SUPPORTING INFORMATION

Simple and efficient modification of Golden Gate design standards and parts using oligo stitching

Jonas De Saeger[1,2], Mattias Vermeersch[1,2], Christophe Gaillochet[1,2], and Thomas B. Jacobs[1,2,*]
*Corresponding author

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, (Technologiepark 71,) 9052 Ghent, Belgium

[2]VIB Center for Plant Systems Biology, (Technologiepark 71,) 9052 Ghent, Belgium

Corresponding author email address: thomas.jacobs@psb.ugent.be

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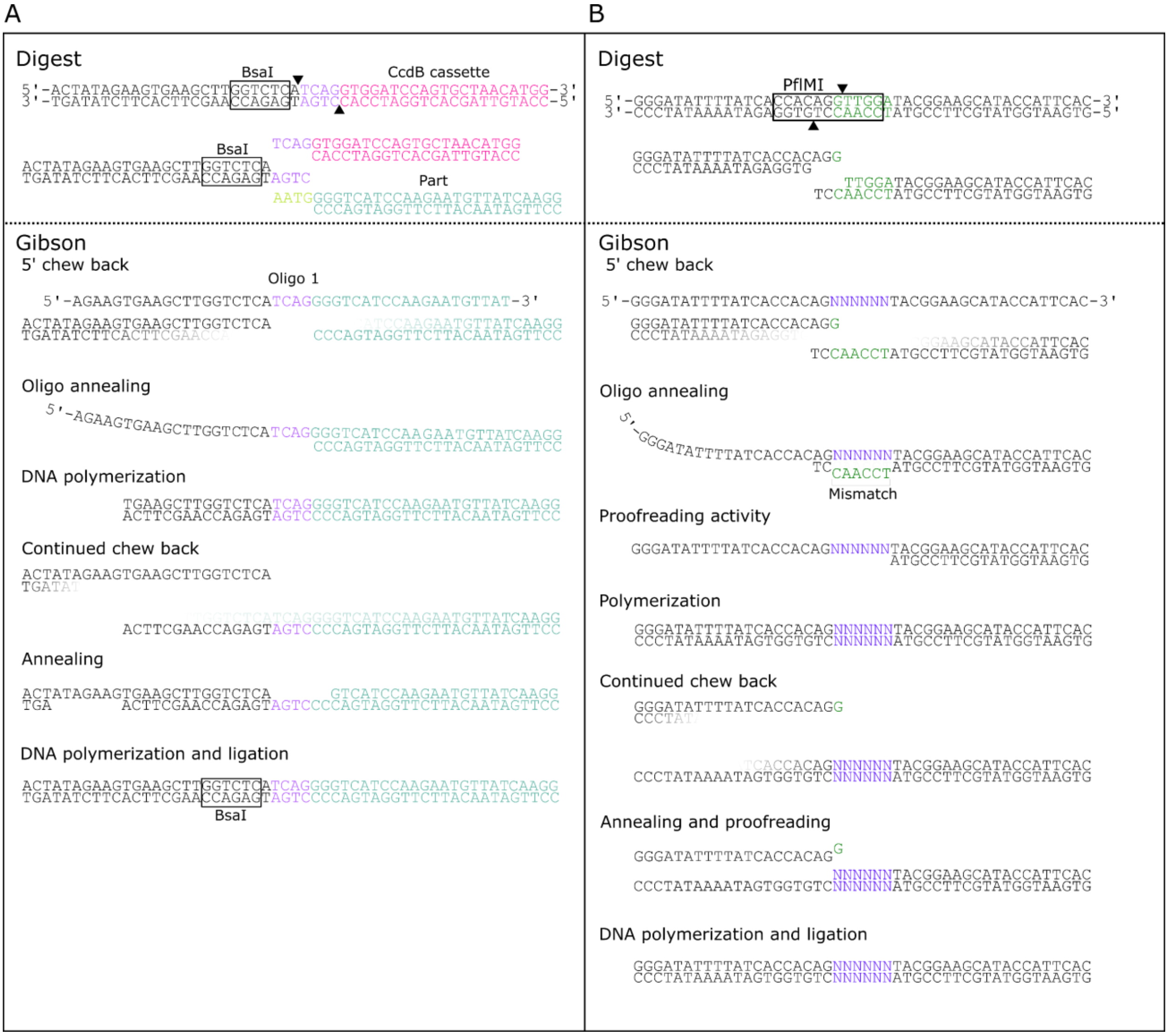
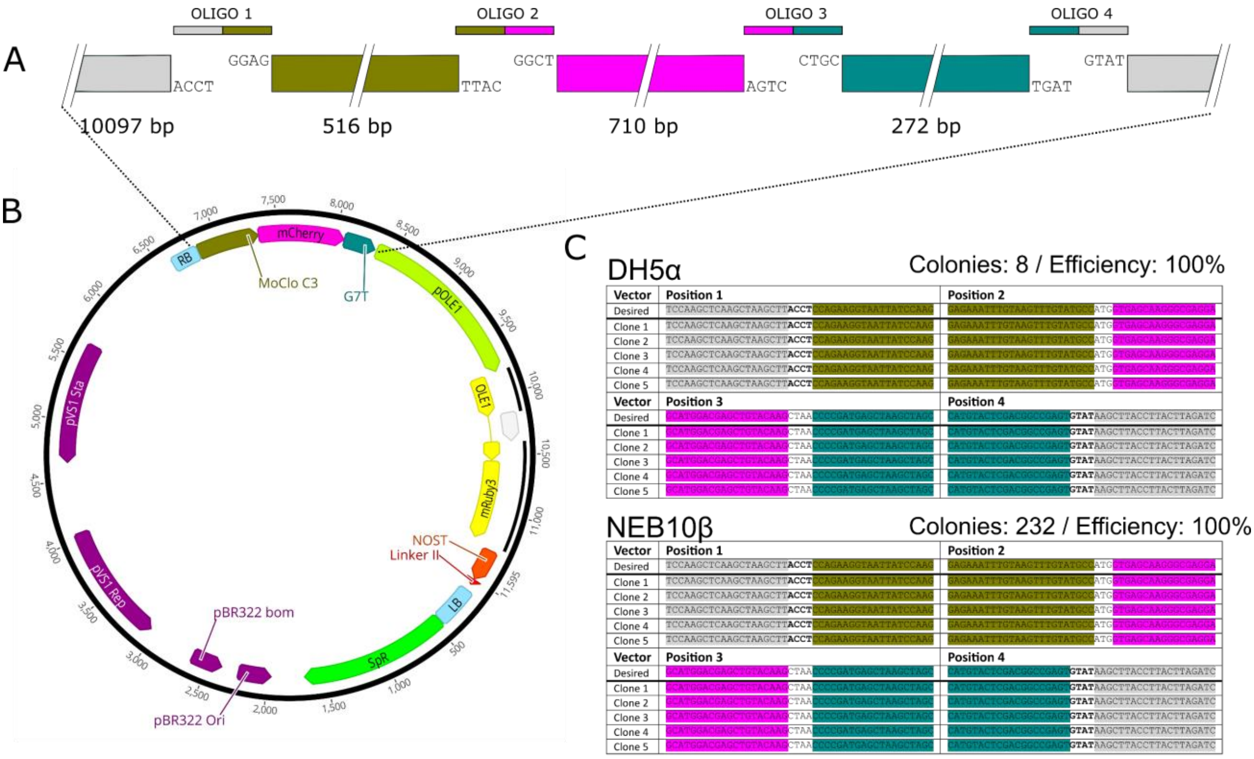


Supplementary Figure 1. Mechanism of Gibson assembly using oligos. (A) Oligo stitching mechanism to change a Type IIS restriction enzyme generated overhangs. Only the 5’ side of the reaction is shown for simplicity. The acceptor vector containing a CcdB-cassette and the donor vector (backbone is omitted) are digested by BsaI. The digested vectors are mixed with two oligos that have homology to both the backbone (20 bp in black) and the part (20 bp in teal). Between these regions is the 4-bp overhang (purple). In the Gibson assembly reaction, the 5’ ends will be chewed back by the 5’ DNA exonuclease. The oligos will then anneal to the complimentary sequences and used as a template by DNA polymerase. The exonuclease continues to chew back the 5’ ends, both of the newly synthesized sequence, as well as of the backbone. These two sequences can then anneal, polymerize and be ligated together to form a new double stranded flank containing the new overhang. (B) Mechanism for reactions where 3’ chew-back from the proofreading activity of the DNA polymerase is needed. In this example the reaction is shown that was used for the generation of the saturation mutagenesis screen of amilCP_Orange. The same reactions take place as in A, but because PflMI cuts upstream of where the modifications are needed, a 3’ chew back is needed.



