Supplementary Material

Table S1. Primers used for cloning and qPCR. Lower-case letters represent nucleotide overhangs used for Gibson cloning. Upper-case letters represent nucleotides binding to the amplicon.

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| Primer | Sequence | Description |
| DN\_39 | gcatggatgaactctacaaataATAGAGGGACAAACTCAAGGTC | Opt\_Syn42\_GFP backbone (fwd) |
| DN\_40 | cctttatgaattcccatgaTCATTAGAAAACCTCCTTAGCATG | Opt\_Syn42\_GFP backbone (rev) |
| DN\_41 | CATGCTAAGGAGGTTTTCTAATGATCATGGGAATTCATAAAGG | Opt\_Syn42/Syn35\_GFP insert (fwd) |
| DN\_42 | GACCTTGAGTTTGTCCCTCTATTATTTGTAGAGTTCATCCATGC | Opt\_Syn42/Syn35/SPA75\_GFP insert (rev) |
| DN\_55 | gaactctacaaataaAACGAGAAAAGCCAACCTGCGGGTTGG | Tra\_Syn42 backbone (fwd) |
| DN\_56 | cattagaaaacctcCTCCTAGGCGTGCAATTATACCTGGCCGC | Tra\_Syn42 backbone (rev) |
| DN\_57 | taattgcacgcctaggAGGAGGTTTTCTAATGATCATGGG | Tra\_Syn42\_GFP insert (fwd) |
| DN\_58 | gttggcttttctcgttTTATTTGTAGAGTTCATCCATGCCG | Tra\_Syn42\_GFP insert (rev) |
| DN\_247 | AACGAGAAAAGCCAACCTG | Tra\_Syn35/SPA75\_GFP; Tra\_Syn42/Syn35/SPA75\_mCherry backbone (fwd) |
| DN\_248 | TAGAAAACCTCCTCCTAGGCG | Tra\_Syn42\_mCherry backbone (rev) |
| DN\_270 | CCTAGGCCCCAAATTATAATTCTAAAC | Tra\_Syn35\_GFP; Tra\_Syn35\_mCherry backbone (rev) |
| DN\_274 | CCTAGGCGTGCAATTATAGTATC | Tra\_SPA75\_GFP; Tra\_SPA75\_mCherry backbone (rev) |
| DN\_249 | gcctaggaggaggttttctaATGGTGAGCAAGGGCGAG | Tra\_Syn42\_mCherry insert (fwd) |
| DN\_250 | gcaggttggcttttctcgttTTACTTGTACAGCTCGTCCATG | Tra\_Syn42/SPA75\_mCherry insert (rev) |
| DN\_271 | attataatttggggcctaggAGGAGGTTTTCTAATGATC | Tra\_Syn35\_GFP insert (fwd) |
| DN\_272 | gcaggttggcttttctcgttTTATTTGTAGAGTTCATCCATG | Tra\_Syn35/SPA75\_GFP insert (rev)  |
| DN\_281 | attataatttggggcctaggAGGAGGTTTTCTAATGGTG | Tra\_Syn35\_mCherry insert (fwd) |
| DN\_250 | gcaggttggcttttctcgttTTACTTGTACAGCTCGTCCATG | Tra\_Syn35\_mCherry insert (rev) |
| DN\_275 | actataattgcacgcctaggAGGAGGTTTTCTAATGATC | Tra\_SPA75\_GFP insert (fwd) |
| DN\_276 | actataattgcacgcctaggAGGAGGTTTTCTAATGGTG | Tra\_SPA75\_mCherry (fwd) |
| DN\_229 | TAGAGGGACAAACTCAAG | Opt\_Syn35/SPA75\_GFP; Opt\_Syn42/Syn35/SPA75\_mCherry backbone (fwd) |
| DN\_230 | TAGAAAACCTCCTTAGCATG | Opt\_Syn35\_GFP; Opt\_Syn42/Syn35/SPA75\_mCherry backbone (rev) |
| DN\_267 | TTAATTAAGACGTCTTGACATAAGC | Opt\_SPA75\_GFP backbone (rev) |
