, 5/9/17

- was plated on wrong plates (think have no antibiotic)
- saved ligation so just retransform

Eut S/MN/SM/LK induction

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-09-04 to 2017-09-05

TUESDAY, 5/9/17

- boil in PCR machine (95C 10 min)

SDS page gel

- load 30uL sol + 20uL ins (for those with low od) - run 7.5uL sol and 5uL insol for others - and run **(we ran for too long - 15 marker is off the gel)**
- Use 18% orf 12 % gel (not 10% as proteins are too small)
- Keep one gel for imaging (use coomassie blue)
- Take picture of gel
- *order for soluble sample SDS: Ladder, S-, S+, MN-, MN+, LK-, LK+, SMN-, SMN+, empty*
- *order for in soluble sample SDS: empty, Ladder, S-, S+, MN-, MN+, LK-, LK+, SMN-, SMN+*
- *order for western blots: Ladder S+, MN+, LK+, SMN+, empty, S+sol, MN+sol, LK+sol, SMN+sol*

Western Blot

- 2 x Western Blots Flag and His
- Using I-bind system (protocol|: https://tools.thermofisher.com/content/sfs/manuals/ibind_qrc.pdf)
- Take image of western blots

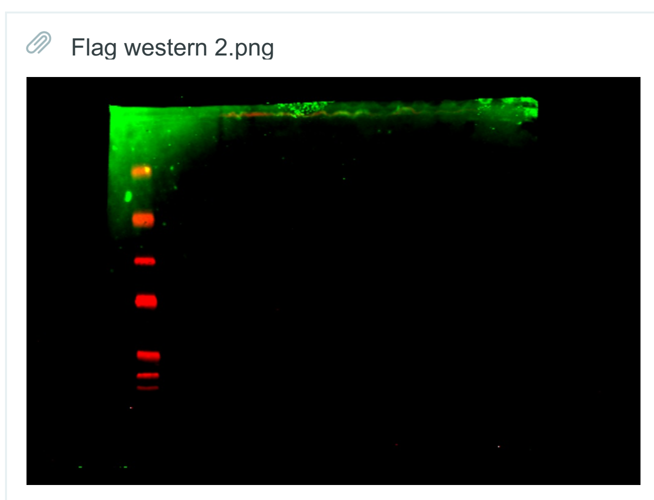
Predicted sizes of proteins and tags associated with them

Table1

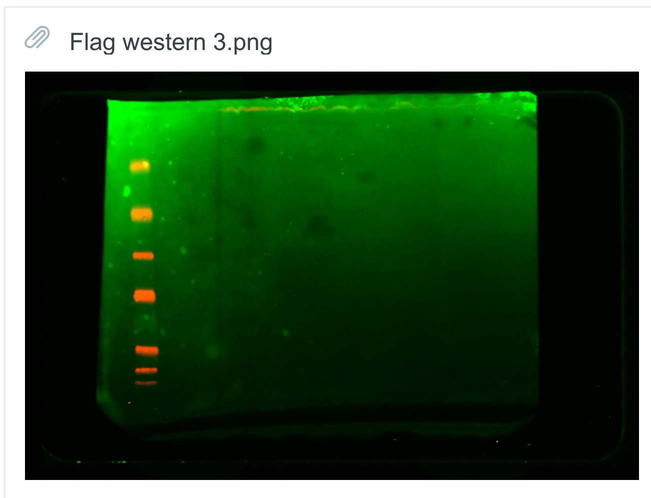
	Protein	Size kDa	Tag
1	S	12.9	N-terminal His
2	M	37.5 (GFP mutant attached to eut M)	N-terminal His tag (on end of GFP)
3	N	11.3	C-terminal Flag
4	L	24.0	N-terminal His
5	K	19.1	C-terminal Flag

saw nothing...

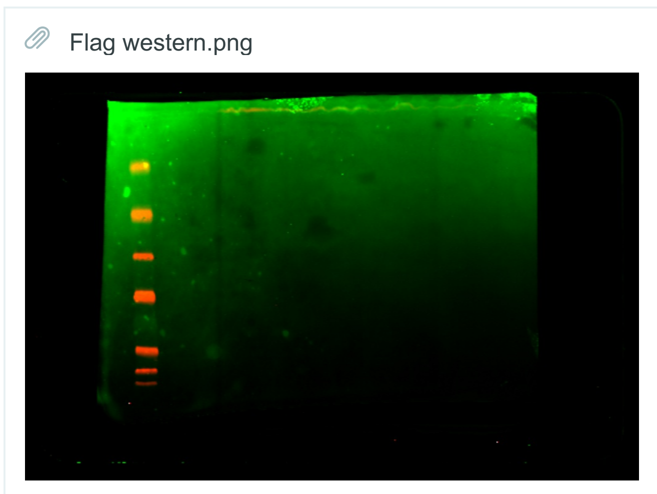
Flag western 2.png



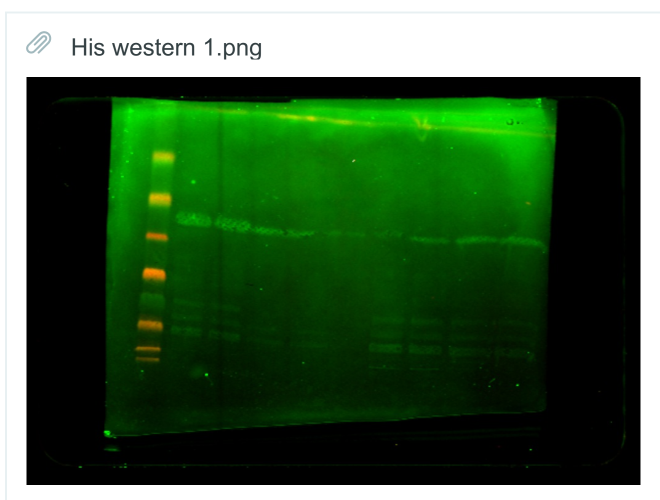
Flag western 3.png



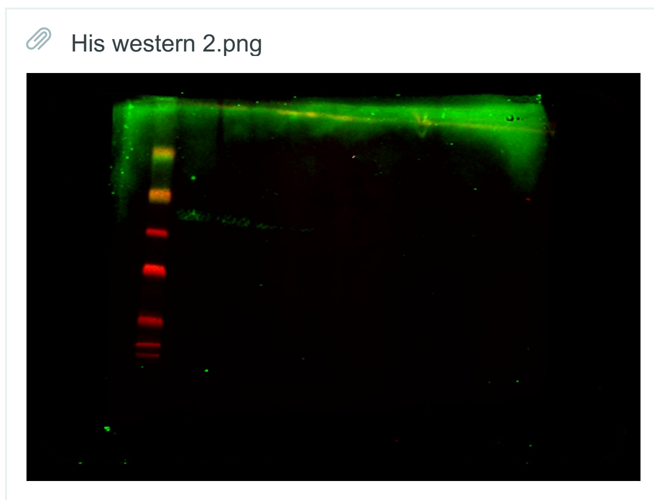
Flag western.png



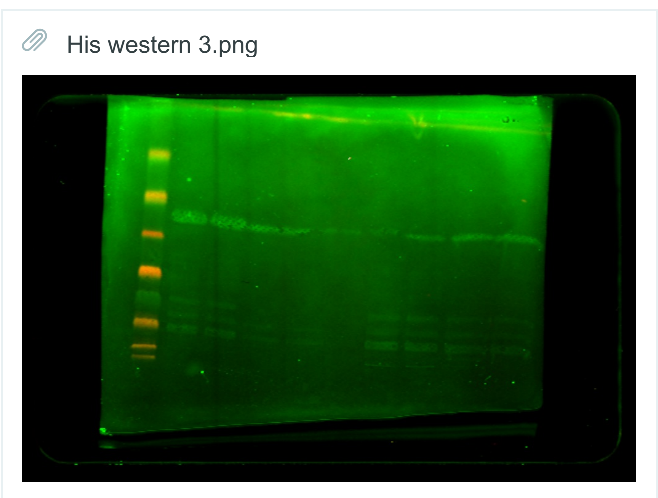
His western 1.png



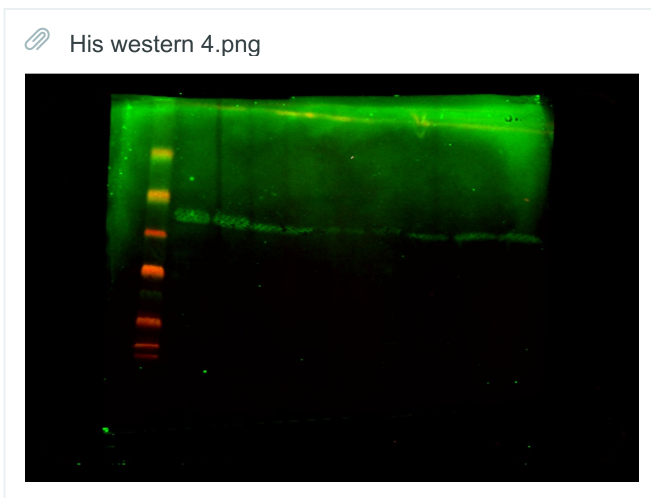
His western 2.png



His western 3.png



His western 4.png



Assemble LKSMN (in pSB1C3)

Project: Manchester iGEM 2017 Shared Project
Authors: Amber Hall
Dates: 2017-09-04 to 2017-09-20

WEDNESDAY, 6/9/17

results = no colonies

--> redo from begining:

restriction digest as above.

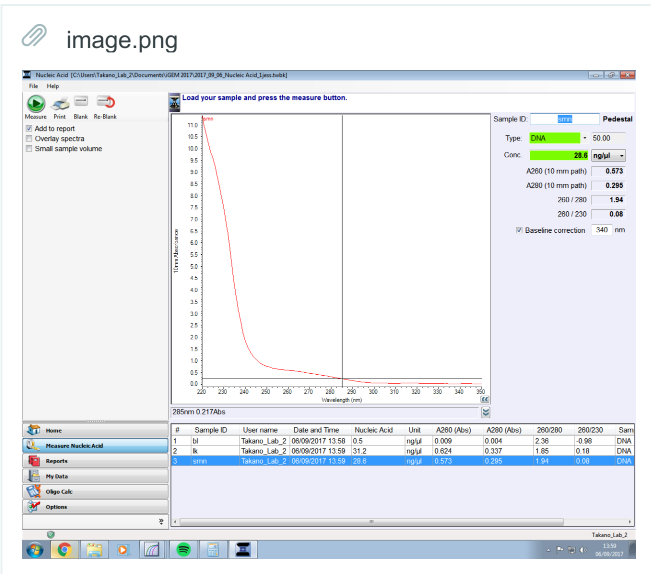
run gel:

UVP06893Sept62017.jpg

extract bands at 6383bp and 2708bp:

nanodrop:

image.png



ligation:

- room temp 2hr, 65C 10 min

Table2

	A	B
1	**Component**	**Volume (μl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA: SMN (50ng)	1.8
4	Insert DNA: LK (63.64ng)	2.05
5	Nuclease-free water	13.15
6	T4 DNA Ligase	1
7	Total	20

transformation.

Assemble LKSMN (in pSB4A5)

Project: Manchester iGEM 2017 Shared Project
Authors: Adam Hannaford
Date: 2017-09-07

THURSDAY, 7/9/17

- room temp for 1hr, 65C for 10minDigest eutLK with **EcoRI + SpeI**
- Digest pSB4A5-EutSMN with **EcoRI + XbaI**
- LK at 367ng/uL
- 4A5-SMN at 257

pSB1C3-eutLK digest

	A	B
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	0.5uL each
3	MilliQ water	14.3
4	DNA	2.7

pSB4A5-eutSMN digst

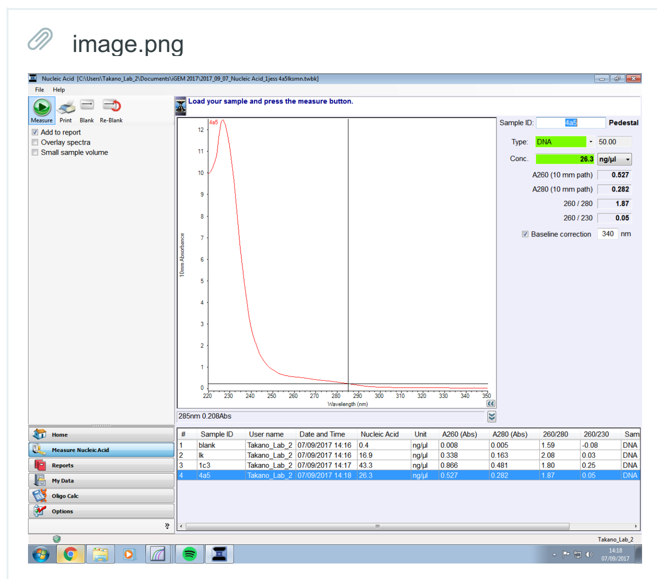
	A	B
1	10X CutSmart Buffer	2uL
2	Restriction enzymes	0.5uL each
3	MilliQ water	13
4	DNA	4

Run gel and extract LK at 2708bp (lanes 2+3), pSB1C3 at 6383bp (lane 4) and pSB1A5-SMN at 7708bp (lane 5)

