Supplementary Materials

Start-Stop Assembly: a functionally scarless DNA assembly system optimised for metabolic engineering.

George M. Taylor, Paweł M. Mordaka and John T. Heap*
Imperial College Centre for Synthetic Biology, Department of Life Sciences, Imperial College London, London, SW7 2AZ, United Kingdom.

*Email: j.heap@imperial.ac.uk

SUPPLEMENTARY FIGURES

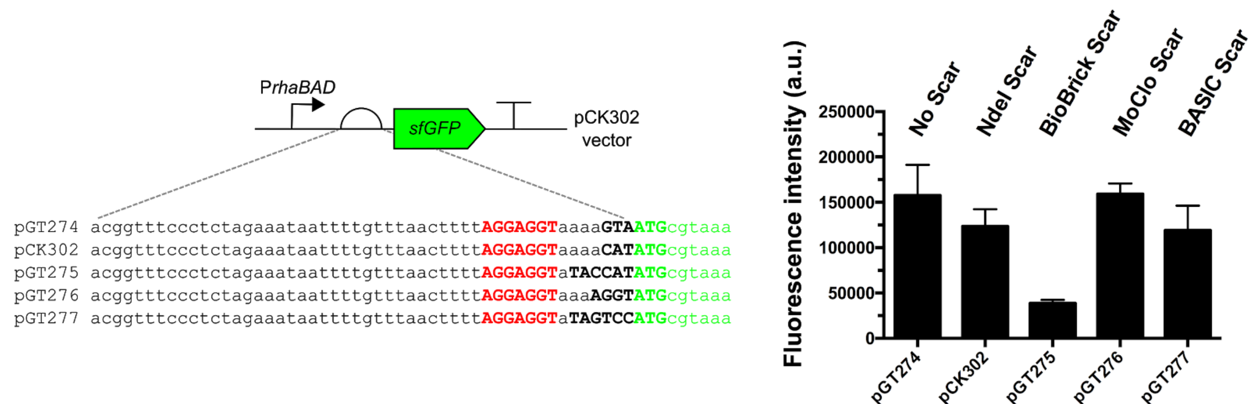


Figure S1. Effect of DNA assembly scars immediately upstream of the start codon on expression of *sfGFP*. The scar sequences (shown in **bold text**) that would result from from NdeI cloning, BioBrick assembly, MoClo assembly or BASIC assembly were each incorporated into derivatives of the reporter plasmid pCK302 (1) between the Shine-Dalgarno sequence (red text) of the RBS and the start codon (ATG) of *sfGFP* (green text) using inverse PCR. *E. coli* was independently transformed with each plasmid and transformants were characterised by flow cytometry. Fluorescence values presented represent the fluorescence of cells in mid-exponential phase of growth measured using flow cytometry with WT background subtracted. The error bars shown represent the standard deviation of three biological repeats.

WebLogo representation of alignment of 3764 *E. coli* TSSs

64 possible 3 bp sequences reduced using frequencies observed among TSSs →

16 sequences equally compatible with TSS frequencies:

TCA	GCA	ACA	CCA
TCG	GCG	ACG	CCG
TTA	GTA	ATA	CTA
TTG	GTG	ATG	CTG

↓ Removed sequences which matched the previously-defined γ and δ fusion sites at two positions

6 sequences equally compatible with TSS frequencies:

		ACA	CCA
TCG	GCG		CCG
			CTA

↓ β fusion site chosen based on frequency of occurrence of 3 bp sequence at *E. coli* TSS

β fusion site defined as:

CCA

↓ β fusion site in Level 1 expression unit

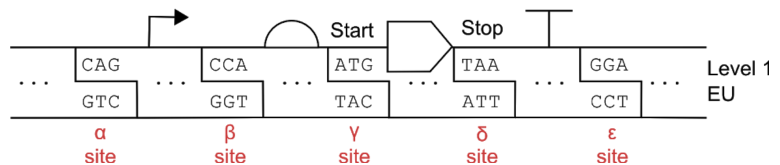


Figure S2. Choice of β fusion site. In an attempt to minimise the introduction of new sequences by the β fusion site at the junction between the promoter and the UTR/RBS, we tried to identify a consensus sequence for *E. coli* transcriptional start sites (TSS). We aligned 3746 previously-described *E. coli* MG1655 TSSs (2) which are visualised in the figure using WebLogo (3). The alignment showed a preference for A or G at the TSS +1 position, a preference for C or T at the -1 position, and no preference at the -2 position. First, we limited the candidate sequences for the β fusion site to the 16 sequences equally compatible with the observed preferences in the TSS. Next, to avoid misassembly we removed any of the candidate sequences which matched the already defined γ (ATG) and δ (TAA) fusion sites at two positions, which left six potential fusion sites. Finally, we compared the frequencies of these six candidate sequences among the 3746 previously-described *E. coli* MG1655 TSSs. The most frequently-occurring were ACA, which occurred 178 times, and CCA, which occurred 175 times. ACA is palindromic so would be a poor choice of fusion site, therefore we defined the β fusion site as CCA.

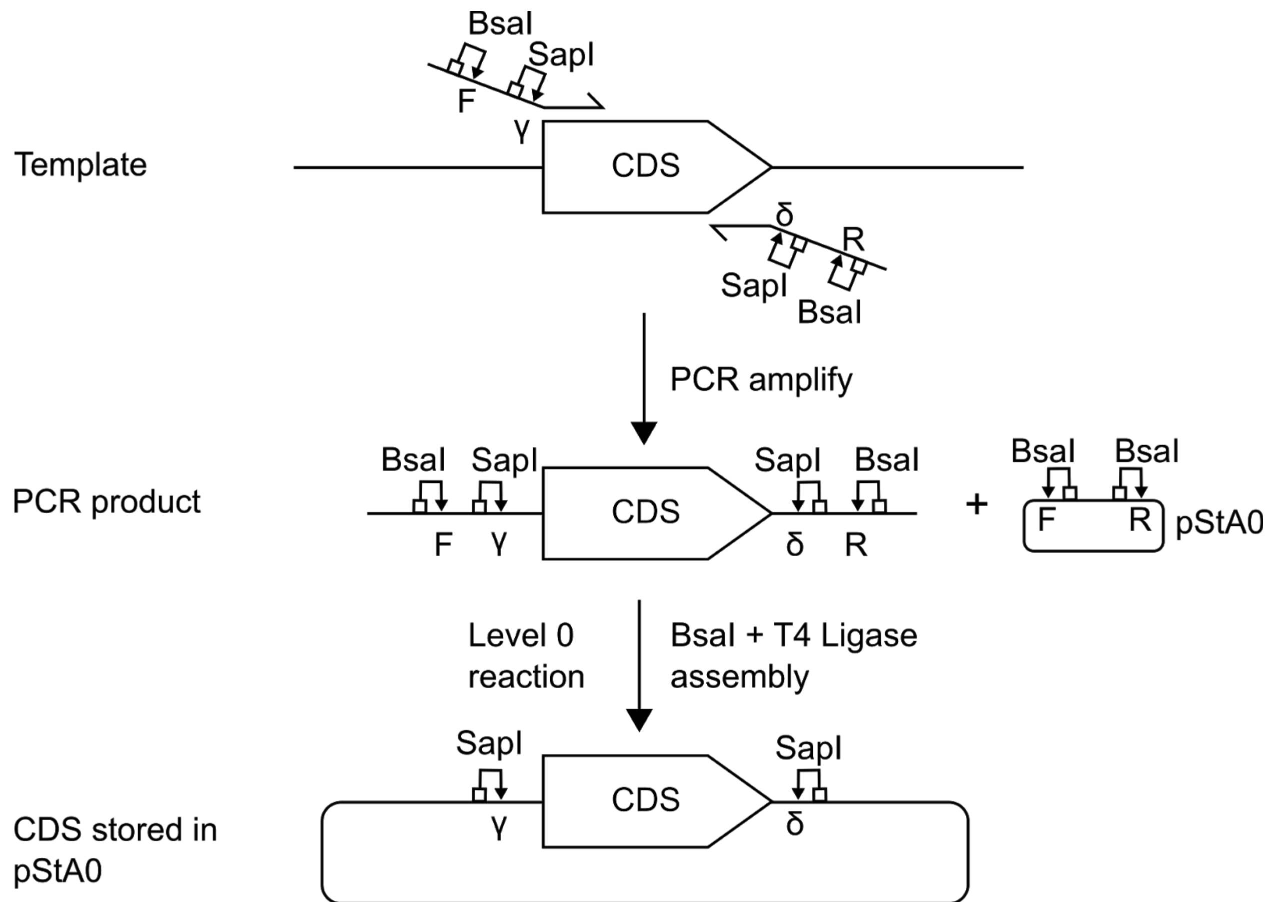


Figure S3. Strategy for PCR-amplification and cloning of genetic parts into Level 0 vector pStA0. To be used in Start-Stop Assembly, each genetic part must be flanked by the appropriate prefix and suffix sequence (Table S1), which can be added by PCR using primers with 5' tails. Different types of genetic part use different prefix and suffix sequences, because they include the differing fusion sites (Table S1). Primer tail sequences for different types of genetic parts are outlined in Table S2. In each instance the prefix and suffix sequences contain the inward-facing BsaI recognition sites and corresponding storage donor fusion sites F (TGTG) and R (GACC), which are used to clone parts into Level 0 vector pStA0. Between the BsaI restriction sites are inward-facing SapI restriction sites and corresponding donor fusion sites which are not used during the storage cloning shown, but are later used for excising cloned parts from pStA0 for use in Level 1 assembly.

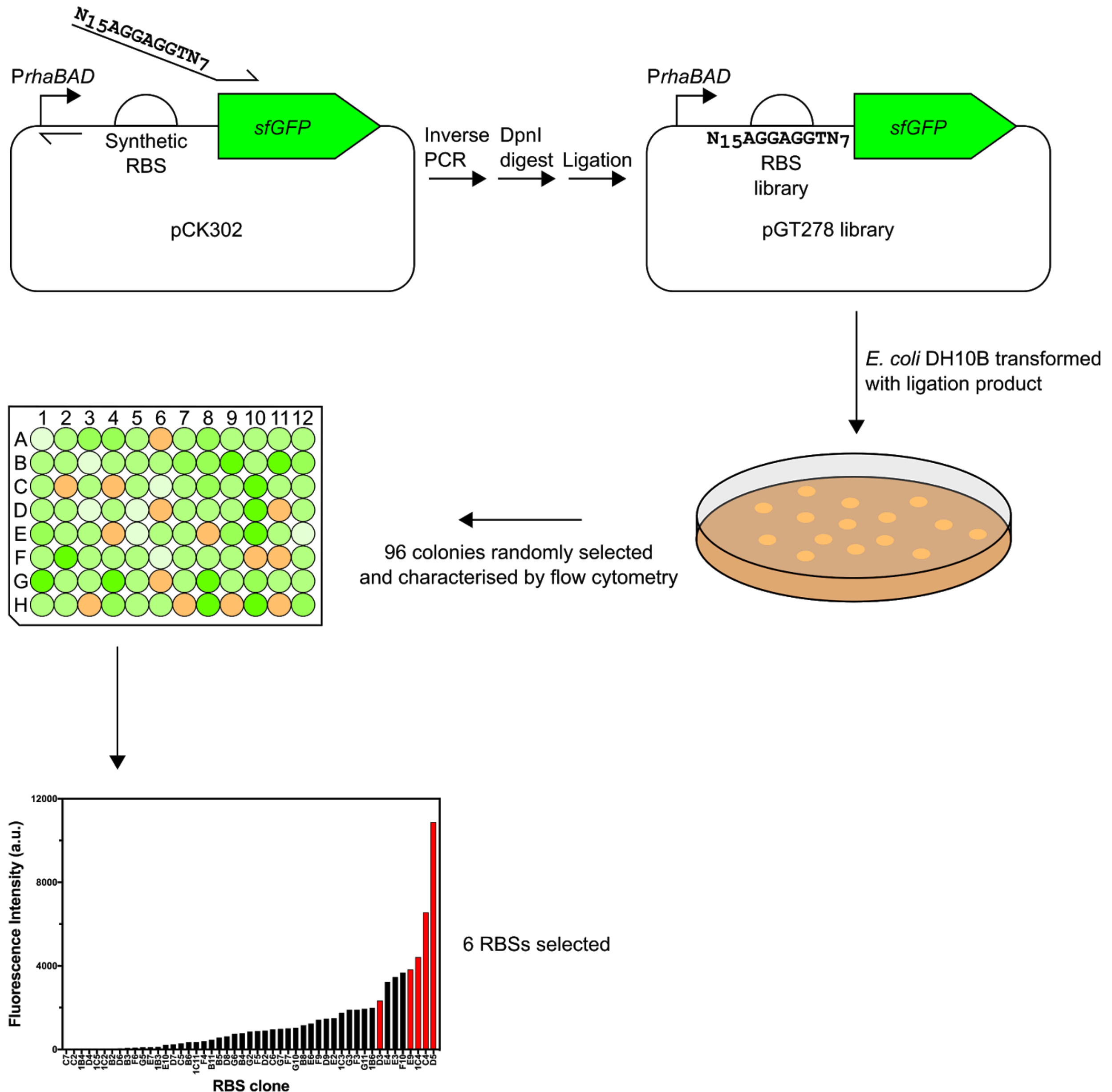


Figure S4. Design, generation and characterisation of a synthetic RBS library. The RBS library design conserved the *E. coli* Shine-Dalgarno (SD) consensus sequence (AGGAGGT) but randomised (N) 15 bp upstream and 7 bp downstream of the SD by incorporating the four nucleotides A, T, G and C at approximately the same 25% frequency at each N position. The RBS library was introduced to pCK302 upstream of *sfGFP* by inverse PCR using primers oligoGT448 and oligoGT463. The PCR reaction product was treated with DpnI to remove the template plasmid DNA. PCR fragments of the expected size (5.6 kbp) were excised and purified following gel electrophoresis, circularised by ligation and *E. coli* was transformed with the resultant ligation product by electroporation. 96 *E. coli* transformant colonies were randomly

selected and characterised in mid-exponential phase of growth by flow cytometry (as described in Supplementary Materials and Methods - Flow cytometry analysis). The fluorescence intensity of each clone is plotted with the background fluorescence subtracted. From this RBS library, six RBSs were chosen to give a wide and evenly-spaced distribution of expression strengths (shown in red here, and separately in Figure S5b).

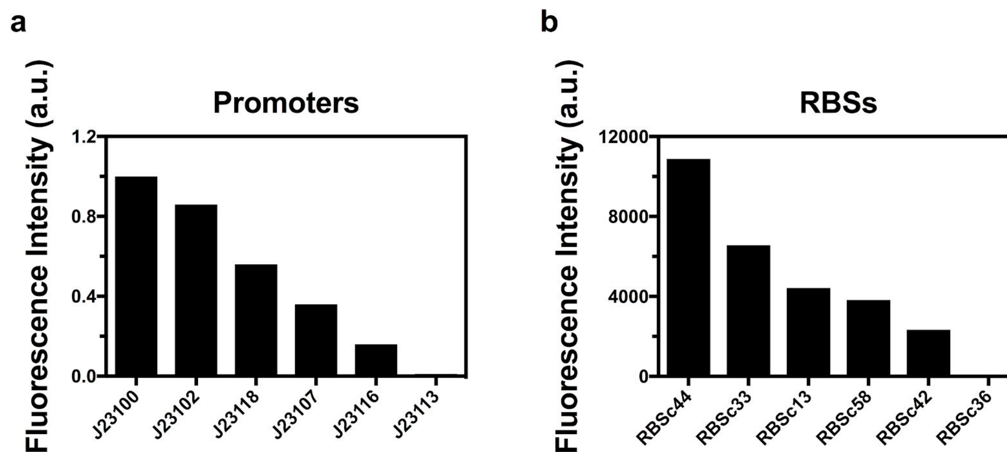


Figure S5. Promoters and RBSs stored in pStA0 in this study. (a) Fluorescence intensities reported by Anderson (4) for the six promoters we selected, relative to promoter J23100. **(b)** Fluorescence intensities of the six RBSs chosen from the characterised library described in Figure S4. These six RBSs give a wide and evenly-spaced distribution of expression strengths, and were stored in Level 0 vector pStA0. Promoters, in descending order of strength: P1 = J23100, P2 = J23102, P3 = J23118, P4 = J23107, P5 = J23116, P6 = J23113. RBSs, in descending order of strength: R1 = RBSc44, R2 = RBSc33, R3 = RBSc13, R4 = RBSc58, R5 = RBSc42, R6 = RBSc36.