| DN\_245 | catgctaaggaggttttctaATGGTGAGCAAGGGCGAG | Opt\_Syn42/Syn35/SPA75\_mCherry insert (fwd) |
| DN\_236 | accttgagtttgtccctctaTTACTTGTACAGCTCGTCCATG | Opt\_Syn42/Syn35/SPA75\_mCherry insert (rev) |
| DN\_269 | tgtcaagacgtcttaattaaGCCCATTGACAACACTATTTTTTGATACTATAATTGCACGCCTAGGAGCTGTCACCGG | Opt\_SPA75\_GFP insert (fwd) |
| DN\_127 | CGGCCGCGCTAGCACTGA | *nag*R\_Tra/Opt\_GFP/mCherry/ilvB\_aldB backbone (fwd) |
| DN\_288 | CCGACGTCGCATGCTCCT | *nag*R\_Tra/Opt\_GFP/mCherry/ilvB\_aldB backbone (rev) |
| DN\_289 | agaggagcatgcgacgtcggAGGAGGTTTTCTAATGATC | *nag*R\_Tra\_GFP insert (fwd) |
| DN\_142 | ggtcagtgctagcgcggccgTTATTTGTAGAGTTCATCCATGC | *nag*R\_Tra\_GFP insert (rev) |
| DN\_290 | agaggagcatgcgacgtcggAGCTGTCACCGGATGTGC | *nag*R\_Opt\_GFP/mCherry insert (fwd) |
| DN\_291 | ggtcagtgctagcgcggccgAAGAAGGTCAATCATAAAGGCCAC | *nag*R\_Opt\_GFP/mCherry/*aldB* insert (rev) |
| DN\_292 | agaggagcatgcgacgtcggAGGAGGTTTTCTAATGGTG | *nag*R\_Tra\_mCherry insert (fwd) |
| DN\_293 | ggtcagtgctagcgcggccgTTACTTGTACAGCTCGTC | *nag*R\_Tra\_mCherry insert (rev) |
| DN\_298 | agaggagcatgcgacgtcggACAGGAGACTTTCTAATGGCC | *nag*R\_Tra\_*ilvB* insert (fwd) |
| DN\_299 | ctaggcgtgcTCACTCGCCGACCATCTC | *nag*R\_Tra\_*ilvB* insert (rev) |
| DN\_300 | cggcgagtgaGCACGCCTAGGAGGAGGTTTTC | *nag*R\_Tra\_*aldB* insert (fwd) |
| DN\_301 | ggtcagtgctagcgcggccgTCACTTGCGCTCGCTTTC | *nag*R\_Tra\_*aldB* insert (rev) |
| DN\_302 | agaggagcatgcgacgtcggAGTCCGTAGTGGATGTGTATC | *nag*R\_Opt\_*ilvB* insert (fwd) |
| DN\_303 | ctaggcgtgcAAAGTGATAATCATAAAGGCCAC | *nag*R\_Opt\_*ilvB* insert (rev) |
| DN\_294 | ttatcactttGCACGCCTAGGAGCTGTC | *nag*R\_Opt\_*aldB* insert (fwd) |
| DN\_537 | AGGAGGTTTGTATCTCTAATG | *att*Tn7\_Tra\_GFP backbone (fwd) |
| DN\_534 | AGCTGTCACCGGATGTGC | *att*Tn7\_Opt\_GFP backbone (fwd) |
| DN\_415 | TTGACAGCTTATCATCGATAAAC | *att*Tn7\_Tra/Opt\_GFP backbone (rev) |
| DN\_532 | tatcgatgataagctgtcaaTTGACACCATCGAATGGTGC | *att*Tn7\_Tra/Opt\_GFP insert (fwd) |
| DN\_538 | attagagatacaaacctcctGCGGCCTAGGGTGTGAAATTG | *att*Tn7\_Tra\_GFP insert (rev) |
| DN\_539 | aagcacatccggtgacagctGCGGCCTAGGGTGTGAAATTG | *att*Tn7\_Opt\_GFP insert (rev) |
| DN\_392 | CACCGCAGACAAACAGAAGA | qPCR primer msfGFP (fwd) (Otto et al., 2019) |
| DN\_393 | ACTGGGTGGACAGGTAGTGG | qPCR primer msfGFP (rev) (Otto et al., 2019) |
| DN\_549 | TTGGCCCAGAGGAAATCAC | qPCR primer rpoB (fwd) |
| DN\_550 | GGCACCGACGTAGACAATAC | qPCR primer rpoB (fwd) |