UVP06898Sept72017.jpg

nanodrop results:

image.png



Ligation:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	2X quick ligase buffer	10
3	Vector DNA: 4A5SMN (50ng)	1.1
4	Insert DNA: LK (52.7ng)	3.1
5	Nuclease-free water	3.8
6	quick ligase	1
7	ATP	1
8	Total	20

Table2

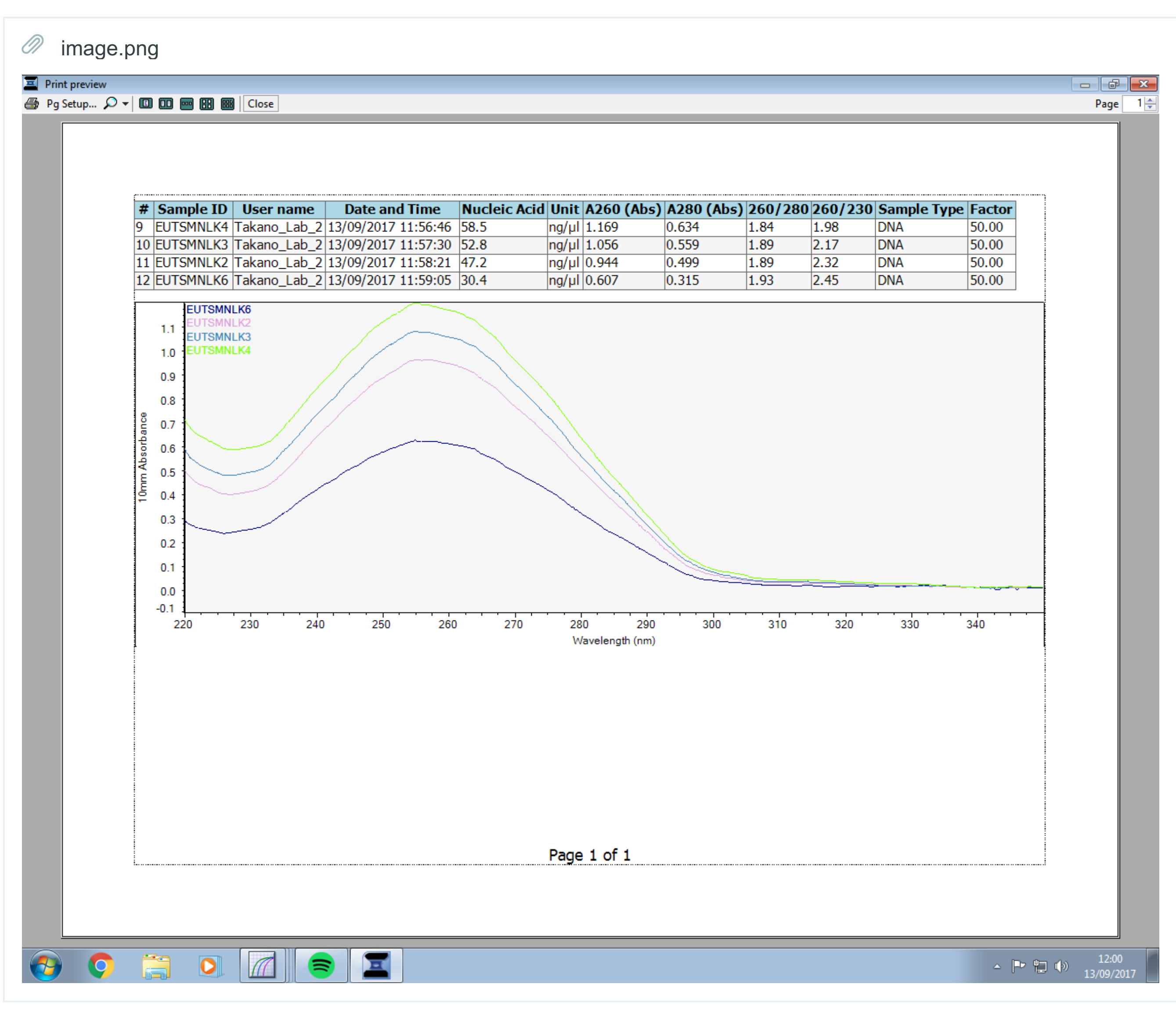
	A	B
1	**Component**	**Volume (µl)**
2	2X quick ligase buffer	10
3	Vector DNA: 1C3SMN (50ng)	1.9
4	Insert DNA: LK (63.64ng)	3.7
5	Nuclease-free water	2.4
6	quick ligase	1
7	ATP	1
8	Total	20

room temp for 1hr, 65C for 10min

transformation, plate

nanodrop overnights

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
9	EUTSMNLK4	Takano_Lab_2	13/09/2017 11:56:46	58.5	ng/µl	1.169	0.634	1.84	1.98	DNA	50.00
10	EUTSMNLK3	Takano_Lab_2	13/09/2017 11:57:30	52.8	ng/µl	1.056	0.559	1.89	2.17	DNA	50.00
11	EUTSMNLK2	Takano_Lab_2	13/09/2017 11:58:21	47.2	ng/µl	0.944	0.499	1.89	2.32	DNA	50.00
12	EUTSMNLK6	Takano_Lab_2	13/09/2017 11:59:05	30.4	ng/µl	0.607	0.315	1.93	2.45	DNA	50.00

Table3

	A	B	C	D	E	F	G
1	#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs
2	9	EUTSMNLK4	Takano_Lab_2	13/09/2017 11:56	58.5	ng/µl	
3	10	EUTSMNLK3	Takano_Lab_2	13/09/2017 11:57	52.8	ng/µl	
4	11	EUTSMNLK2	Takano_Lab_2	13/09/2017 11:58	47.2	ng/µl	
5	12	EUTSMNLK6	Takano_Lab_2	13/09/2017 11:59	30.4	ng/µl	

digest to run on a gel:

Sample2,3,4:
0.5ul EcoRI
0.5ul PstI
2ul cutsmart buffer
10ul DNA 400ng ish
7ul water (to 20ul)

Sample6:
0.5ul EcoRI
0.5ul PstI
2ul cutsmart buffer
14ul DNA 400ng ish
3ul water (to 20ul)

LK seq

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Date: 2017-09-07

THURSDAY, 7/9/17

Table1

	Construct	Stock Concentration (ng/uL)	DNA vol needed (uL)	Water vol needed (uL)
1	pSB1C3_ EutLK	367	3.4059945504	21.5940054496

eut S/MN/SMN/LK induction

Project: Manchester iGEM 2017 Shared Project

Authors: Jessica Burns

Dates: 2017-09-07 to 2017-09-11

THURSDAY, 7/9/17

overnights of:

pSB1C3-eutS

pSB1C3-eutMN

pSB1C3-eutLK

pSB1C3-eutSMN

eut S/MN/SMN/LK induction

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-09-07 to 2017-09-11

FRIDAY, 8/9/17

- Inoculate flask of 50mL LB (+chl) with 200uL of the overnight
- Grow at 37C (put in at 9:30) until reaches OD 0.5

Table3

	Time (mins)	OD S -	OD S +	OD - MN	OD - MN+	OD SMN -	OD SMN +
1	120	0.062	0.065	0.03	0.05	0.123	0.125
2	180	0.170	0.170	0.088	0.115	0.406	0.379
3	210	0.300	0.278	0.159	0.201	0.6 - induce	0.75 - induce
4	240	0.502 - induce	0.505 - induce	0.34	0.43		
5	270						

Table2

	Inducer	Stock conc (mM)	Desired conc (uM)	Vol to add to 50ml (uL)
1	IPTG	100	250	125
2	Tetracycline	0.214	0.1	23.364485…
3	Arabinose	100	1000	500

- 4hr incubation (post-induction) **(SMN, LK in at 13:30, S in at 14:15, MN in at)**
- harvest samples - measure GFP and OD in plate reader (200uL to each well)

Well1

	1	2	3	4	5	6	7	8	9	10	11	12
A	s-	s+	smn-	smn+	lk-	lk+	blank (LB)	**mn-**	**mn+**			
B	s-	s+	smn-	smn+	lk-	lk+	blank (LB)	**mn-**	**mn+**			
C	s-	s+	smn-	smn+	lk-	lk+	blank (LB)	**mn-**	**mn+**			
D												
E												
F												
G												
H												

Table4

	Flask Name	Construct	Inducer + conc	OD600 from plate reader	GFP
1	S-	eutS	none		
2	S+	eutS	250uM IPTG		
3	MN-	eutMN	none		
4	MN+	eutMN	100nM Tet		
5	SMN-	eutSMN	none		
6	SMN+	eutSMN	100nmTet and 250uM IPTG		
7	LK-	eutLK	none		
8	LK+	eutLK	500uL arabinose		

Harvest cells:

- split culture into 2 falcon tubes (~25ml in each)
- centrifuge for 10min at 8000rpm
- pour off supernatant - and freeze

eut S/MN/SMN/LK induction

Project: Manchester iGEM 2017 Shared Project
Authors: Jessica Burns
Dates: 2017-09-07 to 2017-09-11

MONDAY, 11/9/17

- resuspend pellet in 1.25ml buffer (on ice)
- pool samples (on ice)
- sonicate each sample at 2mins, 30%, 40% power
- split into two 2ml eppendorf tubes (= 2 samples) - freeze one lot, proceed with the other
- centrifuge on max at 10min
- sepearate sol and insol fractions
- resuspend insol in same vol water (same vol as sol sample)
- add equal amounts sample and SDS loading buffer (30uL + 30uL)
- boil in PCR machine (95C 10 min)

SDS page gel

- load 30uL sol + 20uL ins (for those with low od) - run 7.5uL sol and 5uL insol for others - and run
- Use 18% or 12 % gel (not 10% as proteins are too small)
- Keep one gel for imaging (use coomassie blue)
- Take picture of gel

Western Blot

- 2 x Western Blots Flag and His
- Using I-bind system (protocol|: https://tools.thermofisher.com/content/sfs/manuals/ibind_qrc.pdf)
- Take image of western blots

Predicted sizes of proteins and tags associated with them

Table1

	Protein	Size kDa	Tag
1	S	12.9	N-terminal His
2	M	37.5 (GFP mutant attached to eut M)	N-terminal His tag (on end of GFP)
3	N	11.3	C-terminal Flag
4	L	24.0	N-terminal His
5	K	19.1	C-terminal Flag

eut S/MN/SMN/LK induction 4 hours

Project: Manchester iGEM 2017 Shared Project
Authors: Alice Fraser
Date: 2017-09-13

WEDNESDAY, 13/9/17

- resuspend pellet in 1.25ml buffer on ice (25mMHepes and 500mM NaCl)
- pool samples (on ice)
- sonicate each sample at 2mins, 30%, power
- split into 2ml eppendorf tubes (= 1 samples) - rest of sample used for GFP plate reading
- centrifuge on max at 10min
- sepearate sol and insol fractions
- resuspend insol in same vol water (same vol as sol sample)
- add equal amounts sample and SDS loading buffer (30uL + 30uL)
- boil in PCR machine (95C 10 min)

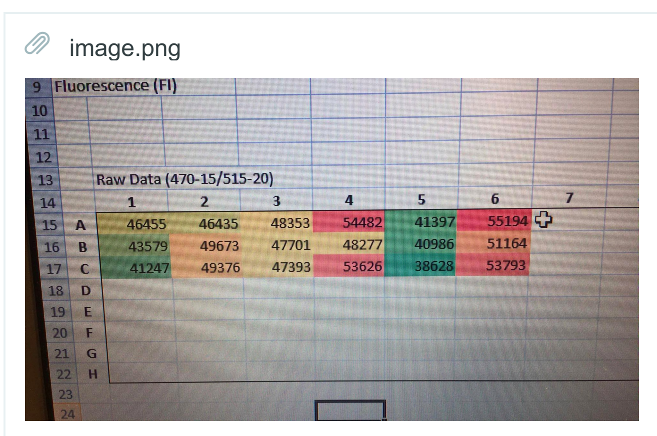
GFP reading

Well1

	1	2	3	4	5	6	7	8	9	10	11	12
A	s-	s+	mn-	mn+	smn-	smn+	lk+	lk-				
B	s-	s+	mn-	mn+	smn-	smn+	lk+	lk-				
C	s-	s+	mn-	mn+	smn-	smn+	lk+	lk-				
D												
E												
F												
G												
H												

Results (different gains)
Readings for LK missed off.

image.png

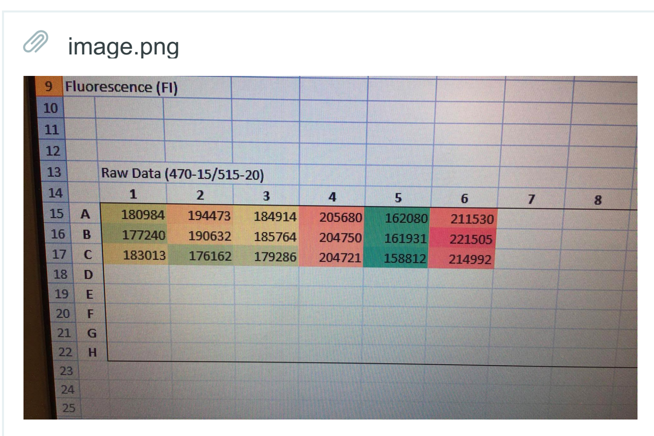


Fluorescence (FI)

Raw Data (470-15/515-20)

	1	2	3	4	5	6	7
A	46455	46435	48353	54482	41397	55194	
B	43579	49673	47701	48277	40986	51164	
C	41247	49376	47393	53626	38628	53793	
D							
E							
F							
G							
H							

image.png



Fluorescence (FI)

Raw Data (470-15/515-20)

	1	2	3	4	5	6	7	8
A	180984	194473	184914	205680	162080	211530		
B	177240	190632	185764	204750	161931	221505		
C	183013	176162	179286	204721	158812	214992		
D								
E								
F								
G								
H								

20 hour induction + GFP reading in buffer.

