Supplementary materials

Expanding the CRISPR-Cas9 toolkit for *Pichia pastoris* with efficient donor integration and alternative resistance makers

S1: Primers and gBlocks

Primers and gBlocks used in this study are listed here. The generation of some plasmids has previously been described in: Weninger *et al*., (2016) "Combinatorial optimization of CRISPR/Cas9 expression enables precision genome engineering in the methylotrophic yeast *Pichia pastoris*", Journal of Biotechnology, 235:139-149.

Primer name	**Sequence 5' --> 3'**
Primers for the amplification of the *GUT1* locus	
GUTout3prR1	ATGAAGTTAGTAAGGTTCTTGATGAAGC
PGUTseq2	GGTACTTTGCCGACTCCTC
5prGUT1_fwd	tgggttcaatggcgtttgagttag
GUT1-3-fw	GAAAAGGTTTACTATCCCGATTTAGGCGAAAAGAG
GUT1-438-seq-fw	Cttcagagagcttgccgtagaaagaacg
GUT1-525-seq-rv	Gttggaatcccagtaaggggcaaacaatcctg
CRISPR/Cas9 nickase constructs	
RZ-GUT1-gRNA0-RZ	CCAGTTCAAGTTACCTAAACAAATCAAATCTGTGCTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTCcacagatatatctactataaGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGGTCCCAGC CTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTCAAGAGGA TGTCAGAATGCC
RZ-GUT1-gRNA4-RZ	CCAGTTCAAGTTACCTAAACAAATCAAAGCCTTTCTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTCaaaggcaaagggctggcttaGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTA GTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGGTCCCAG CCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTCAAGAGG ATGTCAGAATGCC
DAS1TT-hsCas9-rv	TAAAACTGTAAAGACTTCCCGTCTCGAGTTAAACTTTTCTTTTCTTCTTTGGGTCTCCAC
HsCas9-D10A-fw	TATATTAAAACTACAACAGAATTCCGAAACGATGGACAAGAAGTACTCCATTGGGCTCGCT ATCGGCACAAACAGCGTCG
hf-HsCas9	
P_AOX1_syn_pUCORI _Gibs_fv	CTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGGTACCC
HsCas9_rev	CTCGATTCACGCATGAACACCAA
HsCas9_fv	CTTATCCACGACTTCCTCGAAGTTC
HDV_AOX1TT_Gibs_r ev	CAAATGGCATTCTGACATCCTCTTGAGTCCCATTCGCCATGCCGAA