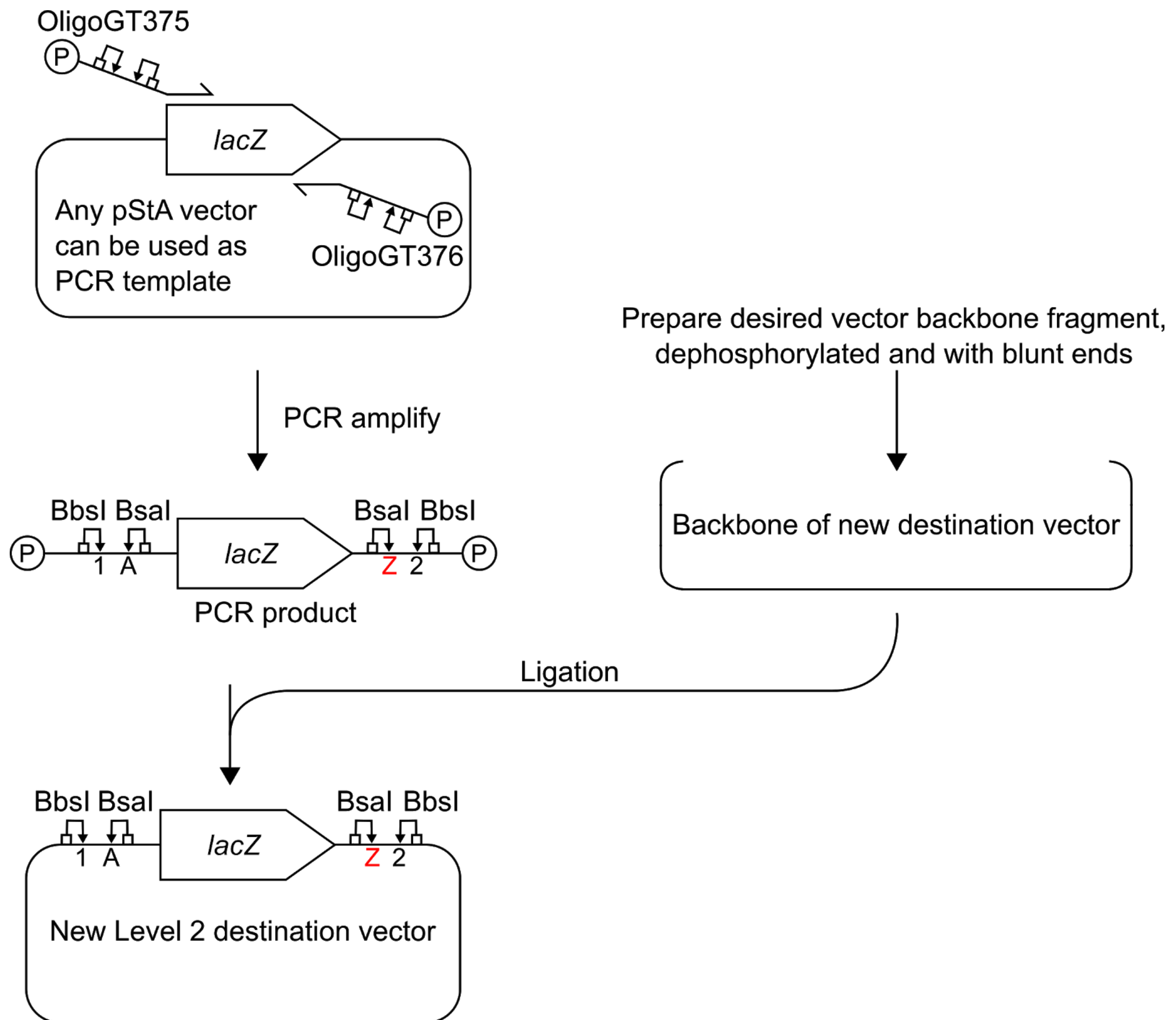


Figure S6. Strategy for construction of an alternative Level 2 destination vector. Prepare a linear, blunt-ended, dephosphorylated backbone fragment for the desired destination vector, for example by PCR-amplification. PCR-amplify the *lacZα* gene with primers oligoGT375 and oligoGT376 using any of the vectors in Table 1 as template. The primer tails contain outward-facing BsaI recognition sites, with corresponding A and Z acceptor fusion sites, and inward-facing BbsI recognition sites, with corresponding 1 and 2 donor fusion sites. This arrangement is the same as the assembly cassette of pStA212. Purify the PCR product and ligate it with the backbone vector fragment. Ligation of blunt-ended DNA fragments is not orientation-specific. Transform a suitable cloning strain of *E. coli* with the ligation product and use blue/white screening to identify blue colonies containing the desired plasmid with the assembly cassette. Verify the plasmid by DNA sequencing. BsaI sites and/or BbsI sites in the vector backbone should be removed.

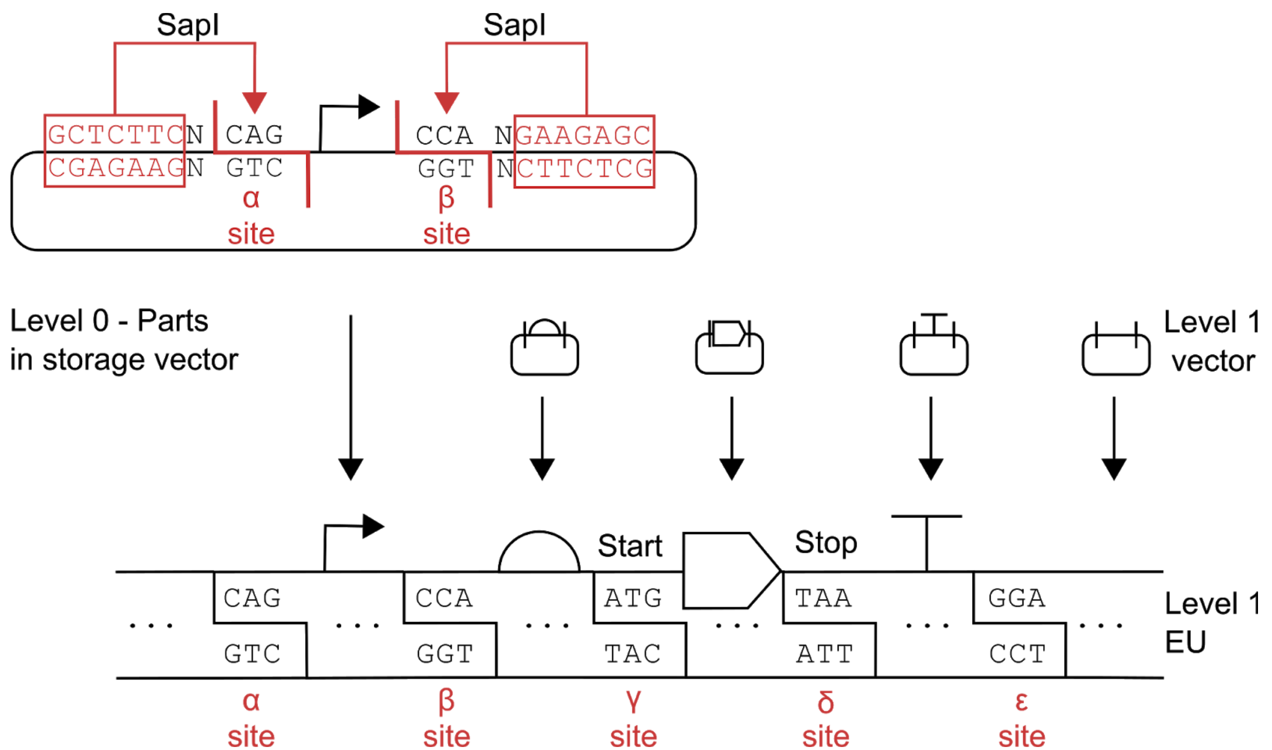


Figure S7. Level 1 assembly of an expression unit showing detail of Level 0 promoter part. Genetic parts stored in pStA0 are flanked by inward-facing SapI recognition sites (red boxes) with corresponding donor fusion sites (staggered red lines) for Level 1 assembly of expression units. Promoters use the α site (CAG) and β site (CCA).

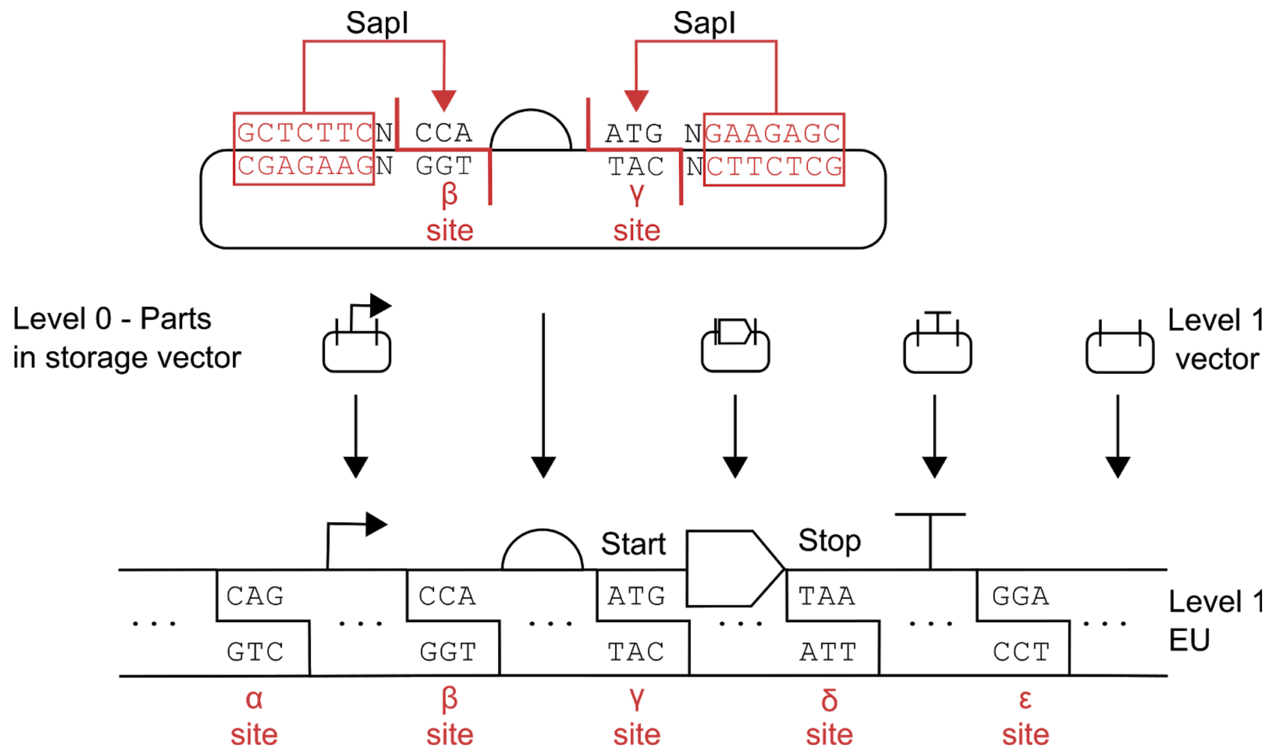


Figure S8. Level 1 assembly of an expression unit showing detail of Level 0 RBS part. Genetic parts stored in pStA0 are flanked by inward-facing SapI recognition sites (red boxes) with corresponding donor fusion sites (staggered red lines) for Level 1 assembly of expression units. RBSs use the β site (CCA) and γ site (ATG, start codon).

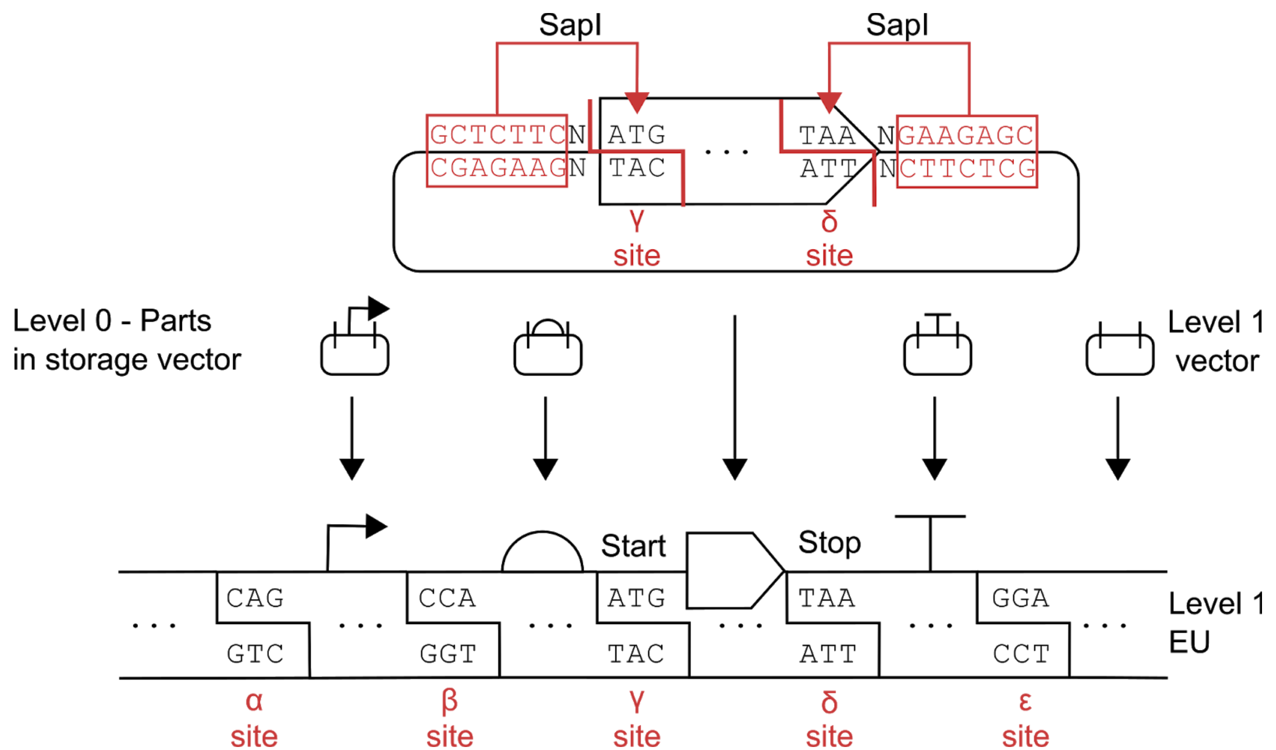


Figure S9. Level 1 assembly of an expression unit showing detail of Level 0 CDS part. Genetic parts stored in pStA0 are flanked by inward-facing SapI recognition sites (red boxes) with corresponding donor fusion sites (staggered red lines) for Level 1 assembly of expression units. CDSs use the γ site (ATG, start codon) and δ site (TAA, stop codon).

