B. subtilis Optimized ORF's

21/06/2021

Linearization of ECE59-mCherry plasmid and addition of overlap sequences for gBlocks via PCR (see cloning protocol):

The ECE59-mCherry plasmid that was prepared on 07.04.21 was linearized via a PCR reaction, with primers containing overlap sequence to each gBlock and that excludes the original mCherry gene. (see Primers document).

The PCR was performed with Q5- HF 2x Master Mix from NEB, and according the following procedure:

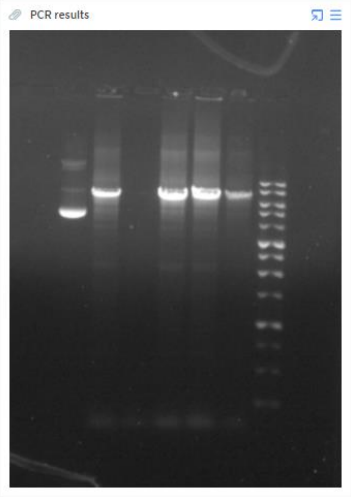
Each PCR tube contained:

- 25ul of Q5- HF 2x Master Mix
- 2.5ul of 10uM Forward and Reversed primers
- 10ng of DNA template (ECE59-mCherry plasmid)
- Nuclease-free water to make 50ul total volume

Thermocycling conditions:

- Initial Denaturation: 98°C, 30sec
- 30 cycles:
    1. 98°C, 30 sec
    2. 65°C, 30 sec
    3. 72°C, 30 sec/kbp
- Final extension: 72°C, 2min
- Hold: 10°C

Following PCR, 10ul from each reaction were taken for analysis in DNA electrophoresis. The rest was treated with 1ul DpnI+ 4.5ul of NEB buffer #4, at 37°C for 1hr (to degrade the original vector).



Loading scheme (left-right):
Unmodified plasmid (cnt), CAI, TDR difference, TDR ratio, TAI difference, TAI ratio, Ladder.

Next, all PCR products were cleaned with and according to the protocol of Monarch PCR&DNA Cleanup Kit. The concentration of linearized plasmids was measured with Nanodrop:

Concentration of PCR products following cleaning

	A	B	C	D
1	Sample	Concentration (ng/ul)	260/280	260/230
2	CAI	148.5	1.80	1.67
3	TAI- D	196.3	1.79	1.84
4	TAI- R	182.0	1.85	1.92
5	TDR- D	38.4	1.97	1.73
6	TDR- R	184.6	1.84	1.93

Additionally, concentrations of gBlocks (inserts designed by our software and synthesized by IDT) were determined with Nanodrop after dissolving them with 100ul Nuclease-free water:

gBlocks Concentration

	A	B
1	gBlock	Concentration (ng/ul)
2	CAI	11.9
3	TAI- D	4.7
4	TAI- R	5.6
5	TDR- D	8.1
6	TDR- R	9.2

Then, Gibson assembly reaction was performed to insert each gBlock to its corresponding linearized vector, as follows:

Vector (from PCR) and gBlock in a molar ratio of 1:2 (0.13pmols of gBlock and 0.07pmols of the vector) were added to 10ul of HiFi DNA Assembly Master Mix (NEB) and deionized water to make 20ul. Samples were incubated at 50°C for 1hr.
2ul from each Gibson assembly reaction product was mixed with NEB competent cells for bacterial transformation. Then, bacteria were spread over pre-warmed LB plates with 10ug/ml chloramphenicol. The plates were incubated at 37°C ON.

22/06/2021

A single colony from each plate was picked and transferred into 7ml LB for ON starter.

23/06/2021

Plasmids were prepared from ON bacterial starters with Monarch Miniprep kit. Plasmids concentration was measured via Nanodrop as follows:

Table6

	A	B	C	D
1	Sample	Conc. (ng/ul)	260/280	260/230
2	TAI- Ratio NEB	84.6	1.78	1.59
3	TDR- Ratio NEB	162	1.83	2.00
4	TDR- Diff NEB	235.4	1.86	1.74
5	CAI (K-12)	302.9	1.89	1.29
6	TDR-Diff (K-12)	269.5	1.86	2.06
7	TAI-Ratio (K-12)	272.6	1.85	1.98

New colonies of CAI-NEB, TDR-R-NEB, TAI-D-NEB, TDR-D, TDR-R, TAI-D, and CAI were picked to an ON starter, as performed the day before

24/06/2021

Plasmids were prepared from ON bacterial starters with Monarch Miniprep kit. Plasmids concentration was measured via Nanodrop as follows:

Table7

	A	B	C	D
1	Smp	Conc. (ng/ul)	260/280	260/230
2	CAI-NEB	115.6	1.82	1.83
3	TDR-R-NEB	338.4	1.81	1.27
4	TAI-D-NEB	218.5	1.90	2.37
5	TDR-D	26.3	2.93	0.64
6	TDR-R	12.9	1.56	0.78
7	TAI-D	35.4	1.56	0.72
8	CAI	270.6	1.85	2.13

Plasmids prepared on the previous day were sent for validation by Sanger sequencing.