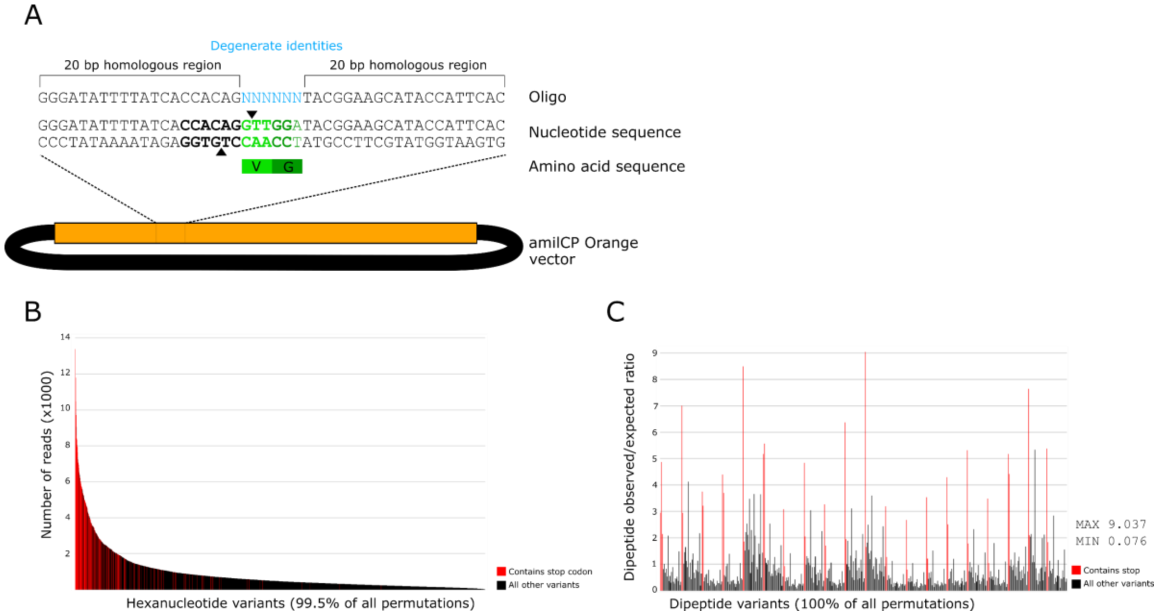
DH5α — Colonies: 8 / Efficiency: 100%

Vector	Position 1	Position 2
Desired	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 1	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 2	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 3	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 4	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 5	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Vector	**Position 3**	**Position 4**
Desired	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 1	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 2	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 3	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 4	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 5	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC

NEB10β — Colonies: 232 / Efficiency: 100%

Vector	Position 1	Position 2
Desired	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 1	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 2	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 3	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 4	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Clone 5	TCCAAGCTCAAGCTAAGCTT**ACCT**CCAGAAGGTAATTATCCAAG	GAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAGGA
Vector	**Position 3**	**Position 4**
Desired	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 1	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 2	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 3	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 4	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC
Clone 5	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	CATGTACTCGACGGCCGAGT**GTAT**AAGCTTACCTTACTTAGATC

Supplementary Figure 2. Incompatible library assembly. (A) The different incompatible parts assembled in this experiment. The circular backbone (10,097 bp) is depicted in gray, the Cassava Vein Mosaic Virus promoter (516 bp) in brown, the mCherry CDS (710 bp) in pink and the G7T terminator (272 bp) in teal. Note that none of the overhangs are compatible for Golden Gate assembly. (B) Fully assembled 11,595 bp construct. (C) Sequence comparison of desired sequence at the four junctions and the sequences of 5 randomly picked clones. Both DH5α chemically competent cells and NEB10B electrocompetent cells were used. The number of colonies and the cloning efficiency is also indicated.



Supplementary Figure 3. Supporting information for saturation mutagenesis experiment. (A) Schematic overview of the amilCP_Orange chromoprotein vector showing the nucleotides centered around the codons V63 and G64. The recognition site of the restriction enzyme PflMI is indicated in bold and its cutting position by triangles. The mutagenic oligo is also indicated and was composed out of 20 nt homology on either end of the six degenerate nucleotides. (B) Distribution of the hexanucleotide variants present in the reads and ranked according to abundance. Data shown in red indicates that the hexanucleotide encoded at least one stop codon in either of the two codons. The original variant is not included in the graph. (C) Ratio of observed and expected of each of the possible 441 dipeptides/amino acid-stop codon/stop codon-stop codon combinations. Data shown in red indicate that at least one stop codon was present. The maximum and minimum observed/expected ratio is also indicated. The original codon combination of the original variant is not included in the analysis.

SUPPLEMENTARY METHODS

The theory

Oligos can be used to modify Golden Gate parts in various ways with Gibson assembly in a process called "oligo stitching". Oligo stitching can be used to change the overhang sequence between standards (e.g. MoClo to GreenGate) and also within a standard. Oligos are designed to have 20 bp homology to the backbone and 20 bp to the part, with 4 bp in the middle specifying the new overhang and/or other elements (tags, start/stop codons, etc.). The orientation of the oligos does not appear to have an effect.

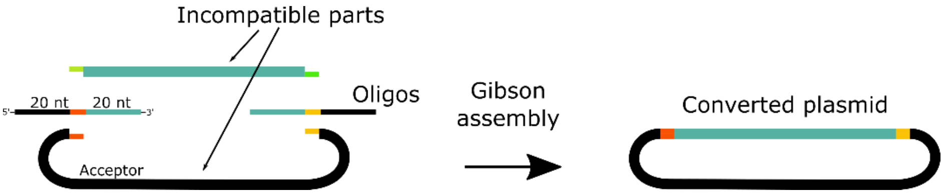
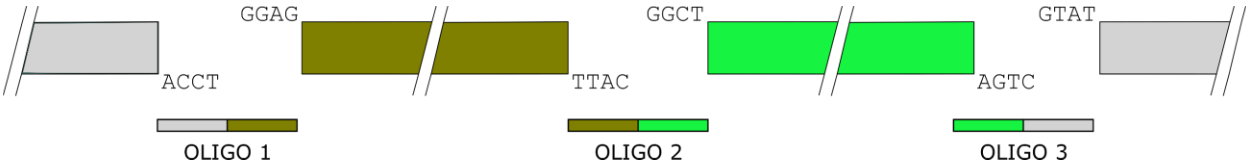
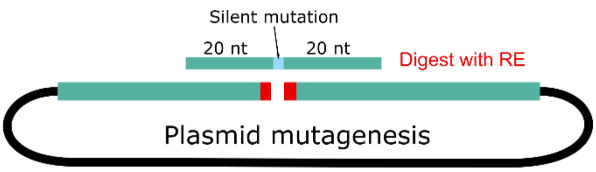


Figure 1. Oligo stitching to change overhangs on Golden Gate Plasmid.

Oligo stitching can also be used to assemble non-compatible parts allowing one to mix and match different Golden Gate standards.



Oligos can be directly assembled into a synthetic DNA sequence. For sequences of ≥300 bp, DNA synthesis is probably a more economic option, but for sequences composed of a combination of standard (fixed) parts and variable parts, this is a convenient solution. For example, prime editing gRNAs are composed of a variable 60 bp spacer sequence (20 bp backbone homology--20 bp spacer--20 bp scaffold homology), a fixed scaffold and variable RT+PBS (20 bp scaffold homology--X bp of RT+PBS--20 bp of backbone homology). As oligos longer than 60 bp are significantly more expensive and take more time to arrive, the homologies can be shortened (e.g., 17 bp on both sides instead of 20 bp). This will reduce efficiency but still yield sufficient colonies. Note that we have only performed these assemblies swapping sense and antisense orientations. The design is based on Kweon et al., 2021.



Plasmids can be mutagenized to remove an unwanted restriction site (domestication) or perform saturation mutagenesis. Note that success depends on the restriction enzyme used (BsaI led to more contaminating original plasmid than with XbaI and EcoRI). Alternatively, by using oligos containing ambiguous bases a library of plasmid variants can be produced. For mutating one codon for example, take 20 bp upstream of the codon, add 3 ambiguous bases, and then add 20 bp downstream of the codon for the oligo design. Multiple mutagenic oligos can also be used in one reaction.

Step-by-step protocol

Oligos: Resuspend the oligos to 100 µM and take 1 µL (100 pmol) of each and add to 331 µL of water. Per oligo concentration 0.3 pmol/ul = 300 fmol.

For ≥2 oligos, add 1 µL per *n* oligo part to 333-*n* µL of water.

For DNA assembly: Dilute oligos to 100 nM. Add 1 µL (100 fmol) of each oligo to Gibson assembly reaction below. Note: For 3-4 oligos, 10^6 competent cells can be used, for >4 oligos higher efficiencies are recommended.

Plasmids and dsDNA parts may be prepared by digest or PCR.

Component	Amount
Part Plasmid	1 µg
10X Buffer*	2 µL
Enzyme	0.5 µL
H_2O	variable
Total	20 µL

Component	Amount
Acceptor Plasmid	1 µg
10X Buffer*	2 µL
Enzyme	0.5 µL
H_2O	variable
Total	20 µL

*Don't forget to add Bovine Serum Albumin (BSA) if using Promega enzymes

Digest for ~4 hours.

Heat inactivate according to the manufacturer's instructions, typically 60 °C or 80°C for 20 minutes.

Prepare PCR parts as any other standard cloning reaction.

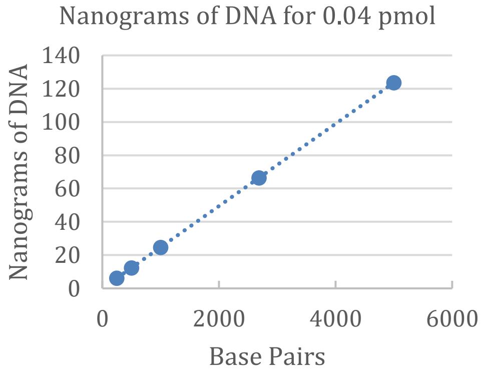
PCR parts may be used unpurified or may require gel purification if multiple bands are present. Gel extract plasmid parts with the same resistance marker to avoid picking up the original vector. Alternatively, we have GreenGate entry vectors containing Gm and TetR resistance, as well as SpecR for non-GreenGate.

Gibson Assembly

Determine the ng of plasmid needed for 0.04 pmol of parts.

$$ng\ of\ plasmid\ needed = bp\ of\ plasmid\ \times 0.0264$$

Use the graph at the left to quickly estimate →



Prepare the NEBuilder reaction as follows

Component	Amount	Volume (µL)
Oligo mix	0.30 pmol	1
Part 1	0.04 pmol	variable
Part 2…	0.04 pmol	variable
Acceptor	0.04 pmol	variable
2XNEBuilder		5
H_2O		variable
Total		10 µL

Incubate at 50°C for one hour and transform ~2 µl into ~25 µl *E. coli*. Reactions can be scaled up or down as necessary.

Notes: NEBuilder is essential for reactions that chew back 3' ends. 1 µL of each digested plasmid (~50 ng) will also yield satisfactory results. We obtained good results with up to three parts, but cells with higher transformation efficiency (>10^6) may be needed when more parts are used.

Quality Control: Sanger sequencing. Errors may occur at the junctions, so clones should be sequenced at all junctions.