Project: Manchester iGEM 2017 Shared Project

Authors: Alice Fraser

Date: 2017-09-14

THURSDAY, 14/9/17

OD readings before induction

NB Ampilcillin growin v slow but waited to induce

Table1

	s-	s+	mn-	mn+	smn-	smn+	lk-
1	0.62	0.63	0.65	0.67	0.66	0.63	

Table2

	Inducer	Stock conc (mM)	Desired conc (uM)	Vol to add to 50ml (uL)
1	IPTG	100	250	125
2	Tetracycline	0.214	0.1	23.364485…
3	Arabinose	100	1000	500

Table4

	Flask Name	Construct	Inducer + conc
1	S-	eutS	none
2	S+	eutS	250uM IPTG
3	MN-	eutMN	none
4	MN+	eutMN	100nM Tet
5	SMN-	eutSMN	none
6	SMN+	eutSMN	100nmTet and 250uM IPTG
7	LK-	eutLK	none
8	LK+	eutLK	500uL arabinose

GFP Induction

Project: Manchester iGEM 2017 Shared Project
Authors: Alice Fraser
Date: 2017-09-14

THURSDAY, 14/9/17

Table2

	Induction levels	B	C	D	E	F	G
1	**TET/IPTG-->**						
2	**100/250**	s, 0.1	s, 0.2	s, 0.4	s, 0.6	mn, 0.1	mn, 0.2
3	**0/250**	s, 0.1	s, 0.2	s, 0.4	s, 0.6	mn, 0.1	mn, 0.2
4	**100/0**	s, 0.1	s, 0.2	s, 0.4	s, 0.6	mn, 0.1	mn, 0.2
5	**50/125**	s, 0.1	s, 0.2	s, 0.4	s, 0.6	mn, 0.1	mn, 0.2
6	**0/125**	s, 0.1	s, 0.2	s, 0.4	s, 0.6	mn, 0.1	mn, 0.2
7	**50/0**	s, 0.1	s, 0.2	s, 0.4	s, 0.6	mn, 0.1	mn, 0.2
8	**0/0**	s, 0.1	s, 0.2	s, 0.4	s, 0.6	mn, 0.1	mn, 0.2
9	**blank lb**	blank lb	blank lb	blank lb	blank lb	blank lb	blank lb

making the concentrations
1.100/250 and 0/250 and 100/0

then dilute by half in the petridish to get:
50/125 and 0/125 and 50/0

Table3

	Inducer	Stock conc (mM)	Desired conc (uM)	Vol to add to 50ml (uL)
1	IPTG	100	250	125
2	Tetracycline	0.214	0.1	23.364485…

Table1

	Desired IPTG conc (mM)	Amount IPTG to add (uL)	Desired Tet con (nM)	Amount Tet to add (uL)
1	125		50	
2	250		100	

confirming EUT LKSMN assembly with PCR and gel run

Project: Manchester iGEM 2017 Shared Project
Authors: Adam Hannaford
Date: 2017-09-14

THURSDAY, 14/9/17

Forward primer: EutL eq primer forward

UVP06937Sept152017.jpg

Reverse primer: eutS seq primer reverse

1. Set up PCR on ice using cloneamp master mix

PCR reaction

	A	B	C
1	**Reaction total**	**25**	**Done???**
2	10 uM Fwd	0.625	yeah
3	10 uM Rev	0.625	yeah g
4	Plasmid template	0.6	oh yeah
5	2x cloneamp master mix	12.5	
6	ddH20	10.65	yeah

PCR Cycles

120s 98 ^{o}C

34 cycles of:

10s 98 ^{o}C

5s 55 ^{o}C

15s at 72°C - (1kb - 5s)

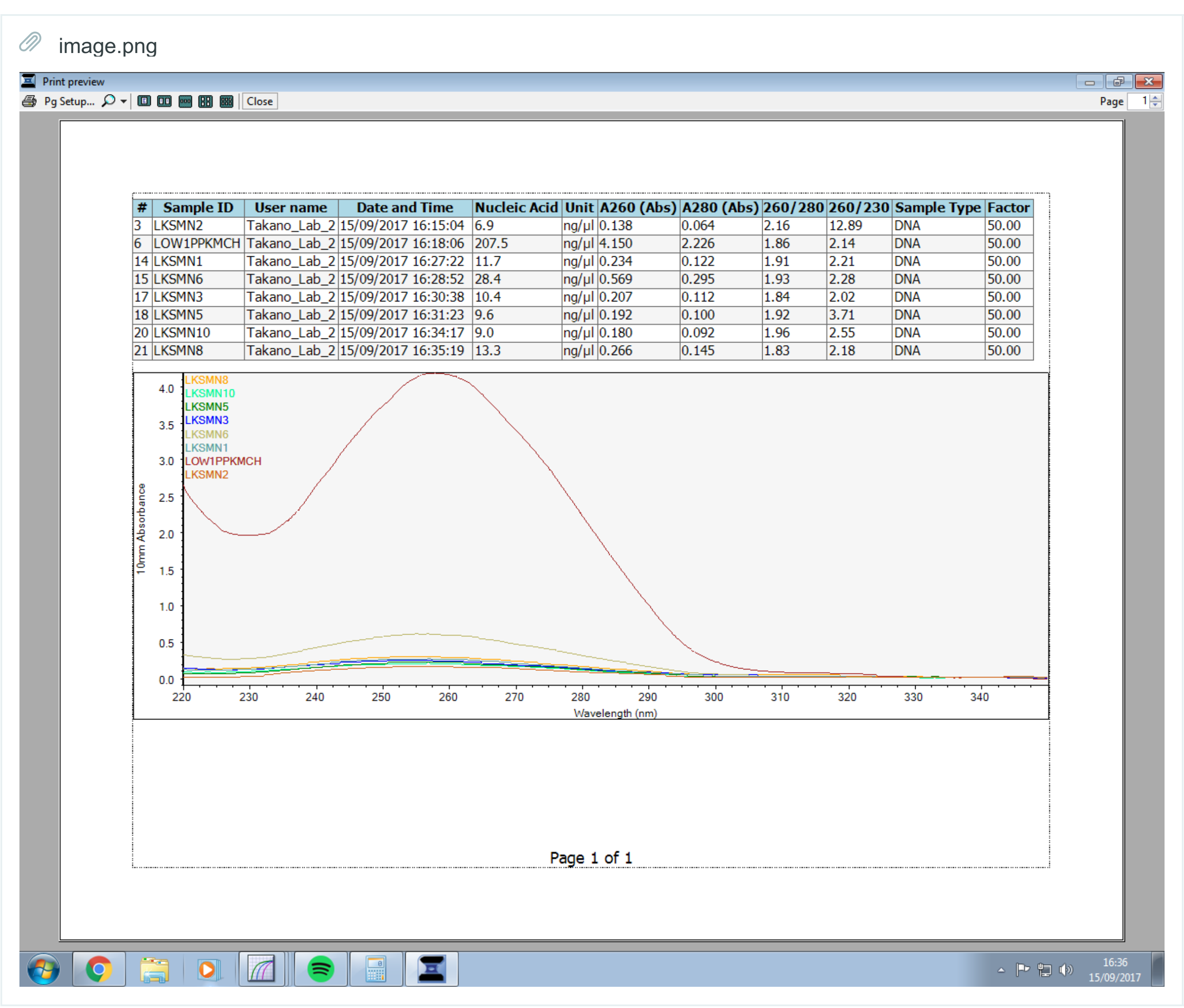
1 min at 72 ^{o}C

expecting 2.5kb band

ran on gel:

Owen Miniprep 15/09/2017 LKSMN + Low1MCherryPPK

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
3	LKSMN2	Takano_Lab_2	15/09/2017 16:15:04	6.9	ng/µl	0.138	0.064	2.16	12.89	DNA	50.00
6	LOW1PPKMCH	Takano_Lab_2	15/09/2017 16:18:06	207.5	ng/µl	4.150	2.226	1.86	2.14	DNA	50.00
14	LKSMN1	Takano_Lab_2	15/09/2017 16:27:22	11.7	ng/µl	0.234	0.122	1.91	2.21	DNA	50.00
15	LKSMN6	Takano_Lab_2	15/09/2017 16:28:52	28.4	ng/µl	0.569	0.295	1.93	2.28	DNA	50.00
17	LKSMN3	Takano_Lab_2	15/09/2017 16:30:38	10.4	ng/µl	0.207	0.112	1.84	2.02	DNA	50.00
18	LKSMN5	Takano_Lab_2	15/09/2017 16:31:23	9.6	ng/µl	0.192	0.100	1.92	3.71	DNA	50.00
20	LKSMN10	Takano_Lab_2	15/09/2017 16:34:17	9.0	ng/µl	0.180	0.092	1.96	2.55	DNA	50.00
21	LKSMN8	Takano_Lab_2	15/09/2017 16:35:19	13.3	ng/µl	0.266	0.145	1.83	2.18	DNA	50.00

Table1

	A	B	C	D	E	F	G
1	#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs
2	3	LKSMN2	Takano_Lab_2	15/09/2017 16:15	6.9	ng/µl	
3	6	LOW1PPKMCH	Takano_Lab_2	15/09/2017 16:18	207.5	ng/µl	
4	14	LKSMN1	Takano_Lab_2	15/09/2017 16:27	11.7	ng/µl	
5	15	LKSMN6	Takano_Lab_2	15/09/2017 16:28	28.4	ng/µl	
6	17	LKSMN3	Takano_Lab_2	15/09/2017 16:30	10.4	ng/µl	
7	18	LKSMN5	Takano_Lab_2	15/09/2017 16:31	9.6	ng/µl	
8	20	LKSMN10	Takano_Lab_2	15/09/2017 16:34	9.0	ng/µl	
9	21	LKSMN8	Takano_Lab_2	15/09/2017 16:35	13.3	ng/µl	

ash mCherry deletion

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-15 to 2017-09-27

FRIDAY, 15/9/17

Primer resuspension

287 µl and 216 µl of milliQ water was added to primer Eut del-Rev and Eut del-fwd respectively to make a concentration of 100 µM. The mixture was then spun for 5s on a table top centrifuge. 90 µl was then added to 10 µl primer to make a final concentration of 10 µM.

PCR

The Cloneamp protocol was used for this PCR. In an Eppendorf tube, we mixed:

Table1

	A	B
1	Reagent	Volume used (µl)
2	2x Cloneamp	25
3	Water	22.3
4	Eut del-fwd	1.25
5	Eut del-rev	1.25
6	Eut SMNLK pduD 1-20 mCherry plasmid	0.2

The mixture was subsequently put on the thermocycler and left to run for 30 minutes.

Gel electrophoresis and Analysis

5µl of the mixture was run on a 1% agarose gel alongside a 1kb ladder at 120mV for 30 minutes. The gel was then analyzed under UV light. Analysis revealed a 7kb fragment, which corresponds with the size of our Eut SMNLK construct.

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PCR cleanup

DNA 1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.
For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.
If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube.
Collection tubes are re-used to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back in the same tube.
Centrifuge the column for an additional 1 min.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Gibson Assembly® Protocol (E5510)

Introduction

This is the protocol for Gibson Assembly using the Gibson Assembly® Cloning Kit (E5510). More information from NEB can be found here.

Materials

- Gibson Assembly Cloning Kit
  - Gibson Assembly® Master Mix
  - NEBuilder® Positive Control
  - NEB® 5-alpha Competent *E. coli* (High Efficiency)
  - SOC Outgrowth Medium
  - pUC19 Transformation Control Plasmid
- DNA Polymerases (for generating PCR products)
  - Recommended: Q5® High-Fidelity DNA Polymerase, Q5 Hot Start High-Fidelity DNA Polymerase, or Q5 Hot Start High-Fidelity 2X Master Mix
- LB (Luria-Bertani) plates with appropriate antibiotic

Procedure

Set up the following reaction on ice:

1. Reaction volumes: *Use this table to calculate reaction volumes and set up the reaction. Remember to* ***input your total DNA fragment volume in cells B3 and C3*** *for assemblies with 2-3 fragments and 4-6 fragments, respectively.*

   - NEB recommends a total of ***0.02–0.5 pmols of DNA fragments when 1 or 2 fragments*** *are being assembled into a vector and* ***0.2–1.0 pmoles of DNA fragments when 4–6 fragments*** *are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend using NEB's online tool, NEBioCalculator.*
   - The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm, or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Table1

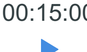
	A	B	C
1		**2-3 Fragments Assembly**	**4-6 Fragments Assembly**
2	**Concentration Range of DNA fragments**	02 - .5 pmols*	.2 - 1.0 pmols*
3	**Total Volume of Fragments (µl)**		
4	**Gibson Assembly Master Mix (2x) (µl)**	10	
5	**Deionized Water (µl)**	10	
6	**Total Volume (µl) *** **	20	

**Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in Gibson Assembly reaction should not exceed 20%.*
*** Control reagants are provided for 5 experiments with the Gibson Asembly Kit.*
**** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.*

2. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled.

   Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section).
   **Select the pencil icon and change the timer to 60 minutes if working with 4-6 fragments*

   00:15:00



3. Store samples on ice or at –20°C for subsequent transformation.

4. Transform NEB 5-alpha Competent E. coli cells (provided with the kit) with 2 µl of the assembly reaction, following the chemcial transformation protocol or electro competent cells transformation protocol

ash mCherry deletion

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-15 to 2017-09-27

MONDAY, 18/9/17

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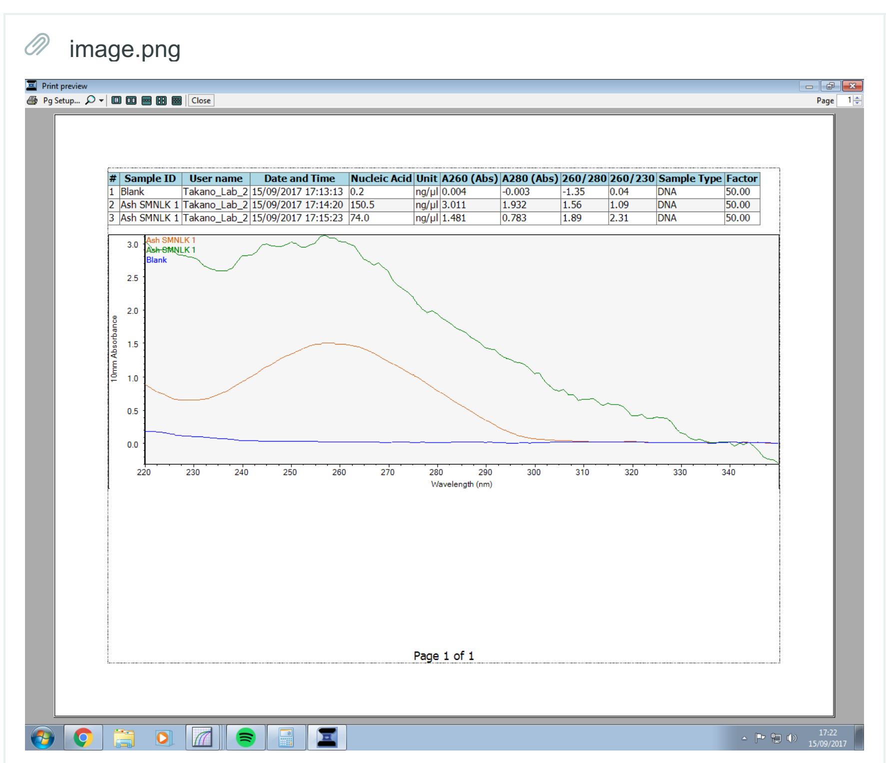
DpnI treated for 1 hour

Eut del miniprep results

image.png



#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	Blank	Takano_Lab_2	15/09/2017 17:13:13	0.2	ng/µl	0.004	-0.003	-1.35	0.04	DNA	50.00
2	Ash SMNLK 1	Takano_Lab_2	15/09/2017 17:14:20	150.5	ng/µl	3.011	1.932	1.56	1.09	DNA	50.00
3	Ash SMNLK 1	Takano_Lab_2	15/09/2017 17:15:23	74.0	ng/µl	1.481	0.783	1.89	2.31	DNA	50.00

-Ligation:
-4.5 ul DNA
-5ul 2x buffer
-0.5ul Ligase
-Leave for 30min
-Put back on ice

-Transformation:
-Add 5ul of ligation to competent cells
-Leave on ice for 30min
-Heat shock for 30s at 42°C
-Add 200ul SOC
-Shake incubate for 1h at 37°C
-Plate on Amp plate

Overnights were made

optimising GFP expression

Project: Manchester iGEM 2017 Shared Project
Authors: Adam Hannaford
Dates: 2017-09-18 to 2017-09-20

MONDAY, 18/9/17

1. take 2 overnights from 37C incubator (TB and LB)
2. create 2, 1 litre conical flask of TB and LB inoculated with 1ml of overnight.
3. Grow until reach an OD of 0.1
4. when an OD of 0.1 is reached in both the flasks, create 10ml cultures in 5oml conical flasks.
5. induce these flasks at varying Tet and IPTG concentrations (see table) and put them in the approriate incubators.

DoE design:

responses: maximise GFP florescence

Design:

Table1

	A	B	C	D	E	F
1	flask number	Temperature	Tet nM	IPTG uM	Medium	harvest time
2	1	20	0	250	LB	1
3	7	30	100	0	LB	1
4	2	20	0	0	LB	4
5	4	25	50	125	LB	4
6	6	25	50	125	LB	4
7	18	30	0	250	LB	4
8	19	20	100	250	LB	4
9	9	30	100	250	LB	20
10	12	20	100	0	LB	20
11	16	20	0	250	LB	20
12	17	30	0	0	LB	20
13	3	30	100	250	TB	1
14	14	30	0	0	TB	1
15	15	20	100	0	TB	1
16	5	20	0	250	TB	4
17	10	25	50	125	TB	4
18	11	25	50	125	TB	4
19	21	30	100	0	TB	4
20	8	20	0	0	TB	20
21	13	30	100	0	TB	20
22	20	20	100	250	TB	20
23	22	30	0	250	TB	20

to create the correct concentrations the table below was used

Table2				
	Inducer	Stock conc (mM)	Desired conc (uM)	vol to add to 10ml (ul)
1	IPTG	100	250	25
2	IPTG	100	125	12.5
3	Tetracycline	0.214	0.1	4.6728971…
4	Tetracycline	0.214	0.05	2.3364485…

This is the well layout that was used:

Well1												
	1	2	3	4	5	6	7	8	9	10	11	12
A	T3			T20	T21	T22						
B	T5			T21	T22	T23						
C	T8			T22	T23	T24						
D	T10						L4	L4				
E	T11						L2	L2				
F	T13						L6	L6				
G	T14						L18	L18				
H	T15						L19	L19				

Assemble LKSMN (in pSB1C3)

Project: Manchester iGEM 2017 Shared Project

Authors: Amber Hall

Dates: 2017-09-04 to 2017-09-20

TUESDAY, 19/9/17

Overnight cultures (50ml) set up with colonies from the transformation in LB or TB

optimising GFP expression

Project: Manchester iGEM 2017 Shared Project

Authors: Adam Hannaford

Dates: 2017-09-18 to 2017-09-20

TUESDAY, 19/9/17

20 hour harvest

In clean plates, the cultures were aliquotted into a 96 deep well plate like this:

Well2

	1	2	3	4	5	6	7	8	9	10	11	12
A	8	8	8									
B	9	9	9									
C	12	12	12									
D	13	13	13									
E	16	16	16									
F	17	17	17									
G	20	20	20									
H												

- Flask 22 could not be used, it was empty so had probably spilled over

Assemble LKSMN (in pSB1C3)

Project: Manchester iGEM 2017 Shared Project

Authors: Amber Hall

Dates: 2017-09-04 to 2017-09-20

WEDNESDAY, 20/9/17

No growth in TB. LB cultures miniprepped. Yields (nanodrop) as follows:

Table3

	A	B	C	D	E	F	G	H	
1	#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	26
2	1	Blank	Takano_Lab_2	#########	2.2	ng/µl	0.043	0.032	
3	2	EutSMNLK_1	Takano_Lab_2	#########	8.5	ng/µl	0.17	0.091	
4	3	EutSMNLK_1	Takano_Lab_2	#########	9.7	ng/µl	0.193	0.102	
5	4	EutSMNLK_1	Takano_Lab_2	#########	9	ng/µl	0.181	0.087	
6	5	EutSMNLK_2	Takano_Lab_2	#########	17	ng/µl	0.339	0.178	
7	6	EutSMNLK_2	Takano_Lab_2	#########	17.3	ng/µl	0.346	0.18	
8	7	EutSMNLK_2	Takano_Lab_2	#########	18.6	ng/µl	0.372	0.194	
9									
10		Averages	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280		
11		EutSMNLK_1	9.066667	ng/µl	0.181333	0.0933…	1.943333		
12		EutSMNLK_2	17.63333	ng/µl	0.352333	0.184	1.92		

ash mCherry deletion

Project: Manchester iGEM 2017 Shared Project

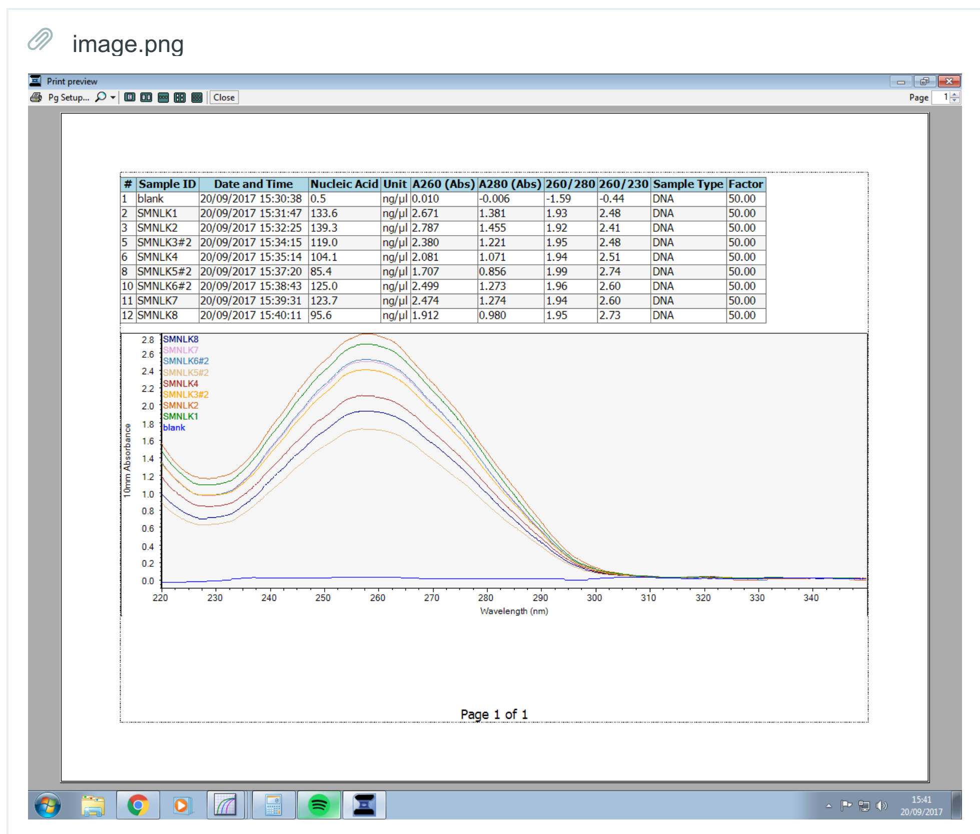
Authors: james engleback

Dates: 2017-09-15 to 2017-09-27

WEDNESDAY, 20/9/17

Overnights were miniprepped and nanodropped:

image.png



#	Sample ID	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	blank	20/09/2017 15:30:38	0.5	ng/µl	0.010	-0.006	-1.59	-0.44	DNA	50.00
2	SMNLK1	20/09/2017 15:31:47	133.6	ng/µl	2.671	1.381	1.93	2.48	DNA	50.00
3	SMNLK2	20/09/2017 15:32:25	139.3	ng/µl	2.787	1.455	1.92	2.41	DNA	50.00
5	SMNLK3#2	20/09/2017 15:34:15	119.0	ng/µl	2.380	1.221	1.95	2.48	DNA	50.00
6	SMNLK4	20/09/2017 15:35:14	104.1	ng/µl	2.081	1.071	1.94	2.51	DNA	50.00
8	SMNLK5#2	20/09/2017 15:37:20	85.4	ng/µl	1.707	0.856	1.99	2.74	DNA	50.00
10	SMNLK6#2	20/09/2017 15:38:43	125.0	ng/µl	2.499	1.273	1.96	2.60	DNA	50.00
11	SMNLK7	20/09/2017 15:39:31	123.7	ng/µl	2.474	1.274	1.94	2.60	DNA	50.00
12	SMNLK8	20/09/2017 15:40:11	95.6	ng/µl	1.912	0.980	1.95	2.73	DNA	50.00

Next step was a digest so the products could be run on a gel to see if mCherry was successfully removed

2ul cutsmart buffer

1ul EcoRI

1ul PstI

10ul water

6ul DNA

Made a master mix including all of the above minus the DNA:

