Fast and efficient assembly of Targeting Vectors by *in vivo* 4-Way Recombineering (4WR) using *E. coli* GB05-dir

Introduction

4-Way Recombineering (4WR) offers a new fast and streamlined way to assemble plasmids in a single *in vivo* recombination step from up to four DNA fragments, e.g. PCR products. This is facilitated by co-electroporation of all fragments to Gene Bridges' popular *E. coli* stain GB05-dir (Gene Bridges, Cat. No. K008).

The 4WR system greatly complements existing technologies such as CRISPR/Cas9. In recent years CRISPR/Cas9 has become the system of choice for the editing of eukaryotic chromosomes. There are many elaborated strategies to address a genomic site by the guiding RNA and mutant Cas9 nucleases in a strikingly precise manner. But when it comes to allelic exchange, gene fusion, or protein tagging operations methods to construct corresponding donor vectors are still traditional, thus, time consuming and tricky. The power of 4WR was impressively demonstrated by BAKER and coworkers (2016, Nature *Scientific Reports* **6**, 25529), who used 4WR for the very rapid construction of donor vectors with short Homology Regions (~1 kb). Such constructs work remarkably efficient for mono-allelic applications, such as fluorescent protein tagging, targeting the male Y-chromosome, or heterozygous targeting. This combination of straightforward technologies was termed **Recombineering And Cas9-assisted tagging (RAC-tagging)**.

The applications of 4WR also extends to laboratory standard operations like the cloning of whole plasmid series differing in a specific component, e.g. promoter libraries driving an invariant reporter gene, or exchanging of antibiotic resistance markers or protein tags. Because the 4WR is modular by design, implementation of such tasks is quite easy.

Together with the earlier developed **Direct Cloning (DC)** technique, the 4WR further allows for handling of large portions of DNA, e.g. whole gene clusters. Operons which were sub-cloned from genomic DNA in an initial DC step, can easily combined with non-native promoters or protein tags in subsequent 4WR operations.