SUPPLEMENTARY TABLES

Supplementary Table 1: Clone conversion

Partial sequences of the original sequence, the desired sequence and five sequenced clones are shown in each table. The BsaI recognition motif is underlined. The 4-bp overhangs that are generated by BsaI digestion are shown in bold, flanked by 20 bp of sequence at both sides. The overhang is shown in blue for the original MoClo sequence and in green for the desired GreenGate sequence. Additional bases that were added, such as start codons or bases needed to keep the sequences in frame after assembly are also highlighted in green. The sequence highlighted in gray is the backbone sequence. Mutations that deviate from the desired sequence are highlighted in red. The number of colonies and the cloning efficiency is also indicated. Experiment number is indicated in top left corner.

1) MoClo Plant Part A10 (CDS1 module) to GreenGate pGGD000

Colonies: 1072 / **Efficiency**: 100%

Vector	Position 1	Position 2
Original	TTAATCACTCTGTGGTCTCA**AATG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**GCTT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT

2) MoClo Plant Part A12 (3U + Ter module) to GreenGate pGGE000

Colonies: 177 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	TTAATCACTCTGTGGTCTCA**GCTT**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**CGCT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTC-**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT

3) MoClo Plant Part C3 (PRO + 5U module) to GreenGate pGGA000

Colonies: 2296 / **Efficiency**: 100%

Vector	Position 1	Position 2
Original	TTAATCACTCTGTGGTCTCA**GGAG**CCAGAAGGTAATTATCCAAG	AGAGAAATTTGTAAGTTTGT**AATG**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**ACCT**CCAGAAGGTAATTATCCAAG	AGAGAAATTTGTAAGTTTGT**AACA**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**ACCT**CCAGAAGGTAATTATCCAAG	AGAGAAATTTGTAAGTTTGT**AACA**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**ACCT**CCAGAAGGTAATTATCCAAG	AGAGAAATTTGTAAGTTTGT**AACA**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**ACCT**CCAGAAGGTAATTATCCAAG	AGAGAAATTTGTAAGTTTGT**AACA**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**ACCT**CCAGAAGGTAATTATCCAAG	AGAGAAATTTGTAAGTTTGT**AACA**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**ACCT**CCAGAAGGTAATTATCCAAG	AGAGAAATTTGTAAGTTTGT**AACA**TGAGACCGAATTCTCGCCCT

4) MoClo Plant Part C7 (NT1 module) to GreenGate pGGC000

Colonies: 1476 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	GAAGAGCCACTGTGGTCTCA**CCAT**GGTGAGCAAGGGCGAGGAGC	AAAAACGCGGCTATTAGATC**AATG**TGAGACCACGAGTGATTAAT
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGGTGAGCAAGGGCGAG	AAAACGCGGCTATTAGATCA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGGTGA-CAAGGGCGAG	AAAACGCGGCTATTAGATCA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGGTGAGCAAGGGCGAG	AAAACGCGGCTATTAGATCA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGGTGAGCAAGGGCGAG	AAAACGCGGCTATTAGATCA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGGTGAGCAAGGGCGAG	AAAACGCGGCTATTAGATCA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGGTGAGCAAGGGCGAG	AAAACGCGGCTATTAGATCA**TCAG**TGAGACCGAATTCTCGCCCT

5) MoClo Plant Part A10 (CDS1 module) to GreenGate pGGD000 (Sense/Sense)

Colonies: 180 / **Efficiency**: 100%

Vector	Position 1	Position 2
Original	TTAATCACTCTGTGGTCTCA**AATG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**GCTT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT

6) MoClo Plant Part A10 (CDS1 module) to GreenGate pGGD000 (Antisense/Antisense)

Colonies: 414 / **Efficiency**: 100%

Vector	Position 1	Position 2
Original	TTAATCACTCTGTGGTCTCA**AATG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**GCTT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT

Clone 3	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
7) MoClo Plant Part A10 (CDS1 module) to GreenGate pGGD000 (Sense/Antisense) **Colonies:** 136 / **Efficiency**: 100%		
Vector	**Position 1**	**Position 2**
Original	TTAATCACTCTGTGGTCTCA**AATG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**GCTT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
8) MoClo Plant Part A10 (CDS1 module) to GreenGate pGGD000 (Antisense/Sense) **Colonies:** 142 / **Efficiency**: 100%		
Vector	**Position 1**	**Position 2**
Original	TTAATCACTCTGTGGTCTCA**AATG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**GCTT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TGAGACCGAATTCTCGCCCT
9) MoClo Plant Part A12 (3U + Ter module) to GreenGate pGGE000 (Sense/Sense) **Colonies:** 368 / **Efficiency**: 100%		
Vector	**Position 1**	**Position 2**
Original	TTAATCACTCTGTGGTCTCA**GCTT**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**CGCT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
10) MoClo Plant Part A12 (3U + Ter module) to GreenGate pGGE000 (Antisense/Antisense) **Colonies:** 492 / **Efficiency**: 100%		
Vector	**Position 1**	**Position 2**
Original	TTAATCACTCTGTGGTCTCA**GCTT**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**CGCT**TGAGACCACGAAGTGGCTCT

Desired	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT

11) MoClo Plant Part A12 (3U + Ter module) to GreenGate pGGE000 (Sense/Antisense)

Colonies: 208 / **Efficiency**: 100%

Vector	**Position 1**	**Position 2**
Original	TTAATCACTCTGTGGTCTCA**GCTT**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**CGCT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT

12) MoClo Plant Part A12 (3U + Ter module) to GreenGate pGGE000 (Antisense/Sense)

Colonies: 471 / **Efficiency**: 100%

Vector	**Position 1**	**Position 2**
Original	TTAATCACTCTGTGGTCTCA**GCTT**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**CGCT**TGAGACCACGAAGTGGCTCT
Desired	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**CTGC**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**ACTA**TGAGACCGAATTCTCGCCCT

Supplementary Table 2: Clone conversion within standard

Partial sequences of the original sequence, the desired sequence and five sequenced clones are shown in each table. The BsaI recognition motif is underlined. The 4-bp overhangs that are generated by BsaI digestion are shown in green, flanked by 20 bp of sequence at both sides. The sequence highlighted in gray is the backbone sequence. Mutations that deviate from the desired sequence are highlighted in red. The number of colonies and the cloning efficiency is also indicated. Experiment number is indicated in top left corner.

13) GreenGate pGG-D-GFP*-E to GreenGate pGG-B-GFP-C

Colonies: 88 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	AGAAGTGAAGCTTGGTCTCA**TCAG**CTATGGTGAGCAAGGGCGAG	TGGACGAGCTGTACAAGTAA**CTGC**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**AACA**CTATGGTGAGCAAGGGCGAG	GCATGGACGAGCTGTACAAG**GGCT**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**AACA**CTATGGTGAGCAAGGGCGAG	GCATGGACGAGCTGTACAAG**GGCT**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**AACA**CTATGGTGAGCAAGGGCGAG	GCATGGACGAGCTGTACAAG**GGCT**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**AACA**CTATGGTGAGCAAGGGCGAG	GCATGGACGAGCTGTACAAG**GGCT**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**AACA**CTATGGTGAGCAAGGGCGAG	GCATGGACGAGCTGTACAAG**GGCT**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**AACA**CTATGGTGAGCAAGGGCGAG	TGGACGAGCTGTACAAGTAA**CTGC**TGAGACCGAATTCTCGCCCT

14) GreenGate pGG-E-MBP*-F to GreenGate pGG-B-MBP-C

Colonies: 327 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	AGAAGTGAAGCTTGGTCTCA**CTGC**AAAATCGAAGAAGGTAAACTG	TGAAAGACGCGCAGACTTAG**ACTA**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**AACA**GCATGAAAATCGAAGAAGGTAAACTG	CCCTGAAAGACGCGCAGACT**GGCT**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**AACA**GCATGAAAATCGAAGAAGGTAAACTG	CCCTGAAAGACGCGCAGACT**GGCT**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**AACA**GCATGAAAATCGAAGAAGGTAAACTG	CCCTGAAAGACGCGCAGACT**GGCT**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**AACA**GCATGAAAATCGAAGAAGGTAAACTG	CCCTGAAAGACGCGCAGACT**GGCT**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**AACA**GCATGAAAATCGAAGAAGGTAAACTG	CCCTGAAAGACGCGCAGACT**GGCT**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**AACA**GCATGAAAATCGAAGAAGGTAAACTG	CCCTGAAAGACGCGCAGACT**GGCT**TTAGACCGAATTCTCGCCCT

15) GreenGate pGG-B-GST-C to GreenGate pGG-A-GST-B (Gel extraction + pGGC000 backbone)

Colonies: 29 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	AGAAGTGAAGCTTGGTCTCA**AACA**CCATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGT**GGCT**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGT**TCAA**CATGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGT**TCAA**CATGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGT**TCAA**CATGAGACCGAATTCTCGCCCT

Clone 3	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCACGT**TCAA**CATGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGT**TCAA**CATGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGT**TCAA**CATGAGACCGAATTCTCGCCCT

16) GreenGate pGG-A-MtU6-B to GreenGate pGG-B-MtU6-C

Colonies: 27 / **Efficiency**: 20%

Vector	**Position 1**	**Position 2**
Original	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**AACA**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**GGCT**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**AACA**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**GGCT**TGAGACCGAATTCTCGCCCT
Clone 3	Mix of AACA and ACCT	Mix of AACA and ACCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**GGCT**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT

17) GreenGate pGG-A-MtU6-B to GreenGate pGG-B-MtU6-C (pGGC000 GmR)

Colonies: 39 / **Efficiency**: 40%

Vector	**Position 1**	**Position 2**
Original	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**AACA**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**GGCT**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**AACA**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**GGCT**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCT-A**AACA**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**GGCT**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**AACA**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**GGCT**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCT-A**AACA**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**GGCT**TGAGACCGAATTCTCGCCCT

18) GreenGate pGG-A-MtU6-B to GreenGate pGG-C-MtU6-D

Colonies: 28 / **Efficiency**: 40%

Vector	**Position 1**	**Position 2**
Original	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT

19) GreenGate pGG-A-MtU6-B to GreenGate pGG-C-MtU6-D (pGGC000 GmR)

Colonies: 72 / **Efficiency**: 40%

Vector	**Position 1**	**Position 2**
Original	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT

Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**TCAG**TG-GACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**GGCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGTTCTCA**GGCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**ACCT**ATGCCTATCTTATATGATCA	CTTGTACAAAGTTGGCATTA**AACA**TGAGACCGAATTCTCGCCCT

Supplementary Table 3: Replacement of BsaI recognition site with other TypeIIS site

Partial sequences of the original sequence, the desired sequence and five sequenced clones are shown in each table. The positions in the table refer to the junctions in final tagged clone, for the original untagged vector the end that is tagged is shown twice in the table. The BsaI/AarI/SapI recognition motifs are underlined. The overhangs that are generated the restriction enzymes are shown in bold. The sequence highlighted in gray is the backbone sequence. Mutations that deviate from the desired sequence are highlighted in red. The number of colonies and the cloning efficiency is also indicated. Experiment number is indicated in top left corner.