20ul cutsmart buffer

10ul EcoRI

10ul PstI

100ul water

For each tube 14ul of master mix was added plus 6ul of each sample

optimising GFP expression

Project: Manchester iGEM 2017 Shared Project

Authors: Adam Hannaford

Dates: 2017-09-18 to 2017-09-20

WEDNESDAY, 20/9/17

The data that we collected can be seen in the table below

Table3

	A	B	C	D	E	F	G
1	flask number	temperature	tet	iptg	growth medium	harvest time	OD1
2	1	20	0	250	LB	1	
3	7	30	100	0	LB	1	
4	2	20	0	0	LB	2	
5	4	25	50	125	LB	2	
6	6	25	50	125	LB	2	
7	18	30	0	250	LB	2	
8	19	20	100	250	LB	2	
9	9	30	100	250	LB	20	
10	12	20	100	0	LB	20	
11	16	20	0	250	LB	20	
12	17	30	0	0	LB	20	
13	3	30	100	250	TB	1	
14	14	30	0	0	TB	1	
15	15	20	100	0	TB	1	
16	5	20	0	250	TB	3	
17	10	25	50	125	TB	3	
18	11	25	50	125	TB	3	
19	21	30	100	0	TB	3	
20	8	20	0	0	TB	20	
21	13	30	100	0	TB	20	
22	20	20	100	250	TB	20	
23	22	30	0	250	TB	20	
24	extra data points						
25	5	20	0	250	TB	1	
26	10	25	50	125	TB	1	
27	11	25	50	125	TB	1	
28	21	30	100	0	TB	1	
29	8	20	0	0	TB	1	
30	13	30	100	0	TB	1	
31	20	20	100	250	TB	1	
32	22	30	0	250	TB	1	

ash mCherry deletion

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-15 to 2017-09-27

THURSDAY, 21/9/17

UVP06957Sept212017.jpg

Colony PCR of Ash's EUT plasmid tranformation

Project: Manchester iGEM 2017 Shared Project
Authors: Theodore Reinhard Glenaldo
Date: 2017-09-26

TUESDAY, 26/9/17

Primers:
VR - in our stocks (will be 10ul working solution
lacUV5_Ins_Rev01 (in ash's box, look at ash's excel document [think its called ash's primer list] to find the location of this primer)
If deletion successful:
2 bands at 2920 and 2358 bp
If deletion unsuccessful:
3 bands at 2920, 2358, and 5249 bp
VR: attaccgcctttgagtgagc
LacUV5_Ins_Rev01: ccgctcacaattccacac
make up this stock solution in an epindorf:

Table3

	A	B	C
1	**Reaction vol:**	**10**	
2	**Number Reactions:**	1	**20**
3	10 uM Fwd Primer	0.2	4
4	10 uM Rev Primer	0.2	4
5	Template	0	0
6	2x OneTaq Mix	5	100
7	ddH20	4.6	92
8			

1. line up 20 PCR tubes and fill them with 10ul each of the stock solution.
2. with a sterile tip take a sample of a colony and put the sample on an index plate and then with THE SAME TIP dab inside the pcr tube.
3. label the PCR tube with the same number on the index plate and put the pcr tube on ice
4. do this for all the PCR tubes.
5. put the tubes in the PCR machine and run this with the settings below

Table2

	A	B	C
1		**Temp**	**Time**
2	**Initial Denaturating**	95 oC	30 s
3	**40 cycles:**	95 oC	15-30s
4		~ 55 oC	15-60 s
5		68 oC	2 min
6	**Final Extension**	68 oC	5 mins
7	**Hold**	4-10 oC	Inf

run these samples on a gel, image and upload the gel image below
band should be 2350bp

ash mCherry deletion

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-15 to 2017-09-27

TUESDAY, 26/9/17

UVP06977Sept262017.jpg

Theo attemped a colony pcr on the colonies from the transformation plate. He PCR'd two of eight colonies, shown on the gel to the left. It looks like the bands might be a primer dimer.

Digest to confirm SMN +LK cotransformation

Project: Manchester iGEM 2017 Shared Project

Authors: james engleback

Dates: 2017-09-27 to 2017-10-04

WEDNESDAY, 27/9/17

create overnights

ash mCherry deletion

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-15 to 2017-09-27

WEDNESDAY, 27/9/17

Colony PCR of the deletion plate

Theo said yesterday that there were eight colonies on the transformation plate. This morning, two of the colonies were surrounded by satelite colonies. It's not clear whether these are contaminants. I'm going to set up 20 colony PCR reactions with the VR and lacUV5 primers (more info)

If deletion successful:
2 bands at 2920 and 2358 bp
If deletion unsuccessful:
3 bands at 2920, 2358, and 5249 bp
VR: attaccgcctttgagtgagc
LacUV5_Ins_Rev01: ccgctcacaattccacac

PCR was done according to these condions:

Table2

	A	B	C	D	E
1	**Component**	**10 µl reaction**			
2	**10 µM VR Primer**	0.2 µl			
3	**10 µM Reverse Primer**	0.2µl			
4	**Template DNA**	variable			
5	**OneTaq 2X MasterMix with Standard Buffer**	5 µl			
6	**Nuclease-free water**	to 25 µl			

Table3

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	95	30 seconds
3	30 Cycles	95	30 sec
4		55	60 sec
5		68	120 sec
6	Final Extension	68°C	5 minutes
7	Hold	4-10°C	

One control was set up with no template.
The PCR product was run on a 1% TAE agarose gel (with syperview) at 100V for 40 minutes and visulaised with UV:

UVP06980Sept272017.jpg

Lane 1 + 20: 1kb Ladder. Lane 2 - control (PCR with no template) Lanes 3-19 - PCR reactions. All we can see are primers, the PCR hasn't worked at all.

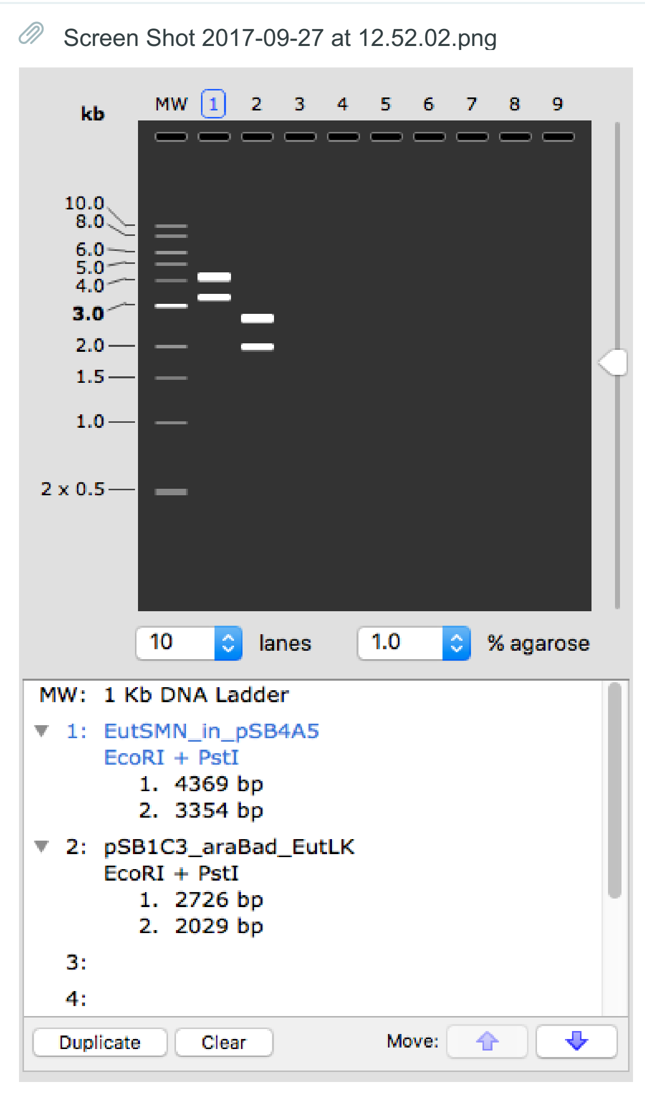
Digest to confirm SMN +LK cotransformation

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-27 to 2017-10-04

THURSDAY, 28/9/17

cut with EcoRI and PstI to get this gel image but in 1 lane:

Screen Shot 2017-09-27 at 12.52.02.png



Digest mix:
0.5ul EcoRI
0.5ul PstI
2ul cutsmart
DNA (700ng ish) and water

Do this for each overnight sample

gibson assembly of eutSMNLK(ash) and mcherry PPK

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-28 to 2017-10-09

THURSDAY, 28/9/17

.independently make the 2 PCR mixes below.

PCR of ash's plasmid
primers:
- igem_gibson_vector_FOR
- igem_gibson_vector_REV

PCR reaction

	A	B	C
1	**Reaction total**	**25**	
2	10 uM Fwd	1.25	
3	10 uM Rev	1.25	
4	Plasmid template	0.2	
5	2x master mix q5	12.5	
6	ddH20	9.8	

PCR Cycles Q5
120s 98 ^{o}C
34 cycles of:

10s 98 ^{o}C

30s 69 ^{o}C

4mins at 72°C - (pSB1C3 is 2kb - 10s)

1 min at 72 ^{o}C

PCR of tag mcherry ppk
primers:
igem_gibson_fragment_FOR
igem_gibson_fragment_REV

Table1	A	B	C
1	**Reaction total**	**25**	
2	10 uM Fwd	1.25	
3	10 uM Rev	1.25	
4	Plasmid template	0.2	
5	2x master mix q5	12.5	
6	ddH20	9.8	

PCR Cycles Q5

120s 98 ^{o}C

34 cycles of:

10s 98 ^{o}C

30s 68 ^{o}C

1mins at 72°C - (pSB1C3 is 2kb - 10s)

1 min at 72 ^{o}C

Dpn1 Digest:

1ul of DPN1 added to each PCR reaction, incubated for 50 minutes at 37°C.

Both reactions were cleaned up with QIAgen PCR kits according to the QIAgen protocol

Table2	A	B	C	D	E
1	Reaction	Conc. (ng/ul)	A260/280		
2	Vector (pETDUET_EutSMLK)	110	1.8		
3	Insert (PduD(1-20)_mCherry_PPK)	110	1.8		

Both fragments were rectrospectively run on a TAE 1% agarose gel (110V, 60min) to confirm the reaction worked:

UVP06981Sept292017.jpg

Lanes: 1 - 1kb Ladder, 2: Vector (>3kb) , 3: insert (1.5-2kb) Should have run the gel for longer

The stocks were diluted in QIAgen EB buffer to 25ng/ul each and added to the following reaction:

Table3

	Component	Vol (ul)
1	NEB Gibson master mix	5
2	Insert (25ng/ul)	1
3	Vector (25ng/ul)	1
4	ddH2O	3

The reaction was incubated at 50°C for 30 mins and then cooled to 4°C overnight

Digest to confirm SMN +LK cotransformation

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-27 to 2017-10-04

FRIDAY, 29/9/17

Miniprep of overnights as per QIAgen protocol. Colony 4 did not grow. Here are the miniprep results:

Table1

	A	B	C	D	E	F	G
1	#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs
2	1	blank	Takano_Lab_2	29/09/2017 12:29	0.3	ng/µl	
3	2	smnklk1	Takano_Lab_2	29/09/2017 12:29	465.6	ng/µl	
4	3	smnklk2	Takano_Lab_2	29/09/2017 12:30	602.3	ng/µl	1
5	4	smnklk3	Takano_Lab_2	29/09/2017 12:30	105.8	ng/µl	
6	5	smnklk5	Takano_Lab_2	29/09/2017 12:40	558.1	ng/µl	1
7	6	smnklk6	Takano_Lab_2	29/09/2017 12:41	462.3	ng/µl	
8	7	smnklk7	Takano_Lab_2	29/09/2017 12:43	419.1	ng/µl	
9	8	smnklk8	Takano_Lab_2	29/09/2017 12:45	589.5	ng/µl	1

Digest was done like this:

0.5ul EcoRI
0.5ul PstI
2ul cutsmart
17ul miniprepped DNA
Incubated at 37 for 1 hour
Ran on TAE 1% agarose gel (w/ syperveiw) at 110V for 80mins:

UVP06983Sept292017.jpg

cloning tag-mCherry-PPK into eut LK

Project: Manchester iGEM 2017 Shared Project
Authors: Adam Hannaford
Date: 2017-09-29

FRIDAY, 29/9/17

eut LK apears to have amutation in the XbaI site so we cannot use t hisin the assembly

LK digest

- [x] 0.5ul SpeI
- [x] 0.5ul PstI
- [x] 1ul rSAP
- [x] 2ul cutsmart
- [x] 1.3ul LK DNA
- [x] 14.7 Water

Lp1 digest

- [x] 0.5ul XbaI
- [x] 0.5ul PstI
- [x] 2ul cutsmart
- [x] 4.3ul Lp1 DNA
- [x] 12.7ul Water

PUT AT 37 FOR 2 HOUR DIGEST

Ligation

2.5ul LK digest
2.5ul Lp1 digest
1ul of 10x buffer
1ul ATP
0.5ul ligase
2.5ul water

gibson assembly of eutSMNLK(ash) and mcherry PPK

Project: Manchester iGEM 2017 Shared Project

Authors: james engleback

Dates: 2017-09-28 to 2017-10-09

FRIDAY, 29/9/17

2ul of the reaction product was transformed into an aliquot of the NEB Gibson competent cells like this:

- Defrost on ice
- mix with 2ul of reaction product
- incubate on ice for 1 hour
- Heat shock @ 42degC for 30 seconds
- Transfer to ice for >5mins
- Mix cells with 1ml of sterile SOC medium, incubate in shaker at 37degC and 180rpms for 1 hour
- Plate onto Ampicillin plate, leave on bench over weekend

before the transformation, a dab of the cell aliquot was plated onto a antibiotic-free plate to act as a negative for the colony PCR on Monday

gibson assembly of eutSMNLK(ash) and mcherry PPK

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-28 to 2017-10-09

MONDAY, 2/10/17

No colonies on the transformation plates. I had poured them fresh on Friday from an LBA bottle on the bench. Today I saw a half empty bottle of LBA on the bench (maybe the one I used) that said it had chloramphenicol in. That's probably the problem. There wasn't even growth on the control plate. Made some fresh plates.
Re-Transformed 2ul of the Gibson reaction into NEB ultracompetent cells from Ross (specific Gibson cells)

- Defrost on ice
- mix with 2ul of reaction product
- incubate on ice for 1 hour
- Heat shock @ 42degC for 30 seconds
- Transfer to ice for >5mins
- Mix cells with 1ml of sterile SOC medium, incubate in shaker at 37degC and 180rpms for 1 hour
- Plate onto (fresh) Ampicillin plate, leave in 37°C incubator overnight

gibson assembly of eutSMNLK(ash) and mcherry PPK

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-28 to 2017-10-09

TUESDAY, 3/10/17

Colonies on both the (+ve)control and the Gibson transformation plate.

