CRISPR journal

Project: CRISPR in Vibrio

Authors: Michelle Scharte

MONDAY, 8/5/2019

CRISPR plasmid

Culture E.coli strain with plasmid on LB + kanamycin agar (45 of 20 mg/ml to achieve 30 ug/ml)

Grow overnight at 37C

started o/n culture in BHIv2 to make electrocompetent cells

TUESDAY, 8/6/2019

CRISPR plasmid

Pick colonies from the plate to grow on LB broth for plasmid isolation (2 from Amanda's plate: A1, A2; 2 from Nurul's plate: N1, N2)

WEDNESDAY, 8/7/2019

CRISPR plasmid

Isolate plasmid [CRISPR A1, A2, N1, N2 at -20C]

Make glycerol stock [CRISPR E. coli JM109. pJ08999 A1, A2, N1, N2 stored at -80C]

Concentrations of working solutions:

A1 23 ng/mcl

A2 31 ng/mcl

N1 22 ng/mcl

N2 19.8 ng/mcl

TUESDAY, 9/10/2019

Plasmid digestion:

CRISPR N1 DNA 0.5 mcg - 25mcl

BsaI HF v2 Enzyme - 0.5mcl

SmartCut buffer - 2.5

Total: 28mcl

WEDNESDAY, 9/11/2019

Gel purification of digested plasmid:

QIAGEN kit protocol

Digested isolated vector concentration 9.15 ng/mcl

Annealing of gRNA oligos:

Oligos F&R - 1mcl each

10x T4 Ligase buffer - 1mcl

H2O - 7mcl

Total: 10mcl

PCR: cooling down 0.1 °C per second from 95 to 25 °C (~20mins)

Concentrations of gRNAs:

gRNA1 1567 ng/mcl

gRNA2 1185 ng/mcl
gRNA3 1117 ng/mcl

Ligation:
Annealed oligos 1:200 -3mcl
Digested CRISPR vector - 3mcl
T4 ligase - 2mcl
T4 ligase buffer - 1.5mcl
H2O - 5.5 mcl
Total: 15mcl

Overnight ligation at 4 degrees

THURSDAY, 9/12/2019

Transformation to E.coli:
Competent cells (E.coli) - 60mcl
Insert DNA - 6mcl

30mins on ice
30secs heatshock 42 degrees
5mins on ice

Add 850mcl recovery medium (SOC)
Let grow for 1h

Plate on agar with Kan to identify positive clones

FRIDAY, 9/13/2019

The plan was to do colony PCR on grown clones, outgrowth of positive clones, Plasmid purification, and inoculation of V. natriegens (for competent cells), but there were no colonies on plates. We repeated the protocol starting with plasmid digestion 5 times without any success.

WEDNESDAY, 10/2/2019

We decided to skip the step of gel purification and only used a small amount of DNA to determine the plasmid size

THURSDAY, 10/3/2019

Ligation protocol --> overnight

FRIDAY, 10/4/2019

Transformation to E.coli & plating on Kan plate

MONDAY, 10/7/2019

cPCR with gRNA3 (overgrown) colonies --> nothing on gel

TUESDAY, 10/8/2019

cPCR of 5 colonies per gRNA insert (15 in total) and seeding a liquid culture with Kan

Reaction setup

	A	B	C
1	**component**	**1 rxn**	**16 rxns**
2	water	10	160
3	PCR buffer	2.5	40
4	dNTP	0.5	8
5	taq Polymerase	0.125	2
6	primers (F & R)	1+1	16+16
7	**total**	**15.125**	**242**

Thermocycling conditions

	A	B	C	D
1	**step**	**temp**	**time**	
2	initial denaturation	94	7 min	
3	denaturation	94	30 sec	30 cycles
4	annealing	50	30 sec	
5	elongation	72	1 min	
6	final extension	72	10 min	
7	hold	4		

cPCR --> nothing on gel

WEDNESDAY, 10/9/2019

Plasmid DNA extraction using QIAprep spin miniprep kit (QIAGEN) from two liquid cultures per gRNA (6 in total)

Concentrations:

1.1 29.9 ng/ul
1.3 17.2 ng/ul
2.6 19.1 ng/ul
2.8 16.1 ng/ul
3.11 16.8 ng/ul
3.13 18.2 ng/ul

Sequiencing (Eurofins): 2 tubes per sample

Plasmid DNA 7 ul [around 100 ng/ul]
Forward or Reverse primer 10 ul [10x dilution]

10 out of 12 samples had inserts

MONDAY, 10/14/2019

Homologous parts PCR

- Resuspend gBlocks (1000ng):

spin down the tube
add molecular grade water 100 ul (--> 10ng/ul)
vortex
incubate at 50 deg 15-20 mins
vortex, centrifuge
concentration NanoDrop --

- Make working solution for F and R primers (1500 and 600bp)

10ul F + 10ulR + 80ul water

Reaction setup Q5 pol

	A	B	C
1	**COMPONENT**	**25 µl REACTION**	**FINAL CONCENTRATION**
2	10 µM F + R primer	1.25 µl	0.5 µM
3	Template DNA	1ul	< 1,000 ng (10ng is recommended)
4	2x Q5 master mix	12.5 ul	
5	Water	10.35 ul	

PCR 1500bp

	A	B	C	D
1	**STEP**	**TEMP**	**TIME**	
2	Initial Denaturation	98°C	30 seconds	
3	denaturation	98°C	5–10 seconds	25–35 Cycles
4	annealing	57	10–30 seconds	
5	elongation	72°C	90 seconds	
6	Final Extension	72°C	2 minutes	
7	Hold	4–10°C		

PCR 600bp

	A	B	C	D
1	**STEP**	**TEMP**	**TIME**	
2	Initial Denaturation	98°C	30 seconds	
3	denaturation	98°C	5–10 seconds	25–35 Cycles
4	annealing	60	10–30 seconds	
5	elongation	72°C	40 seconds	
6	Final Extension	72°C	2 minutes	
7	Hold	4–10°C		

- PCR purification (QIAGEN PCR purification kit)

TUESDAY, 10/15/2019

Transformation to E.coli and V.natriegens

Transformation to E.coli:
Competent cells (E.coli) - 60ul
Plasmid DNA - 2ul
Homologous part 12ul

30min on ice
30sec heatshock 42 degrees
5mins on ice
Add 850mcl recovery medium (SOC)
Let grow for 1h

Transformation to V.natriegens:
Competent cells - 60 ul
Plasmid DNA - 2ul
Homologous part 12ul

10min on ice
2.5min heatshock 42 degrees
5mins on ice
Add 3ml recovery medium
Let grow for 2h

Both E.coli and V.natriegens were plated on LB (or LBV for V.natriegens) agar plates containing 5 μg ml−1 kanamycin and 0.2% mannose for induction of cas9 under the control of PmanP. Plates were first incubated at 30°C.

THURSDAY, 10/17/2019

After 2 days colonies were placed with a toothpick on LB plates without antibiotics and E.coli plates were incubated at 50°C.

FRIDAY, 10/18/2019

To identify whether we reached auxotrophy in bacteria we replated colonies on M17 and M9 (without histidine) minimal media plates