20) MoClo Plant Part A10 (CDS1 module) to GreenGate (AarI overhangs)
Colonies: 750 / **Efficiency**: 100%

Vector	Position 1	Position 2
Original	TGGCCGATTCATTAATCACTCTGTGGTCTCA**AATG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**GCTT**TGAGACCACGAAGTGGCTCTTCAGTGGACGA
Desired	ACACTATAGAAGTGAAGCTTCACCTGCAATA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TCGTGCAGGTGGAATTCTCGCCCTATAGTGA
Clone 1	ACACTATAGAAGTGAAGCTTCACCTGCAATA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TCGTGCAGGTGGAATTCTCGCCCTATAGTGA
Clone 2	ACACTATAGAAGTGAAGCTTCACCTGCAATA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TCGTGCAGGTGGAATTCTCGCCCTATAGTGA
Clone 3	ACACTATAGAAGTGAAGCTTCACCTGCAATA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TCGTGCAGGTGGAATTCTCGCCCTATAGTGA
Clone 4	ACACTATAGAAGTGAAGCTTCACCTGCAATA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TCGTGCAGGTGGAATTCTCGCCCTATAGTGA
Clone 5	ACACTATAGAAGTGAAGCTTCACCTGCAATA**TCAG**GGGTCATCCAAGAATGTTAT	GCCACCATCTGTTCCTTTAA**CTGC**TCGTGCAGGTGGAATTCTCGCCCTATAGTGA

21) MoClo Plant Part A12 (3U + Ter module) to GreenGate (SapI overhangs)
Colonies: 1520 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	CGATTCATTAATCACTCTGTGGTCTCA**GCTT**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**CGCT**TGAGACCACGAAGTGGCTCTTCAGTGG
Desired	ACACTATAGAAGTGAAGCTTGCTCTTCA**TGA**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**GAT**TGAAGAGCGAATTCTCGCCCTATAGTGA
Clone 1	ACACTATAGAAGTGAAGCTTGGTCTTCA**TGA**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**GAT**TGAAGAGCGAATTCTCGCCCTATAGTGA
Clone 2	ACACTATAGAAGTGAAGCTTGCTCTTCA**TGA**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**GAT**TGAAGAGCGAATTCTCGCCCTATAGTGA
Clone 3	ACACTATAGAAGTGAAGCTTGCTCTTCA**TGA**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**GAT**TGAAGAGCGAATTCTCGCCCTATAGTGA
Clone 4	ACACTATAGAAGTGAAGCTTGCTCTTCA**TGA**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**GAT**TGAAGAGCGAATTCTCGCCCTATAGTGA
Clone 5	ACACTATAGAAGTGAAGCTTGCTCTTCA**TGA**GCTCTCAAGATCAAAGGCTT	ATTTTAAGATCGCACCATTT**GAT**TGAAGAGCGAATTCTCGCCCTATAGTGA

Supplementary Table 4: Clone tagging with PCR products or donor plasmids

Partial sequences of the original sequence, the desired sequence and five sequenced clones are shown in each table. The positions in the table refer to the junctions in final tagged clone, for the original untagged vector the end that is tagged is shown twice in the table. The BsaI recognition motif is underlined. The 4-bp overhangs that are generated by BsaI digestion are shown in bold. The sequence that is tagged is highlighted in blue, the tag in green. The sequence highlighted in gray is the backbone sequence. The sequence highlighted in yellow is a linker that was included in the oligo. Mutations that deviate from the desired sequence are highlighted in red. The number of colonies and the cloning efficiency is also indicated. Experiment number is indicated in top left corner.

24) GUS C-terminal GFP-fusion (PCR product) to pGGC0000 (CbR)

Colonies: 184 / **Efficiency**: 80%

Vector	Position 1	Position 2	Position 3
Original	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	CGCAGCAGGGAGGCAAACAA**TCAG**TGAGACCGAATTCTCGCCCT	CGCAGCAGGGAGGCAAACAA**TCAG**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	GAAGTGAAGCTTGGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT

25) Integrase 4 C-terminal V5-tag-fusion (donor vector) to pGGC0000 (CbR)

Colonies: 94 / **Efficiency**: 60%

Vector	Position 1	Position 2	Position 3
Original	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT

26) Csy4 N-terminal mCherry-fusion (donor vector) to pGGC0000 (CbR)

Colonies: 29 / **Efficiency**: 0%

Vector	Position 1	Position 2	Position 3
Original	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGATCATTATCTTGATAT	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGTGAGCAAGGGCGAGGA	GACGAGCTGTACAAG**GGAGGTTCA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT
Clone 1	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT

Clone 4	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	AAGTGAAGCTTGGTCTCA**GGCTCC**ATGGTGAGCAAGGGCGAGGA	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT

27) GUS C-terminal GFP-fusion (PCR product) to pGGC0000 (GmR)

Colonies: 112/ **Efficiency**: 80%

Vector	Position 1	Position 2	Position 3
Original	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	CGCAGCAGGGAGGCAAACAA**TCAG**TGAGACCGAATTCTCGCCCT	CGCAGCAGGGAGGCAAACAA**TCAG**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**T-AG**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**GGCT**CAACAATGGTCCGTCCTGTA	GGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGG	GCATGGACGAGCTGTACAAG**TCAG**TGAGACCGAATTCTCGCCCT

28) Integrase 4 C-terminal V5-tag-fusion (vector) to pGGC0000 (GmR)

Colonies: 12 / **Efficiency**: 100%

Vector	Position 1	Position 2	Position 3
Original	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCCTCTC	TCGGACTCGACTCAACCTAA**TCAG**TGAGACCGAATTCTCGCCCT

29) Csy4 N-terminal mCherry-fusion (vector) to pGGC0000 (GmR)

Colonies: 29 / **Efficiency**: 100%

Vector	Position 1	Position 2	Position 3
Original	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGATCATTATCTTGATAT	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGTGAGCAAGGGCGAGGA	GACGAGCTGTACAAG**GGAGGTTCA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGTGAGCAAGGGCGAGGA	GACGAGCTGTACAAG**GGAGGTTCA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGTGAGCAAGGGCGAGGA	GACGAGCTGTACAAG**GGAGGTTCA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGTGAGCAAGGGCGAGGA	GACGAGCTGTACAAG**GGAGGTTCA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGTGAGCAAGGGCGAGGA	GACGAGCTGTACAAG**GGAGGTTCA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCA**AACA**ATGGTGAGCAAGGGCGAGGA	GACGAGCTGTACAAG**GGAGGTTCA**ATGGATCATTATCTTGATAT	TTGAAGAAAATCCTGGACCC**GGCT**TGAGACCGAATTCTCGCCCT

Supplementary Table 5: One PCR product to many entries

Partial sequences of the original sequence, the desired sequence and five sequenced clones are shown in each table. The BsaI recognition motif is underlined. The 4-bp overhangs that are generated by BsaI digestion are shown in green, flanked by 20 bp of sequence at both sides. The sequence highlighted in gray is the backbone sequence. Mutations that deviate from the desired sequence are highlighted in red. The number of colonies and the cloning efficiency is also indicated. Experiment number is indicated in top left corner.

30) pGG-A-GST-B (from pEN-R2-GST-L3)
Colonies: 336 / **Efficiency**: 100%

Vector	**Position 1**	**Position 2**
Original	TACAAAGTGGGCGGAGGTGGCAGCATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTAAGCAACTTTATTATACAAAGTTGG
Desired	AGAAGTGAAGCTTGGTCTCAACCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAACATGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCAACCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAACATGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCAACCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAACATGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCAACCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAACATGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCAACCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAACATGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCAACCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAACATGAGACCGAATTCTCGCCCT

31) pGG-B-GST-C (from pEN-R2-GST-L3)
Colonies: 410 / **Efficiency**: 100%

Vector	**Position 1**	**Position 2**
Original	TACAAAGTGGGCGGAGGTGGCAGCATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTAAGCAACTTTATTATACAAAGTT
Desired	AGAAGTGAAGCTTGGTCTCAAACAATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTGGCTTGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCAAACAATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTGGCTTGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCAAACAATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTGGCTTGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCAAACAATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTGGCTTGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCAAACAATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTGGCTTGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCAAACAATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTGGCTTGAGACCGAATTCTCGCCCT

32) pGG-C-GST-D (from pEN-R2-GST-L3)
Colonies: 840 / **Efficiency**: 80%

Vector	**Position 1**	**Position 2**
Original	TACAAAGTGGGCGGAGGTGGCAGCATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTAAGCAACTTTATTATACAAAGTT
Desired	AGAAGTGAAGCTTGGTCTCAGGCTCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAGTGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCAGGCTCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAGTGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCAGGCTCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAGTGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCAGGCTCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAGTGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCAGGCTCTATGTCCCCTATACTAGGTTA	AATCGGAT-TAGTTCCGCGTTCAGTGAGACCGAATTCTCGCCCT

Clone 5	AGAAGTGAAGCTTGGTCTCAGGCTCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTCAGTGAGACCGAATTCTCGCCCT

33) pGG-D-GST-E (from pEN-R2-GST-L3)

