# ANNEX

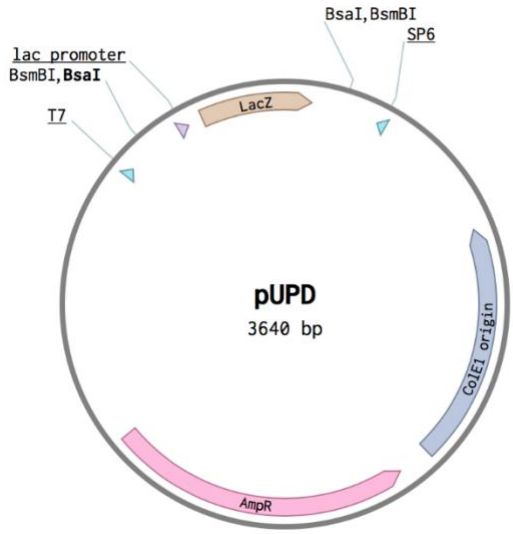


**Figure S1. Graphic representation of the pUPD vector.** It contains the bacterial ampicillin resistance gene (AmpR) for counterselection, the bacterial replication origin ColE1 and the *lacZ* gene for blue-white colony screening. *BsmI* and *BsaI* Type IIS restriction enzymes flank the *lacZ* gene. T7 and SP6 sequences flank the expected insert.


**Figure S2. Graphic representation of pDGB3α1 and pDGBΩ1 vectors.** Destination pCAMBIA-based vectors. pCAMBIA backbone contains the bacterial origin of replication pBR322 for *E. coli* propagation and pVS1-Rep and pVS1 STA for *A.tumefaciens* replication and stability, respectively. pDGBα vectors contain the kanamycin resistance gene (KanR), whilst pDGBΩ contain the spectinomycin resistance gene (SpmR). *BsmBI* and *BsaI* recognition sites are depicted as green and orange, respectively. GB barcodes flank the *lacZ* gene. LB and RB refers to the Left and Right Borders of the T-DNA region.

