# Promoters Optimization

05/09/2021

**Cloning of optimized promoters for *E. coli* and *B. subtilis* into the original plasmid, by replacing the original promoter with PCR amplification and Gibson Assembly:**

PCR amplification of mCherry plasmid and exclusion of the p43 promoter:

First, six and five optimized promoters for *B. subtilis* and *E. coli*, respectively, were chosen and labeled according to the table below:

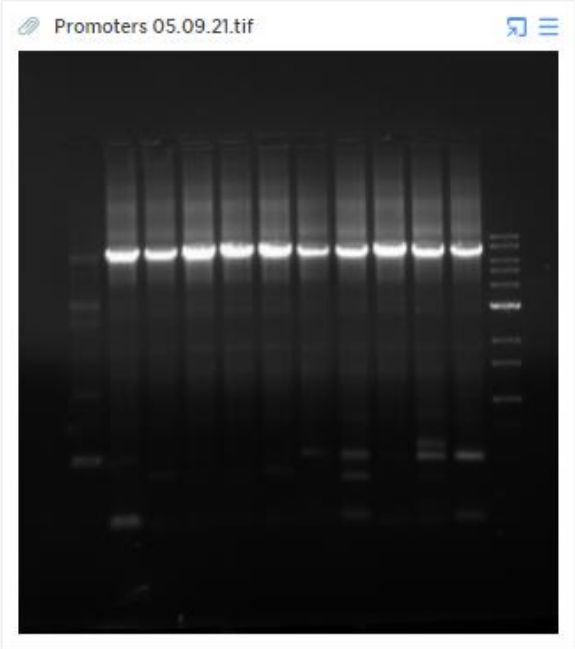
Table16

| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | Bacteria | Code | Name by modeling team | Primers' names | gBlock |
| 2 | B.subtilis | A1-1 | ribosomal protein s10 | rpsj Fwd (Rev same as rpsj syn!) | IDT |
| 3 | B.subtilis | A1-2 | ribosomal protein s10 synthetic version | rpsj syn Fwd + Rev | IDT |
| 4 | B.subtilis | D1 | L-lactate permease | lactate Fwd + Rev | twist (D1) |
| 5 | B.subtilis | A2 | ribosomal protein L4 | L4 Fwd + Rev | twist (A2) |
| 6 | B.subtilis | B2 | rpld2 (synthetic) | rpld syn Fwd (Rev same as A2 [L4]) | twist (B2) |
| 7 | B.subtilis | E1 | lctp4 | lctp syn (primers same as D1 [lactate]) | twist (E1) |
| 8 | | | | | |
| 9 | E.coli | C2 | cAMP phospho | cAMP Fwd + Rev | twist (C2) |
| 10 | E.coli | D2 | cpda synthetic | cpda syn (same primers as C2 [cAMP]) | twist (D2) |
| 11 | E.coli | M1 | RNA polymerase transcription factor DK (dksa) | dksa Fwd + Rev | IDT |
| 12 | E.coli | T1 | 16S rRNA pseudo | rsua Fwd + Rev | IDT |
| 13 | E.coli | K1 | rpma | rpma Frw+Rev | IDT |

The mCherry vector was PCR-amplified with corresponding primers containing overlapping tails for its gBlock (=promoters),(see Primers document), in the following thermocycling conditions:

- Initial Denaturation: 98°C, 3 sec
- 30 cycles:
  1. 98°C, 10 sec
  2. 57°C, 30 sec
  3. 72°C, 3:50 min
- Final extension: 72°C, 2min
- Hold: 10°C
- Total volume: 50ul

10ul from each PCR product was examined by a DNA electrophoresis gel as below:
The rest of the volume was kept at -20°C.




Loading scheme: T1, A1S, K1, A2, A1, B2, M1, C2, D1, E1 & D2.

