Note S3. Start-Stop Assembly Lab Protocol

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

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Level 0 Start-Stop Assembly to store genetic parts

- Design genetic part. Generate by PCR or synthesis. For part and primer design see Figure S3, Note S2, Table S1 and S2.
- Gel-purify PCR products.
- Level 0 Start-Stop Assembly reactions contain 20 fmol of Level 0 vector pStA0 plasmid DNA, 40 fmol of insert (PCR product or synthetic DNA), T4 DNA Ligase buffer, 400 units of T4 DNA Ligase (1 µl of typical 400,000 units/ml stock) and 10 units of BsaI (1 µl of typical 10,000 units/ml stock) in a total volume of 20 µl.
- Incubate reactions using a thermocycler for 30 two-step cycles of 37 °C for 5 minutes then 16 °C for 5 minutes, before a single final denaturation step at 65 °C for 20 minutes.
- Transform *E. coli* with assembly reaction product and plate onto LB agar plates containing ampicillin (100 µg ml^{-1}), IPTG (0.1 mM) and X-Gal (40 µg ml^{-1}).
- Pick single white colonies, sequence inserts using primers oligoGT234 and oligoGT235.

Level 1 Start-Stop Assembly

- Refer to Figure 4 and Table 1 for choice of Level 1 vector.
- Level 1 Start-Stop Assembly reactions contain 20 fmol of Level 1 vector plasmid DNA, 40 fmol of each insert (plasmid DNA or annealed oligonucleotides), T4 DNA Ligase buffer, 400 units of T4 DNA Ligase (1 µl of typical 400,000 units/ml stock) and 10 units of SapI (1 µl of typical 10,000 units/ml stock) in a total volume of 20 µl.
- For combinatorial assembly use a mixture of parts at an overall concentration of 40 fmol.
- Incubate reactions using a thermocycler for 30 two-step cycles of 37 °C for 5 minutes then 16 °C for 5 minutes, before a single final denaturation step at 65 °C for 20 minutes.
- Transform *E. coli* with assembly reaction product and plate onto LB agar plates containing tetracycline (10 µg ml-1), IPTG (0.1 mM) and X-Gal (40 µg ml^{-1}).
- Pick single white colonies. For combinatorial assembly, pick or scrape all white colonies from transformation plates, pool them (e.g. in Qiagen P1 buffer), miniprep, use pool in subsequent steps.

Level 2 Start-Stop Assembly

- Refer to Figure 4 and Table 1 for choice of Level 2 vector.
- Level 2 Start-Stop Assembly reactions contain 20 fmol of Level 2 vector plasmid DNA, 40 fmol of each insert (plasmid DNA), T4 DNA Ligase buffer, 400 units of T4 DNA Ligase (1 µl of typical 400,000 units/ml stock) and 10 units of BsaI (1 µl of typical 10,000 units/ml stock) in a total volume of 20 µl.
- Incubate reactions using a thermocycler for 30 two-step cycles of 37 °C for 5 minutes then 16 °C for 5 minutes, before a single final denaturation step at 65 °C for 20 minutes.
- Transform *E. coli* with assembly reaction product and plate onto LB agar plates containing kanamycin (50 µg ml^{-1}), IPTG (0.1 mM) and X-Gal (40 µg ml^{-1}).

Level 3 Start-Stop Assembly

- Refer to Figure 4 and Table 1 for choice of Level 3 vector.
- Level 3 Start-Stop Assembly reactions contain 20 fmol of Level 3 vector plasmid DNA, 40 fmol of each insert (plasmid DNA), T4 DNA Ligase buffer, 400 units of T4 DNA Ligase (1 µl of typical 400,000 units/ml stock) and 10 units of BbsI (1 µl of typical 10,000 units/ml stock) in a total volume of 20 µl.
- Reactions were incubated using a thermocycler for 30 two-step cycles of 37 °C for 5 minutes then 16 °C for 5 minutes, before a single final denaturation step at 65 °C for 20 minutes.
- Transform *E. coli* with assembly reaction product and plate onto LB agar plates containing chloramphenicol (25 µg ml^{-1}), IPTG (0.1 mM) and X-Gal (40 µg ml^{-1}).