Experimental Procedure

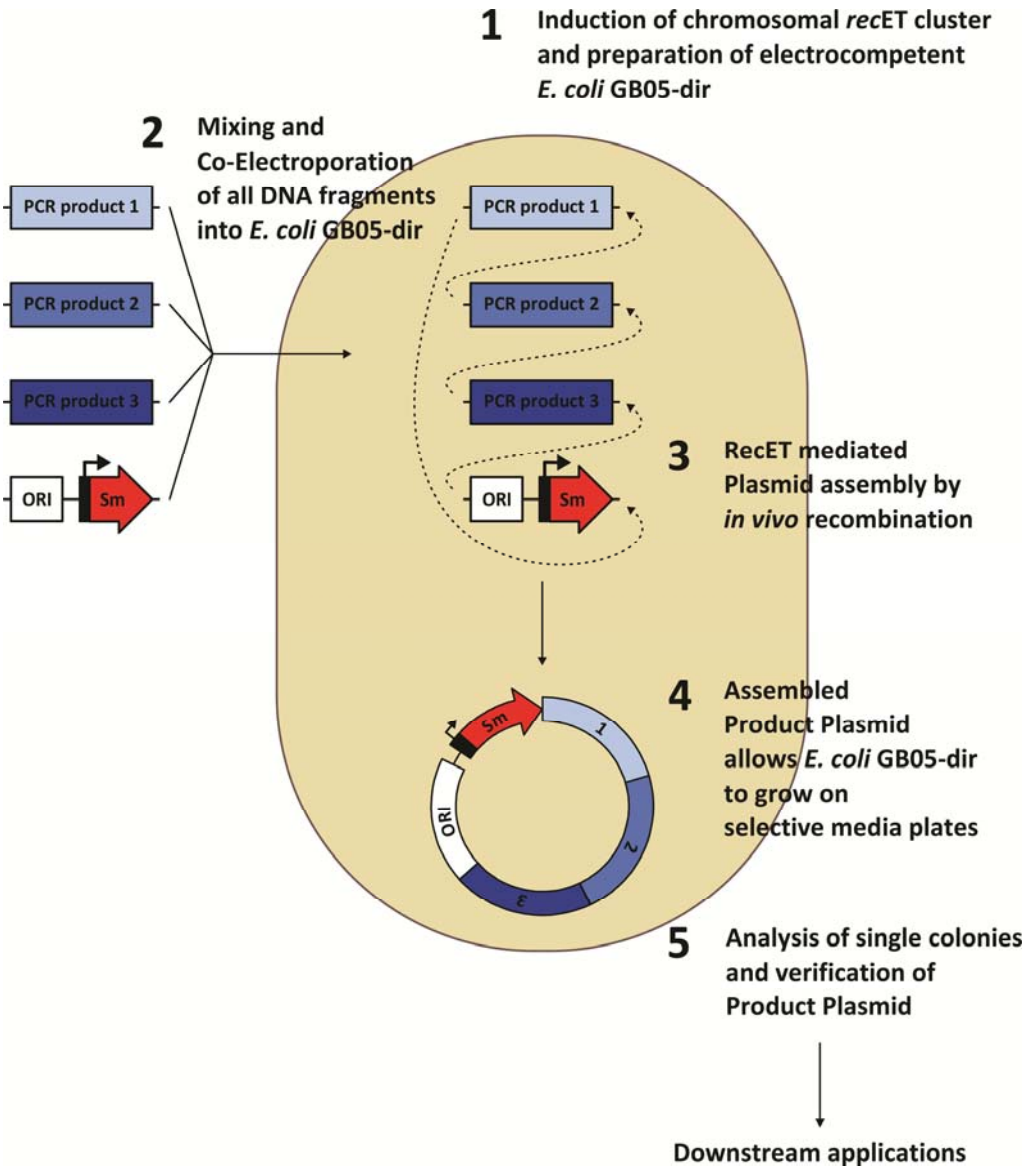

Figure 1: Work Flow of RecET *in vitro* 4-Way Recombineering.

PCR products to be assembled by 4WR need to feature terminal homologies, e.g. stretches of identical sequence. Homologies of 50 bp length work best, while sequences shorter than 30 bp significantly lower the cloning efficiency. These regions may easily be added by primer extension technique. It is recommended to designing the primers *in silico* on the sequence of the desired final construct.
The PCR products may be generated by standard PCR techniques. According to the product size the use of a polymerase with proofreading activity is advisable.

One of the PCR products has to harbor an origin of replication (ORI) and a selectable marker gene (see Figure 1). The original *E. coli* GB05-dir from Kit K008 is free of any plasmid and supports most common ORIs (e.g. ColE1, pUC, pMB, p15A, pSC101, CloDF13).

Protocol

Preparation of PCR products

PCR products were purified by agarose gel-electrophoresis and subsequently eluted from the gel using a column-based kit that allows for elution with small volumes. 500 ng of each of the PCR products was mixed in a volume of 4 µL (up to 12 µL were applied successfully). This mix can be stored at -20°C.

Transformation of *E. coli* GB05-dir

A small culture of *E. coli* GB05-dir was gown overnight at 37°C. The following day 1.3 mL of fresh LB broth was inoculated with 50 µL of the overnight culture and again incubated at 37°C/800 rpm until OD_{600} reached about 0.3. At this time point expression of the genomic *rec*ET cluster was induced by the addition of 50 µL of a sterile 10% L-Arabinose solution, followed by further incubation (37°C/800 rpm) for 35'.

Then cells were pelleted by centrifugation for 30'' with 11.2k rcf at 4°C. Cells were prepared for electroporation by three consecutive washes with 1 mL of ice-cold 10% glycerol (30''/11.2k rcf/4°C). The supernatant was discarded each time. After the third wash the pellet was re-suspended in the residual glycerol (30-50 µL) and the mix of PCR products (containing 500 ng of each PCR products) was added.