09/09/2021

The rest of the PCR products were treated with DpnI in a total volume of 50ul, 10% CutSmart buffer for 1hr at 37°C.
Since PCR amplification for promoters cloning resulted in unspecific bands (see the gel above), we ran the total volume of PCR products following degradation by DpnI in a wide-wells agarose gel, and relevant bands (~7400bp) were extracted by GenElute Gel Extraction Kit from Sigma, according to manufacturer's instructions. Then, concentrations of linearized plasmids and gBlocks (promoters from IDT and Twist= inserts) were measured by Nanodrop:

Concentrations of PCR products and gBlockes and calculation for Gibson Assembly reaction volumes

| | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| 1 | PCR product | PCR Conc | Gibson | gBlock | Conc | Gibson | Vector+insert | UPW |
| 2 | A2 | 18.1 | 6.0 | A2 | 8.7 | 2.5 | 8.5 | 1.5 |
| 3 | B2 | 5.5 | 9.1 | B2 | 11.6 | 0.5 | 9.6 | 0.4 |
| 4 | C2 | 24.4 | 4.4 | C2 | 11.1 | 2.0 | 6.4 | 3.6 |
| 5 | D1 | 23 | 4.7 | D1 | 12.3 | 1.8 | 6.5 | 3.5 |
| 6 | D2 | 25.7 | 4.2 | D2 | 11 | 2.0 | 6.2 | 3.8 |
| 7 | E1 | 19.2 | 5.6 | E1 | 10.4 | 2.1 | 7.7 | 2.3 |
| 8 | K1 | 28.3 | 3.8 | K1 | 15.8 | 1.4 | 5.2 | 4.8 |
| 9 | M1 | 17.7 | 6.1 | M1 | 13.3 | 1.7 | 7.8 | 2.2 |
| 10 | T1 | 13.5 | 8.0 | T1 | 15.3 | 1.4 | 9.4 | 0.6 |
| 11 | A1 | 11.5 | 9.4 | A1 | 19.7 | 1.1 | 10.5 | 0 |
| 12 | A1S | 14 | 7.7 | A1S | 14.7 | 1.5 | 9.2 | 0.8 |

The table above shows measured concentrations of vectors and inserts, as well as the amount in µl needed for Gibson assembly reaction, in a molar ratio of 1:2 in favor of the insert.

13/09/2021

**Gibson Assembly for promoters:**

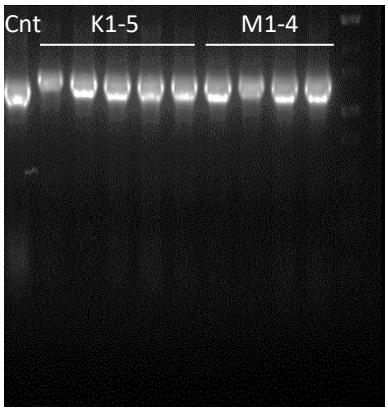
Gibson assembly was performed as follows:
Vector (from 09.09.21): gBlock ratio was 1:2, in which 0.05pmols of gBlock and 0.025pmols of vector were added to 10ul of HiFi DNA Assembly Master Mix (NEB) and deionized water to make 20ul. Samples were incubated at 50°C for 1hr. Then, 3ul from each reaction was used for bacterial transformation according to NEB's protocols. 100ul of cells were spread over an LB- agar + chloramphenicol 10ug/ul plates and incubated ON at 37°C.

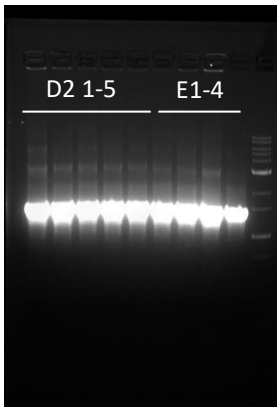
17/09/2021

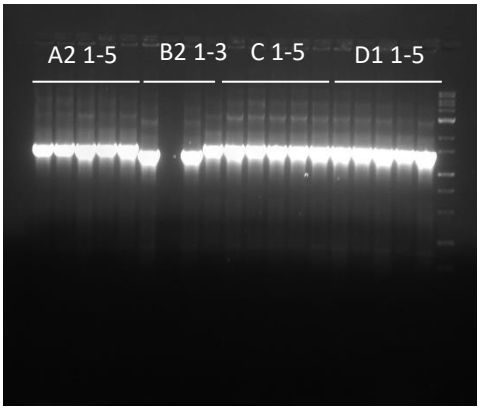
**Screening new primer-positive colonies by colony PCR:**