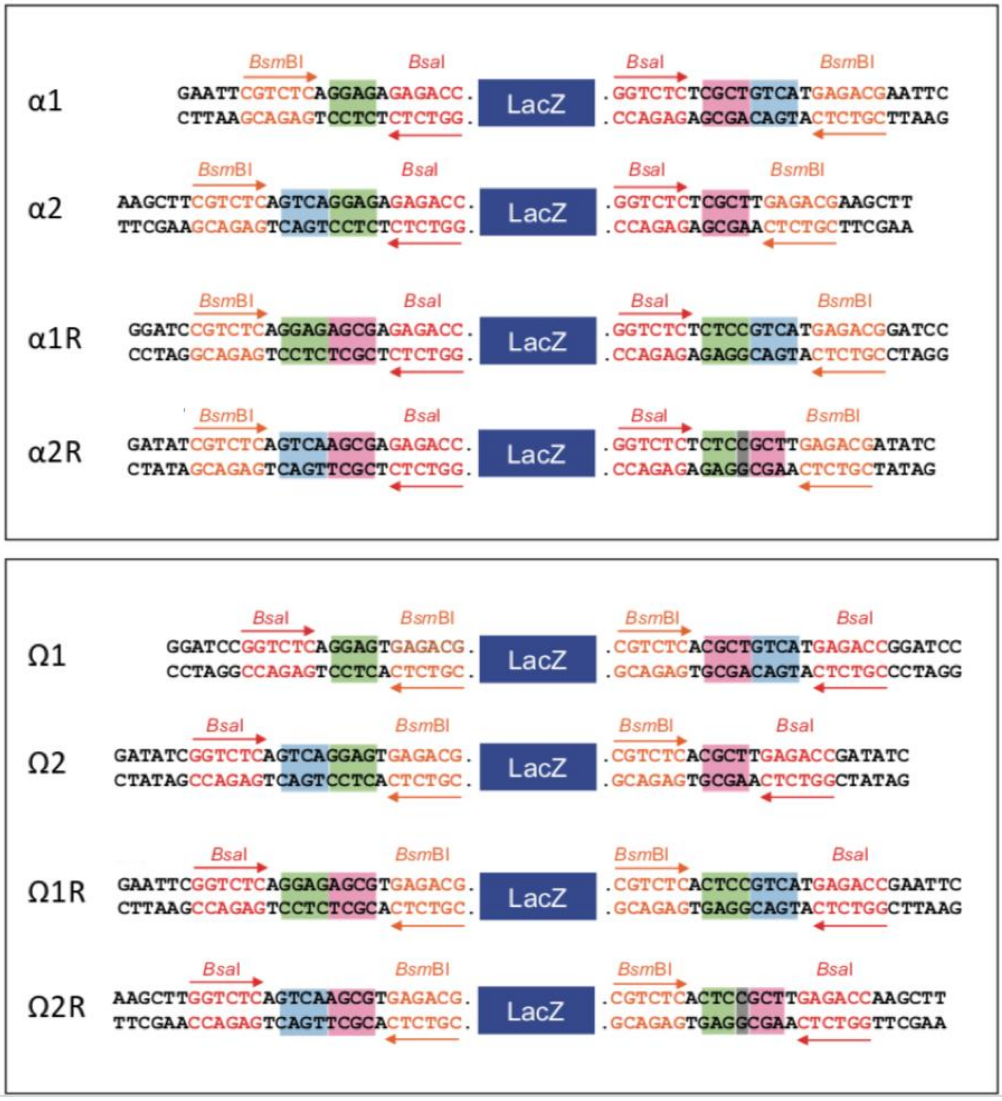


**Figure S3. pDGB3α y pDGB3Ω destination vectors for Level 1 and Level 2 GB3.0. assembly**. There are eight GB3.0. destination vectors: four alpha and four omega plasmids, identified as 1 and 2 (direct orientation) or 1R or 2R (reverse orientation). This toolkit has been developed to carry out a theoretically endless number of binary assemblies through a "loop" design. The four vector options allow both the selection of a desired order (1 precedes 2) and orientation (1R and 2R for the reverse orientation of a genetic construct). *BsmBI* (orange) and *BsaI* (red) recognition sites are rationally positioned to carry out simultaneous digestion/ligation ("one-pot" reaction), so once a plasmid is cut it cannot be ligated to its original backbone. Standardized overhangs (barcodes) as a result of *BsmBI* or *BsaI* digestions are coloured in green, blue and pink. Modified from Supplemental Figure S1 of *Hernanz-Koers et al.* (2018).


**Figure S4. Sanger sequencing of an expected FB120 construct. (A)** Sequencing with pCambia FW primer (P6) and alignment against the expected FB120 sequence.