Colony PCR

- 19 coloniesfrom the Gibson plate and some of the control colony were transferred to individual PCR tubes with 20ul of QIAgen EB buffer
- Dabs of each tube were transferred to an Ampicillin LBA index plate, which was sent to the incubator at 37degC
- The PCR tubes were heated at 98degC for 5 minutes to break the cells
- 20 PCR tubes were set up with the following mix:

Table4

	A	B
1	**Reaction vol: (ul)**	**10**
2	**Number Reactions:**	1
3	10 uM Fwd Primer	0.2
4	10 uM Rev Primer	0.2
5	Template	1
6	2x OneTaq Mix	5
7	ddH20	3.6

Where the Forward primer was pET_DUET_Vec_MCS2_For (TM=53.7**Tm: 62 °C according to the NEB calculator**) and the Reverse Primer was T7 Terminator Primer (Tm=52.9, **Tm: 54 °C according to the NEB calculator**)

The reactions were put in the thermocycler with these settings:

Table5

	A	B	C
1		**Temp**	**Time**
2	**Initial Denaturating**	95 oC	30 s
3	**40 cycles:**	95 oC	30s
4		55 oC	15-60 s
5		68 oC	2 min
6	**Final Extension**	68 oC	5 mins
7	**Hold**	4-10 oC	Inf

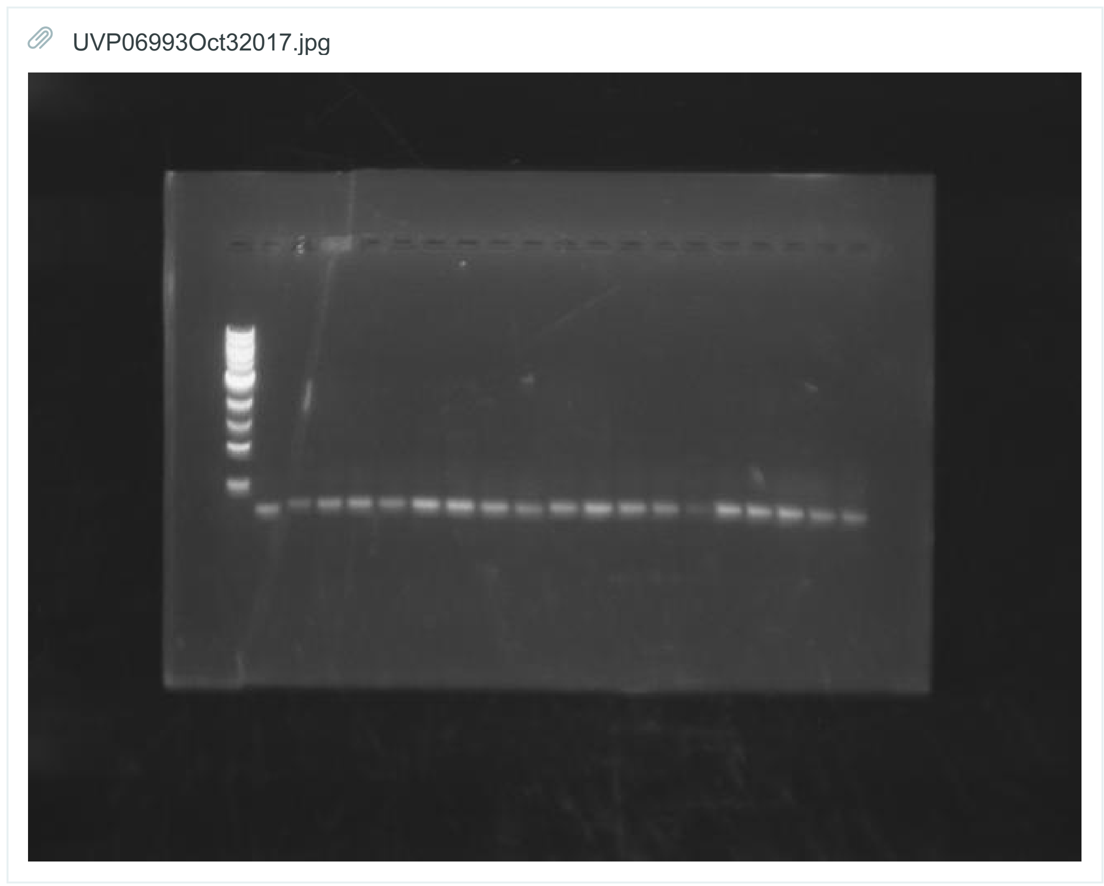
This was a blunder, so I set up another reaction with the mCherry_For01 and T7 Terminator Primer (**Tm: 61 °C and Tm: 56 °C respectively, annealing temp: 51 °C) this time with 51degC annealing temp.**

According to the plasmid map of pTE_1183_pET_DUET_1_eutSMNLK_lacUV5_pduP1_18_mCherry there cloned fragment should be 645bp. When I Imported our PPK construct into benchling to check the band size, the the mCherry_For01 primer didn't bind.

That's really annoying so I made an allignment to see what's going on pTE_1183_pET_DUET_1_eutSMNLK_lacUV5_pduP1_18_mCherry and it turns out that we have a different mCherry sequence to Ash.

A gel of Both PCRs was run. PCR 1 didn't work at all, no bands, not even the ladder, no idea what happened. Didn't bother saving the image. PCR 2 showed me this:

UVP06993Oct32017.jpg



Primers!

Digest to confirm SMN +LK cotransformation

Project: Manchester iGEM 2017 Shared Project

Authors: james engleback

Dates: 2017-09-27 to 2017-10-04

WEDNESDAY, 4/10/17

Table2

	A	B	C	D	E	F	G
1	#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Ab
2	1	blank	Takano_Lab_2	04/10/2017 14:08	0.2	ng/µl	
3	2	LK1	Takano_Lab_2	04/10/2017 14:11	433.8	ng/µl	
4	3	LK2	Takano_Lab_2	04/10/2017 14:12	292.9	ng/µl	
5	4	LK3	Takano_Lab_2	04/10/2017 14:12	322.4	ng/µl	
6	5	LK4	Takano_Lab_2	04/10/2017 14:13	268.4	ng/µl	
7	6	LK5	Takano_Lab_2	04/10/2017 14:13	291.8	ng/µl	
8	7	LK6	Takano_Lab_2	04/10/2017 14:14	439.9	ng/µl	
9	8	LK7	Takano_Lab_2	04/10/2017 14:14	199.5	ng/µl	
10	9	LK8	Takano_Lab_2	04/10/2017 14:15	285.8	ng/µl	
11	10	LK9	Takano_Lab_2	04/10/2017 14:15	271.6	ng/µl	
12	11	LK10	Takano_Lab_2	04/10/2017 14:16	259.0	ng/µl	
13	12	LK11	Takano_Lab_2	04/10/2017 14:16	145.6	ng/µl	
14	13	LK12	Takano_Lab_2	04/10/2017 14:17	173.3	ng/µl	
15	14	LK13	Takano_Lab_2	04/10/2017 14:17	348.2	ng/µl	
16	15	LK14	Takano_Lab_2	04/10/2017 14:18	150.1	ng/µl	
17	16	LK15	Takano_Lab_2	04/10/2017 14:18	199.2	ng/µl	
18	17	C1	Takano_Lab_2	04/10/2017 14:19	100.9	ng/µl	
19	18	C2	Takano_Lab_2	04/10/2017 14:20	123.6	ng/µl	
20	19	C3	Takano_Lab_2	04/10/2017 14:20	166.0	ng/µl	

digest of LK+tag+mCherry+PPK to check assembly

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-10-04 to 2017-10-11

WEDNESDAY, 4/10/17

for **each** of the miniprepped samples create in a pcr tube:

0.5ul EcoRI
0.5ul PstI
2ul Cutsmart
17ul of DNA

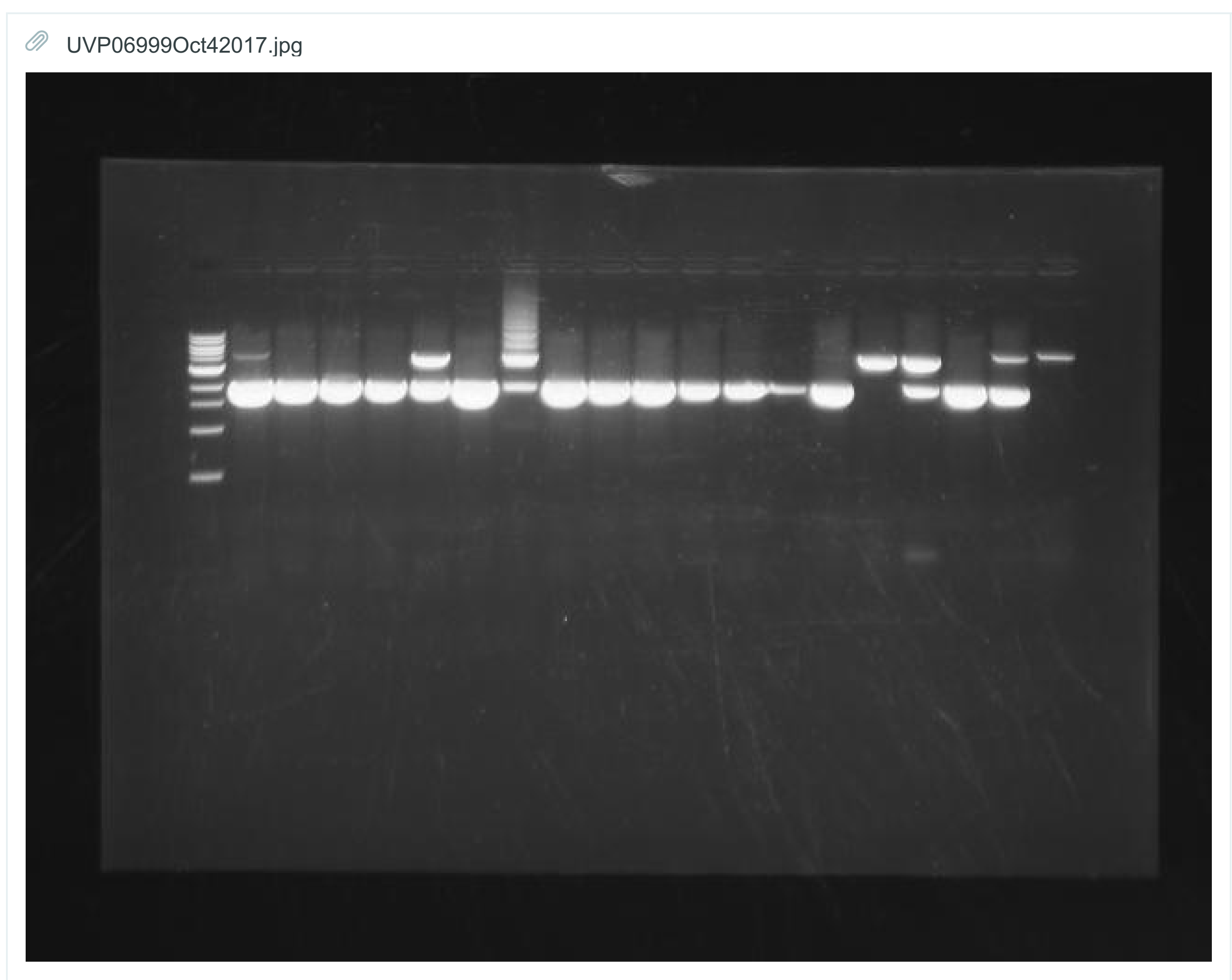
digest at 37C for 1 hour

no need to do a heat inactivation as we're only running it on a gel

load of the the PCR sample onto a gel

bands should be at
4639bp and 2029bp

UVP06999Oct42017.jpg



ok I'm going to do lane 6 with the ~2 and 4kb bands. Tube 5

gibson assembly of eutSMNLK(ash) and mcherry PPK

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-28 to 2017-10-09

WEDNESDAY, 4/10/17

Going to try the Colony PCR again today based on Gibson_AshEut_PPK using the primers EutL seq primer forward **Tm: 58 °C and** T7 Terminator Primer **Tm: 56 °C (according to the NEB OneTaq Calculator) TAnneal = 51 °C**

The Amplified region should be 2452bp. We don't have enough OneTaq to get started just yet.