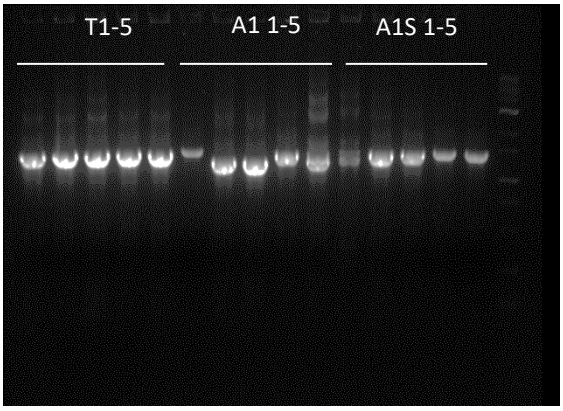
Colonies were picked for colony PCR assessment in order to find positive colonies having the new insert. Since the size of the vector with the insert is larger by ~360bp than the original vector, colony screening was based on the vector's size instead of insert-specific primers.

Colony PCR was performed on four or five colonies from each transformation, according to the protocol. Additionally, bacterial suspensions from picked colonies were streaked over an LB-agar plate.



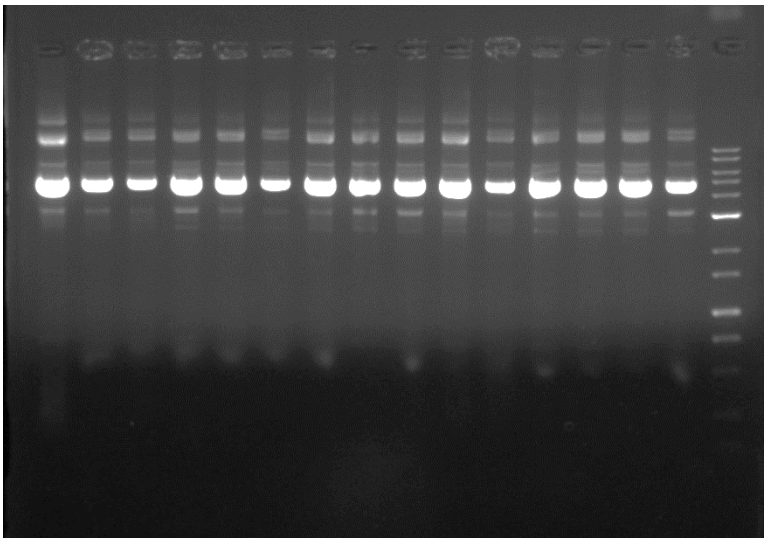

18/09/2021

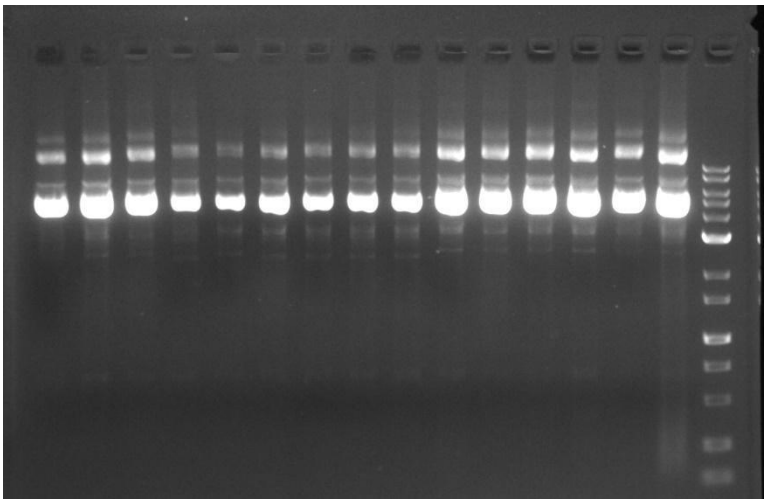
Insert-positive colonies were transferred to ON Starters, except A1S and B2 that failed to grow.