27/06/2021

Sequencing results:

Only TDR-ratio and TAI-ratio transferred in NEB competent cells contained the correct insert.

28/06/2021

Plasmids prepared on 24.06 were sent for sequencing.

29/06/2021

Sequencing results:

TDR-R and CAI from NEB were positive for the correct sequences.

07/07/2021

Transformation of CAI and TAI-R into *B. subtilis* and *E. coli*:

Since the extracted TDR-R plasmids showed additional band when examined by DNA electrophoresis, we transform bacteria with CAI and TAI-R plasmids only. In parallel, colonies obtained from the Gibson reaction are screening for TDR-R, TDR-D, and TAI-D.

Approximately 300ng or 50ng of each plasmid were transformed into *B. subtilis* and *E. coli* respectively, according to the protocols. LB plates were incubated ON at 37°C.

08/07/2021

Colonies from the transformed bacteria were picked for ON starters to make glycerol stocks.

09/07/2021

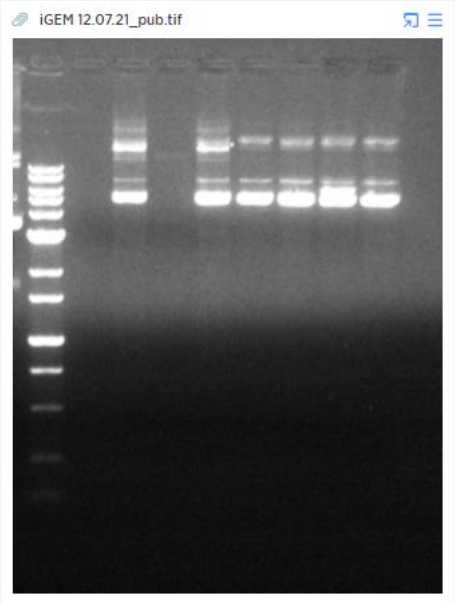
560ul from each ON starter (*E. coli* and *B. subtilis* containing either CAI or TAI-R modified plasmid) were mixed with 240ul of 50% glycerol. Stocks were stored at -80°C.

11/07/2021

Colonies obtained from the following Gibson reaction were picked and transferred into 6ml LB for ON growth (for TAI-D, TDR-D, and TDR-R).
Additionally, starters of *E. coli* and *B. subtilis* containing either CAI or TAI-R were prepared directly from glycerol stocks.

12/07/2021

Plasmid minipreps were obtained from all starters of the previous day, except TAI-D that failed to grow. Plasmid purification from *B. subtilis* was failed since cells weren't pretreated with Lysozyme to break cells wall.



Loading scheme from left to right:
B. subtilis CAI, *E. coli* CAI, *B. subtilis* TAI-R, *E. coli* TAI-R, TDR-R2, TDR-D2, TDR-R1 and TDR-D1.

Plasmids concentrations were measured via Nanodrop:

Table8

	A	B	C	D
1	Smp	Conc. (ng/ul)	260/280	260/230
2	TDR-D1	174.3	1.86	2.48
3	TDR-R1	235.1	1.86	2.11
4	TDR-D2	173.9	1.86	2.04
5	TDR-R2	223.6	1.84	2.08
6	Transformed E.coli -TAI-R	310.4	1.80	2.05
7	Transformed E.coli -CAI	306.3	1.89	2.11
8	TAI-D 1	121.6	1.86	1.92
9	TAI-D 2	171.7	1.89	2.04
10				

13/07/2021

Plasmids were sent for Sanger sequencing.

14/07/2021

Sequencing results:

Table9

	A	B	C	D	E
1	name	Sequencing with Frw primer	Sequencing with Rev primer	Original (to F and R)	result
2	TDR R2	Aligned!	Aligned!	not aligned	V
3	TDR D2	Aligned!	Aligned!	not aligned	V
4	TDR R1	one mutation	same thing	not aligned	X
5	TDR D1	Aligned!	Aligned!	not aligned	V
6	Transformed E.coli with CAI	not aligned	not aligned	aligned	X
7	Transformed E.coli with TAI-R	Aligned!	Aligned!	not aligned	V
8					
9					
10					

18/07/2021

E. coli was transformed with all optimization results (but CAI-NEB, of which we already have a glycerol stock).

Additionally, W.T. *B. subtilis* was plated for transformation.