The whole mixture was transferred to a pre-cooled (4°C) 1 mm gapped electroporation cuvette, electroporation was carried out at 1350 V, 10 µF, and 600 Ω applying an Eppendorf Electroporator 2510. The measured time constant generally was in the range of 5.6 to 6.0 ms. 1 mL of ice-cold LB broth was used to flush the cuvette and cells were transferred to a fresh, lid-punctured 2 mL reaction tube. After 3 hours of incubation (37°C/800 rpm) cells were spread to a LB agar plate appropriately supplemented with antibiotic. The plate was incubated at 37°C overnight. The next day >100 colonies had formed on the antibiotic-supplemented LB agar plate.

Verification of successfully assembled plasmids

To verify successfully assembled plasmids, small scale cultures (1.3 mL LB + antibiotic) were inoculated from 12 single colonies from which subsequently plasmid DNA was isolated and subjected to restriction analysis (see Figure 2). All analyzed clones matched the *in silico* prediction. Finally Sanger DNA sequencing confirmed these to be the desired Product Plasmids.

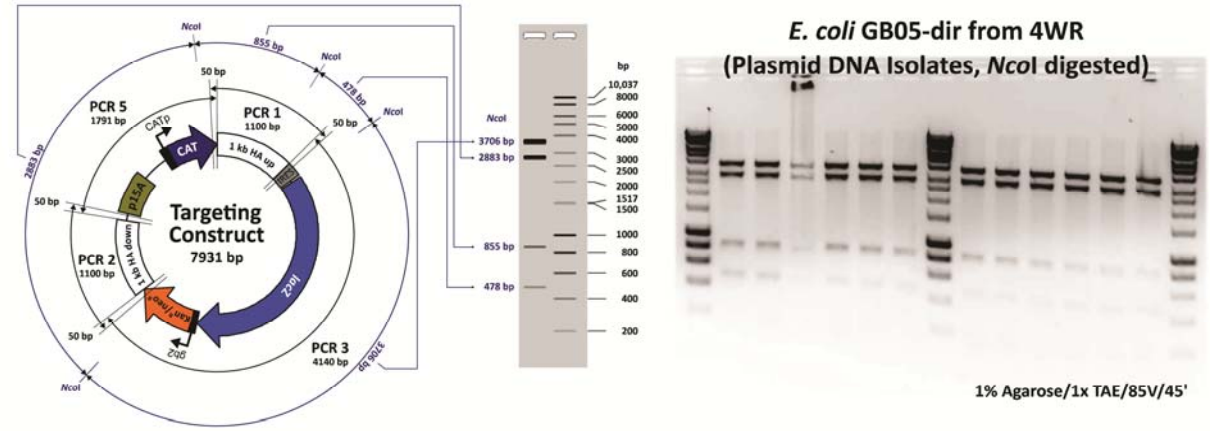

Figure 2: Analytical restriction digestion of 4WR Targeting Constructs. The plasmid to the left shows the desired 4WR product, a Targeting Construct, and the *in silico* prediction of an *Nco*I restriction digestion thereof. On the right plasmid DNA isolated from twelve Cm^R/Kan^R clones from a 4WR to achieve such Targeting Construct was subjected to an analytical *Nco*I digestion. In agarose gel-electrophoresis all candidates feature the banding pattern expected for a correct Plasmid Product.

Key Advantages of 4WR with *E. coli* GB05-dir

The *E. coli* GB05-dir strain comes free of any "tool-plasmids". This makes it an eminently suited host cell for the *in vivo* assembly of novel plasmids:

- No other plasmids present than the product plasmid.
- Product plasmids get multiplied and maintained in *E. coli* GB05-dir which is a K-12 derivative closely related to DH10β.
- No need for (re-)transformation to other maintaining *E. coli* strains.
- Product plasmids may be directly prepared from *E. coli* GB05-dir and applied to downstream applications.

Red/ET©-mediated homologous recombination occurs in a highly specific, precise, and accurate manner and is, thus, predictable, reliable, and reproducible. **Red/ET© is completely independent of the presence or absence of restriction endonuclease recognition sites, e.g. Type II-S restriction enzymes like *Bsa*I** and is, thus, free from limitations characteristic for Golden Gate technique.