19/09/2021

Plasmids were prepared from all ON starters with PureYield plasmid miniprep system (Promega). And plasmids concentrations were measured via NanoDrpo and examined in DNA electrophoresis gel:

| Sample | conc. (ng/ul) | 260/280 | 270/230 |
|---|---|---|---|
| **M-1** | 435.8 | 1.88 | 2.3 |
| **M-4** | 438.5 | 1.89 | 2.28 |
| **M-5** | 472 | 1.89 | 2.34 |
| **A1-2** | 226.8 | 1.88 | 2.25 |
| **A1-4** | 176.3 | 1.87 | 2.19 |
| **A1-5** | 262.2 | 1.89 | 2.28 |
| **TAI-D1** | 238.8 | 1.83 | 1.68 |
| **TAI-D2** | 223.4 | 1.88 | 2.27 |
| **TAI-D3** | 239 | 1.88 | 2.25 |
| **T-1** | 433.8 | 1.89 | 2.29 |
| **T-3** | 378 | 1.87 | 2.19 |
| **T-4** | 442.3 | 1.87 | 2.14 |
| **E-1** | 474.9 | 1.88 | 2.29 |
| **E-2** | 412.7 | 1.87 | 2.27 |
| **E-3** | 600.8 | 1.91 | 2.32 |
| **K-1** | 630.3 | 1.9 | 2.3 |
| **K-2** | 372.3 | 1.87 | 2.3 |
| **K-4** | 263.5 | 1.88 | 2.15 |
| **D1-2** | 390.8 | 1.891 | 2.33 |
| **D1-3** | 375.2 | 1.88 | 2.27 |
| **D1-4** | 251.1 | 1.84 | 2.01 |
| **C-1** | 382.6 | 1.88 | 2.25 |
| **C-4** | 334.7 | 1.86 | 2.11 |
| **C-5** | 380.6 | 1.88 | 2.22 |
| **A2-1** | 453.3 | 1.9 | 2.3 |
| **A2-4** | 373.8 | 1.87 | 2.07 |
| **A2-5** | 364.1 | 1.88 | 2.2 |
| **D2-2** | 367.3 | 1.87 | 2.19 |
| **D2-3** | 371.8 | 1.87 | 2.06 |
| **D2-5** | 232.1 | 1.87 | 2.11 |





Order in gel from left to right is identical to the list in the table.

22/09/2021

Plasmids were sent for sequencing in hylabs.

23/09/2021

From each plasmid preparation, at least one was positive for the new promoter version. One plasmid from each version was transformed into *E. coli* and *B. subtilis* following the cloning protocols.

24/09/2021

One colony from each transformation and bacterial species was picked for ON starter.

25/09/2021

Glycerol stock was prepared from each starter.

03/10/2021

**Assay for promoters:**

Bacteria containing the new promoter versions (from glycerol stocks) were streak over an LB-agar plate with 10mg/ml chloramphenicol.

04/10/2021

Bacteria containing the new promoter versions (from glycerol stocks) were streak over an LB-agar plate with 10mg/ml chloramphenicol, for a second biological repeat.

Colonies picked to ON starters from plates of the previous day.