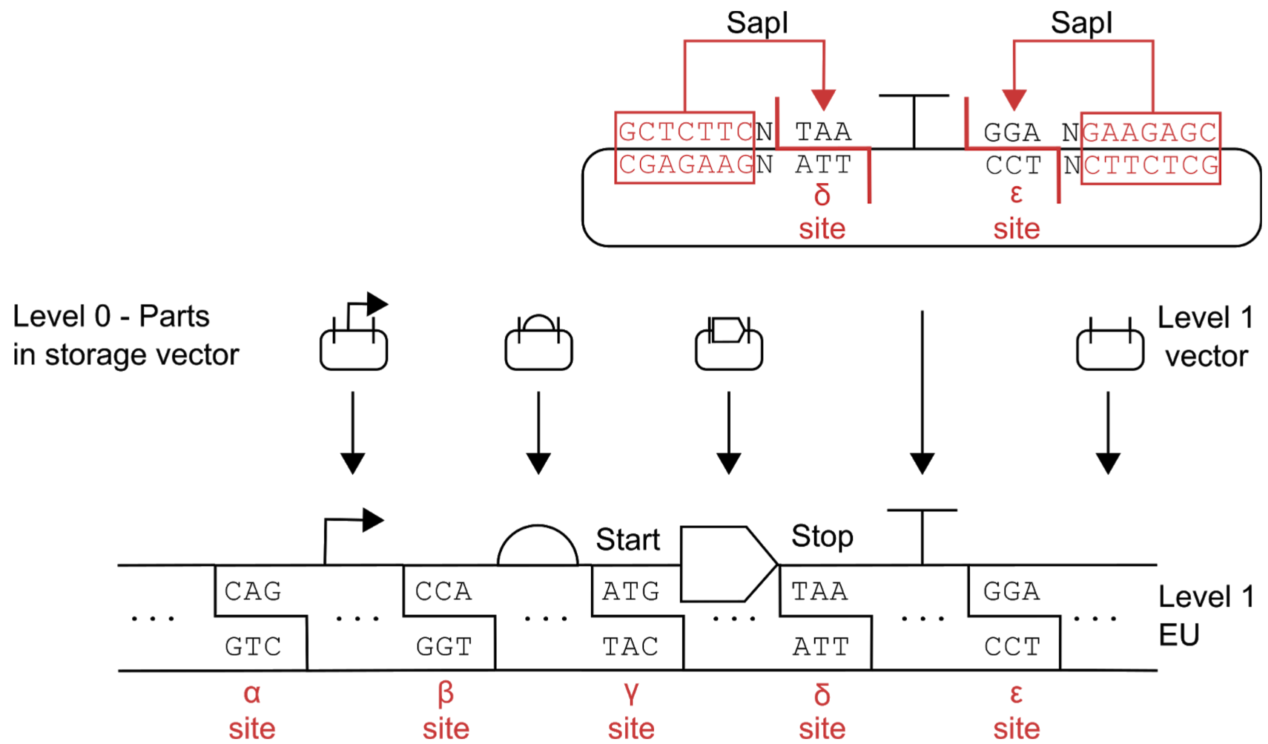


Figure S10. Level 1 assembly of an expression unit showing detail of Level 0 terminator part. Genetic parts stored in pStA0 are flanked by inward-facing SapI recognition sites (red boxes) with corresponding donor fusion sites (staggered red lines) for Level 1 assembly of expression units. Terminators use the δ site (TAA, stop codon) and ε site (GGA).

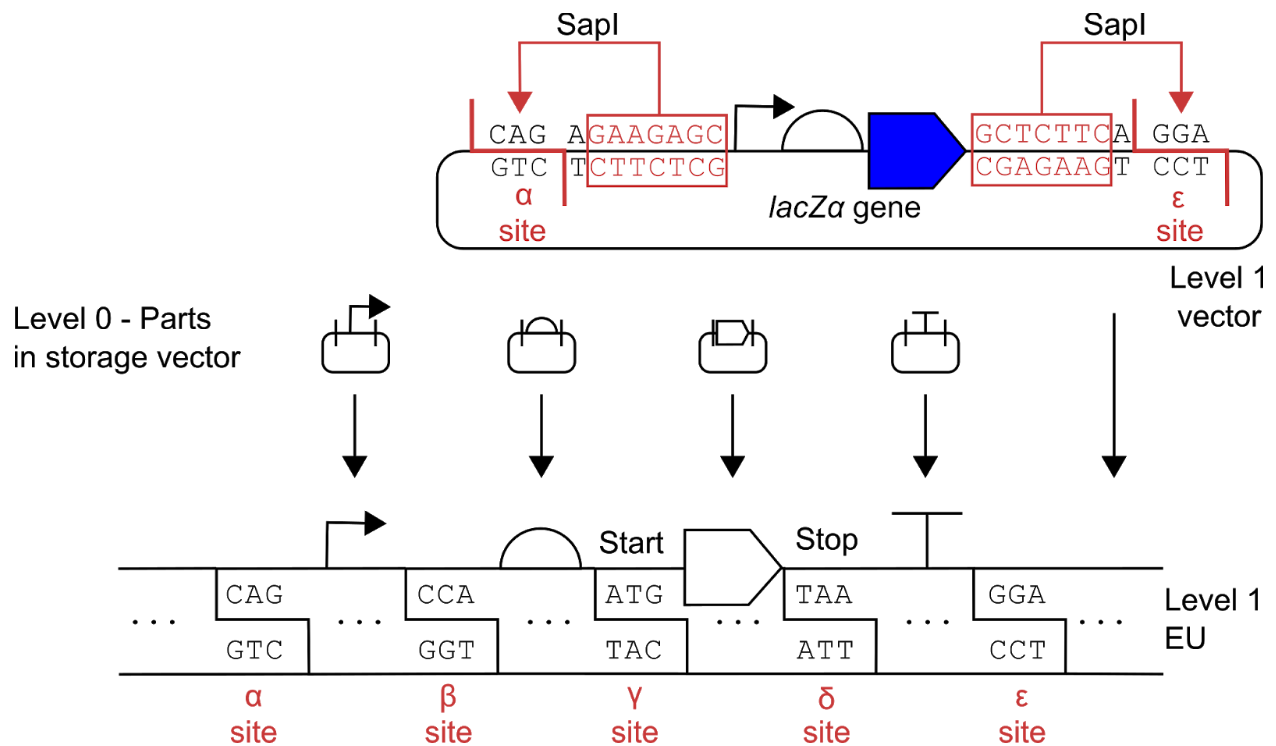
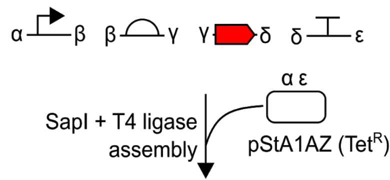
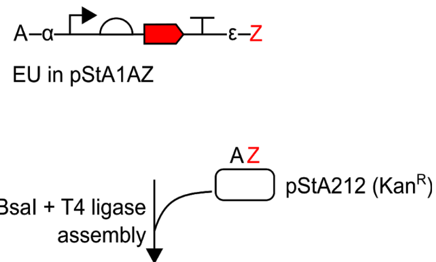


Figure S11. Level 1 assembly of an expression unit showing detail of Level 1 vector. Level 1 vectors contains two outward-facing SapI recognition sites (red boxes) with corresponding α (CAG) and ε (GGA) acceptor fusion sites (staggered red lines) for Level 1 assembly of expression units. Between the SapI sites in a Level 1 empty vector is a *lacZα* gene which can be used for blue/white screening.

Level 0 - Parts in storage vector pStA0, Amp^R



Level 1 - Expression unit (EU), Tet^R



Level 2 - Up to 5 EUs, Kan^R

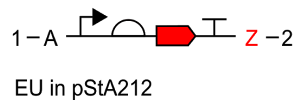
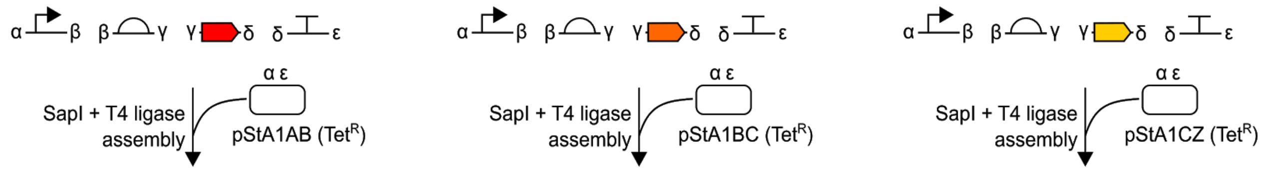
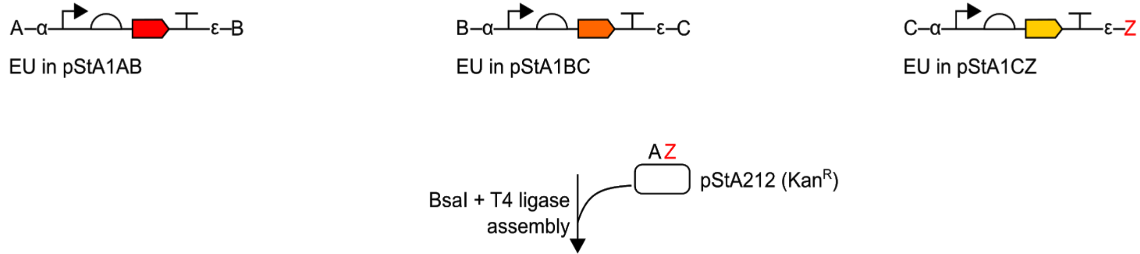


Figure S12. General strategy for assembling one expression unit using Start-Stop Assembly. One expression unit is assembled in Level 1 vector pStA1AZ from Level 0 parts by Level 1 assembly using the fusion sites α, β, γ, δ and ε. For cases in which only a single expression unit is required, either a Level 1 vector can be used directly for expression, or the expression unit can be transferred to a Level 2 destination vector via a Level 2 assembly reaction.

Level 0 - Parts in storage vector pStA0, Amp^R



Level 1 - Expression units (EU), Tet^R



Level 2 - Up to 5 EUs, Kan^R

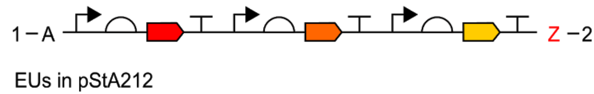
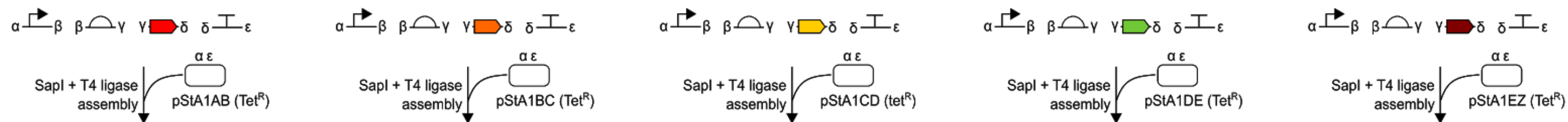


Figure S13. General strategy for assembling three expression units in a single construct using Start-Stop Assembly. Three expression units are independently assembled in the Level 1 vectors pStA1AB, pStA1BC and pStA1CZ from Level 0 parts by Level 1 assembly using the fusion sites α, β, γ, δ and ε. The three expression units can be assembled as a single construct in a Level 2 vector (in this case pStA212) by Level 2 assembly using the fusion sites A, B, C and Z.



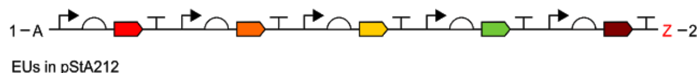


Figure S14. General strategy for assembling five expression units in a single construct using Start-Stop Assembly. Five expression units are independently assembled in the Level 1 vectors pStA1AB, pStA1BC, pStA1CD, pStA1DE and pStAEZ from Level 0 parts by Level 1 assembly using the fusion sites α, β, γ, δ and ε. The five expression units can be assembled as a single construct in a Level 2 vector (in this case pStA212) by Level 2 assembly using the fusion sites A, B, C, D, E and Z.

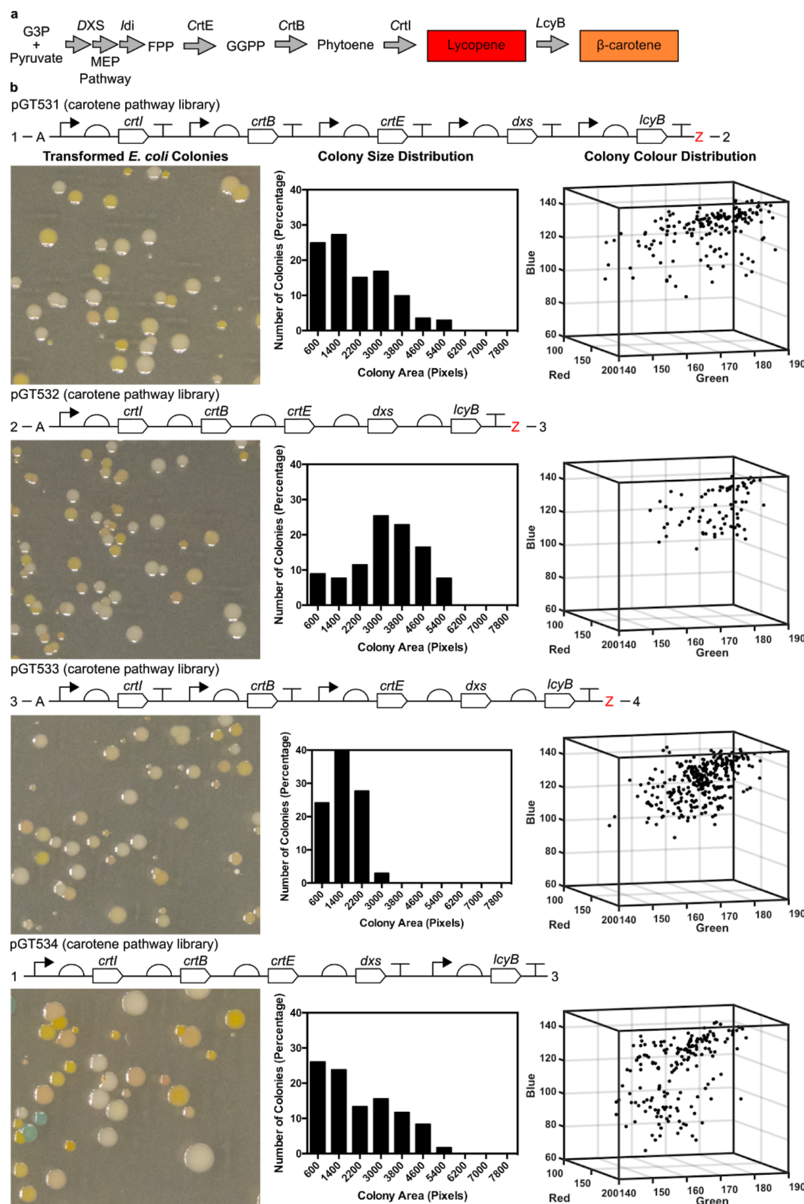
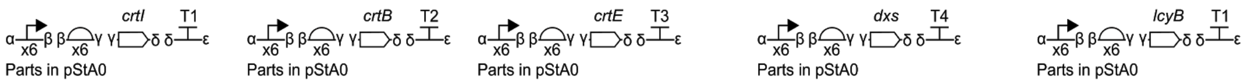


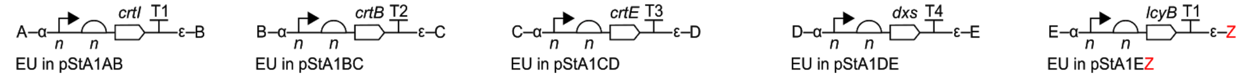
Figure S15. Analysis of the four β-carotene pathway libraries. (a) β-carotene pathway showing the endogenous enzymes of the *E. coli* MEP pathway and heterologous enzymes. Coloured products are shown in boxes, enzymes are shown above arrows. Abbreviations: G3P (glyceraldehyde 3-phosphate), FPP (Farnesyl pyrophosphate), GGPP (Geranylgeranyl pyrophosphate). **(b)** Designs of β-carotene pathway libraries (pGT531-534) and phenotypic variation among *E. coli* clones from those libraries. Variation was compared between the four β-carotene combinatorial pathway libraries (pGT531-534) and the control plasmids, pStA314 and pGT536 (data for the controls is shown in Figure 5c). Phenotypic variation is shown using representative pictures of the transformation plates, histograms of colony size (measured as cell area, *x*-axis represents the upper limit of each histogram bin) and the distributions of colony colours (measured using colony red, green and blue values extracted from colony images).

Assembly of pGT531 (β-carotene pathway library)

Level 0

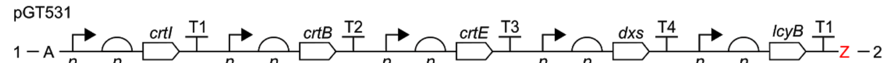


Level 1



Level 2

pGT531



Total combinatoiral library size of 6.05×10^7

Figure S16. Combinatorial assembly of monocistronic β-carotene pathway library pGT531. A library of constructs each encoding a β-carotene pathway was assembled hierarchically and combinatorially as shown in the figure and as described in the main text. The destination vector was pStA212. Equimolar mixtures of six promoter parts or six RBS parts (Figure S5) labelled as 'x6' were used in Level 1 assemblies. The uncertain representation of each promoter and RBS at each position following assembly is represented by '*n*'. The total combinatorial design space was $6^{10} = 6.05\times10^7$. T1 = terminator L3S2P55, T2 = terminator L3S2P21, T3 = terminator ECK120033737, T4 = terminator ECK120019600.