19/07/2021

According to the sequencing results, the TAI-D1 contained a mutation so we continued using TAI-D2 for the *B. subtilis* transformation and plating.
Additionally, all *E. coli* transformations have worked.

20/07/2021

Only a small number of colonies were present on the *B. subtilis* plate- we should consider adding more plasmid (maybe 400 nanograms), or reduce antibiotic concentration. However, there were a few colonies (1-4) for each optimization.

21/07/2021

Made a glycerol stock for all optimizations in both bacteria (but *E. coli* CAI-NEB).
We conducted the assay with the bacteria at O.D. 0.2 in M9 medium according to the POC assay protocol. Unfortunately, we inaccurately prepared the M9 solution, so bacteria weren't growing.

29/07/2021

Plasmid extraction (miniprep) from *E. coli* and *B. subtilis* (*E. coli* protocol using the regular kit, and *B. subtilis* extraction was performed using a Geneaid kit from Ido's lab, in which the bacteria were treated with 100ul of 10mg/ml Lysozyme to break cell wall following resuspension step).

Plasmids were sent for Sanger sequencing.

01/08/2021

Our lab *E. coli* strain was transformed with either TAI-R and CAI plasmids (prepared on 23.06 and 24.06 respectively) once again since the sequencing of plasmids prepared from previously *E. coli* transformed cells weren't contained the correct insert.

04/08/2021

One colony from *E. coli* CAI and TAI-R transformations was picked and transferred to a 5ml LB starter and incubated ON at 37°C.

05/08/2021

Glycerol stocks for *E. coli* CAI and TAI-R were made from the starters.

Plasmids were prepared from lab strain ON starters with Monarch miniprep kit.
Plasmidsconcentrations were evaluated via Nanodrop:

Sample	Conc. (ng/ul)	260/280	260/230
E. coli CAI	111.3	1.83	2.29
E. coli TAI R	225.4	1.85	2.15

Moreover, plasmids were examined by DNA electrophoresis.

07/08/2021

Assay for *B. subtilis* optimized mCherry gene:

Sequencing confirmation for transformed *E. coli* CAI and TAI-R (from 05/08/21) was done in parallel to the assay: starters prepared from colonies of *E. coli* CAI and TAI-R (streak on the previous day), to extract the plasmid for sequencing.

The assay was repeated **three times** in the following manner:

- Bacterial streaking over LB plates at 07,08 and 09.08.21
- Starters from colonies at 08, 09, and 10.08.21.
- Bacterial growth and intensity measurements at 09, 10, and 11.08.21.

Streaking bacteria:

Glycerol stocks of *E. coli* and *B. subtilis* containing the following plasmids were streaked over an LB+10mg/ml Chloramphenicol:

- ECE-59 (lacking mCherry gene)
- ECE-59-mCherry (original mCherry)

And the following modified mCherry genes:

- TDR-R
- TDR-D
- TAI-R
- TAI-D
- CAI

08/08/2021

Plasmids were prepared from ON starters of *E. coli* CAI and TAI-R (new transformation from 05/08/21) and sent for Sanger sequencing.

Assay for *B. subtilis* optimized mCherry gene (continue):

One colony from each plate was picked and transferred to 5ml LB+10mg/ml Chloramphenicol for ON starter.

Moreover, new plates were streaked with bacteria for a second biological repeat of the assay, as described on 07/08/21.

09/08/2021

Sequencing results for *E. coli* CAI and TAI-R were positive for the correct plasmids.

Assay for *B. subtilis* optimized mCherry gene (continue):

The assay was performed according to the protocol. Since the bacteria were highly concentrated (according to OD600nm measurements prior to plate loading), the dilution to a final OD600nm of 0.15 was not accurate among all bacterial samples.