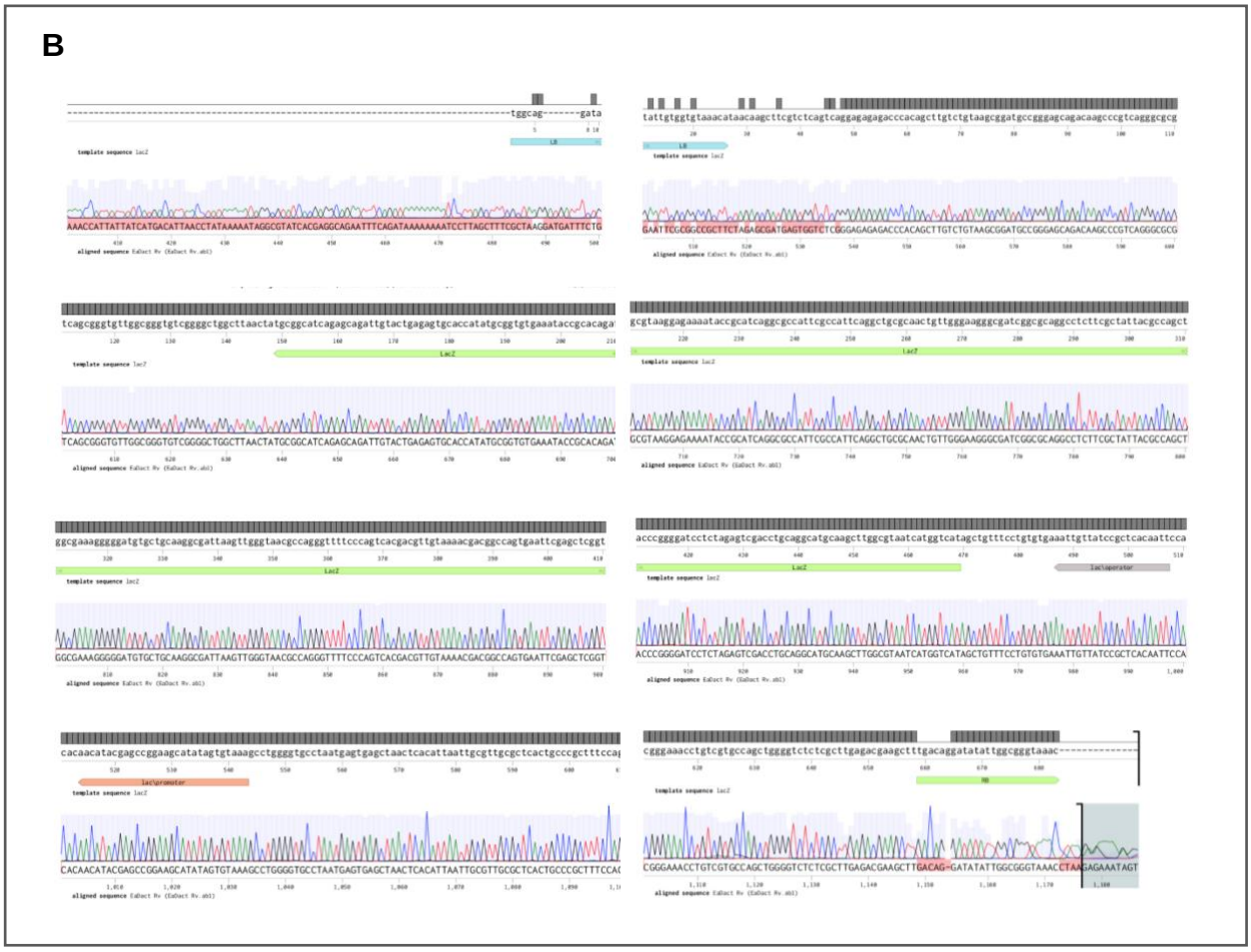


**Figure S4. Sanger sequencing of an expected FB120 construct. (B)** Sequencing with pCambia RV (P7) primer and alignment against the GB lacZ cassette. The overall alignment resulted in the identification of a truncated sequence, with the aberrant ligation of a partial FB120 sequence with the *lacZ* cassette.

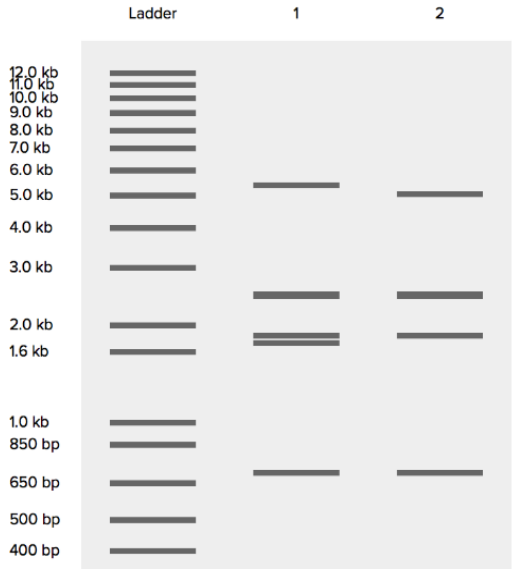


**Figure S5. Expected band patter for the restriction analysis with *NdeI*.**
**(1)** FB126 **(2)** Aberrant ligation of FB126 without the FB120 partner.

**Table S1. List of primer pairs used for Sanger sequencing.**

| Primer ID | Sequence (5'-3') | Use |
|---|---|---|
| **P1** | TAATACGACTCACTATAGGG | pUPD T7 |
| **P2** | GATTTAGGTGACACTATAGAATAC | pUPD SP6 |
| **P3** | GCTTTCGCTAAGGATGATTTCTGG | pUPD2 FW |
| **P4** | CAGGGTGGTGACACCTTGCC | pUPD2 RV |
| **P5** | TTGTCTCCCCATAACAATTA | EaDAcT CDS FW |
| **P6** | GGTGGCAGGATATATTGTGG | pCAMBIA FW |
| **P7** | CGCCCTTTTAAATATCCGATT | pCAMBIA RV |