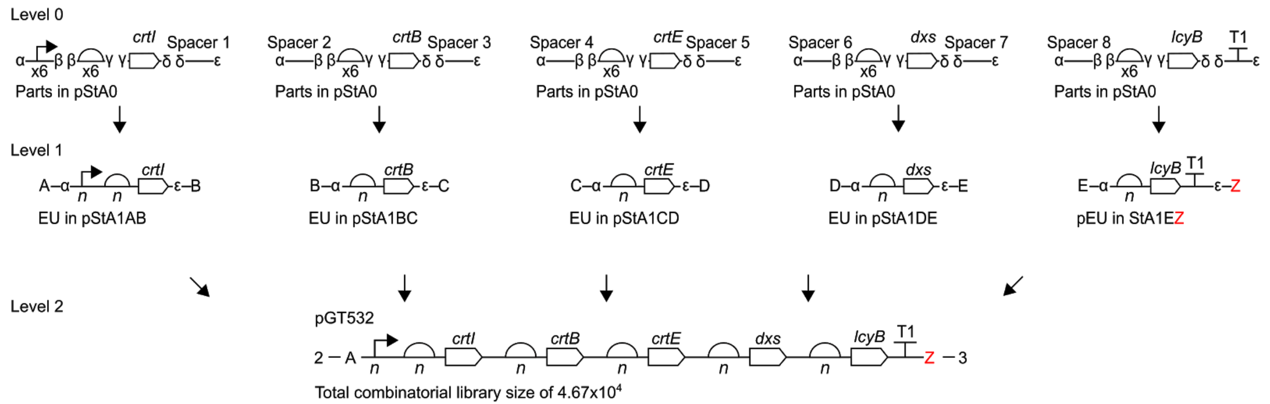


Figure S17. Combinatorial assembly of operon-based β-carotene pathway library pGT532. A library of constructs each encoding a β-carotene pathway was assembled hierarchically and combinatorially as shown in the figure and as described in the main text. The destination vector was pStA223. Equimolar mixtures of six promoter parts or six RBS parts (Figure S5) labelled as ‘x6’ were used in Level 1 assemblies where shown. Spacer sequences were used in Level 1 assemblies (as linkers, Table S5) in place of promoters and/or terminators where shown. The uncertain representation of each promoter and RBS at each position following assembly is represented by ‘*n*’. The total combinatorial design space was 6^6 = 4.6×10^4. T1 = terminator L3S2P55.

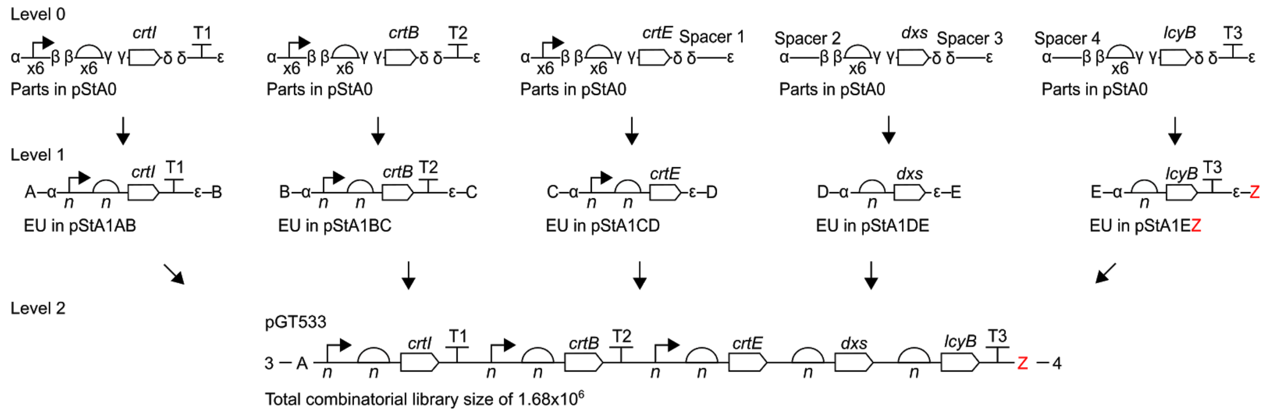


Figure S18. Combinatorial assembly of hybrid β-carotene pathway library pGT533.

A library of constructs each encoding a β-carotene pathway was assembled hierarchically and combinatorially as shown in the figure and as described in the main text. The destination vector was pStA234. Equimolar mixtures of six promoter parts or six RBS parts (Figure S5) labelled as 'x6' were used in Level 1 assemblies where shown. Spacer sequences were used in Level 1 assemblies (as linkers, Table S5) in place of promoters and/or terminators where shown. The uncertain representation of each promoter and RBS at each position following assembly is represented by '*n*'. The total combinatorial design space was $6^8 = 1.68 \times 10^6$. T1 = terminator L3S2P55, T2 = terminator L3S2P21, T3 = terminator ECK120033737.

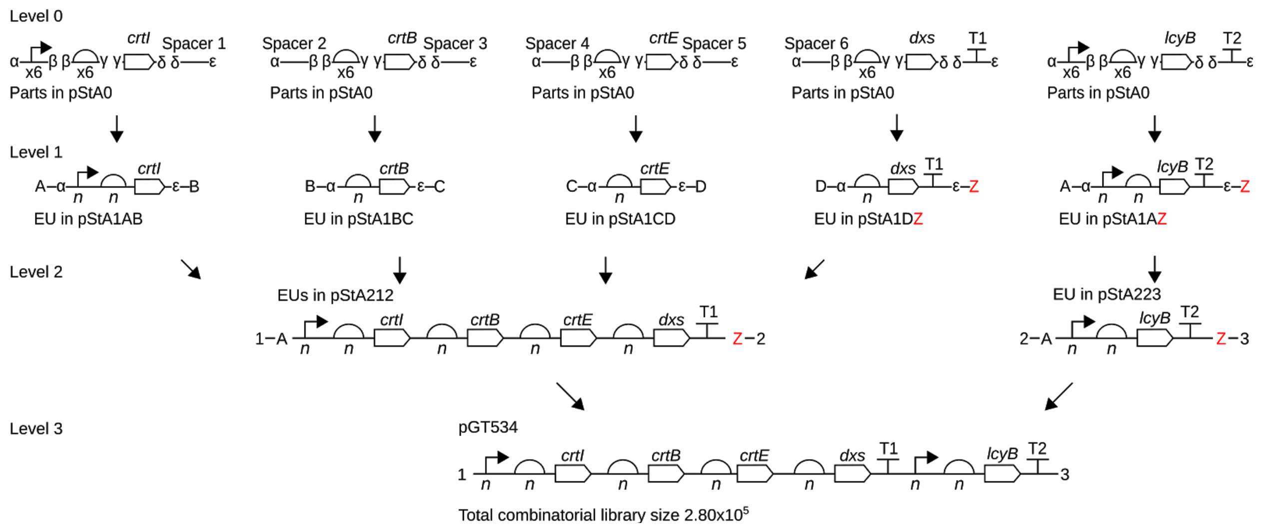


Figure S19. Combinatorial assembly of hybrid β-carotene pathway library pGT534.

A library of constructs each encoding a β-carotene pathway was assembled hierarchically and combinatorially as shown in the figure and as described in the main text. The destination vector was pStA313. Equimolar mixtures of six promoter parts or six RBS parts (Figure S5) labelled as 'x6' were used in Level 1 assemblies where shown. Spacer sequences were used in Level 1 assemblies (as linkers, Table S5) in place of promoters and/or terminators where shown. The uncertain representation of each promoter and RBS at each position following assembly is represented by '*n*'. The total combinatorial design space was $6^7 = 2.80 \times 10^5$. T1 = terminator L3S2P55, T2 = terminator L3S2P21.

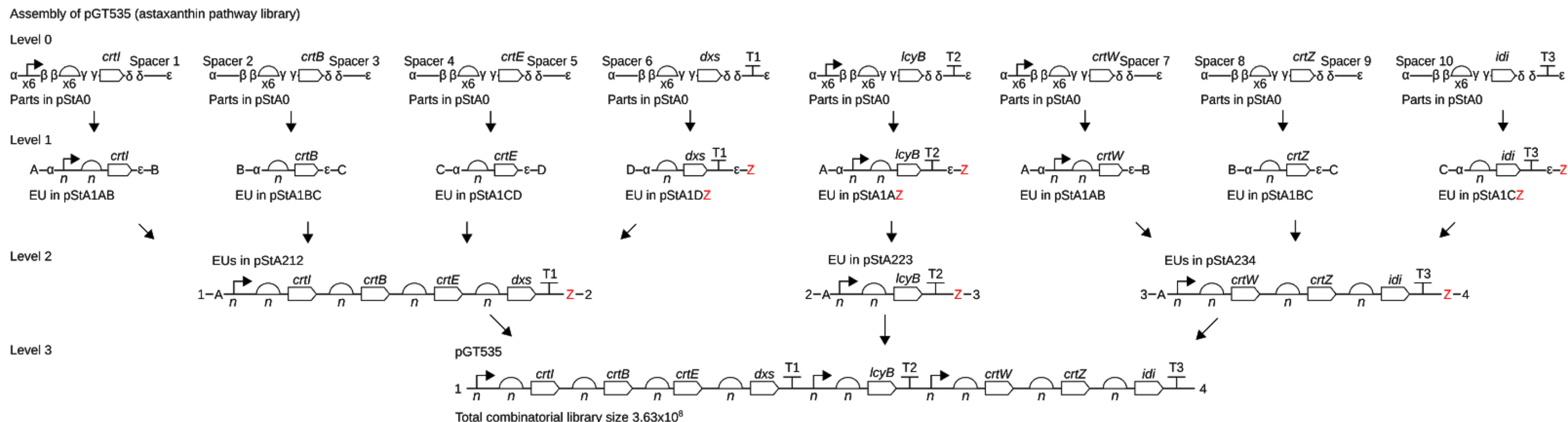


Figure S20. Combinatorial assembly of hybrid astaxanthin pathway library pGT535. A library of constructs each encoding an astaxanthin pathway was assembled hierarchically and combinatorially as shown in the figure and as described in the main text. The destination vector was pStA314. Equimolar mixtures of six promoter parts or six RBS parts (Figure S5) labelled as 'x6' were used in Level 1 assemblies where shown. Spacer sequences were used in Level 1 assemblies (as linkers, Table S5) in place of promoters and/ or terminators where shown. The uncertain representation of each promoter and RBS at each position following assembly is represented by '*n*'. The total combinatorial design space was $6^{11} = 3.63 \times 10^8$. T1 = terminator L3S2P55, T2 = terminator L3S2P21, T3 = terminator ECK120033737.

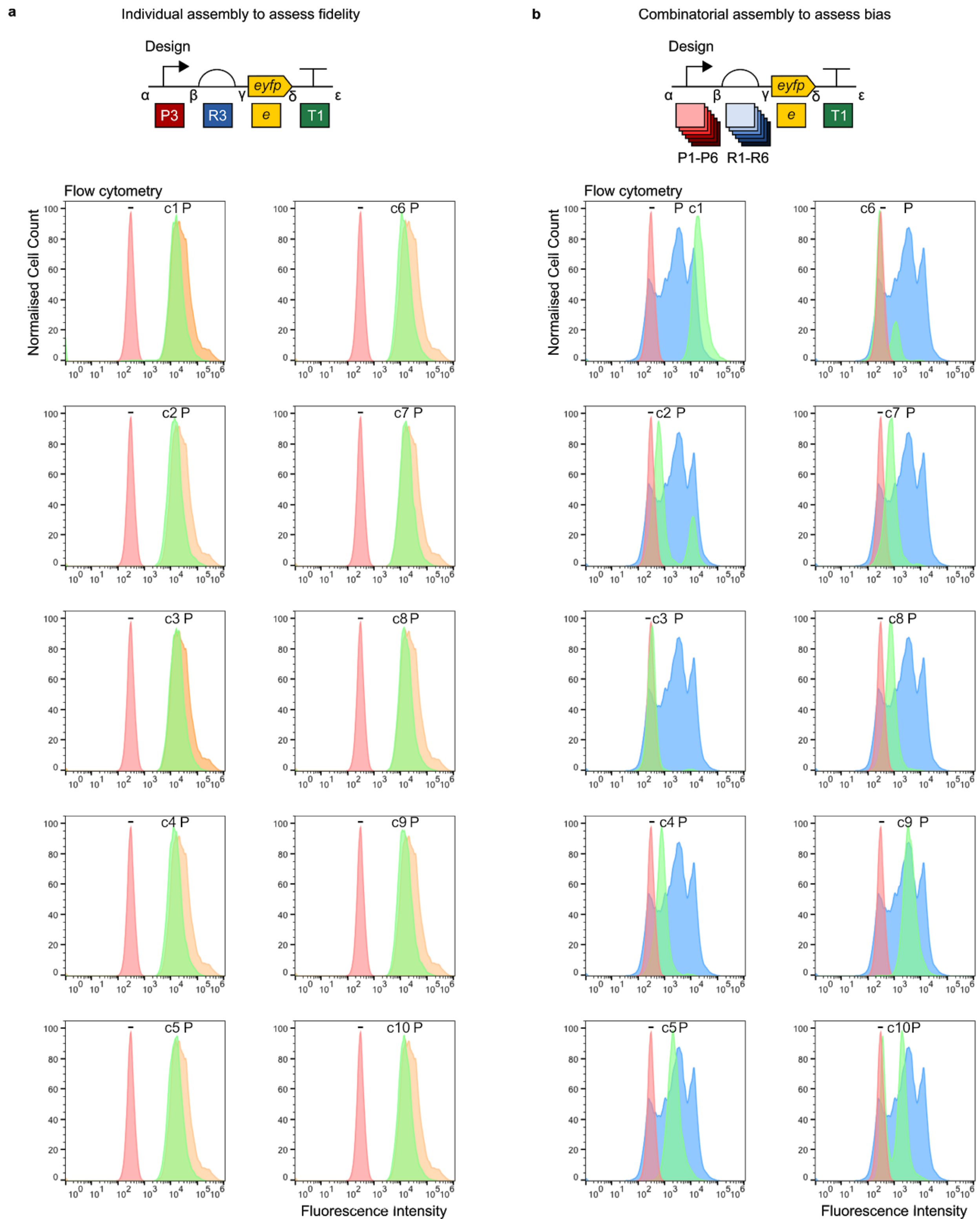


Figure S21. Flow cytometry data for individual clones used in assessment of fidelity and bias of multi-part Level 1 assembly. The experiments are described in the main text and Figure 2. Flow cytometry histograms show fluorescence intensity of 10,000 events (cells) normalised to the maximum (in order to visualise distribution rather than absolute values) for

wild-type *E. coli* DH10B as a negative reference (-), each of the ten clones (c1-c10) and a pool of several hundred transformants (P). **(a)** Assessment of assembly fidelity using assembly of an individual P3-R3-*e*-T1 expression unit. The ten clones showed similar fluorescence intensity to one another. **(b)** Assessment of assembly bias by combinatorial assembly of EYFP expression units using six promoters P1-P6, six RBSs R1-R6, *eyfp* and terminator T1. The ten clones showed widely differing fluorescence intensity values.

