**EasyClone-MarkerFree - DTU Orbit (06/05/2018)**

**EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into *Saccharomyces cerevisiae* via CRISPR-Cas9**

*Saccharomyces cerevisiae* is an established industrial host for production of recombinant proteins, fuels and chemicals. To enable stable integration of multiple marker-free overexpression cassettes in the genome of *S. cerevisiae*, we have developed a vector toolkit EasyClone-MarkerFree. The integration of linearized expression cassettes into defined genomic loci is facilitated by CRISPR/Cas9. Cas9 is recruited to the chromosomal location by specific guide RNAs (gRNAs) expressed from a set of gRNA helper vectors. Using our genome engineering vector suite, single and triple insertions are obtained with 90–100% and 60–70% targeting efficiency, respectively. We demonstrate application of the vector toolkit by constructing a haploid laboratory strain (CEN.PK113-7D) and a diploid industrial strain (Ethanol Red) for production of 3-hydroxypropionic acid, where we tested three different acetyl-CoA supply strategies, requiring overexpression of three to six genes each. Among the tested strategies was a bacterial cytosolic pyruvate dehydrogenase complex, which was integrated into the genome in a single transformation. The publicly available EasyClone-MarkerFree vector suite allows for facile and highly standardized genome engineering, and should be of particular interest to researchers working on yeast chassis with limited markers available.

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