Colonies: 676 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	TACAAAGTGGGCGGAGGTGGCAGCATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTAAGCAACTTTATTATACAAAGTT
Desired	AGAAGTGAAGCTTGGTCTCATCAGCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTCTGCTGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCATCAGCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTCTGCTGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCATCAGCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTCTGCTGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCATCAGCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGT-CCGC-TCTGCTGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCATCAGCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTCTGCTGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCATCAGCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTCTGCTGAGACCGAATTCTCGCCCT

34) pGG-E-GST-F (from pEN-R2-GST-L3)

Colonies: 346 / **Efficiency**: 60%

Vector	Position 1	Position 2
Original	TACAAAGTGGGCGGAGGTGGCAGCATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTAAGCAACTTTATTATACAAAGTTGG
Desired	AGAAGTGAAGCTTGGTCTCACTGCCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTGAACTATGAGACCGAATTCTCGCCC
Clone 1	AGAAGTGAAGCTTGGTCTCACTGCCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTGAACTATGAGACCGAATTCTCGCCC
Clone 2	AGAAGTGAAGCTTGGTCTCACTGCCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTGAACTATGAGACCGAATTCTCGCCC
Clone 3	AGAAGTGAAGCTTGGTCTCACTGC-TATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTGAACTATGAGACCGAATTCTCGCCC
Clone 4	AGAAGTGAAGCTTGGTCTCACTGCCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTGAACTATGAGACCGAATTCTCGCCC
Clone 5	AGAAGTGAAGCTTGGTCTCACTGCCTATGTCCCCTATACTAGGTTA	AATCGGATCTAGTTCCGCGTTAAGCAACTTTATTATACAAAGTTGG**

35) pGG-A-3xHA-B (from pEN-R2-3xHA-L3)

Colonies: 1373 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	TACAAAGTGGGTGGAGGCGGTTCAGCATGATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGATCAACTTTATTATACAAAGTTGG
Desired	AGAAGTGAAGCTTGGTCTCAACCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAACATGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCAACCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTAC-CTTCAACATGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCAACCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAACATGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCAACCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAACATGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCAACCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAACATGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCAACCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAACATGAGACCGAATTCTCGCCCT

36) pGG-B-3xHA-C (from pEN-R2-3xHA-L3)

Colonies: 1584 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	TACAAAGTGGGTGGAGGCGGTTCAGCATGATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGATCAACTTTATTATACAAAGTT
Desired	AGAAGTGAAGCTTGGTCTCAAACAATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTGGCTTGAGACCGAATTCTCGCCCT

Clone 1	AGAAGTGAAGCTTGGTCTCAAACAATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTGGCTTGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCAAACA-TGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTGGCTTGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCAAACAATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTGGCTTGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCAAACAATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTGGCTTGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCAAACAATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTGGCTTGAGACCGAATTCTCGCCCT

37) pGG-C-3xHA-D (from pEN-R2-3xHA-L3)

Colonies: 1845 / **Efficiency**: 100%

Vector	Position 1	Position 2
Original	TACAAAGTGGGTGGAGGCGGTTCAGCATGATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGATCAACTTTATTATACAAAGTT
Desired	AGAAGTGAAGCTTGGTCTCAGGCTCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAGTGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCAGGCTCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAGTGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCAGGCTCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAGTGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCAGGCTCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAGTGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCAGGCTCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAGTGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCAGGCTCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTCAGTGAGACCGAATTCTCGCCCT

38) pGG-D-3xHA-E (from pEN-R2-3xHA-L3)

Colonies: 936 / **Efficiency**: 80%

Vector	Position 1	Position 2
Original	TACAAAGTGGGTGGAGGCGGTTCAGCATGATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGATCAACTTTATTATACAAAGTT
Desired	AGAAGTGAAGCTTGGTCTCATCAGCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTCTGCTGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCATCAGCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTCTGCTGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCATCAGCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTCTGCTGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCATCAGCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTCTGCTGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCATCAGCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTCTGCTGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCATCAGCTATGGCATACCCTTACGATGT †	ACGACGTTCCAGATTACGCTCTGCTGAGACCGAATTCTCGCCCT

39) pGG-E-3xHA-F (from pEN-R2-3xHA-L3)

Colonies: 1257 / **Efficiency**: 100%

Vector	Position 1	Position 2
Original	TACAAAGTGGGTGGAGGCGGTTCAGCATGATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGATCAACTTTATTATACAAAGTTGGC
Desired	AGAAGTGAAGCTTGGTCTCACTGCCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGAACTATGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCACTGCCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGAACTATGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCACTGCCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGAACTATGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCACTGCCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGAACTATGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCACTGCCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGAACTATGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCACTGCCTATGGCATACCCTTACGATGT	ACGACGTTCCAGATTACGCTTGAACTATGAGACCGAATTCTCGCCCT

*attL3

† part of 3xHA is missing

Supplementary Table 6: Clone tagging with oligo-encoded sequences

Partial sequences of the original sequence, the desired sequence and five sequenced clones are shown in each table. The positions in the table refer to the junctions in final tagged clone, for the original untagged vector the end that is tagged is shown twice in the table. The BsaI recognition motif is underlined. The 4-bp overhangs that are generated by BsaI digestion are shown in bold. The sequence that is tagged is highlighted in blue, the tag in green. The sequence highlighted in gray is the backbone sequence. Mutations that deviate from the desired sequence are highlighted in red. The number of colonies and the cloning efficiency is also indicated. Experiment number is indicated in top left corner.

40) ER signal peptide-Integrase 4 fusion (oligo-encoded) to pGGC0000 (GmR)

Colonies: 118 / **Efficiency**: 80%

Vector	**Position 1**
Original	AGAAGTGAAGCTTGGTCTCA**GGCT**CC ATGATTACGACCAGAAAG
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 2	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 5	AGAAGTGAAGCTTGGTCT---GCTCCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG

Vector	**Position 2**
Original	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Desired	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	TAGATATACATTGGACCTTT**TCAG**TGAGACCGAATTCTCGCCCT

41) Integrase 4-SV40 fusion (oligo-encoded) to pGGC0000 (TetR)

Colonies: 29 / **Efficiency**: 100%

Vector	**Position 1**
Original	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG
Clone 2	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG
Clone 5	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGATTACGACCAGAAAG

Vector	**Position 2**
Original	TAGATATACATTGGACCTTT **TCAG**TGAGACCGAATTCTCGCCCT
Desired	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT

42) ER signal peptide-Integrase 4-SV40 fusion (oligo-encoded) to pGGC0000 (SpecR)

Colonies: 45 / **Efficiency**: 80%

Vector	**Position 1**
Original	AGAAGTGAAGCTTGGTCTCA**GGCT**CC ATGATTACGACCAGAAAG
Desired	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 1	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 2	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 3	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATAC-CAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 4	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Clone 5	AGAAGTGAAGCTTGGTCTCA**GGCT**CCATGAAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGGATGATTACGACCAGAAAG
Vector	**Position 2**
Original	TAGATATACATTGGACCTTT **TCAG**TGAGACCGAATTCTCGCCCT
Desired	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 1	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 2	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 3	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 4	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT
Clone 5	TAGATATACATTGGACCTTTCCTAAGAAGAAGAGGAAGGTT**TCAG**TGAGACCGAATTCTCGCCCT

Supplementary Table 7: Restriction enzyme recognition site removal

Partial sequences of the original sequence, the desired sequence and five sequenced clones are shown in each table. The restriction enzyme recognition sites are shown in bold, flanked by backbone at both sides. The base that is targeted for replacement is highlighted in blue in the original sequence and in green in the desired final sequence. Mutations that deviate from the desired sequence are highlighted in red. The number of colonies and the cloning efficiency is also indicated. Experiment number is indicated in top left corner.

47) Removal of XbaI in A-OLE-P-B

Colonies: 673 / **Efficiency**: 100%

Vector	Position 1 (2525-2568)	Position 2 (3162-3205)	
Original	CGATCTGATACTGATAACG**TCTAGA**TTTTTAGGGTTAAAGCAAT	TATCCATTTTCTTCATTGT**TCTAGA**ATGTCGCGGAACAAATTTT	
Desired	CGATCTGATACTGATAACG**TCTCGA**TTTTTAGGGTTAAAGCAAT	TATCCATTTTCTTCATTGT**TATAGA**ATGTCGCGGAACAAATTTT	
Clone 1	CGATCTGATACTGATAACG**TCTCGA**TTTTTAGGGTTAAAGCAAT	TATCCATTTTCTTCATTGT**TATAGA**ATGTCGCGGAACAAATTTT	
Clone 2	CGATCTGATACTGATAACG**TCTCGA**TTTTTAGGGTTAAAGCAAT	TATCCATTTTCTTCATTGT**TATAGA**ATGTCGCGGAACAAATTTT	

48) Removal of EcorI in B-Csy4-C

Colonies: 97 / **Efficiency**: 60%

Vector	Position 1 (199-242)	Position 2 (412-455)	Position 3 (662-705)
Original	AGACTTGGAGAAAGACTTA**GAATTC**ATGCTTCTGCTGATGATCT	GAAGAAGAAGCTAGAAAAA**GAATTC**CTGATACTGTTGCTAGAGC	CTGGACCCGGCTTGAGACC**GAATTC**TCGCCCTATAGTGAGTCGT
Desired	AGACTTGGAGAAAGACTTA**GGATTC**ATGCTTCTGCTGATGATCT	GAAGAAGAAGCTAGAAAAA**GAGTTC**CTGATACTGTTGCTAGAGC	CTGGACCCGGCTTGAGACC**GAACTC**TCGCCCTATAGTGAGTCGT
Clone 1	AGACTTGGAGAAAGACTTA**G-ATTC**ATGCTTCTGCTGATGATCT	GAAGAAGAAGCTAGAAAAA**GAGTTC**CTGATACTGTTGCTAGAGC	CTGGACCCGGCTTGAGACC**GAACTC**TCGCCCTATAGTGAGTCGT
Clone 2	AGACTTGGAGAAAGACTTA**GGATTC**ATGCTTCTGCTGATGATCT	GAAGAAGAAGCTAGAAAAA**GAGTTC**CTGATACTGTTGCTAGAGC	CTGGACCCGGCTTGAGACC**GAACTC**TCGCCCTATAGTGAGTCGT
Clone 3	AGACTTGGAGAAAGACTTA**GGATTC**ATGCTTCTGCTGATGATCT	GAAGAAGAAGCTAGAAAAA**GAGTTC**CTGATACTGTTGCTAGAGC	CTGGACCCGGCTTGAGACC**GAACTC**TCGCCCTATAGTGAGTCGT
Clone 4	AGACTTGGAGAAAGACTTA**GGATTC**ATGCTTCTGCTGATGATCT	GAAGAAGAAGCTAGAAAAA**GAGTTC**CTGATACTGTTGCTAGAGC	CTGGACCCGGCTTGAGACC**GAACTC**TCGCCCTATAGTGAGTCGT
Clone 5	AGACTTGGAGAAAGACTTA**GGATTC**ATGCTTCTGCTGATGATCT	AAGAAGAAGCTAGGAAAAA**GAGTTC**CTGATACTGTTGCTAGAGC	CTGGACCCGGCTTGAGACC**GAACTC**TCGCCCTATAGTGAGTCGT