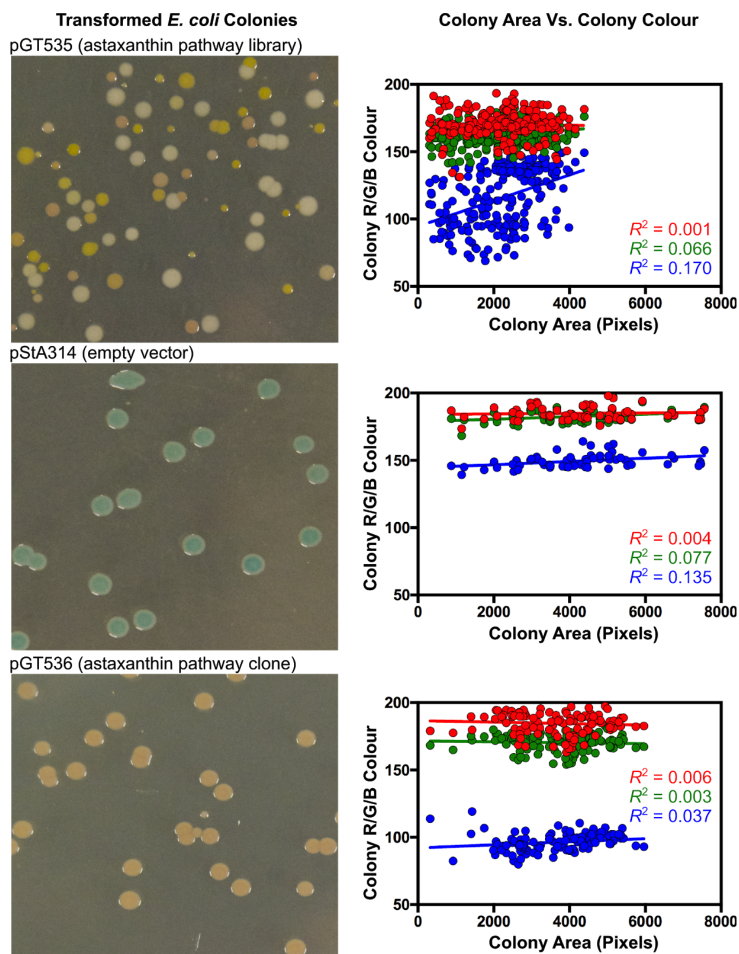
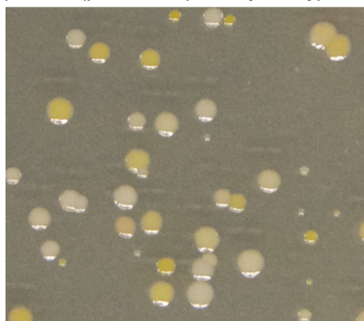


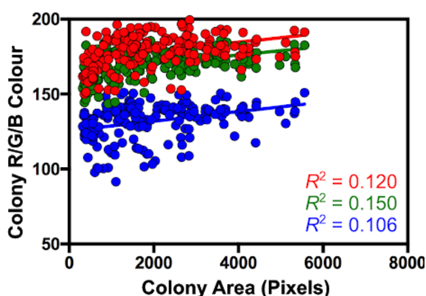
Figure S22. Relationship between colony area and colony colour in the astaxanthin pathway library. The experiment is described in the main text and Figure 5. Phenotypic variation among *E. coli* clones of the astaxanthin pathway library (pGT535) was compared to controls pStA314 (empty vector) and an isolated clone pGT536 from the astaxanthin pathway library pGT535. The representative images of colonies shown are the same as those in Figure 5c. Colony area and RGB colour (red, green, blue) values were extracted from images. For each colony, the three colour values are plotted separately in the corresponding colour. Linear regressions for each R/G/B colour are also shown in the corresponding colour (solid lines and R^2 values). There is little or no association between colony area and colony colour, reflected by low R^2 values.

Transformed *E. coli* Colonies

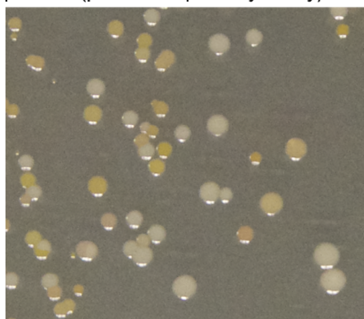
Colony Area Vs. Colony Colour

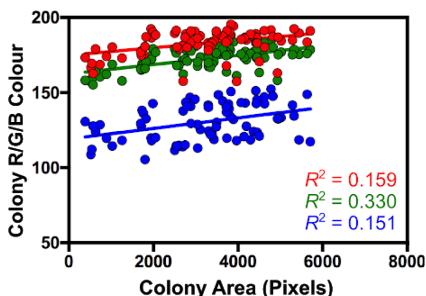
pGT531 (β-carotene pathway library)



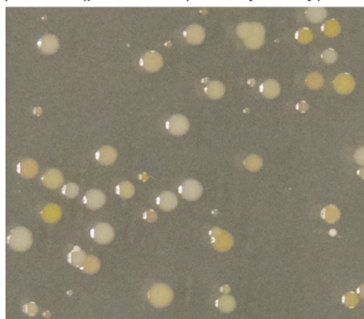


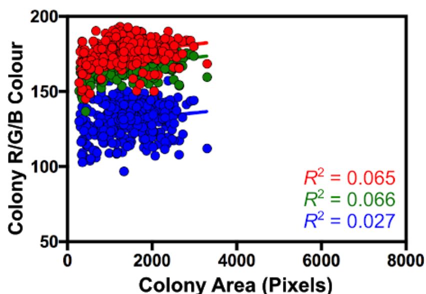
pGT532 (β-carotene pathway library)



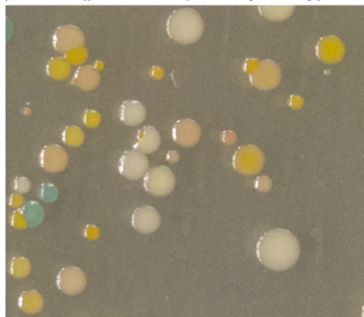


pGT533 (β-carotene pathway library)





pGT534 (β-carotene pathway library)



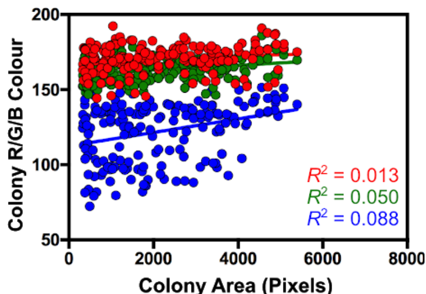


Figure S23. Relationship between colony area and colony colour in the β-carotene pathway libraries. The experiment is described in the main text and Figure S15. The representative images of colonies shown are the same as those in Figure 15. Colony area and RGB colour (red, green, blue) values were extracted from images. For each colony, the three colour values are plotted separately in the corresponding colour. Linear regressions for each

R/G/B colour are also shown in the corresponding colour (solid lines and R^2 values). There is little or no association between colony area and colony colour, reflected by low R^2 values.

SUPPLEMENTARY NOTES

Note S1: Start-Stop Assembly Quick-Start Guide

See separate file.

Note S2. Cloning parts into Level 0 vector pStA0

We suggest three approaches for cloning parts into Level 0 vector pStA0:

1. **Inverse PCR adding part sequence using primer tails**
   Use pStA0 as the PCR template and primers which anneal outside the BsaI sites of pStA0 (removing the BsaI sites and *lacZα* from resultant construct). See Table S8 for primer annealing regions and SapI sites and corresponding fusion sites to be included in primer tails. The part that is being cloned into pStA0 is added using primer tail(s) outside the SapI sites and fusion sites. The part can be added to one primer or split between both to avoid excessively long primers. Use a DNA polymerase which yields blunt DNA ends, suitable for ligation. At least one end of the PCR product must include a 5' phosphate group to allow ligation, which is most conveniently achieved by obtaining a primer with a 5' phosphate group from the manufacturer. This approach is particularly useful for small parts and when no template for the part is available.

2. **PCR amplification followed by one-pot assembly with pStA0**
   See Figure S3. PCR-amplify the part of interest using primers with tails shown in Table S2. The purified PCR product can be used directly in a Level 0 one-pot assembly reaction with pStA0 as described in Materials and Methods.

3. **DNA synthesis followed by one-pot assembly with pStA0**
   Design the part with prefix and suffix sequences shown in Table S1. The synthesised DNA fragment can be used directly in a Level 0 one-pot assembly reaction with pStA0 as described in Materials and Methods.

Note S3: Start-Stop Assembly Lab Protocol

See separate file.

SUPPLEMENTARY MATERIALS AND METHODS

Plasmid construction

Plasmids, oligonucleotides and synthetic DNA used in this study are shown in Tables 1, S2, S3 and S5. All oligonucleotides and gBlock linear DNA fragments were synthesised by Integrated DNA Technologies Inc. All Start-Stop Assembly vector sequences have been deposited in GenBank (accession numbers shown in Table 1 and Table S3).

Construction of Level 0 vector pStA0

Level 0 vector pStA0 was derived from pUC19. To silence a BsaI site in the ampicillin-resistance cassette the pUC19 backbone was amplified in two fragments, the first using primers oligoGT249 and oligoGT252, and the second using oligoGT250 and oligoGT251. OligoGT251 and oligoGT252 include overlapping regions with a single nucleotide mutation to silence the BsaI site. The two PCR products served as a template for overlap extension PCR (5) using oligoGT249 and oligoGT250 to generate a blunt-ended vector backbone fragment lacking the BsaI site. This vector backbone PCR product was ligated with 5'-phosphorylated gblock dnaGT6 to generate pGT421. The pGT421 backbone was PCR-amplified using oligoGT253 and oligoGT254 and ligated with a *lacZα* fragment amplified from pUC19 using oligoGT255 and oligoGT256 to generate pStA0.

Construction of Level 1 vectors

The nine Level 1 vectors (Table 1) were derived from pACYC184. To silence a BbsI site in the tetracycline-resistance cassette the pACYC184 backbone was amplified in two fragments, the first using primers oligoGT257 and oligoGT259, and the second using oligoGT258 and oligoGT260. OligoGT259 and oligoGT260 include overlapping regions with a single nucleotide mutation to silence the BbsI site. The two PCR products served as a template for overlap extension PCR using oligoGT257 and oligoGT258 to generate a blunt-ended vector backbone fragment lacking the BbsI site. This vector backbone PCR product was ligated with a *lacZα* fragment amplified from pUC19 using oligoGT261 and oligoGT262 to generate pGT422.

Plasmids pStA1AZ, pStA1BC, pStA1CD and pStA1DE were generated using overlap extension PCR and pGT422 as the template. The pStA1AZ *lacZα* fragment was amplified using oligoGT356 and oligoGT357 and the backbone was amplified using oligoGT265 and oligoGT266. The pStA1BC *lacZα* fragment was amplified using oligoGT363 and oligoGT366 and the backbone was amplified using oligoGT268 and oligoGT270. The pStA1CD *lacZα* fragment was amplified using oligoGT364 and oligoGT367 and the backbone was amplified using oligoGT268 and oligoGT273. The pStA1DE *lacZα* fragment was amplified using oligoGT365 and oligoGT368 and the backbone was amplified using oligoGT268 and oligoGT276. Each pair of overlapping PCR products was extended by overlap extension PCR to

generate a single PCR product, which was subsequently phosphorylated and ligated using the NEB site-directed mutagenesis kit to generate the corresponding complete vector.

Plasmids pStA1AB, pStA1BZ, pStA1CZ, pStA1DZ and pStA1EZ were generated using inverse PCR and pStA1AZ as the PCR template. pStA1AB was generated using oligoGT268 and oligoGT362, pStA1BZ was generated using oligoGT279 and oligoGT358, pStA1CZ was generated using oligoGT279 and oligoGT359, and pStA1DZ was generated using oligoGT279 and oligoGT360. PCR products were ligated to obtain circular vectors.

Construction of Level 2 vectors

The three Level 2 vectors (Table 1) were derived from pACYC177. First, the pACYC177 backbone was PCR-amplified using primers oligoGT482 and oligoGT483, and ligated with a *lacZα* fragment amplified from pStA0 using primers oligoGT261 and oligoGT262, to generate pGT410. Next, the three Level 2 vectors were each generated by overlap extension PCR using pGT410 as template. For pGT411 the *lacZα* fragment was amplified with oligoGT377 and oligoGT378 and the backbone was amplified with oligoGT289 and oligoGT265. For pGT412 the *lacZα* fragment was amplified with oligoGT379 and oligoGT381 and the backbone was amplified with oligoGT380 and oligoGT265. For pGT413 the *lacZα* fragment was amplified with oligoGT382 and oligoGT384 and the backbone was amplified with oligoGT265 and oligoGT383. Each pair of overlapping PCR products was extended by overlap extension PCR to generate a single PCR product, which was subsequently phosphorylated and ligated using the NEB site-directed mutagenesis kit to generate the corresponding complete vector.

pGT411, pGT412 and pGT413 had a BsaI site in the backbone that was silenced via inverse PCR using primers oligoGT569 and oligoGT570. The PCR products were each phosphorylated and ligated using the NEB site-directed mutagenesis kit to generate final three Level 2 plasmids pStA212, pStA223 and pStA234.

Construction of Level 3 vectors

The Level 3 vectors (Table 1) were derived from pACYC184. First the vector backbone was PCR-amplified from pACYC184 using oligoGT297 and oligoGT298 and ligated with a *lacZα* fragment amplified from pStA0 using oligoGT261 and oligoGT262 to generate pGT414. To construct pStA313, the vector backbone of pGT414 was PCR-amplified using primers oligoGT265 and oligoGT299, and the *lacZα* fragment was PCR-amplified using primers oligoGT385 and oligoGT386. The two overlapping PCR products were extended by overlap extension PCR to generate a single PCR product, which was subsequently phosphorylated and ligated using the NEB site-directed mutagenesis kit to generate the vector pStA313.

Vector pStA314 was generated by inverse PCR using primers oligoGT265 and oligoGT387 with pStA313 as the template. The PCR product was phosphorylated and ligated using the NEB site-directed mutagenesis kit to generate the vector pStA314.

Parts cloned into pStA0

To construct pStA0::*eyfp* the *eyfp* coding sequence was PCR-amplified from the reporter plasmid pATM2 (6) using primers oligoGT614 and oligoGT615. The gel-purified PCR product was directly used in a Level 0 Start-Stop Assembly reaction with pStA0. The Level 0 reaction product was then used to transform *E. coli* and the plasmids of isolated transformant colonies were purified and sequence verified. To generate the eight plasmids pStA0::*crtI*, pStA0::*crtB*, pStA0::*crtE*, pStA0::*dxs*, pStA0::*crtW*, pStA0::*crtZ*, pStA0::*lcyB* and pStA0::*idi* codon-optimised CDSs (Table S6) were synthesised as linear gBlock DNA fragments (Integrated DNA Technologies) and cloned into pStA0. Linear gBlock DNA fragments were used directly in Level 0 Start-Stop Assembly reactions with pStA0. The Level 0 reaction product was then used to transform *E. coli* and the plasmids of transformant colonies were purified and sequence verified.

The promoters, RBSs and terminators were cloned into pStA0 by inverse PCR using pStA0 as the template and the primers shown in Table S7. The PCR products were phosphorylated and ligated using the NEB site-directed mutagenesis kit and *E. coli* was transformed with the ligation product.

Additional plasmids used in study

pGT274-pGT277 were generated by inverse PCR using pCK302 (1) as a template. pGT274 was PCR-amplified using oligoGT468 and oligoGT472. pGT275 was PCR amplified using oligoGT469 and oligoGT472. pGT276 was generated using oligoGT470 and oligoGT472. pGT277 was amplified using primers oligoGT471 and oligoGT472. Each PCR product was phosphorylated and ligated using the NEB site-directed mutagenesis kit and *E. coli* was transformed with the ligation product.

Annealing of oligonucleotide linkers

Double-stranded linkers containing spacers were generated by annealing complementary pairs of oligonucleotides (Table S5). Pairs of oligonucleotides were mixed together to a final concentration of 10 µM. Oligonucleotide mixtures were heated to 95 °C for 5 mins, then allowed to cool slowly to room temperature. These annealed linkers were used directly as parts in Level 1 assembly reactions at a concentration of 40 fmol.

Analysis of DNA scars and characterisation of RBS library by flow cytometry analysis

E. coli DH10B cells were transformed with the appropriate plasmids and individual transformant colonies were used to inoculate 200 µl LB supplemented with ampicillin, which was incubated overnight (16 h) in plates of 96 U-shaped 1.2 ml wells covered with sterile breathable sealing film (Breathe Easy) at 37 °C with shaking at 700 r.p.m on a Multitron shaker (Infors-HT). For flow cytometer assays, overnight cultures were subcultured 1:1000 into 200 µl fresh LB medium that contained ampicillin and the *rhaBAD* promoter inducer L-rhamnose (0.6 mg ml^{-1}), in

deep-well plates and grown for 6 h at 37 °C with shaking at 700 r.p.m. Cultures were diluted 1:50 in filtered PBS and immediately subjected to flow cytometer analysis. GFP fluorescence was measured using an Attune NxT flow cytometer (Invitrogen) as described in Materials and Methods.