OD measurements after incubation and dilution calculations- First repetition

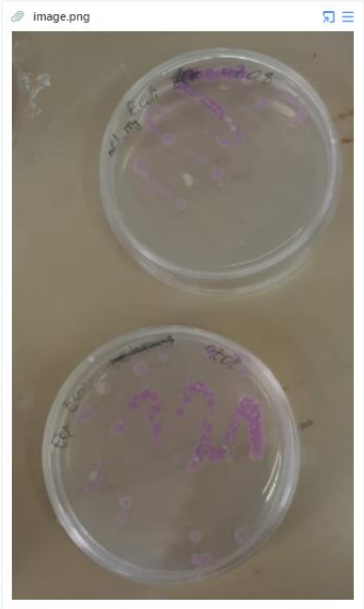
	A	B	C	D	E	F
1	Bacteria	Sample name	OD600nm	Volume of sample to dilute to an OD600=0.15	M9 for dilution to an OD600=0.15	
2	*E. coli*	cai	2.08	72.11538	1927.885	
3		tai r	1.905	78.74016	1921.26	
4		tai d	1.804	83.14856	1916.851	
5		tdr r	0.124	1209.677	790.3226	
6		tdr d	1.478	101.4885	1898.512	
7						
8	*B. subtilis*	cai	0.664	225.9036	1774.096	
9		tai r	0.763	196.5924	1803.408	
10		tai d	1.123	133.5708	1866.429	
11		tdr r	1.019	147.2031	1852.797	
12		tdr d	0.639	234.7418	1765.258	
13						
14	Ctrl plasmids	*E. coli mcherry*	1.786	83.98656	1916.013	
15		E. coli ECE	1.599	93.80863	1906.191	
16		*B. subtilis mcherry*	1.378	108.8534	1891.147	
17		*B. subtilis ECE*	1.052	142.5856	1857.414	

Plate loading scheme:

Plate loading scheme: 9.8

	A	B	C	D	E	F	G
1	mcherry ecoli	mcherry ecoli	mcherry ecoli		ece bac	ece bac	ece bac
2	ece ecoli	ece ecoli	ece ecoli		mcherry bac	mcherry bac	mcherry bac
3	cai ecoli	cai ecoli	cai ecoli		cai bac	cai bac	cai bac
4	tai r ecoli	tai r ecoli	tai r ecoli		tai r bac	tai r bac	tai r bac
5	tai d ecoli	tai d ecoli	tai d ecoli		tai d bac	tai d bac	tai d bac
6	tdr r ecoli	tdr r ecoli	tdr r ecoli		tdr r bac	tdr r bac	tdr r bac
7	tdr d ecoli	tdr d ecoli	tdr d ecoli		tdr d bac	tdr d bac	tdr d bac
8	blank	blank	blank		blank	blank	blank

Additionally, there has probably been a mistake in the *E. coli* ECE59 streaking since the colonies were pink and exhibited fluorescence as mCherry sample.



Therefore, in the final calculations, we used the *B. subtilis* ECE-59 as the negative fluorescence control for both bacteria. And the assay will be repeated one more.

- Colonies were picked from the second repetition plates to LB starters as described at 08.08.21.
- Moreover, new plates were streaked with bacteria for the third biological repeat of the assay, as described on 07/08/21.

10/08/2021

Assay for *B. subtilis* optimized mCherry gene (continue):

The second repetition of the assay was performed according to the protocol. Bacteria were diluted to OD600nm of 0.265 (corresponding to the OD value of *B. subtilis* ECE59 sample). Since OD of *E. coli* TDR-R was very low compared to all bacterial samples, it wasn't further diluted and was loaded as is in the 96-well plate.

OD measurements after incubation and dilution calculations- second repetition

	A	B	C	D
1	Sample	OD values	Volume of sample to dilute to an OD600=0.265	M9 for dilution to an OD600=0.265
2	EC CAI	1.679	0.315664086	1.68433591
3	EC TAI-R	1.673	0.316796175	1.68320383
4	EC TAI-D	1.707	0.310486233	1.68951377
5	EC TDR-R	0.026	20.38461538	-18.384615
6	EC TDR-D	1.428	0.371148459	1.62885154
7	BS CAI	0.341	1.554252199	0.4457478
8	BS TAI-R	0.466	1.137339056	0.86266094
9	BS TAI-D	0.428	1.238317757	0.76168224
10	BS TDR-R	0.459	1.154684096	0.8453159
11	BS TDR-D	0.405	1.308641975	0.69135802
12	EC ECE	2.099	0.252501191	1.74749881
13	EC mCherry	1.498	0.353805073	1.64619493
14	BS ECE	0.265	2	0
15	BS mCherry	0.32	1.65625	0.34375

Plate loading scheme:

Plate loading scheme- second repetition 10.8

	A	B	C	D	E	F	G
1	ece bac	ece bac	ece bac		mcherry ecoli	mcherry ecoli	mcherry ecoli
2	mcherry bac	mcherry bac	mcherry bac		ece ecoli	ece ecoli	ece ecoli
3	cai bac	cai bac	cai bac		cai ecoli	cai ecoli	cai ecoli
4	tai r bac	tai r bac	tai r bac		tai r ecoli	tai r ecoli	tai r ecoli
5	tai d bac	tai d bac	tai d bac		tai d ecoli	tai d ecoli	tai d ecoli
6	tdr r bac	tdr r bac	tdr r bac		tdr r ecoli	tdr r ecoli	tdr r ecoli
7	tdr d bac	tdr d bac	tdr d bac		tdr d ecoli	tdr d ecoli	tdr d ecoli
8	blank	blank	blank		blank	blank	blank

Colonies were picked from the third repetition plates to LB starters as described at 08.08.21.

11/08/2021

Assay for *B. subtilis* optimized mCherry gene (continue):

The third repetition of the assay was performed according to the protocol. Bacteria were diluted to OD600nm of 0.3. Since OD of *E. coli* TDR-R was very low compared to all bacterial samples, it wasn't further diluted and was loaded as is in the 96-well plate.

OD measurements after incubation and dilution calculations- third repetition

	A	B	C	D
1	Sample	OD values	V	M9
2	E.coli	OD600nm	to OD600nm=0.3	M9
3	ECE59	1.609	0.37	1.63
4	mCherry	1.519	0.39	1.61
5	CAI-w	1.972	0.30	1.70
6	CAI-p	1.943	0.31	1.69
7	TAI-D	1.688	0.36	1.64
8	TAI-R	1.949	0.31	1.69
9	TDR-D	1.479	0.41	1.59
10	TDR-R	0.09	na	na
11	B.subtilis			
12	ECE59	0.57	1.05	0.95
13	mCherry	0.659	0.91	1.09
14	CAI	0.542	1.11	0.89
15	TAI-D	0.693	0.87	1.13
16	TAI-R	0.712	0.84	1.16
17	TDR-D	0.601	1.00	1.00
18	TDR-R	0.669	0.90	1.10

Plate loading scheme:

loading plate 12.8

	A	B	C	D	E	F	G
1	ece ecoli	ece ecoli	ece ecoli	Blank	ece bac	ece bac	ece bac
2	mcherry ecoli	mcherry ecoli	mcherry ecoli	Blank	mcherry bac	mcherry bac	mcherry bac
3	tai d ecoli	tai d ecoli	tai d ecoli	Blank	tai d bac	tai d bac	tai d bac
4	tai r ecoli	tai r ecoli	tai r ecoli		tai r bac	tai r bac	tai r bac
5	tdr d ecoli	tdr d ecoli	tdr d ecoli		tdr d bac	tdr d bac	tdr d bac
6	tdr r ecoli	tdr r ecoli	tdr r ecoli		tdr r bac	tdr r bac	tdr r bac
7	cai ecoli	cai ecoli	cai ecoli		cai bac	cai bac	cai bac
8	blank	blank	blank				

15/08/2021

Forth biological repeat of the assay:

Described here briefly. For more details see protocol.

Bacteria were streaked over LB-agar plates.

16/08/2021

Colonies were transferred to ON starters.

17/08/2021

The assay was performed according to the protocol.

Plate loading scheme:

Well5

	1	2	3	4	5	6
A	B. subtilis - CAI	B. subtilis - CAI	B. subtilis - CAI	E.coli-CAI	E.coli-CAI	E.coli-CAI
B	B. subtilis - TDR R	B. subtilis - TDR R	B. subtilis - TDR R	E.coli-TDR R	E.coli-TDR R	E.coli-TDR R
C	B. subtilis - TDR D	B. subtilis - TDR D	B. subtilis - TDR D	E.coli-TDR D	E.coli-TDR D	E.coli-TDR D
D	B. subtilis - TAI R	B. subtilis - TAI R	B. subtilis - TAI R	E.coli-TAI R	E.coli-TAI R	E.coli-TAI R
E	B. subtilis - TAI D	B. subtilis - TAI D	B. subtilis - TAI D	E.coli-TAI D	E.coli-TAI D	E.coli-TAI D
F	B. subtilis - mCherry	B. subtilis - mCherry	B. subtilis - mCherry	E.coli-mCherry	E.coli-mCherry	E.coli-mCherry
G	B. subtilis - ECE	B. subtilis - ECE	B. subtilis - ECE	E.coli-ECE	E.coli-ECE	E.coli-ECE
H	Blank	Blank	Blank	Blank	Blank	Blank