05/10/2021

The assay was performed according to the protocol. Briefly, bacteria from ON starters were washed with PBSx1, and LB was replaced with M9 medium. Following 4hrs incubation, OD600nm was measured and bacteria were diluted to OD600nm of 0.2, according to the table below:

| Bacteria | Sample | D600nm | Bacterial suspension for dilution | M9 for dilution |
|---|---|---|---|---|
| ***E. coli*** | **ECE** | 0.817 | 171 | 529 |
| | **mCherry** | 1.149 | 122 | 578 |
| | **A2** | 0.829 | 169 | 531 |
| | **C** | 0.752 | 186 | 514 |
| | **D1** | 1.059 | 132 | 568 |
| | **D2** | 1.003 | 140 | 560 |
| | **E** | 1.035 | 135 | 565 |
| | **K** | 1.054 | 133 | 567 |
| | **M** | 0.794 | 176 | 524 |
| | **T** | 1.279 | 109 | 591 |
| ***B. Subtilis*** | **ECE** | 0.805 | 174 | 526 |
| | **mCherry** | 0.731 | 192 | 508 |
| | **A2** | 0.657 | 213 | 487 |
| | **C** | 0.747 | 187 | 513 |
| | **D1** | 0.366 | 383 | 317 |
| | **D2** | 0.925 | 151 | 549 |
| | **E** | 0.273 | 513 | 187 |
| | **K** | 0.51 | 275 | 425 |

| | | | | |
|---|---|---|---|---|
| | **M** | 0.622 | 225 | 475 |
| | **T** | 1.004 | 139 | 561 |

Then, 200 µl of each bacterial sample were loaded into 96 well plates in triplicates, as described in the plating scheme:

Well6

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Coli-ECE | Coli-ECE | Coli-ECE | Coli-M | Coli-M | Coli-M | Bac-ECE | Bac-ECE | Bac-ECE | Bac-M | Bac-M | Bac-M |
| B | Coli-mCherry | Coli-mCherry | Coli-mCherry | Coli-T | Coli-T | Coli-T | Bac-mCherry | Bac-mCherry | Bac-mCherry | Bac-T | Bac-T | Bac-T |
| C | Coli-A2 | Coli-A2 | Coli-A2 | Blank | Blank | Blank | Bac-A2 | Bac-A2 | Bac-A2 | | | |
| D | Coli-C | Coli-C | Coli-C | | | | Bac-C | Bac-C | Bac-C | | | |
| E | Coli-D1 | Coli-D1 | Coli-D1 | | | | Bac-D1 | Bac-D1 | Bac-D1 | | | |
| F | Coli-D2 | Coli-D2 | Coli-D2 | | | | Bac-D2 | Bac-D2 | Bac-D2 | | | |
| G | Coli-E | Coli-E | Coli-E | | | | Bac-E | Bac-E | Bac-E | | | |
| H | Coli-K | Coli-K | Coli-K | | | | Bac-K | Bac-K | Bac-K | | | |

Moreover, colonies picked to ON starters from plates of the previous day.

06/10/2021

The assay was performed as mention above.

OD600nm measurements and dilution to OD600nm=0.2 calculations:

| Bacteria | Sample | D600nm | Bacterial suspension for dilution | M9 for dilution |
|---|---|---|---|---|
| ***E. coli*** | **ECE** | 0.893 | 157 | 543 |
| | **mCherry** | 1.676 | 84 | 616 |
| | **A2** | 0.889 | 157 | 543 |
| | **C** | 1.188 | 118 | 582 |
| | **D1** | 0.949 | 148 | 552 |
| | **D2** | 1.133 | 124 | 576 |
| | **E** | 1.371 | 102 | 598 |
| | **K** | 1.055 | 133 | 567 |
| | **M** | 1.808 | 77 | 623 |
| | **T** | 1.046 | 134 | 566 |
| ***B. Subtilis*** | **ECE** | 0.866 | 162 | 538 |
| | **mCherry** | 0.967 | 145 | 555 |
| | **A2** | 1.086 | 129 | 571 |
| | **C** | 0.749 | 187 | 513 |
| | **D1** | 0.889 | 157 | 543 |
| | **D2** | 0.86 | 163 | 537 |
| | **E** | 1.153 | 121 | 579 |
| | **K** | 0.802 | 175 | 525 |
| | **M** | 0.894 | 157 | 543 |
| | **T** | 0.814 | 172 | 528 |

Then, 200 µl of each bacterial sample was loaded into 96 well plates in triplicates, as shown in the plating scheme above.