SUPPLEMENTARY TABLES

Table S1. Prefix and suffix sequences. Prefix and suffix sequences should be added to genetic parts to allow cloning into Level 0 storage vector pStA0 and subsequent use in Start-Stop Assembly. They can be added by PCR using primer tails (Table S2) or included in the design of synthetic DNA sequences. The prefix and suffix sequences include inward-facing BsaI recognition sites (grey box) and corresponding storage fusion sites (F and R; **bold**) for cloning parts into Level 0 storage vector pStA0 using a Level 0 assembly reaction, as well as inward-facing SapI recognition sites (grey box) with corresponding donor fusion sites (α, β, γ, δ or ε depending on the type of part; **bold**) for subsequent multi-part assembly of expression units in Level 1 assembly.

Part	Prefix		Suffix
Promoter	GGTCTCA**TGTG**GCTCTTCG**CAG** BsaI F SapI α	-Promoter-	**CCA**TGAAGAGC**GACC**AGAGACC β SapI R BsaI
RBS	GGTCTCA**TGTG**GCTCTTCG**CCA** BsaI F SapI β	-RBS-	**ATG**TGAAGAGC**GACC**AGAGACC γ SapI R BsaI
CDS	GGTCTCA**TGTG**GCTCTTCG**ATG** BsaI F SapI γ	-CDS-	**TAA**TGAAGAGC**GACC**AGAGACC δ SapI R BsaI
Terminator	GGTCTCA**TGTG**GCTCTTCG**TAA** BsaI F SapI δ	-Terminator-	**GGA**TGAAGAGC**GACC**AGAGACC ε SapI R BsaI

Table S2. PCR primer tails for amplification of genetic parts to be cloned in Level 0 vector pStA0 using a Level 0 assembly reaction. These primer tails contain the prefix and suffix sequences described in Table S1, which include inward-facing BsaI recognition sites (grey box) and corresponding storage fusion sites (F and R; **bold**) for cloning parts into Level 0 storage vector pStA0 using a Level 0 assembly reaction, as well as inward-facing SapI recognition sites (grey box) with corresponding donor fusion sites (α, β, γ, δ or ε depending on the type of part; **bold**) for subsequent multi-part assembly of expression units in Level 1 assembly. In order to improve restriction digestion, additional nucleotides are included at the 5' end of each primer tail so that the BsaI site will located away from the end of the resultant PCR product.

Primer tail	Sequence (5′-3′) of primer tail
Promoter Fw primer tail	AAGGGGTTGGTCTCA**TGTG**GCTCTTCG**CAG**…Annealing region BsaI F SapI α
Promoter Rv primer tail	CAGTGTTGGGTCTCT**GGTC**GCTCTTCA**TGG**…Annealing region BsaI R SapI β
RBS Fw primer tail	AAGGGGTTGGTCTCA**TGTG**GCTCTTCG**CCA**…Annealing region BsaI F SapI β
RBS Rv primer tail	CAGTGTTGGGTCTCT**GGTC**GCTCTTCA**CAT**…Annealing region BsaI R SapI γ
CDS Fw primer tail	AAGGGGTTGGTCTCA**TGTG**GCTCTTCG**ATG**…Annealing region BsaI F SapI γ
CDS Rv primer tail	CAGTGTTGGGTCTCT**GGTC**GCTCTTCA**TTA**…Annealing region BsaI R SapI δ
Terminator Fw primer tail	AAGGGGTTGGTCTCA**TGTG**GCTCTTCG**TAA**…Annealing region BsaI F SapI δ
Terminator Rv primer tail	CAGTGTTGGGTCTCT**GGTC**GCTCTTCA**TCC**…Annealing region BsaI R SapI ε

Table S3. Genetic parts stored in Level 0 vector pStA0 and other plasmids used in this study.

Plasmid Name	Accession Number	Comments (Part, selection marker(s), replicon, ID number)
pStA0::J23100	MG649435	Promoter P1 = J23100, AmpR, pMB1, ID = pGT323
pStA0::J23102	MG649436	Promoter P2 = J23102, AmpR, pMB1, ID = pGT324
pStA0::J23107	MG649437	Promoter P4 = J23107, AmpR, pMB1, ID = pGT326
pStA0::J23113	MG649438	Promoter P6 = J23113, AmpR, pMB1, ID = pGT328
pStA0::J23116	MG649439	Promoter P5 = J23116, AmpR, pMB1, ID = pGT327
pStA0::J23118	MG649440	Promoter P3 = J23118, AmpR, pMB1, ID = pGT336
pStA0::RBSc13	MG649441	RBS R3 = RBSc13, AmpR, pMB1, ID = pGT330
pStA0::RBSc33	MG649442	RBS R2 = RBSc33, AmpR, pMB1, ID = pGT331
pStA0::RBSc44	MG649445	RBS R1 = RBSc44, AmpR, pMB1, ID = pGT332
pStA0::RBSc58	MG649446	RBS R4 = RBSc58, AmpR, pMB1, ID = pGT333
pStA0::RBSc36	MG649443	RBS R6 = RBSc36, AmpR, pMB1, ID = pGT334
pStA0::RBSc42	MG649444	RBS R5 = RBSc42, AmpR, pMB1, ID = pGT335
pStA0::L3S2P55	MG649450	Terminator T1 = L3S2P55, AmpR, pMB1, ID = pGT337
pStA0::L3S2P21	MG649449	Terminator T2 = L3S2P21, AmpR, pMB1, ID = pGT338
pStA0::ECK120033737	MG649448	Terminator T3 = ECK120033737, AmpR, pMB1, ID = pGT339
pStA0::ECK120019600	MG649447	Terminator T4 = ECK120019600, AmpR, pMB1, ID = pGT340
pStA0::*crtI*	-	*crtI* CDS, AmpR, pMB1, ID = pGT356
pStA0::*crt*B	-	*crtB* CDS, AmpR, pMB1, ID = pGT357
pStA0::*crtE*	-	*crtE* CDS, AmpR, pMB1, ID = pGT358
pStA0::*dxs*	-	*dxs* CDS, AmpR, pMB1, ID = pGT359
pStA0::*crtW*	-	*crtW* CDS, AmpR, pMB1, ID = pGT360
pStA0::*crtZ*	-	*crtZ* CDS, AmpR, pMB1, ID =pGT361
pStA0::*lcyB*	-	*lcyB* CDS, AmpR, pMB1, ID = pGT362
pStA0::*id*i	-	*idi* CDS, AmpR, pMB1, ID = pGT363
pStA0::*eyfp*	-	*efyp* CDS, AmpR, pMB1, ID = pGT431
Plasmid Name	**Accession Number**	**Comments (Selection marker(s), replicon, comments)**
pCK302	KU555410	AmpR, pBR322 (1)
pGT274	-	AmpR, pBR322, pCK302 with 'No scar'
pGT275	-	AmpR, pBR322, pCK302 with BioBrick scar

pGT276	-	AmpR, pBR322, pCK302 with MoClo scar
pGT277	-	AmpR, pBR322, pCK302 with BASIC scar
pUC19	M77789	AmpR, pMB1
pACYC184	X06403	CamR, TetR, p15A
pACYC177	X06402	AmpR, KanR, p15A
pGT421	-	AmpR, pMB1, plasmid construction intermediate
pGT422	-	TetR, p15A, plasmid construction intermediate
pGT410	-	KanR, p15A, plasmid construction intermediate
pGT414	-	CamR, p15A, plasmid construction intermediate
pGT411	-	KanR, p15A, plasmid construction intermediate
pGT412	-	KanR, p15A, plasmid construction intermediate
pGT413	-	KanR, p15A, plasmid construction intermediate

Table S4. List of oligonucleotides used in this study.
5'-phosphorylated oligonucleotides denoted /5Phos/.

Primer	Sequence (5'-3')	Comments
OligoGT234	GGGGAAACGCCTGGTATCT	pStA0 Fw sequencing primer
OligoGT235	AGCAAAAACAGGAAGGCAAA	pStA0 Rv sequencing primer
OligoGT339	GTTGAGGACCCGGCTAGG	pStA1 Fw sequencing primer
OligoGT340	TGTGACGGAAGATCACTTCG	pStA1 Rv sequencing primer
OligoGT573	CCTCGGTGAGTTTTCTCCTTC	pStA2 Fw sequencing primer
OligoGT486	GATTACGCGCAGACCAAAAC	pStA2 Rv sequencing primer and pStA3 Fw sequencing primer
OligoGT487	AAACGGTTAGCGCTTCGTTA	pStA3 Rv sequencing primer
OligoGT249	TGGAGTTCTGAGGTCATTACTGGATCTATCAACAGGAGTCCAAGGCG CTCGGTCGTTCGGCT	
OligoGT250	AAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGAT GCCCGAAAGGGCCTCGTGATACGCC	
OligoGT251	CCGGTGAGCGTGGGTCCCGCGGTATCATTGCAGCAC	
OligoGT252	GTGCTGCAATGATACCGCGGGACCCACGCTCACCGG	
OligoGT253	TTGGTCTCTCACACTGGATTCTCACC	
OligoGT254	GGTCTCAGACCTCTAGGGCG	
OligoGT255	CTATGCGGCATCAGAGCAGATTG	
OligoGT256	GGGCAGTGAGCGCAACGC	
OligoGT257	CAAAAGTTGGCCCAGGGCT	
OligoGT258	GCTCAGGTCGCAGACGT	
OligoGT259	GTTGCATGATAAAGAAAACAGTCATAAGTGC	
OligoGT260	CGCACTTATGACTGTTTTCTTTATCATGCAA	
OligoGT261	/5Phos/GCCGCAGCCGAACGAC	
OligoGT261	/5Phos/GCCGCAGCCGAACGAC	
OligoGT262	/5Phos/GCGTATCACGAGGCCCT	
OligoGT265	/5Phos/ATTTGTCCTACTCAGGAGAGCGT	
OligoGT266	TACTGGCTCTTCTCTGCTCCTGAGACCATTCTCACCAATAAAAAACG CCCG	
OligoGT268	/5Phos/AGAGACCATTTGTCCTACTCAGGAGAG	
OligoGT270	TACTGGCTCTTCTCTGCATTTGAGACCATTCTCACCAATAAAAAACG CC	
OligoGT273	TACTGGCTCTTCTCTGACCTTGAGACCATTCTCACCAATAAAAAACG CC	

OligoGT276	TACTGGCTCTTCTCTGAAGCTGAGACCATTCTCACCAATAAAAAACG CC
OligoGT279	/5Phos/TGAGACCATTCTCACCAATAAAAAACGCC
OligoGT289	TACTGGGTCTCTCTCCGGCATTGTCTTCATTCTCACCAATAAAAAAC GCCCG
OligoGT297	CTTTATCATGCAACTCGTAGGACAGG
OligoGT298	AATTTAACTGTGATAAACTACCGCATTAAAGCT
OligoGT299	TACTGGAAGACTTGGCAATTCTCACCAATAAAAAACGCCCG
OligoGT329	ACGCTGAAAAGCGTCTTTTTTCGTTTTGGTCCGGACGAAGAGCGACC TCTAGGGCGGCGG
OligoGT330	CTTATTGTTCGTCTTTGGTACCGAGTTATGAAGAGCCACACTGGATT CTCACCAATAAAAAACGC
OligoGT331	CCCGAAAGGGGGGCCTTTTTTCGTTTTGGTCCGGACGAAGAGCGACC TCTAGGGCGGCGG
OligoGT332	AGGCCTCTTTTCTGGAATTTGGTACCGAGTTATGAAGAGCCACACTG GATTCTCACCAATAAAAAACGC
OligoGT333	TGACAGTGCGGGCTTTTTTTTTcgaccaaaggGGACGAAGAGCGACC TCTAGGGCGGCGG
OligoGT334	GGTGCGGGCTTTTTTctgtgtttccTTATGAAGAGCCACACTGGATT CTCACCAATAAAAAACGC
OligoGT335	GTAATGCGGTGGACAGGATCGGCGGTTTTCTTTTCTCTTCTCAAGGA CGAAGAGCGACCTCTAGGGCGGCGG
OligoGT336	TGCAAGGTAGTGGACAAGACCGGCGGTCTTAAGTTTTTTGGCTGAAT TATGAAGAGCCACACTGGATTCTCACCAATAAAAAACGC
OligoGT356	GGTCTCAGGAGCAGAGAAGAGCCAGTAGGGCAGTGAGCGCAACG
OligoGT357	GGTCTCTAGTATCCTGAAGAGCGGAAACTATGCGGCATCAGAGCAGA TTG
OligoGT358	aatGCAGAGAAGAGCCAGTAGGGC
OligoGT359	aggtCAGAGAAGAGCCAGTAGGGC
OligoGT360	gcttCAGAGAAGAGCCAGTAGGGC
OligoGT361	cgctCAGAGAAGAGCCAGTAGGGC
OligoGT362	CATTTCCTGAAGAGCGGAAACTATGCG
OligoGT363	ACCTTCCTGAAGAGCGGAAACTATGCG
OligoGT364	AAGCTCCTGAAGAGCGGAAACTATGCG
OligoGT365	AGCGTCCTGAAGAGCGGAAACTATGCG
OligoGT366	GGTCTCAAATGCAGAGAAGAGCCAGTAGGGCAGTGAGCGCAAC
OligoGT367	GGTCTCAAGGTCAGAGAAGAGCCAGTAGGGCAGTGAGCGCAAC

OligoGT368	GGTCTCAGCTTCAGAGAAGAGCCAGTAGGGCAGTGAGCGCAAC
OligoGT375	/5Phos/GAAGACAATGCCGGAGAGAGACCCAGTACCAGTAGGGCAG TGAGCGCAAC
OligoGT376	/5Phos/GAAGACTTTAGTAGTATGAGACCGGAAAGGAAACTATGCG GCATCAGAGC
OligoGT377	GAAGACAATGCCGGAGAGAGACCCAGTAGGGCAGTGAGCGCAACGC
OligoGT378	GAAGACTTTAGTAGTATGAGACCGGAAACTATGCGGCATCAGAGCAG ATTG
OligoGT379	GAAGACAAACTAGGAGAGAGACCCAGTAGGGCAGTGAGCGCAACGC
OligoGT380	TACTGGGTCTCTCTCCTAGTTTGTCTTCATTCTCACCAATAAAAAAC GCCCG
OligoGT381	GAAGACTTGTAAAGTATGAGACCGGAAACTATGCGGCATCAGAGCAG ATTG
OligoGT382	GAAGACAATTACGGAGAGAGACCCAGTAGGGCAGTGAGCGCAACGC
OligoGT383	TACTGGGTCTCTCTCCGTAATTGTCTTCATTCTCACCAATAAAAAAC GCCCG
OligoGT384	GAAGACTTCTCGAGTATGAGACCGGAAACTATGCGGCATCAGAGCAG ATTG
OligoGT385	TGCCAAGTCTTCCAGTAGGGCAGTGAGCGCAACGC
OligoGT386	GTAATTGTCTTCGGAAACTATGCGGCATCAGAGCAGATTG
OligoGT387	CTCGTTGTCTTCGGAAACTATGCGGCATCAGAGCAGATTG
OligoGT448	tacgaccagtctaaaaagcgcc
OligoGT463	NNNNNNNNNNNNNNNAAAGGAGGTNNNNNNNatgcgtaaaggcgaag agctg
OligoGT468	AAAANNNatgcgtaaaggcgaagagctg
OligoGT469	ATACCATatgcgtaaaggcgaagagctg
OligoGT470	AAAAGGTatgcgtaaaggcgaagagctg
OligoGT471	ATAGTCCatgcgtaaaggcgaagagctg
OligoGT472	acctcctaaaagttaaacaaaattatttctagaggg
OligoGT482	AAATCTGGAGCCGGTGAGCGT
OligoGT483	TAATTTCCCCCAAGATTAGAAAAACTCATCGAGCATCAAATG
OligoGT503	/5Phos/CTGCGAAGAGCCACACTGGATTCTCACCAATAAAAAACG
OligoGT504	ttgacggctagctcagtcctaggtacagtgctagcCCATGAAGAGCG TAAGACCTCTAGGGCGGCG
OligoGT505	ttgacagctagctcagtcctaggtactgtgctagcCCATGAAGAGCG TAAGACCTCTAGGGCGGCG
OligoGT507	tttacggctagctcagccctaggtattatgctagcCCATGAAGAGCG