49) Remove extra BsaI site from pGG-E-tUM140_0016-F* (Oligo 2a)**

Colonies: 189 / **Efficiency**: 0%

Vector	Position 1	Position 2	Position 3
Original	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGTGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT

50) Remove extra BsaI site from pGG-E-tUM140_0016-F (Oligo 2b + GmR)

Colonies: 11 / **Efficiency**: 40%

Vector	**Left Flank**	**Extra BsaI site**	**Right Flank**
Original	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGGCCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 1	Backbone	Backbone	Backbone
Clone 2	Backbone	Backbone	Backbone
Clone 3	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGGCCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGGCCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT

51) Remove extra BsaI site from pGG-E-tUM140_0016-F (Oligo 2d + GmR)

Colonies: 12 / **Efficiency**: 60%

Vector	**Left Flank**	**Extra BsaI site**	**Right Flank**
Original	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGAACAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGAACAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGAACAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGAACAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	AACTCTAGTGTAATCCCAGACTGGTGAGACCAGTGACATTGACACCA	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT
Clone 5	Backbone	Backbone	Backbone

52) mCherry: remove BbsI + transfer to Moclo (pGGC0000 SpecR)

Colonies: 496 / **Efficiency**: 40%

Vector	**Left Flank**	**BbsI site**	**Right Flank**
Original	AGAAGTGAAGCTTGGTCTCATCAGGTATGGTGAGCAAGGGCGAGGA	GGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGC	GCATGGACGAGCTGTACAAGCTGCTGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCATTCGCTATGGTGAGCAAGGGCGAGGA	GGCCCCGTAATGCAGAAGAAAACCATGGGCTGGGAGGCCTCCTCCGAGC	GCATGGACGAGCTGTACAAGGCTTTGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCATTCGCTATGGTGAGCAAGGGCGAGGA	GGCCCCGTAATGCAGAAGAAAACCATGGGCTGGGAGGCCTCCTCCGAGC	GCATGGACGAGCTGTACAAGGCTTTGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCATTCGCTATGGTGAGCAAGGGCGAGGA	GGCCCCGTAATGCAGAAGAAAACCATGGGCTGGGAGGCCTCCTCCGAGC	GCATGGACGAGCTGTACAAGGCTTTGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCATTCGCTATGGTGAGCAAGGGCGAGGA	GGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGC	Backbone
Clone 4	AGAAGTGAAGCTTGGTCTCATTCGCTATGGTGAGCAAGGGCGAGGA	GGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGC	GCATGGACGAGCTGTACAAGGCTTTGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCATTCGCTATGGTGAGCAAGGGCGAGGA	GGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGC	Backbone

53) WUS: remove BbsI + transfer to Moclo pICH41233 (blue-white screen)

Colonies: 24 white, 19 blue/ **Efficiency**: 20%

Vector	**Left Flank**	**BbsI site**	**Right Flank**
Original	AGAAGTGAAGCTTGGTCTCAACCTCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACAACATGAGACCGAATTCTCGCCCT
Desired	TTAATCACTCTGTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGACTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCACGAAGTGGCTCT
Clone 1	TTAATCACTCTGTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCACGAAGTGGCTCT
Clone 2	TTAATCACTCTGTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGACTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCACGAAGTGGCTCT
Clone 3	TTAATCACTCTGTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCACGAAGTGGCTCT

Clone 4	TTAATCACTCTGTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCACGAAGTGGCTCT
Clone 5	TTAATCACTCTGTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCACGAAGTGGCTCT

54) WUS: remove BbsI + transfer to Moclo (with SpecR alternative)

Colonies: 548 / **Efficiency**: 0%

Vector	**Left Flank**	**BbsI site**	**Right Flank**
Original	AGAAGTGAAGCTTGGTCTCAACCTCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACAACATGAGACCGAATTCTCGCCCT
Desired	AGAAGTGAAGCTTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGACTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCGAATTCTCGCCCT
Clone 1	AGAAGTGAAGCTTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCGAATTCTCGCCCT
Clone 2	AGAAGTGAAGCTTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCGAATTCTCGCCCT
Clone 3	AGAAGTGAAGCTTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCGAATTCTCGCCCT
Clone 4	AGAAGTGAAGCTTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCGAATTCTCGCCCT
Clone 5	AGAAGTGAAGCTTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	TACTCAACATGTTCATAAGTACACCTGTCTTCACACTCGTTTCACACA	CAAAAGTCGAATCAAACACACCCATTGAGACCGAATTCTCGCCCT

55) pGG-D-GUS-E: remove both AarI sites

Colonies: 38 / **Efficiency**: 10%

Vector	**Position 1**	**Position 2**	
Original	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACGCTCACA	
Desired	AAGACTGTAACCACGCGTCTGTTGACTGGCAAGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 1	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 2	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 3	AAGACTGTAACCACGCGTCTGTTGACTGGCAAGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 4	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 5	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 6	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 7	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 8	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 9	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	
Clone 10	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGCTCACA	

56) pGG-D-GUS-E: remove both AarI sites (Oligo Aar1b)

Colonies: 20 / **Efficiency**: 20%

Vector	**Position 1**	**Position 2**	
Original	TGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTCAG	CCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACGC	
Desired	TGTAACCACGCGTCTGTTGACTGGCAGGTCGTGGCCAATGGTGATGTCAG	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGC	
Clone 1	TGTAACCACGCGTCTGTTGACTGGCAGGTCGTGGCCAATGGTGATGTCAG	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGC	

Clone 2	TGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTCAG	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGC	
Clone 3	TGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTCAG	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGC	
Clone 4	TGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTCAG	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGC	
Clone 5	TGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTCAG	CCGACGCGTCCGATCACCTGTGTCAATGTAATGTTCTGCGACGC	

Supplementary Table 8: Genotype and color phenotype of selected colonies

Table showing the partial vector sequence (i.e., the six bases that were mutagenized flanked by 20 bp on each end) of the original vector, compared with the 35 picked clones of Figure 2.

Vector	Sequence	AA identity (64-65)	Color
Original	GGGATATTTTATCACCACAG**GTTGGA**TACGGAAGCATACCATTCAC	VG	🟧
Clone 1	GGGATATTTTATCACCACAG**TTTTTA**TACGGAAGCATACCATTCAC	FL	🟪
Clone 2	GGGATATTTTATCACCACAG**CTATGT**TACGGAAGCATACCATTCAC	LC	🟪
Clone 3	GGGATATTTTATCACCACAG**ATGTCT**TACGGAAGCATACCATTCAC	MS	🟪
Clone 4	GGGATATTTTATCACCACAG**TTATGC**TACGGAAGCATACCATTCAC	LC	🟪
Clone 5	GGGATATTTTATCACCACAG**TATACG**TACGGAAGCATACCATTCAC	YT	🟪
Clone 6	GGGATATTTTATCACCACAG**ATTGCC**TACGGAAGCATACCATTCAC	IA	🟪
Clone 7	GGGATATTTTATCACCACAG**TTTGTA**TACGGAAGCATACCATTCAC	FV	🟪
Clone 8	GGGATATTTTATCACCACAG**AATTCT**TACGGAAGCATACCATTCAC	NS	🟪
Clone 9	GGGATATTTTATCACCACAG**TGGTGT**TACGGAAGCATACCATTCAC	WC	🟪
Clone 10	GGGATATTTTATCACCACAG**TGGACC**TACGGAAGCATACCATTCAC	WT	🟪
Clone 11	GGGATATTTTATCACCACAG**TTAATG**TACGGAAGCATACCATTCAC	LM	🟪
Clone 12	GGGATATTTTATCACCACAG**TTAATG**TACGGAAGCATACCATTCAC	LM	🟪
Clone 13	GGGATATTTTATCACCACAG**CTGATG**TACGGAAGCATACCATTCAC	LM	🟪
Clone 14	GGGATATTTTATCACCACAG**TTCATG**TACGGAAGCATACCATTCAC	FM	🟪
Clone 15	GGGATATTTTATCACCACAG**TGTCAA**TACGGAAGCATACCATTCAC	CQ	🟪
Clone 16	GGGATATTTTATCACCACAG**GTAAAT**TACGGAAGCATACCATTCAC	VN	🟦
Clone 17	GGGATATTTTATCACCACAG**GTGAAC**TACGGAAGCATACCATTCAC	VN	🟦
Clone 18	GGGATATTTTATCACCACAG**GTGAAT**TACGGAAGCATACCATTCAC	VN	🟦
Clone 19	GGGATATTTTATCACCACAG**GTCAAT**TACGGAAGCATACCATTCAC	VN	🟦
Clone 20	GGGATATTTTATCACCACAG**CAAGTA**TACGGAAGCATACCATTCAC	QV	🟦
Clone 21	GGGATATTTTATCACCACAG**ATCTTT**TACGGAAGCATACCATTCAC	IF	⬛
Clone 22	GGGATATTTTATCACCACAG**ATCTTT**TACGGAAGCATACCATTCAC	IF	⬛
Clone 23	GGGATATTTTATCACCACAG**ATTTTC**TACGGAAGCATACCATTCAC	IF	⬛
Clone 24	GGGATATTTTATCACCACAG**TGCGAG**TACGGAAGCATACCATTCAC	CE	⬛
Clone 25	GGGATATTTTATCACCACAG**GTGGAA**TACGGAAGCATACCATTCAC	VE	⬛
Clone 26	GGGATATTTTATCACCACAG**TACAGC**TACGGAAGCATACCATTCAC	YS	🟥
Clone 27	GGGATATTTTATCACCACAG**TATAGC**TACGGAAGCATACCATTCAC	YS	🟥
Clone 28	GGGATATTTTATCACCACAG**TATTCA**TACGGAAGCATACCATTCAC	YS	🟥
Clone 29	GGGATATTTTATCACCACAG**TATTCA**TACGGAAGCATACCATTCAC	YS	🟥
Clone 30	GGGATATTTTATCACCACAG**TATTCA**TACGGAAGCATACCATTCAC	YS	🟥
Clone 31	GGGATATTTTATCACCACAG**GTTGGA**TACGGAAGCATACCATTCAC	**VG**	🟧