*aldB, B. brevis*

CCATGGTACCACCGTCAAAAAAAACGGCGCTTTTTAGCGCCGTTTTTATTTTTCAACCTTCGCATACGCTACTTGCATTACAGTTTACGAACCGAACAGGCTTATGTCAAGACGTCTTAATTAAGCCCATTGACAAGGCTCTCGCGGCCAGGTATAATTGCACGCCTAGGAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGCCTCTACAAATTTTGTTTAAGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTTCGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCTAATGAAGAAGAACATTATCACGTCGATTACCAGCTTGGCGTTGGTCGCGGGCCTCAGCTTGACCGCGTTCGCCGCAACGACCGCCACGGTGCCCGCCCCCCCGGCCAAGCAGGAAAGCAAGCCCGCCGTCGCCGCCAACCCGGCTCCTAAGAATGTGCTGTTCCAGTACAGCACCATCAACGCCCTTATGCTGGGCCAGTTCGAAGGCGACCTGACGTTGAAGGATCTGAAGTTGCGCGGCGATATGGGCCTGGGCACGATCAACGATCTTGACGGCGAAATGATCCAAATGGGCACCAAATTCTACCAAATCGACTCCACGGGCAAACTGAGCGAACTCCCAGAATCCGTAAAGACCCCATTCGCCGTCACGACCCACTTCGAGCCAAAGGAGAAGACGACCCTGACCAACGTGCAGGATTACAACCAGCTGACCAAGATGCTGGAGGAAAAATTCGAGAACAAAAACGTCTTCTACGCCGTAAAACTGACCGGGACCTTCAAGATGGTGAAGGCCCGCACCGTGCCGAAGCAAACCCGTCCATACCCACAACTGACCGAGGTGACGAAGAAGCAGAGCGAGTTCGAGTTCAAGAACGTGAAGGGTACGCTGATCGGCTTCTACACGCCGAACTACGCCGCCGCCCTGAATGTCCCCGGTTTCCATTTGCATTTCATCACGGAGGACAAAACGTCCGGCGGTCATGTACTGAACCTTCAGTTCGATAATGCGAACCTGGAGATCAGCCCCATCCACGAGTTCGACGTCCAGCTGCCCCATACCGATGATTTCGCCCATTCGGATTTGACCCAGGTGACCACGTCGCAAGTACATCAGGCCGAAAGCGAGCGCAAGTGATAGAGGGACAAACTCAAGGTCATTCGCAAGAGTGGCCTTTATGATTGACCTTCTTAACGAGAAAAGCCAACCTGCGGGTTGGCTTTTTTATGCAGCGGCCGC**Supplementary Figure 1.** Annotated sequence of the synthetic DNA fragment containing the bidirectional terminators, the synthetic SynPro42 promoter, RiboJ, BCD2, codon optimized *aldB* gene from *B. brevis* and RNase III site R1.1.