	TAAGACCTCTAGGGCGGCG
OligoGT508	ttgacagctagctcagtcctagggactatgctagcCCATGAAGAGCG TAAGACCTCTAGGGCGGCG
OligoGT509	ctgatggctagctcagtcctagggattatgctagcCCATGAAGAGCG TAAGACCTCTAGGGCGGCG
OligoGT510	/5Phos/TGGCGAAGAGCCACACTGGATTCTCACCAATAAAAAACG
OligoGT512	CTACGTTTTTTAGAAAAAGGAGGTATGCGAGATGTGAAGAGCGTAAG ACCTCTAGGGCGGCG
OligoGT513	AAAACACTAGACTGGAAAGGAGGTAGAGAATATGTGAAGAGCGTAAG ACCTCTAGGGCGGCG
OligoGT514	ATCGGATTGGATCCAAAGGAGGTTATACCGATGTGAAGAGCGTAAGA CCTCTAGGGCGGCG
OligoGT515	CATGATCGAATGATTAAAGGAGGTTGGAGGTATGTGAAGAGCGTAAG ACCTCTAGGGCGGCG
OligoGT516	AGCTCCTTAGCTCCTAAAGGAGGTAGTACATATGTGAAGAGCGTAAG ACCTCTAGGGCGGCG
OligoGT517	ACAGGATACATCTGTAAAGGAGGTAACGATGATGTGAAGAGCGTAAG ACCTCTAGGGCGGCG
OligoGT568	ttgacggctagctcagtcctaggtattgtgctagcCCATGAAGAGCG TAAGACCTCTAGGGCGGCG
OligoGT569	/5Phos/CGGTATCATTGCAGCACTGGGG
OligoGT570	CGCTCACCGGCTCCAGATTTG
OligoGT614	AAGGGGTTGGTCTCATGTGGCTCTTCGATGGTGAGCAAGGGCGAG
OligoGT615	AAGGGGTTGGTCTCTGGTCTTACGCTCTTCATTACTTGTACAGCTCG TCCATGCC

Table S5. Spacers implemented as double-stranded linkers. Spacers can be used in either α-β format in place of a promoter, or δ-ε format in place of a terminator. Here we show each of the 16 spacers as double-stranded linkers in both the α-β and δ-ε configurations. Fusion site cohesive ends are shown in **bold**. Spacers are obtained as two single-stranded oligonucleotides and then mixed and annealed together (described in Supplementary Materials and Methods) to generate the spacer as a linker part that can be used directly in Level 1 assembly reactions.

<table>
<tr><th>Spacer</th><th>Spacer sequence in double-stranded linker</th><th>Forward oligonucleotide</th><th>Reverse oligonucleotide</th></tr>
<tr><td>Spacer 1
α-β format</td><td>5′-CAGTGGTCAGCGACT-3′
||||||||||||
3′-ACCAGTCGCTGAGGT-5′</td><td>oligoGT538:
CAGTGGTCAGCGACT</td><td>oligoGT539:
TGGAGTCGCTGACCA</td></tr>
<tr><td>Spacer 1
δ-ε format</td><td>5′-TAATGGTCAGCGACT-3′
||||||||||||
3′-ACCAGTCGCTGACCT-5′</td><td>oligoGT540:
TAATGGTCAGCGACT</td><td>oligoGT541:
TCCAGTCGCTGACCA</td></tr>
<tr><td>Spacer 2
α-β format</td><td>5′-CAGGCTGCCGTGAAT-3′
||||||||||||
3′-CGACGGCACTTAGGT-5′</td><td>oligoGT542:
CAGGCTGCCGTGAAT</td><td>oligoGT543:
TGGATTCACGGCAGC</td></tr>
<tr><td>Spacer 2
δ-ε format</td><td>5′-TAAGCTGCCGTGAAT-3′
||||||||||||
3′-CGACGGCACTTACCT-5′</td><td>oligoGT544:
TAAGCTGCCGTGAAT</td><td>oligoGT545:
TCCATTCACGGCAGC</td></tr>
<tr><td>Spacer 3
α-β format</td><td>5′-CAGGGCACGCTCAAT-3′
||||||||||||
3′-CCGTGCGAGTTAGGT-5′</td><td>oligoGT546:
CAGGGCACGCTCAAT</td><td>oligoGT547:
TGGATTGAGCGTGCC</td></tr>
<tr><td>Spacer 3
δ-ε format</td><td>5′-TAAGGCACGCTCAAT-3′
||||||||||||
3′-CCGTGCGAGTTACCT-5′</td><td>oligoGT548:
TAAGGCACGCTCAAT</td><td>oligoGT549:
TCCATTGAGCGTGCC</td></tr>
<tr><td>Spacer 4
α-β format</td><td>5′-CAGAGTCCGTGCTCA-3′
||||||||||||
3′-TCAGGCACGAGTGGT-5′</td><td>oligoGT550:
CAGAGTCCGTGCTCA</td><td>oligoGT551:
TGGTGAGCACGGACT</td></tr>
<tr><td>Spacer 4
δ-ε format</td><td>5′-TAAAGTCCGTGCTCA-3′
||||||||||||
3′-TCAGGCACGAGTCCT-5′</td><td>oligoGT552:
TAAAGTCCGTGCTCA</td><td>oligoGT553:
TCCTGAGCACGGACT</td></tr>
<tr><td>Spacer 5
α-β format</td><td>5′-CAGATTCTGTGCCGC-3′
||||||||||||
3′-TAAGACACGGCGGGT-5′</td><td>oligoGT554:
CAGATTCTGTGCCGC</td><td>oligoGT555:
TGGGCGGCACAGAAT</td></tr>
<tr><td>Spacer 5
δ-ε format</td><td>5′-TAAATTCTGTGCCGC-3′
||||||||||||
3′-TAAGACACGGCGCCT-5′</td><td>oligoGT556:
TAAATTCTGTGCCGC</td><td>oligoGT557:
TCCGCGGCACAGAAT</td></tr>
<tr><td>Spacer 6
α-β format</td><td>5′-CAGATCAACGCCTGC-3′
||||||||||||
3′-TAGTTGCGGACGGGT-5′</td><td>oligoGT558:
CAGATCAACGCCTGC</td><td>oligoGT559:
TGGGCAGGCGTTGAT</td></tr>
<tr><td>Spacer 6
δ-ε format</td><td>5′-TAAATCAACGCCTGC-3′
||||||||||||
3′-TAGTTGCGGACGCCT-5′</td><td>oligoGT560:
TAAATCAACGCCTGC</td><td>oligoGT561:
TCCGCAGGCGTTGAT</td></tr>
</table>

Spacer 7
α-β format

5′-**CAG**ATCTGCGGCAAC-3′
||||||||||||
3′-TAGACGCCGTTG**GGT**-5′

oligoGT562: CAGATCTGCGGCAAC

oligoGT563: TGGGTTGCCGCAGAT

Spacer 7
δ-ε format

5′-**TAA**ATCTGCGGCAAC-3′
||||||||||||
3′-TAGACGCCGTTG**CCT**-5′

oligoGT564: TAAATCTGCGGCAAC

oligoGT565: TCCGTTGCCGCAGAT

Spacer 8
α-β format

5′-**CAG**TGCGACCTGACT-3′
||||||||||||
3′-ACGCTGGACTGA**GGT**-5′

oligoGT566: CAGTGCGACCTGACT

oligoGT567: TGGAGTCAGGTCGCA

Spacer 8
δ-ε format

5′-**TAA**TGCGACCTGACT-3′
||||||||||||
3′-ACGCTGGACTGA**CCT**-5′

oligoGT616: TAATGCGACCTGACT

oligoGT617: TCCAGTCAGGTCGCA

Spacer 9
α-β format

5′-**CAG**AGGTGTCTCGCA-3′
||||||||||||
3′-TCCACAGAGCGT**GGT**-5′

oligoGT618: CAGAGGTGTCTCGCA

oligoGT619: TGGTGCGAGACACCT

Spacer 9
δ-ε format

5′-**TAA**AGGTGTCTCGCA-3′
||||||||||||
3′-TCCACAGAGCGT**CCT**-5′

oligoGT620: TAAAGGTGTCTCGCA

oligoGT621: TCCTGCGAGACACCT

Spacer 10
α-β format

5′-**CAG**GCTACAGGCTGC-3′
||||||||||||
3′-CGATGTCCGACG**GGT**-5′

oligoGT622: CAGGCTACAGGCTGC

oligoGT623: TGGGCAGCCTGTAGC

Spacer 10
δ-ε format

5′-**TAA**GCTACAGGCTGC-3′
||||||||||||
3′-CGATGTCCGACG**CCT**-5′

oligoGT624: TAAGCTACAGGCTGC

oligoGT625: TCCGCAGCCTGTAGC

Spacer 11
α-β format

5′-**CAG**TCAGACGGCACT-3′
||||||||||||
3′-AGTCTGCCGTGA**GGT**-5′

oligoGT626: CAGTCAGACGGCACT

oligoGT627: TGGAGTGCCGTCTGA

Spacer 11
δ-ε format

5′-**TAA**TCAGACGGCACT-3′
||||||||||||
3′-AGTCTGCCGTGA**CCT**-5′

oligoGT628: TAATCAGACGGCACT

oligoGT629: TCCAGTGCCGTCTGA

Spacer 12
α-β format

5′-**CAG**ATCGCAACTGGC-3′
||||||||||||
3′-TAGCGTTGACCG**GGT**-5′

oligoGT630: CAGATCGCAACTGGC

oligoGT631: TGGGCCAGTTGCGAT

Spacer 12
δ-ε format

5′-**TAA**ATCGCAACTGGC-3′
||||||||||||
3′-TAGCGTTGACCG**CCT**-5′

oligoGT632: TAAATCGCAACTGGC

oligoGT633: TCCGCCAGTTGCGAT

Spacer 13
α-β format

5′-**CAG**GGCAATCGTGCT-3′
||||||||||||
3′-CCGTTAGCACGA**GGT**-5′

oligoGT634: CAGGGCAATCGTGCT

oligoGT635: TGGAGCACGATTGCC

Spacer 13
δ-ε format

5′-**TAA**GGCAATCGTGCT-3′
||||||||||||
3′-CCGTTAGCACGA**CCT**-5′

oligoGT636: TAAGGCAATCGTGCT

oligoGT637: TCCAGCACGATTGCC

Spacer 14
α-β format

5′-**CAG**ATTGCCTGCGTC-3′
||||||||||||
3′-TAACGGACGCAG**GGT**-5′

oligoGT638: CAGATTGCCTGCGTC

oligoGT639: TGGGACGCAGGCAAT

<table>
<tr><td>Spacer 14
δ-ε format</td><td>5′-TAAATTGCCTGCGTC-3′
||||||||||||
3′-TAACGGACGCAGCCT-5′</td><td>oligoGT640:
TAAATTGCCTGCGTC</td><td>oligoGT641:
TCCGACGCAGGCAAT</td></tr>
<tr><td>Spacer 15
α-β format</td><td>5′-CAGGCACCAATCGCT-3′
||||||||||||
3′-CGTGGTTAGCGAGGT-5′</td><td>oligoGT642:
CAGGCACCAATCGCT</td><td>oligoGT643:
TGGAGCGATTGGTGC</td></tr>
<tr><td>Spacer 15
δ-ε format</td><td>5′-TAAGCACCAATCGCT-3′
||||||||||||
3′-CGTGGTTAGCGACCT-5′</td><td>oligoGT644:
TAAGCACCAATCGCT</td><td>oligoGT645:
TCCAGCGATTGGTGC</td></tr>
<tr><td>Spacer 16
α-β format</td><td>5′-CAGAGCAATCCACGC-3′
||||||||||||
3′-TCGTTAGGTGCGGGT-5′</td><td>oligoGT646:
CAGAGCAATCCACGC</td><td>oligoGT647:
TGGGCGTGGATTGCT</td></tr>
<tr><td>Spacer 16
δ-ε format</td><td>5′-TAAAGCAATCCACGC-3′
||||||||||||
3′-TCGTTAGGTGCGCCT-5′</td><td>oligoGT648:
TAAAGCAATCCACGC</td><td>oligoGT649:
TCCGCGTGGATTGCT</td></tr>
</table>

Table S6. List of synthetic DNA sequences used in the study

Synthetic DNA sequence name	Sequence
crtI recoded	atgacatcagctctcccccgcccggcaccaagtccgtacgcacgccgtaaaacggcgttggttattg gcgcaggttttggtgggttggccctgggcattcgtctacagtcgttaggttttgatacaacaatttt ggaacgtctggatggtcctggtggtcgcgcgtatcaaaaacgtaccccagatggctatgtctttgac atgggtccgactgtgctgacggtgccgcattttatcgaagaactgtttgcgcttgaacgtgatcgtg ccggcctggatgcccccgattatcctcctgaagtgttgtcgggcgagcgcgttaaggaaggcgtttc tggtggcccgcatacgagccggtatgtcaccttagtgccgattctgccctttaccgcattgttttt cacgatggcacgtattttgattatgatggcgaccctgaaagtactcggcggcagattgctgaattgg cccctggcgacttagccgggtatgaacgctttcatgccgatgccgaggccatctttcgtcggggctt cctggaactgggctacacgcactttggtgacgtcccgacgatgctgcgggttgtacctgatctgctc aaactggacgccgttcggaccctgttctcctttacgagtaagtactttcagagcgacaaactgcgcc aagtgttctcttttgaaacccttttggtgggtgggaatcctctgagtgtgccggcgatctatgcaat gattcacttcgttgaaaagacttggggggatccactatgccatgggcggcacaggcgcactggtgcgc ggcctagtccaaaaatttgaggagctgggtggcgccattcgttatggcgccggcgtcgatgaagtac tggtggatggcaatctgcctggtaaacgcacagcgcggggtgtgcgcctggaaagcggcgaagaact gcgcgccgacctggtggcgtccaatggcgattgggctaacacgtatctgaaacgcgtccggccatcg gcacgtctggtcaactctgatttacgcgtgaaagccgcatctgaaagtatgagcctgctcgtggttt atttcgggtttcgcggcggtgatgacctgcccctcaaacatcataatattttattaggcccacgcta cgaggctctgctgagcgaaatctttggcacaaaacggttgggcgaagattttagccagtacctgcac gtcccaacgctcaccgatccggctctggcacccgcgggtcatcatgcggcctatacacttgtcccgg tgccgcataatggctcgggtattgattgggacgtggaaggtccaaagcttgccgaagcagccctggc agatatcgagcgccgcggtttgattccgggcctccgtgaacggctcacacattttgaatttattacg ccagattatttcgcaggcactctcgattcctatctggggaacgcgtttggtccggagccgcgtctgg tccagtcggcatttttccgcccgcacaaccgcagcgaggatctccacaacttttacttagtcggggc gggcgcgcagccaggcgcaggcacaccgagcgttatgatgtccgcgaaaatgacagcgcgcctaatc gctgaagatttcggtatccatgctgatatccggcgctaa
crtB recoded	Atgcgtagtcgcgctggtctgagcttacggttacccacgcgtaccttgaccgtgaccgattactccc ccgccctgccctgcaccgaactgcgccgtcctccactggctcaggcggttcgctactgtcgggattt gacccgccagcactcaaagaccttctatctgggttcacagctcttttcgcctccggaacgcgcggca gtttgggcagtgtatgcggcgtgccgcgcgggcgatgacatcgtcgatgaagccggcaacggcgacc gcgaacgcgaattgcgggaatggcgcagccgtattgatgccgcgtttgctggccaaccagcggatga tcccatctcaaccgcgctggcctgggcggcaggtcggtacgccatcccgcactcagctttcgcggaa ctgcatgaaggcctcaacatggatttacgcggtcatgaataccgtgatatggatgacttgttactgt attgccgccgtgtggcaggtgtggttggctttatggtggcaccgatttctggctaccgtgggggggc tgctaccctgaatgatgctctccaactagggcaggcgatgcaactgacgaatattctgcgcgatgtc ggtgaagatctgacccgcggccgcgtatacctgccacagtctctgcttgatgaatatggcctgtctc gcgccgcgttagagcgctggggtcagggtgagcccctgtcaccggcctaccgtgctctcatgactca tcttggcggccttgcacgtgaatggtatgcagcaggtcgtgctggtattcctcaacttgatggacgc ggtcctctcgccgttctgactgccgcccgtgcgtatgagggtattctggacgatttggaacgggccg gctacgacaacttcggtcggcgcgcgtacgtgtcaggtcgtcgtaaacttctgatgttaccgcaggc ctggtgggaactgcgtagtctgggcgctgtccacggctaa
crtE recoded	atgcgcccggaattactcgcacgcgtgttaagcctgttaccggaaacctccgcgacgccggaattgg cacgcttttacgcgctcctgcgcgactatcctcaacgtggtggcaagggcattcggtcagaattact gcttgcctctgctcgtgcgcacggcctgtccgagtcagataccggttgggagtcagcattatggctg