Table6

	A	B	C	D	E
1	Component	10ul reaction	25 µl reaction	x21 working stock	Final Concentration
2	10 µM Forward Primer	0.2	0.5	4.2	0.2 µM
3	10 µM Reverse Primer	0.2	0.5	4.2	0.2 µM
4	Template DNA	variable	variable	variable	< 1,000 ng
5	OneTaq 2X MasterMix with Standard Buffer	5	12.5	105	1X
6	Nuclease-free water	4.6	to 25 µl	96.6	< 1,000 ng

UVP06998Oct42017.jpg

lane 1: PCR product of Ash's pET_Duet EutSNMLK_mCherry(parent) lane 2: un-transformed cells, all other lanes correspond to the PCR products of the gibson assembly cells.

Set up overnights of colonies 5-9 anyway. Mostly for Amber to make slides but I might miniprep and digest

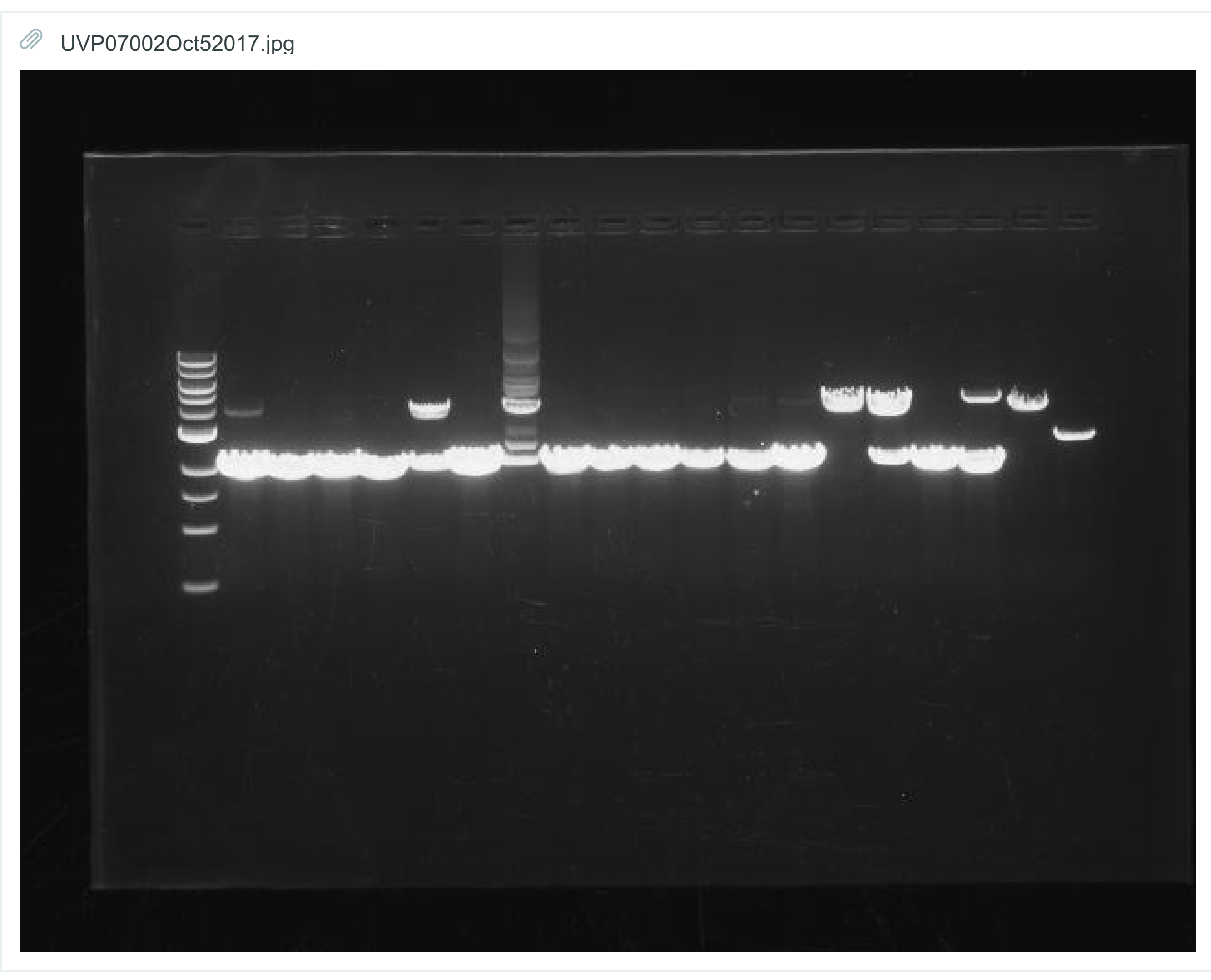
digest of LK+tag+mCherry+PPK to check assembly

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-10-04 to 2017-10-11

THURSDAY, 5/10/17

ok SAME GEL AGAIN, i GOT had doubts about the numbering

UVP07002Oct52017.jpg



gibson assembly of eutSMNLK(ash) and mcherry PPK

Project: Manchester iGEM 2017 Shared Project

Authors: james engleback

Dates: 2017-09-28 to 2017-10-09

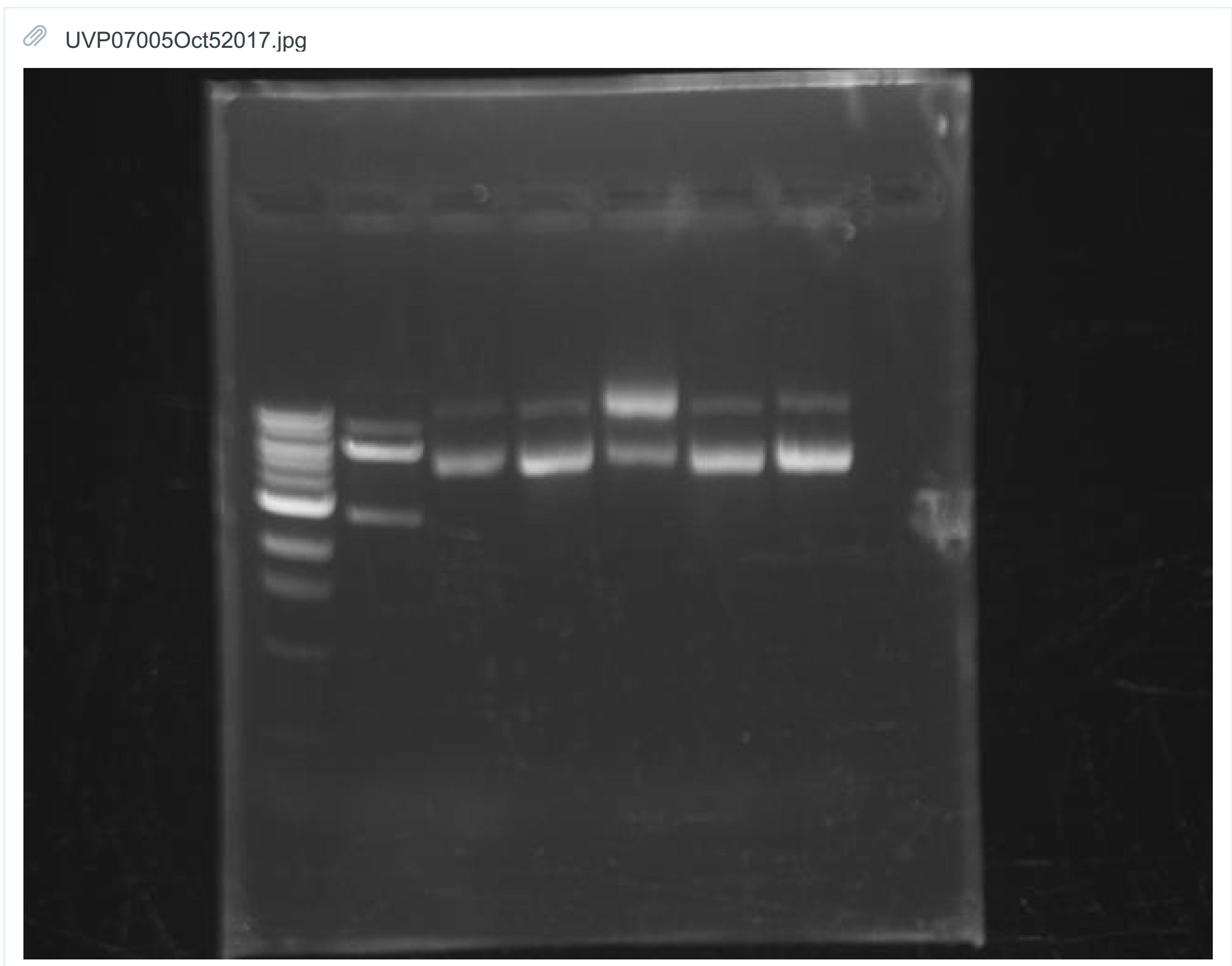
THURSDAY, 5/10/17

I'm going to miniprep those overnights and do a digest to confirm my construct.

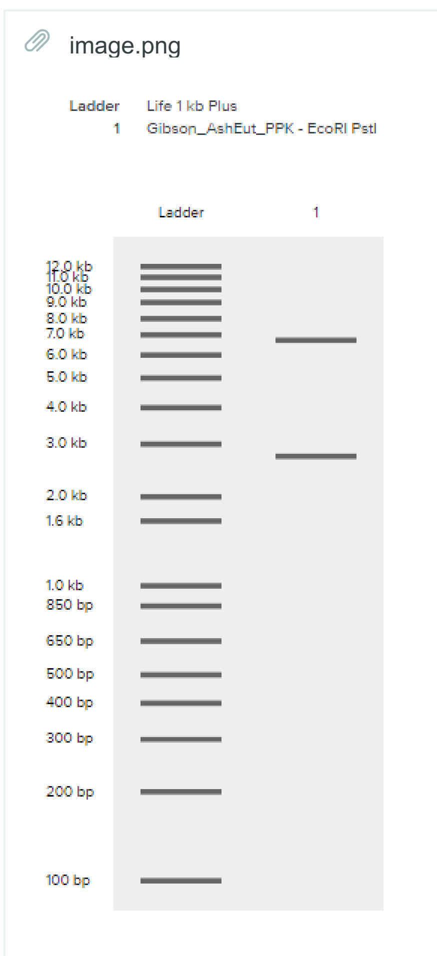
Table7

	A	B	C	D	E	F	G
1	#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs
2	1	BLANK	Takano_Lab_2	05/10/2017 12:31	1.5	ng/µl	
3	2	COL 5	Takano_Lab_2	05/10/2017 12:32	56.0	ng/µl	
4	3	COL 6	Takano_Lab_2	05/10/2017 12:33	114.6	ng/µl	
5	4	COL 7	Takano_Lab_2	05/10/2017 12:33	79.2	ng/µl	
6	5	COL 8	Takano_Lab_2	05/10/2017 12:34	82.0	ng/µl	
7	6	COL 9	Takano_Lab_2	05/10/2017 12:34	94.5	ng/µl	

UVP07005Oct52017.jpg



Lane1: 1kb ladder // 2: Parent plasmid (pETDUET EutSMNLK_PduP1-20_mCherry) // 3-7 digest of col 5-9 DNA



Predicted fragments of Gibson_AshEut_PPK with PstI and EcoRI

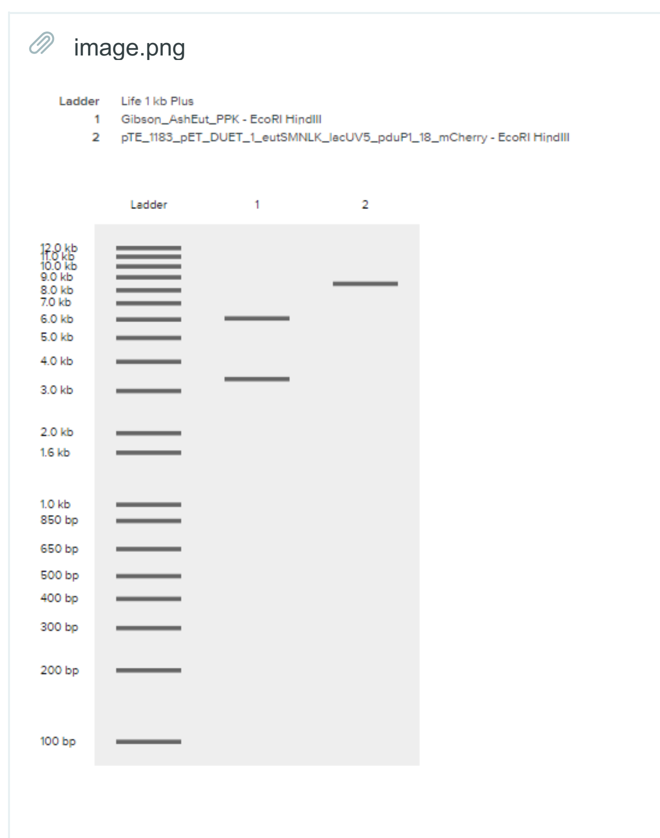
gibson assembly of eutSMNLK(ash) and mcherry PPK

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-28 to 2017-10-09

FRIDAY, 6/10/17

Double digest of the Gibson_AshEut_PPK with EcoR1 and HindIII

image.png



Virtual digest against both the parent plasmid pTE_1183_pET_DUET_1_eutSMNLK_lacUV5_pduP1_18_mCh and Gibson_AshEut_PPK with bands at 6.1 and 3.4 with the successful insert, and at 8.5 for an unsuccessful insert.