*ilvB* C83S*, E. coli* K12

CCATGGAACGAGAAAAGCCAACCTGCGGGTTGGCTTTTTTATGCACGCATACGCTACTTGCATTACAGTTTACGAACCGAACAGGCTTATGTCAAGACGTCTTAATTAATCTACTTGACATCCGACATTCGCGACTGTATAATAAGTTGACCTAGGGAGTCCGTAGTGGATGTGTATCCACTCTGATGAGTCCGAAAGGACGAAACGGACCTCTACAAATAATTTTGTTTAAGGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTTCGTACTGAAACATCTTAATCATGCACAGGAGACTTTCTAATGGCCAGCAGCGGCACCACCAGCACCCGCAAACGCTTCACGGGCGCCGAGTTCATCGTCCACTTCTTGGAGCAGCAGGGCATCAAGATCGTCACCGGCATCCCTGGCGGCAGCATCCTGCCGGTGTACGATGCCCTCAGCCAGAGCACCCAGATCCGCCACATCCTGGCTCGCCATGAACAAGGCGCGGGCTTCATCGCCCAGGGCATGGCCCGCACCGACGGCAAGCCCGCCGTCTGCATGGCGTCGAGCGGTCCGGGCGCCACCAATCTGGTCACCGCAATCGCCGACGCCCGTTTGGATAGCATCCCGCTGATCTGCATCACGGGCCAGGTGCCAGCCAGCATGATAGGCACCGATGCCTTCCAGGAGGTGGACACCTACGGCATCAGCATCCCCATCACCAAGCATAACTACTTGGTGCGCCACATCGAGGAACTCCCGCAGGTGATGTCCGATGCCTTCCGCATCGCCCAGTCGGGTCGGCCAGGCCCAGTTTGGATCGATATCCCGAAAGACGTCCAGACCGCCGTGTTCGAAATCGAAACCCAGCCCGCGATGGCTGAGAAAGCCGCGGCTCCGGCCTTCAGCGAAGAAAGCATCCGCGACGCCGCGGCTATGATCAACGCCGCAAAGCGCCCCGTGCTGTACCTGGGCGGCGGTGTCATCAATGCCCCAGCACGCGTGCGCGAACTGGCCGAGAAGGCCCAGCTTCCGACCACCATGACCCTTATGGCTCTGGGTATGCTGCCGAAGGCTCACCCGCTCTCGCTGGGTATGCTCGGGATGCACGGCGTCCGGAGCACCAACTACATCCTCCAGGAGGCCGACCTGCTGATCGTCCTGGGCGCCCGCTTCGACGACCGTGCCATCGGCAAAACCGAGCAGTTCTGCCCGAACGCCAAAATCATCCATGTTGACATTGACCGCGCGGAGTTGGGCAAGATCAAGCAGCCGCACGTGGCCATCCAGGCGGATGTGGACGACGTGCTGGCCCAGCTCATCCCGCTCGTGGAGGCACAGCCGCGCGCCGAATGGCACCAGCTGGTGGCGGACCTTCAACGCGAGTTCCCTTGCCCCATCCCCAAGGCCTGCGATCCCCTGAGCCATTACGGTCTGATCAACGCTGTGGCCGCGTGCGTCGATGACAACGCGATCATCACCACCGATGTGGGTCAACACCAGATGTGGACCGCTCAGGCGTACCCGCTGAACCGCCCGCGCCAGTGGCTCACCAGCGGCGGCCTGGGCACGATGGGGTTCGGTCTGCCCGCGGCCATCGGGGCTGCCCTGGCTAACCCAGACCGCAAGGTGCTGTGCTTCAGCGGTGACGGGAGCCTGATGATGAACATCCAGGAGATGGCCACCGCCAGCGAGAACCAGCTCGACGTCAAGATCATTCTGATGAACAACGAAGCCCTGGGCTTGGTACACCAGCAGCAGAGCCTGTTCTATGAACAGGGCGTCTTCGCCGCAACCTACCCCGGCAAGATTAACTTCATGCAGATCGCAGCCGGGTTCGGGCTGGAAACCTGCGATCTCAATAATGAGGCTGACCCGCAGGCGTCGCTCCAGGAAATCATCAACCGGCCCGGCCCGGCCCTGATCCATGTCCGTATCGACGCCGAGGAGAAGGTGTATCCAATGGTGCCCCCCGGCGCCGCCAACACGGAGATGGTCGGCGAGTGAAGTGATAGACTCAAGGTCGCTCCTAGCGAGTGGCCTTTATGATTATCACTTTAAATAAAAAAGGCACGTCAGATGACGTGCCTTTTTTCTTGTGCGGCCGC



Supplementary Figure 2. Annotated sequence of the synthetic DNA fragment ordered, containing the bidirectional terminators, the synthetic Syn51 promoter, VtmoJ, BCD1, codon optimized *ilvB* C83S gene from *E. coli* and RNase III site R0.5.

Supplementary Figure 3. Linear regressions of standards of fluorescein in 0.1 mM borate buffer at pH 9.4 and respective fluorescence in arbitrary fluorescence units (AFU). measured with the m2p Biolector (488 nm/520 nm) at the gain 50 (violet), 60 (light blue), 70 (dark blue). The inset shows the linear regression equation and coefficient.

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| --- | --- |
| Slope | -3.525 |
| Efficiency | 0.92171 |

Supplementary Figure 4. qPCR primer pair efficiency for the target gene *msfGFP*.

|  |  |
| --- | --- |
| Slope | -3.287 |
| Efficiency | 1.014785 |

Supplementary Figure 5. qPCR primer pair efficiency for the housekeeping gene *rpoB*.



Supplementary Figure 6. Evaluation of the developed inducible gene expression constructs under induced (dark blue) and non-induced (light blue) conditions: (A) plasmid-based expression of *msfGFP* and *mCherry* under the control of the *nag*R/P*nagAa* promoter, (B) genomic integrated expression of *msfGFP* at the *att*Tn7 site under the control of the P*trc*promoter. Tra, traditional expression cassette; Opt, optimized expression cassette; MFE, µmoles of fluorescein equivalents; AFU, arbitrary fluorescence units.



**Supplementary Figure 7.** Time course of mRNA abundance of the *msfGFP* gene normalized with the transcript level of the housekeeping gene *rpoB* for the traditional (A) and the optimized expression cassette (B).

**References**

Otto, M., Wynands, B., Drepper, T., Jaeger, K.-E., Thies, S., Loeschcke, A., et al. (2019). Targeting 16S ribosomal DNA for stable recombinant gene expression in *Pseudomonas*. *ACS Synth. Biol.*, acssynbio.9b00195. doi:10.1021/acssynbio.9b00195.