gcggcagccttagaactgtttcagaactgggtgctggtgcacgatgatattgaagatgattcggaag
aacgccgtggtcgtccggccctgcaccacttgtgtggtatgccggtcgctcttaacgtgggggacgc
gctgcacgcttacatgtgggctgctgttgggaaagccaatgttccgggagcgtttgaagagtttctg
cagatggtgtaccgcacggcggaaggccagcatctggatctggcatgggtggagggtcgtgaatggg
gcctgcgtcccgcggattatctccagatggttggcctgaaaaccgcacactacacggttatcgtgcc
gttacgtctgggggccctggcggcaggcatggcaccgcaggacgcgttcaccccagcgggtctggcg
ctgggtaccgcgtttcagattcgtgacgatgtcctcaatctggcaggtgatccggtgaagtatggta
aagaaattggtggcgatctgttggaaggtaaacgtactctgattgtcctggactggttgactacggc
gccggatgatcgcaaagccatcttcctggaccagatgcgtcggcaccgcgcagataaagaccctgcg
gtgatcgatgaaattcaccgctggctgcttgaaagcggctctgtggaagcggcgcaggactacgcgc
aggcacaagccgcggaaggtctggacttgcttgaaaaagcattggcagacgcgccggatgcccaggc
cgccgctgccttactcgcttctgttcgggaactggccacccgcgaaaaataa

dxs recoded

atgtcttttgatattgcgaaatatccaaccctggccctagttgactcgactcaggaattacgcctgc
tgccgaaagagagccttccaaagctgtgcgatgagttacgccgctacctcctggattctgttagtcg
tagctccggccacttcgcatcgggactaggtaccgtcgaactgacggttgcactgcattatgtgtat
aacaccccgttcgatcaattaatttgggacgtcggtcatcaagcatatccgcataaaattctgaccg
gtcgtcgcgacaagatcggcacaattcgtcaaaaaggtggattgcatcctttcccgtggcgcggcga
gtccgaatacgatgtgttgagcgttggccattcgtcaacttctatcagcgctggtattggtattgcg
gtcgccgcggagaaagaaggcaaaaatcggcgtacggtttgcgtgatcggggatggtgcaatcaccg
caggcatggcattcgaagcgatgaaccatgcgggggacattcgtccggatatgctagtgattctgaa
cgataacgaaatgagtatttccgagaacgtgggggctcttaataaccacttagcgcagctgctgagc
ggtaaactttattctagcctgcgcgaaggcggtaagaaagtgttctcaggcgtccctccgattaaag
agctccttaagcgtactgaggaacacattaagggtatggtggttccaggcaccctgttcgaagaact
gggttttaattacattggtcctgtggacggccatgacgtgttaggcttaattaccacgttaaaaaac
atgcgcgatctgaaaggacctcagtttctccacatcatgaccaagaaaggtcgcggctatgaaccgg
ccgagaaagatccgattaccttccacgcagttccgaaattcgacccatcctccggctgtctgccgaa
aagcagcggaggcctgccgagctattccaaaatctttggtgactggctgtgcgaaactgcggcaaaa
gataacaaactgatggccatcaccccggcaatgcgcgaaggttcgggaatggtggaattctctcgca
agttcccagaccgttattttgacgttgctatcgccgagcaacatgcagtaacctttgcggctggtct
ggcaattggtggttataagccaattgtggccatctattcaacgttcttacaacgtgcatatgaccag
gtgctacatgatgtggccattcagaaactgccggtcttatttgccatcgatcgtgcggggatcgttg
gggcagatggtcagacgcatcagggcgccttcgacctaagttatctgcgctgtatcccagaaatggt
gattatgacccctagcgatgagaatgaatgtcgtcagatgttatacaccggctatcattacaacgac
ggaccatccgctgtgcgctacccgcgcggaaacgcggtgggcgttgaactgaccccgttagaaaaat
taccgatcggaaaagggattgttaaacgtcgcggagaaaaactcgctattctgaacttcggcacact
tatgcccgaagcggctaaagtagcggaaagcctaaacgcaaccttggtggatatgcgcttcgtaaag
ccactggacgaagccctaatcttagagatggccgcgtcacatgaggcgttagttacggtagaggaaa
acgctattatgggcggagcgggtagtggtgtgaacgaagttctgatggctcaccgcaaaccggtgcc
ggtactgaacatcggcctcccggatttttcattccccaaggtactcaagaggaaatgcgcgccgag
ctcggcttagatgctgcaggaatggaagccaaaattaaggcctggcttgcataa

crtW recoded

atggtccagtgccagccgtcgtcgcttcattctgaaaaactcgttcttctctcgagcactattcgcg
atgataaaaacatcaacaagggcattttcatcgcctgctttattttgtttctctgggccatttctct
gattcttctgctgtccattgatacttcaattattcataaatctctgctgggtatcgcaatgctttgg
cagacattttatataccggcctcttcattacagcgcacgatgctatgcatggcgttgtttatccga
aaaacccgcgcattaataatttcattggaaaactgacgctgattctgtatggtctgttaccgtataa
agatctgttaaaaaagcattggcttcatcatggtcatccgggtaccgatctggatccggattactac
aatggccatccccagaatttttcttatggtacttgcactttatgaaaagctattggcggtggaccc
aaattttcggccttgtgatgattttccacggactgaaaaatctggtacatattccggagaataattt
gatcatcttttggatgattccgtccattttgtccagtgtgcaactcttttactttgggacattctta
ccgcataaaaaactggaaggcggctataccaatccccactgcgctcgctccattccgctcccgctgt
tctggagctttgtgacatgttatcacttcggctatcataaagaacatcacgaatatcctcaactccc

gtggtggaaactgcctgaagcacacaaaatctcgctgtaa

crtZ recoded

atgctgtggatttggaatgctctgatcgtgtttgttaccgtcattggcatggaagttatcgcggctc
tggcccacaaatatattatgcatggttggggatggggttggcatttgtcgcaccatgagccgcgcaa
gggagcgtttgaagtgaacgatctgtatgcagtagtttttgcggcccttagcattcttctgatttat
ctgggctcgaccggcatgtggcccttgcaatggatcggtgcgggcatgacggcttacggcttgcttt
attttatggttcacgatggattagttcaccagcgctggccattccgttatatcccacgcaaaggcta
tttaaaacgtctttacatggcacatcgtatgcatcatgcggttcgggggaaagaaggctgcgtgagc
tttgggtttctctatgcgccgcctctttctaaactgcaagctaccctgcgtgaacggcatggcgcgc
gcgcaggcgcggctcgtgatgcgcagggtggcgaagatgaacccgcatctggcaaataa

lcyB recoded

atggatacgcttctgaaaacgcctaataaccttgagtttctgaatccccatcatggttttgcagtga
aagcgagcaccttccgttccgagaaacatcataatttcgggagtcgcaaattctgtgaaacacttgg
ccgctcggtgtgtgtgaagggctcatcgtcggccttattggaacttgtgccggagactaaaaaagaa
aacttggattttgaactgcccatgtatgacccttctaaaggagtggtggttgatcttgccgtagtag
gagggggcccggcgggcctggccgtagcccagcaggtgagcgaagctggtctgagcgtctgctctat
tgacccgaatccaaaactgatctggccaaacaactacggcgtatgggtggatgagtttgaggcgatg
gatctgctggattgcttagatgcgacctggtcgggcgctgcagtgtatattgatgacaatactgcaa
aagaccttcaccgcccttacggtcgtgttaaccgtaaacagttaaaatccaaaatgatgcagaaatg
tatcatgaacggagtcaaattccaccaggcaaaagttattaaagtgattcacgaagaaagcaaaagc
atgctgatttgtaatgacggcatcaccattcaggcgactgtcgtattagatgcgacaggctttagcc
gctctcttgtacaatatgataagccatataacccaggctatcaagttgcatatggcatccttgcaga
ggtggaagaacacccatttgacgttaataaaatggtttttatggattggcgtgacagccacttaaag
aacaataccgatctgaaagagcgtaatagtcgcattccgactttcctctatgccatgccgtttagta
gcaaccgcatttttctggaggaaacaagcctggtcgcccgtccgggccttcgcatcgatgacattca
ggaacgcatggtggcccgcctgaaccatctgggcatcaaagtcaaaagtattgaagaagatgaacac
tgcctgattccgatgggcggtcccctgccggtgcttccgcaacgcgtcgtgggcattggcgggactg
cgggcatggttcacccttcgaccggatatatggtggcgcggacccttgctgccgcccctgtggtggc
taacgctattattcaatacctgggcagtgaacgtagtcactccggtaatgaactgtcaactgcagtc
tggaaggatctgtggccaattgagcgccgtcgccagcgtgaatttttttgcttcggtatggatattc
tgctgaaactggatctgccggctacccgtcgcttcttcgatgctttttcgatcttgagccccgcta
ttggcatggctttctgtccagccgtttattcctgccggaactcatcgttttcggtttgtccttgttt
tcacatgccagcaacacttctcgttttgaaatcatgacgaaagggacggtacctctggttaatatga
ttaacaacttgttgcaagataaagaataa

idi recoded

atgctgcgctcgcttctgcgcggtttaactcacatcccgcgcgtcaactcggcccaacagccttcat
gcgcacacgcgcgcttacaatttaagctgcgttcgatgcagatgactctgatgcaaccgagtatctc
agctaacctgtcgcgcgccgaagatcgtactgaccacatgcgcggtgcaagtacgtgggctggtggt
caatcccaagatgaactgatgttaaaggacgaatgcattttggttgatgttgaagataacattactg
gacatgcatcaaagttggaatgtcataaatttctgccgcaccaacccgcaggtttgttgcaccgcgc
cttcagtgtatttctgtttgatgaccaaggtcgtctgttgcttcaacaacgggcacgctcgaaaatt
acctttccgagtgtatggaccaatacttgttgcagtcatccattgcacggtcagacgccagatgaag
tggatcaactgagccaggtcgcggacgggaccgtcccgggggctaaggcggccgccatccggaaact
ggaacatgaactgggtatcccggcccatcaactgccagcttcagcgtttcgtttcctcacccgtttg
cattactgtgcggctgacgtgcagccggcggctacgcagtcagcactttgggggaacatgaaatgg
attatatcctgttcattcgtgcgaatgtaacactcgccccgaatcctgacgaagtggacgaggtccg
ttatgtaacgcaggaagaactgcgccagatgatgcaaccggataatggccttcagtggagtccatgg
ttccgtattattgccgcccggtttcttgaacgttggtgggccgatctggacgcagcactgaacacgg
ataaacatgaagattggggtaccgtgcatcatattaatgaggcataa

Table S7. Primers used for cloning parts into Level 0 vector pStA0 by inverse PCR

Plasmid	Fw primer	Rv primer
pStA0::J23100	oligoGT504	oligoGT503
pStA0::J23102	oligoGT505	oligoGT503
pStA0::J23118	oligoGT568	oligoGT503
pStA0::J23107	oligoGT507	oligoGT503
pStA0::J23116	oligoGT508	oligoGT503
pStA0::J23113	oligoGT509	oligoGT503
pStA0::RBSc13	oligoGT510	oligoGT512
pStA0::RBSc33	oligoGT510	oligoGT513
pStA0::RBSc44	oligoGT510	oligoGT514
pStA0::RBSc58	oligoGT510	oligoGT515
pStA0::RBSc36	oligoGT510	oligoGT516
pStA0::RBSc42	oligoGT510	oligoGT517
pStA0::L3S2P55	oligoGT329	oligoGT330
pStA0::L3S2P21	oligoGT331	oligoGT332
pStA0::ECK120033737	oligoGT333	oligoGT334
pStA0::ECK120019600	oligoGT335	oligoGT336

Table S8. Primer designs to introduce suitable parts into Level 0 vector pStA0 using inverse PCR. Annealing regions shown (underlined) do not include the *lacZα* gene and BsaI recognition sites, so these are removed from the resultant plasmid. The partial tails shown include SapI recognition sites (grey box) with corresponding donor fusion sites (α, β, γ, δ or ε depending on the type of part; **bold**) for subsequent multi-part assembly of expression units in Level 1 assembly. The sequence of the part being cloned can be added to the tail outside of the fusion sites where indicated. The part sequence can be added to one primer or split between both to avoid excessively long primers. The PCR product is circularised by ligation to form the complete plasmid. Ligation requires a 5' phosphate group to be present at one or both ends of the PCR product, which can be achieved using a 5'-phosphorylated primer as shown (denoted /5Phos/) or by enzymatic phosphorylation of the PCR product, for example using the NEB site-directed mutagenesis kit.

Primer	Sequence (5′-3′) of primer annealing region and partial tail
Promoter forward primer	/5Phos/...Part Sequence...**CCA**TGAAGAGCGACCTCTAGGGCGGCG β SapI Annealing region
Promoter reverse primer	Part Sequence...**CTG**CGAAGAGCCACACTGGATTCTCACCAATAAAAAACG α SapI Annealing region
RBS forward primer	/5Phos/...Part Sequence...**ATG**TGAAGAGCGACCTCTAGGGCGGCG γ SapI Annealing region
RBS reverse primer	Part Sequence...**TGG**CGAAGAGCCACACTGGATTCTCACCAATAAAAAACG β SapI Annealing region
CDS forward primer	/5Phos/...Part Sequence...**TAA**TGAAGAGCGACCTCTAGGGCGGCG δ SapI Annealing region
CDS reverse primer	Part Sequence...**CAT**CGAAGAGCCACACTGGATTCTCACCAATAAAAAACG γ SapI Annealing region
Terminator forward primer	/5Phos/...Part Sequence...**GGA**TGAAGAGCGACCTCTAGGGCGGCG ε SapI Annealing region
Terminator reverse primer	Part Sequence...**TTA**CGAAGAGCCACACTGGATTCTCACCAATAAAAAACG δ SapI Annealing region

SUPPLEMENTARY REFERENCES

1. Kelly,C.L., Liu,Z., Yoshihara,A., Jenkinson,S.F., Wormald,M.R., Otero,J., Estévez,A., Kato,A., Marqvorsen,M.H.S., Fleet,G.W.J., *et al.* (2016) Synthetic Chemical Inducers and Genetic Decoupling Enable Orthogonal Control of the rhaBAD Promoter. *ACS Synth. Biol.*, **5**, 1136–1145.

2. Kim,D., Hong,J.S.-J., Qiu,Y., Nagarajan,H., Seo,J.-H., Cho,B.-K., Tsai,S.-F. and Palsson,B.Ø. (2012) Comparative analysis of regulatory elements between Escherichia coli and Klebsiella pneumoniae by genome-wide transcription start site profiling. *PLoS Genet.*, **8**, e1002867.

3. Crooks,G.E., Hon,G., Chandonia,J.-M. and Brenner,S.E. (2004) WebLogo: a sequence logo generator. *Genome Res.*, **14**, 1188–1190.

4. Anderson,J.C. (2009) *Registry of Standard Biological Parts*.

5. Bryksin,A.V. and Matsumura,I. (2010) Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. *Biotechniques*, **48**, 463–465.

6. Kelly,C.L., Taylor,G.M., Hitchcock,A., Torres-Méndez,A. and Heap,J.T. (2018) A Rhamnose-Inducible System for Precise and Temporal Control of Gene Expression in Cyanobacteria. *ACS Synth. Biol.*, 10.1021/acssynbio.7b00435.