UVP07013Oct62017.jpg

1kb ladder // pTE_1183_pET_DUET_1_eutSMNLK_lacUV5_pduP1_18_mCherry // Colonies 5-9 from the assembly reaction

It's hard to tell because not all of the ladder is there. If we assume lane 2 is 8.5kb then the faint bands in the other lanes are the same size which suggests that I was unsuccessful, gut then there are bands at 3.5kb which suggests a sucessful insert.

gibson assembly of eutSMNLK(ash) and mcherry PPK

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Dates: 2017-09-28 to 2017-10-09

MONDAY, 9/10/17

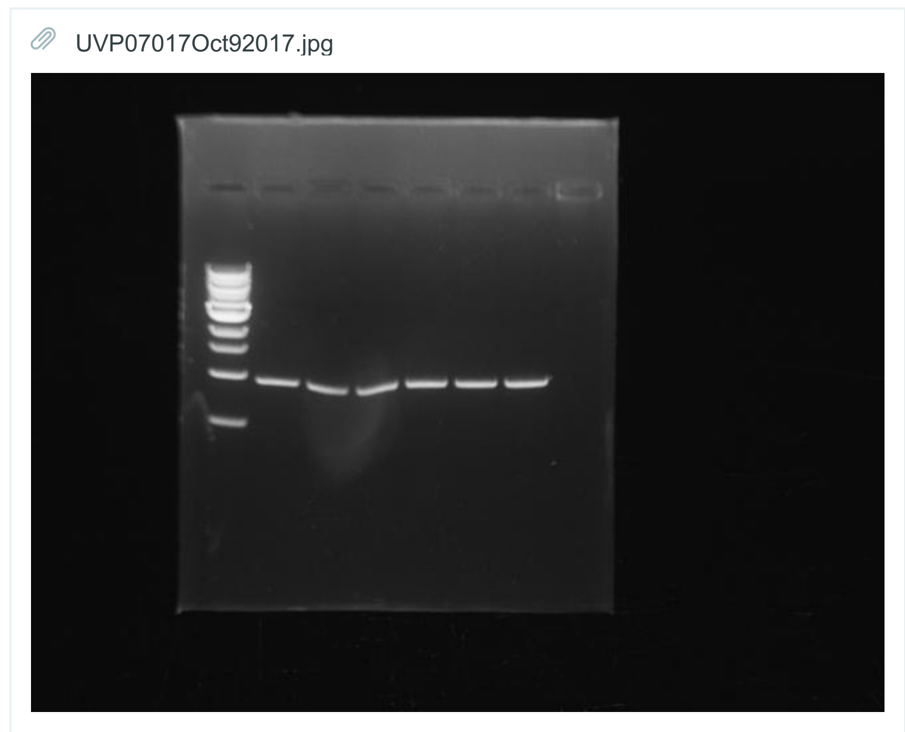
PCR to confirm(?) insert

Table8

	A	B	C	D
1	**Component**	**25 µl Reaction**	**7 reactions**	**Final Concentration**
2	Q5 High-Fidelity 2X Master Mix	12.5	87.5	1X
3	10 µM Forward Primer	1.25	8.75	0.5 µM
4	10 µM Reverse Primer	1.25	8.75	0.5 µM
5	Template DNA	variable	variable	< 1,000 ng
6	Nuclease-Free Water	10	70	

Primers: CP forward seq primer 1 CP reverse seq primer 1 , Stocks - control (pTE_1183_pET_DUET_1_eutSMNLK_lacUV5_pduP1_18_mCherry) then miniprepped DNA from colonies 5-9

UVP07017Oct92017.jpg



1 Kb Ladder, **2:** pTE_1183_pET_DUET_1_eutSMNLK_la **3:** Colony 5; **4:** Colony6; 5: **Colony 7; 6: colony 8; 7: colony 9**

digest of LK+tag+mCherry+PPK to check assembly

Project: Manchester iGEM 2017 Shared Project

Authors: james engleback

Dates: 2017-10-04 to 2017-10-11

WEDNESDAY, 11/10/17

Table1

	A	B	C	D	E	F	G
1	**#**	**Sample ID**	**User name**	**Date and Time**	**Nucleic Acid**	**Unit**	**A260 (Abs**
2	1	blank	Takano_Lab_2	11/10/2017 16:10	1.2	ng/µl	
3	2	1	Takano_Lab_2	11/10/2017 16:11	308.7	ng/µl	
4	3	2	Takano_Lab_2	11/10/2017 16:11	367.0	ng/µl	
5	4	3	Takano_Lab_2	11/10/2017 16:12	387.4	ng/µl	
6	5	4	Takano_Lab_2	11/10/2017 16:12	324.1	ng/µl	
7	6	5	Takano_Lab_2	11/10/2017 16:13	329.8	ng/µl	

digest and gel to confirm cotransformation of SMN and LK+tag+mcherry+ppk

Project: Manchester iGEM 2017 Shared Project

Authors: Adam Hannaford

Date: 2017-10-12

THURSDAY, 12/10/17

SMN is in pSB4A5

LK+tag+mCherry+ppk is in pSB1C3

digest miniprep with

20ul digest mix:

0.5ul EcoRI

0.5ul PstI

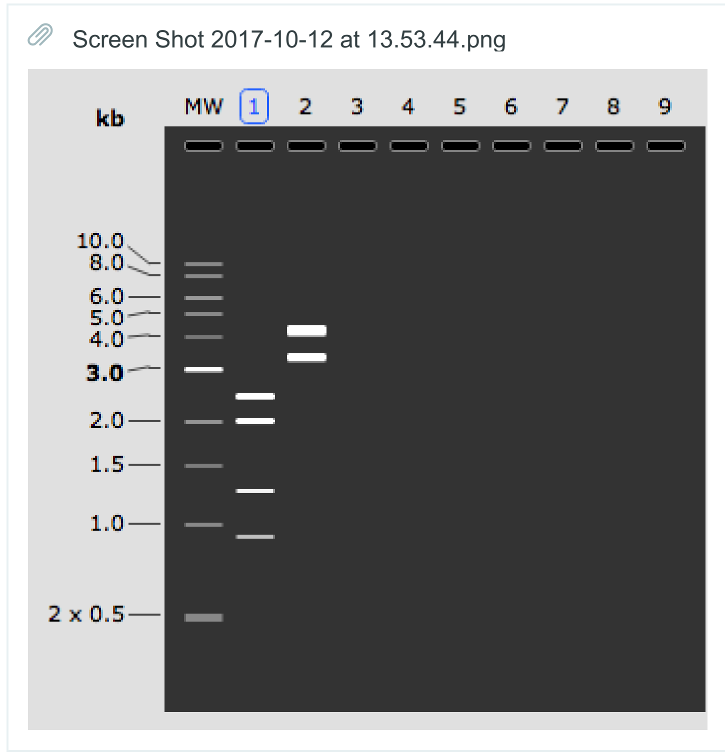
0.5ul BamHI

2ul cutsmart buffer

16.5ul of DNA

expected gel result in 1 lane

Screen Shot 2017-10-12 at 13.53.44.png



Screen Shot 2017-10-12 at 13.55.16.png

MW: 1 Kb DNA Ladder

▼ 1: LK-tag-mCherry-PPK
EcoRI + PstI + BamHI
1. 2454 bp
2. 2029 bp
3. 1260 bp
4. 925 bp

▼ 2: EutSMN_in_pSB4A5
XbaI + PstI
Noncutter: BamHI
1. 4354 bp
2. 3369 bp

Eut SMN + LK + pduD(1-20)_mCherry_cgPPK

Project: Manchester iGEM 2017 Shared Project
Authors: james engleback
Date: 2017-10-17

TUESDAY, 17/10/17

A colony from an index plate for a transformation corresponding to pSB4A5_Eut SMN + pSB1C3_Eut LK+pduD(1-20)_mCherry_cgPPK were grown up in LB + Chloramphenicol (35ng/ml) + Ampicillin (100ng/ml).
The culture was transferred in 5ml aliquots to falcon tubes that contained inducers at the following levels:

Table1

	Tube ID	[IPTG]/uM	[Arabinose]/uM	[Tetracycline]/nM
1	1	0	0	0
2	2	0	0	50
3	3	0	0	100
4	4	0	10	0
5	5	0	10	50
6	6	0	10	100
7	7	0	100	0
8	8	0	100	50
9	9	0	100	100
10	10	125	0	0
11	11	125	0	50
12	12	125	0	100
13	13	125	10	0
14	14	125	10	50
15	15	125	10	100
16	16	125	100	0
17	17	125	100	50
18	18	125	100	100
19	19	125	0	0
20	20	250	0	50
21	21	250	0	100
22	22	250	10	0
23	23	250	10	50
24	24	250	10	100
25	25	250	100	0
26	26	250	100	50
27	27	250	100	100

The 50ml falcon tubes were set up with the inducers so that they would reach the appropriate final concentration when 5ml of culture was added.
The master culture failed to grow today, so i left the tubes on the bench overnight and kept the maste culture at 37dehC overnight.

removing XbaI mutation from LK and LK-tag-mcherry-ppk

Project: Manchester iGEM 2017 Shared Project
Authors: Adam Hannaford
Date: 2017-10-20

FRIDAY, 20/10/17

1. make 10ul working stock of the primers BB-XbaLmut_Fwd and BB-Xbalmut_Rev
2. set up 2 cloneamp PCRs with the same primers but plasmids EutLK and EutLK-tag-mcherry-PPK

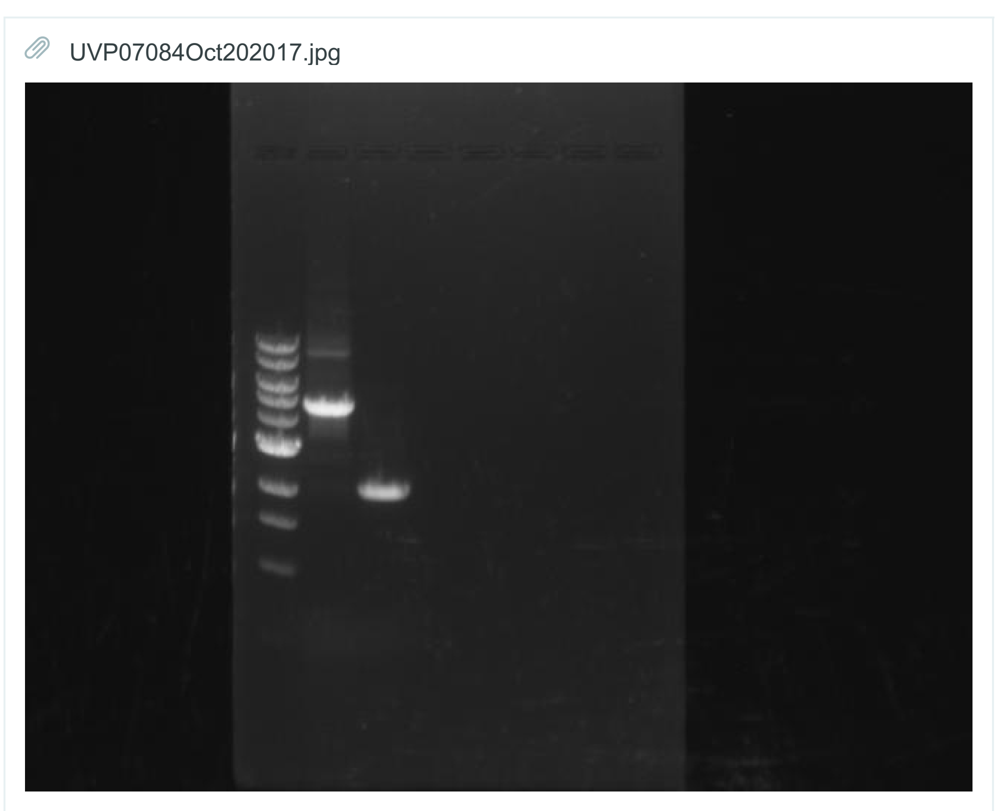
Table1

	A	B
1	Fwd Primer	1.25
2	Rev Primer	1.25
3	plasmid	0.2
4	mastermix	25
5	ddH2O	22.3
6		

PCR

Run on a gel

UVP07084Oct202017.jpg



PCR cleanup and Dpnl treat