Clone 32	GGGATATTTTATCACCACAG**GTTGGA**TACGGAAGCATACCATTCAC	**VG**	🟫
Clone 33	GGGATATTTTATCACCACAG**GTTGGA**TACGGAAGCATACCATTCAC	**VG**	🟫
Clone 34	GGGATATTTTATCACCACAG**GCTGGA**TACGGAAGCATACCATTCAC	AG	🟫
Clone 35	GGGATATTTTATCACCACAG**TGTGGA**TACGGAAGCATACCATTCAC	CG	🟫
Clone 36	GGGATATTTTATCACCACAG**CTTCGG**TACGGAAGCATACCATTCAC	LR	🟨
Clone 37	GGGATATTTTATCACCACAG**CTCCGA**TACGGAAGCATACCATTCAC	LR	🟨
Clone 38	GGGATATTTTATCACCACAG**CAGCGT**TACGGAAGCATACCATTCAC	QR	🟨
Clone 39	GGGATATTTTATCACCACAG**GTCCGA**TACGGAAGCATACCATTCAC	VR	🟨
Clone 40	GGGATATTTTATCACCACAG**AATAGG**TACGGAAGCATACCATTCAC	NR	🟨
Clone 41	GGGATATTTTATCACCACAG**GGGAGA**TACGGAAGCATACCATTCAC	GR	🟨
Clone 42	GGGATATTTTATCACCACAG**AGTCCT**TACGGAAGCATACCATTCAC	SP	🟨
Clone 43	GGGATATTTTATCACCACAG**AGTGAT**TACGGAAGCATACCATTCAC	SD	🟨
Clone 44	GGGATATTTTATCACCACAG**AAAGGA**TACGGAAGCATACCATTCAC	KG	🟨
Clone 45	GGGATATTTTATCACCACAG**AAGTTA**TACGGAAGCATACCATTCAC	KL	🟨
Clone 46	GGGATATTTTATCACCACAG**TAGTGG**TACGGAAGCATACCATTCAC	-W	🟨
Clone 47	GGGATATTTTATCACCACAG**TAGCGG**TACGGAAGCATACCATTCAC	-R	🟨
Clone 48	GGGATATTTTATCACCACAG**TAGGAT**TACGGAAGCATACCATTCAC	-D	🟨
Clone 49	GGGATATTTTATCACCACAG**ACTTAA**TACGGAAGCATACCATTCAC	T-	🟨
Clone 50	GGGATATTTTATCACCACAG**TCGTAA**TACGGAAGCATACCATTCAC	S-	🟨
Clone 51	GGGATATTTTATCACCACAG**CAGTAA**TACGGAAGCATACCATTCAC	Q-	🟨
Clone 52	GGGATATTTTATCACCACAG**CGTTGA**TACGGAAGCATACCATTCAC	R-	🟨
Clone 53	GGGATATTTTATCACCACAG**TGCTAA**TACGGAAGCATACCATTCAC	C-	🟨
Clone 54	GGGATATTTTATCACCACAG**AAATGA**TACGGAAGCATACCATTCAC	K-	🟨
Clone 55	GGGATATTTTATCACCACAG**TGATAG**TACGGAAGCATACCATTCAC	--	🟨

Supplementary Table 9: List of oligos used in this study

Oligo name	Sequence	Purpose
PexR	CAGGCTTTACACTTTATGCTTCCGGC	Sequencing primers
L4440	AGCGAGTCAGTGAGCGAG	
M13F	AGGGTTTTCCCAGTCACGACGTT	
P21	AGGGTTATTGTCTCATGAGCGG	
P39	TACCGCCTTTGAGTGAGCTG	
P147	TTGGGTAACGCCAGGGTTTT	
P156	CGCGTTGGCCGATTCATTAA	
pWUS	GACTATACAAAAGTTGGGTAT	
A10_OL1	AGAAGTGAAGCTTGGTCTCATCAGGGGTCATCCAAGAATGTTAT	Clone conversion: MoClo to GreenGate (new overhang is underlined; extra sequences that were added are shown in bold)
A10_OL2	GCCACCATCTGTTCCTTTAACTGCTGAGACCGAATTCTCGCCCT	
A12_OL1	AGAAGTGAAGCTTGGTCTCACTGCGCTCTCAAGATCAAAGGCTT	
A12_OL2	ATTTTAAGATCGCACCATTTACTATGAGACCGAATTCTCGCCCT	
C3_OL1	AGAAGTGAAGCTTGGTCTCAACCTCCAGAAGGTAATTATCCAAG	
C3_OL2	AGAGAAATTTGTAAGTTTGTAACATGAGACCGAATTCTCGCCCT	
C7_OL1	AGAAGTGAAGCTTGGTCTCAGGCT**CCATG**GTGAGCAAGGGCGAGGAGCT	
C7_OL2	AAAAACGCGGCTATTAGATC**A**TCAGTGAGACCGAATTCTCGCCCT	
A10_OL1_AS	ATAACATTCTTGGATGACCCCTGATGAGACCAAGCTTCACTTCT	Clone conversion: S/AS experiment (new overhang is underlined)
A10_OL2_AS	AGGGCGAGAATTCGGTCTCAGCAGTTAAAGGAACAGATGGTGGC	
A12_OL1_AS	AAGCCTTTGATCTTGAGAGCGCAGTGAGACCAAGCTTCACTTCT	
A12_OL2_AS	AGGGCGAGAATTCGGTCTCATAGTAAATGGTGCGATCTTAAAAT	
GST_FW	ATGTCCCCTATACTAGGTTA	One PCR to many entries (new overhang is underlined; extra sequences that were added are shown in bold)
GST_REV	ACGCGGAACTAGATCCGATT	
A-GST-B_L	AGAAGTGAAGCTTGGTCTCAACCTATGTCCCCTATACTAGGTTA	
A-GST-B_R	AATCGGATCTAGTTCCGCGT**TC**AACATGAGACCGAATTCTCGCCCT	
B-GST-C_L	AGAAGTGAAGCTTGGTCTCAAACAATGTCCCCTATACTAGGTTA	
B-GST-C_R	AATCGGATCTAGTTCCGCGTGGCTTGAGACCGAATTCTCGCCCT	
C-GST-D_L	AGAAGTGAAGCTTGGTCTCAGGCT**CT**ATGTCCCCTATACTAGGTTA	
C-GST-D_R	AATCGGATCTAGTTCCGCGTTCAGTGAGACCGAATTCTCGCCCT	
D-GST-E_L	AGAAGTGAAGCTTGGTCTCATCAG**CT**ATGTCCCCTATACTAGGTTA	

D-GST-E_R	AATCGGATCTAGTTCCGCGTCTGCTGAGACCGAATTCTCGCCCT	
E-GST-F_L	AGAAGTGAAGCTTGGTCTCACTGC**CT**ATGTCCCCTATACTAGGTTA	
E-GST-F_R	AATCGGATCTAGTTCCGCGT**TGA**ACTATGAGACCGAATTCTCGCCCT	
3xHA_FW	ATGGCATACCCTTACGATGT	
3xHA_REV	AGCGTAATCTGGAACGTCG	
A-3xHA-B_L	AGAAGTGAAGCTTGGTCTCAACCTATGGCATACCCTTACGATGT	
A-3xHA-B_R	ACGACGTTCCAGATTACGCT**TC**AACATGAGACCGAATTCTCGCCCT	
B-3xHA-C_L	AGAAGTGAAGCTTGGTCTCAAACAATGGCATACCCTTACGATGT	
B-3xHA-C_R	ACGACGTTCCAGATTACGCTGGCTTGAGACCGAATTCTCGCCCT	
C-3xHA-D_L	AGAAGTGAAGCTTGGTCTCAGGCT**CT**ATGGCATACCCTTACGATGT	
C-3xHA-D_R	ACGACGTTCCAGATTACGCTTCAGTGAGACCGAATTCTCGCCCT	
D-3xHA-E_L	AGAAGTGAAGCTTGGTCTCATCAG**CT**ATGGCATACCCTTACGATGT	
D-3xHA-E_R	ACGACGTTCCAGATTACGCTCTGCTGAGACCGAATTCTCGCCCT	
E-3xHA-F_L	AGAAGTGAAGCTTGGTCTCACTGC**CT**ATGGCATACCCTTACGATGT	
B_GFP_C_L	AGAAGTGAAGCTTGGTCTCAAACACTATGGTGAGCAAGGGCGAG	Clone conversion: GreenGate to GreenGate (new overhang is underlined; extra sequences that were added are shown in bold)
B_GFP_C_R	GCATGGACGAGCTGTACAAGGGCTTGAGACCGAATTCTCGCCCT	
B_MBP_C_L	AGAAGTGAAGCTTGGTCTCAAACAGCATGAAAATCGAAGAAGGTAAACTG	
B_MBP_C_R	CCCTGAAAGACGCGCAGACTGGCTTGAGACCGAATTCTCGCCCT	
B_MtU6_C_L	AGAAGTGAAGCTTGGTCTCAAACAATGCCTATCTTATATGATCA	
B_MtU6_C_R	CTTGTACAAAGTTGGCATTAGGCTTGAGACCGAATTCTCGCCCT	
C_MtU6_D_L	AGAAGTGAAGCTTGGTCTCAGGCTATGCCTATCTTATATGATCA	
C_MtU6_D_R	CTTGTACAAAGTTGGCATTATCAGTGAGACCGAATTCTCGCCCT	
A-GST-B_L	AGAAGTGAAGCTTGGTCTCAACCTATGTCCCCTATACTAGGTTA	
A-GST-B_R	AATCGGATCTAGTTCCGCGT**TC**AACATGAGACCGAATTCTCGCCCT	
A-MBP-B_L	AGAAGTGAAGCTTGGTCTCAACCTATGAAAATCGAAGAAGGTAAACT	
A-MBP-B_R	CCCTGAAAGACGCGCAGACT**TC**AACATGAGACCGAATTCTCGCCCT	
A10_AarI_OL1	ACACTATAGAAGTGAAGCTT**CACCTGC**AATATCAGGGGTCATCCAAGAATGTTAT	Replacement of Type IIS recognition site (new Type IIS recognition site in bold; new overhang is underlined)
A10_AarI_OL2	GCCACCATCTGTTCCTTTAACTGCTCGT**GCAGGTG**GAATTCTCGCCCTATAGTGA	
A12_SapI_OL1	ACACTATAGAAGTGAAGCTT**GCTCTTC**ATGAGCTCTCAAGATCAAAGGCTT	
A12_SapI_OL2	ATTTTAAGATCGCACCATTTGATT**GAAGAGC**GAATTCTCGCCCTATAGTGA	
GFP_StitAmp_F	ATGGTGAGCAAGGGCGAGG	GFP amplification
GFP_StitAmp_R	CTTGTACAGCTCGTCCATGCC	

GUS_GFP_OL1	AGAAGTGAAGCTTGGTCTCAGGCTCAACAATGGTCCGTCCTGTA	Clone tagging with PCR products or donor plasmids (overhang sequence is underlined; extra sequence that was added is in bold)
GUS_GFP_OL2	CGCAGCAGGGAGGCAAACAA**GGTGGATCAGGCGGAAGT**ATGGTGAGCAAGGGCGAGGA	
GUS_GFP_OL3	GCATGGACGAGCTGTACAAGTCAGTGAGACCGAATTCTCGCCCT	
INT4_V5_OL1	AGAAGTGAAGCTTGGTCTCAGGCTCCATGATTACGACCAGAAAG	
INT4_V5_OL2	TAGATATACATTGGACCTTTGGTAAGCCAATCCCTAATCC	
INT4_V5_OL3	TCGGACTCGACTCAACCTAATCAGTGAGACCGAATTCTCGCCCT	
Csy4_mCherry_OL1	AGAAGTGAAGCTTGGTCTCAAACAATGGTGAGCAAGGGCGAGGA	
Csy4_mCherry_OL2	GCATGGACGAGCTGTACAAG**GGAGGTTCA**ATGGATCATTATCTTGATAT	
Csy4_mCherry_OL3	TTGAAGAAAATCCTGGACCCGGCTTGAGACCGAATTCTCGCCCT	
INT4_left	AGAAGTGAAGCTTGGTCTCAGGCTCCATGATTACGACCAGAAAG	Clone tagging with oligo-encoded sequences (overhang sequence is underlined; extra sequence that was added is in bold)
INT4_ER_left	AGAAGTGAAGCTTGGTCTCAGGCTCCATG**AAGGTACAGGAGGGTTTGTTCGTGGTGGCTGTTTTCTACCTTGCTTATACGCAGCTAGTCAAGGGG**ATGATTACGACCAGAAAG	
INT4_SV40_right	TAGATATACATTGGACCTTT**CCTAAGAAGAAGAGGAAGGTT**TCAGTGAGACCGAATTCTCGCCCT	
INT4_right	TAGATATACATTGGACCTTTTCAGTGAGACCGAATTCTCGCCCT	
FT_Oligo	TTGGCCATAAGTAACCTTTAGAGTGATTGATCTATTAAACGGATCAAGAACGTCTCCAACAACTCTGCTTACTATAAGAGGGTCTCTTATATTTATAGACATGCTTCTTGGTGCCGCGCCT	pegRNA assembly
gRNA_Spacer_FT	TAAAGGTTACTTATGGCCAACTCGTGACCACCTTCACCCAGTTTTAGAGCTAGAAATAGC	
gRNA-Scaffold	GCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	
gRNA_RTPBS_TEVOPREQ1	AAGTGGCACCGAGTCGGTGCTGCACGCCGTACGTGAAGGTGGTCACCGCGGTTCTATCTAGTTACG	
TEVOPREQ1_Oligo	TCGGTCTCAATACAAAAAAATTCTAGTTGGTTTAACGCGTAACTAGATAGAACCGCG	
gRNA_Spacer	AGGCGCGGCACCAAGAAGCACTCGTGACCACCTTCACCCAGTTTTAGAGCTAGAAATAGC	
gRNA_RTPBS	AAGTGGCACCGAGTCGGTGCTGCACGCCGTACGTGAAGGTGGTCACTTTTTTTGTATTGAGACCGA	
OLE1P_XbaI_OL1	TATCCATTTTCTTCATTGT**TATAGA**ATGTCGCGGAACAAATTTT	Restriction enzyme recognition site removal
OLE1P_XbaI_OL2	CGATCTGATACTGATAACG**TCTCGA**TTTTTAGGGTTAAAGCAAT	
Csy4_EcorI_OL1	AGACTTGGAGAAAGACTTA**GGATTC**ATGCTTCTGCTGATGATCT	

Csy4_EcorI_OL2	GAAGAAGAAGCTAGAAAAA**GAGTTC**CTGATACTGTTGCTAGAGC	(The modified restriction enzyme motif is shown in bold, with the changed base underlined. The original sequence is “TCTAGA” for XbaI, “GAATTC” for EcorI, “GAGACC” for BsaI, “CACCTGC“ for PaqCI, and “GAAGAC” for BbsI).
Csy4_EcorI_OL3	CTGGACCCGGCTTGAGACC**GAACTC**TCGCCCTATAGTGAGTCGT	
tUM140_1	AGAAGTGAAGCTTGGTCTCACTGCGCCGCGCACAGCTGACGTAG	
tUM140_2a	AACTCTAGTGTAATCCCAGACTGGTG**T**GACCAGTGACATTGACACCA	
tUM140_2b	AACTCTAGTGTAATCCCAGACTGGTGAG**G**CCAGTGACATTGACACCA	
tUM140_2c	AACTCTAGTGTAATCCCAGACTGGTGA**C**ACCAGTGACATTGACACCA	
tUM140_2d	AACTCTAGTGTAATCCCAGACTGGTGAGA**A**CAGTGACATTGACACCA	
tUM140_2e	AACTCTAGTGTAATCCCAGACTGGT**T**AGACCAGTGACATTGACACCA	
tUM140_3	TACATTTGCATGCAAAGTTGACTATGAGACCGAATTCTCGCCCT	
MoClo_mCherry_1	TTAATCACTCTGTGGTCTCATTCGATGGTGAGCAAGGGCGAGGA	
Alt_mCherry_1	AGAAGTGAAGCTTGGTCTCATTCGCTATGGTGAGCAAGGGCGAGGA	
MoClo_mCherry_2	GGCCCCGTAATGCAGAAGAA**A**ACCATGGGCTGGGAGGCCTCCTCCGAGC	
MoClo_mCherry_3	TGGACGAGCTGTACAAGTAGGCTTTGAGACCACGAAGTGGCTCT	
Alt_mCherry_3	GCATGGACGAGCTGTACAAGGCTTTGAGACCGAATTCTCGCCCT	
MoClo_WUS_1	TTAATCACTCTGTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	
Alt_WUS_1	AGAAGTGAAGCTTGGTCTCAGGAGCCCATGTTTACGTTTACGTT	
MoClo_WUS_2	TACTCAACATGTTCATAAGTACACCTG**A**CTTCACACTCGTTTCACACA	
MoClo_WUS_3	CAAAAGTCGAATCAAACACACCCATTGAGACCACGAAGTGGCTCT	
Alt_WUS_3	CAAAAGTCGAATCAAACACACCCATTGAGACCGAATTCTCGCCCT	
GUS_Aar1	AAGACTGTAACCACGCGTCTGTTGACTGGCA**A**GTGGTGGCCAATGGTGATGT	
GUS_Aar1b	AAGACTGTAACCACGCGTCTGTTGACTGGCAGGT**C**GTGGCCAATGGTGATGTCAG	
GUS_Aar2	CCGACGCGTCCGATCACCTG**T**GTCAATGTAATGTTCTGCGACGCTCACA	
IncLib_Oligo1	TCCAAGCTCAAGCTAAGCTTACCTCCAGAAGGTAATTATCCAAG	Stitching oligos for incompatible library assembly
IncLib_Oligo2	AGAGAAATTTGTAAGTTTGTATGCCATGGTGAGCAAGGGCGAG	
IncLib_Oligo3	GCATGGACGAGCTGTACAAGCTAACCCCGATGAGCTAAGCTAGC	
IncLib_Oligo4	CATGTACTCGACGGCCGAGTGTATAAGCTTACCTTACTTAGATC	
amilCPOrange_Mut Oligo	GGGATATTTTATCACCACAG**NNNNNN**TACGGAAGCATACCATTCAC	Saturation mutagenesis of amilCP Orange (ambiguous bases are shown in bold)
Orange_Seq_F	AATAGGCGTATCACGAGGC	Colony PCR primers for saturation mutagenesis experiment
Orange_Seq_R	AGCGAGTCAGTGAGCGAG	
Orange_NGS_F	GGAGCAGACGGTAAAGCTCA	Primers for generation of NGS amplicon of amilCP_Orange
Orange_NGS_R	AGTTGCCTTGGATGCTGGAA	