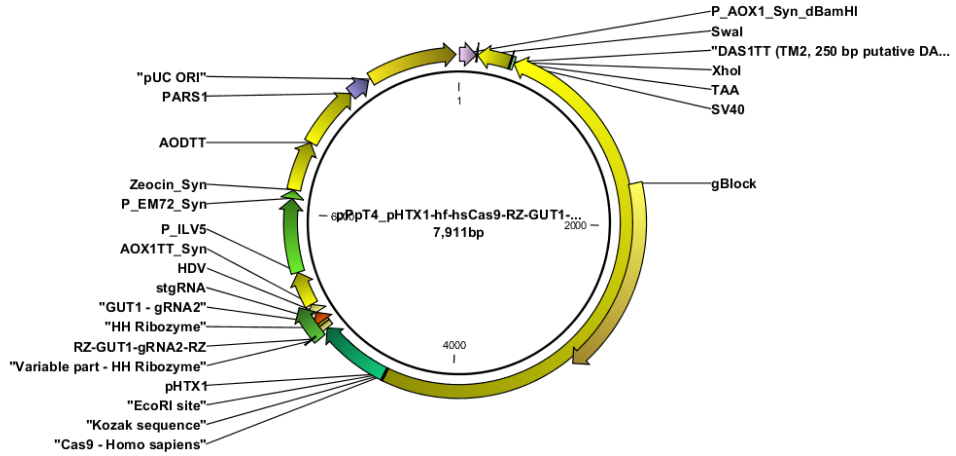
gBlock_hf-HsCas9	TTGGTGTTCATGCGTGAATCGAGAATTTGGGCCACGTGCTTGGTGATAGCGCGTGTCTCA ACAAGCTGCCTTTTGATGAAGCCGGCTTTATCCAACTCAGACAGGCCACCTCGTTCAGCCT TAGTCAGATTATCGAACTTCCGTTGTGTGATCAGTTTGGCGTTCAGCAGCTGCCGCCAATA ATTTTTCATTTTCTTGACAACTTCTTCTGAGGGGACGTTATCACTCTTCCCTCTATTTTTATC GGATCTTGTCAACACTTTATTATCAATAGAATCATCTTTGAGAAAAGACTGGGGCACGATAT GATCCACGTCGTAGTCGGAGAGCCGATTGATGTCCAGTTCCTGATCCACGTACATGTCCC TGCCGTTCTGCAGGTAGTACAGGTAGAGCTTCTCATTCTGAAGCTGGGTGTTTTCAACTGG GTGTTCCTTAAGGATTTGGGACCCCAGTTCTTTTATACCCTCTTCAATCCTCTTCATCCTTT CCCTACTGTTCTTCTGTCCCTTCTGGGTAGTTTGGTTCTCTCGGGCCATCTCGATAACGAT ATTCTCGGGCTTATGCCTTCCCATTACTTTGACGAGTTCATCCACGACCTTAACGGTCTGC AGTATTCCCTTTTTGATAGCTGGGCTACCTGCAAGATTAGCGATGTGCTCGTGAAGACTGT CCCCCTGGCCAGAAACTTGTGCTTTCTGGATGTCCTCCTTAAAGGTGAGAGAGTCATCATG GATCAAAGCCATGAAGTTCCGGTTGGCAAATCCATCGGACTTAAGAAAATCCAGGATTGTC TTTCCACTCTGCTTGTCTCGGATCCCATTGATCAGTTTTCTTGACAGAGCCCCCCATCCTG TATATCGGCGCCTCTTGAGCTGTTTCATGACTTTGTCGTCGAAGAGATGAGCGTAAGTTTT CAAGCGTTCTTCAATCATCTCCCTATCTTCAAACAACGTAAGGGTGAGGACAATGTCCTCA AGAATGTCCTCGTTCTCCTCATTGTCCAGGAAGTCCTTGTCTTTAATGATTTTCAGGAGATC GTGATACGTTCCCAGGGATGCGTTGAAGCGATCCTCCACTCCGCTGATTTCAACAGAGTC GAAACATTCAATCTTTTTGAAATAGTCTTCTTTGAGCTGTTTCACGGTAACTTTCCGGTTCG TCTTGAAGAGGAGGTCCACGATAGCTTTCTTCTGCTCTCCAGACAGGAATGCTGGCTTTCT CATCCCTTCTGTGACGTATTTGACCTTGGTGAGCTCGTTATAAACTGTGAAGTACTCGTAC AGCAGAGAGTGTTTAGGAAGCACCTTTTCGTTAGGCAGATTTTTATCAAAAGCAGTCATCC TTTCGATGAAGGACTGGGCAGAGGCCCCCTTATCCACGACTTCCTCGAAGTTC
3UTRGUTR	GTGTTTGCTGTAGGATGACCTAGATTTAAATATAAGAGGAAACAACGTTCGTATCGTGA
seq-pGUT1-332..308-fwd	tgggttcaatggcgtttgagttag
Episomal donor cassettes	
ARG4TT-ARS-fw	TCGTCTCCAGCAGCATCCTCGCATTGGTACTCGAGATAAGCTGGGGGAACATT
pUORI-ARS-rv	CTACGGGGTCTGACGCTCAGTG TCGACAATTAATATTTACTTATTTTGGTCAAC
Pjet-ARS-fw	AATAGGCGTATCACGAGGCCGCCCCTGCATCGAGATAAGCTGGGGGAACATT
Pjet-ARS-rv	TTATTTGGCAAAAATAATATAATTCGGCTCGACAATTAATATTTACTTATTTTGGTCAAC
CRISPR/Cas9 constructs for DNA deletion at the DAS1/DAS2 locus	
RZ-DAS1/2-gRNA1-RZ	CCAGTTCAAGTTACCTAAACAAATCAAAACCTCACTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTCTGAGGTTACCACTGGTCCCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATA AGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGG TCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTC AAGAGGATGTCAGAATGCC
RZ-DAS1/2-gRNA2-RZ	CCAGTTCAAGTTACCTAAACAAATCAAACTCAACCTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTCGTTGAGGCCTATGTCGCATAGTTTTAGAGCTAGAAATAGCAAGTTAAAATA AGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGG TCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTC AAGAGGATGTCAGAATGCC
Das2_rev	GTCCTACAGGTAAACAGATAGCCACTACG
Das1_fw	CATCAGATATTATCATCGCGGCTTACG
Das2_fw	CGATACTTCTCCACATTCAGTCATAGATGG
Das1_rev	CCTCAGGTCTCGGTTAGCCTCTAGG
CRISPR/Cas9 plasmid bearing the Geneticin resistance cassette	
RZ-GUT1-gRNA1-RZ	CCAGTTCAAGTTACCTAAACAAATCAAATACTCGCTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTCCGAGTACTCTACCTCTGCTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGGT CCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTCA AGAGGATGTCAGAATGCC
RZ-GUT1-gRNA2-RZ	CCAGTTCAAGTTACCTAAACAAATCAAAATTGCACTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTCTGCAATTTCCTCAGCCAGGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATA AGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGG TCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTC AAGAGGATGTCAGAATGCC

RZ-GUT1-gRNA3-RZ	CCAGTTCAAGTTACCTAAACAAATCAAAAACAACCTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTCGTTGTTTGGTCCAAGAAGACGTTTTAGAGCTAGAAATAGCAAGTTAAAATA AGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGG TCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTC AAGAGGATGTCAGAATGCC
Zeocin resistance gene (CDS)	ATGGCTAAACTCACCTCTGCTGTTCCAGTC**CTGACTGCTCGTGATGTTGC**TGGTGCTGTTG AGTTCTGGACTGATAGACTCGGTTTCTCCCGTGACTTCGTAGAGGACGACTTTGCCGGTG TTGTACGTGACGACGTTACCCTGTTCATCTCCGCAGTTCAGGACCAGGTTGTGCCAGACA ACACT**CTGGCATGGGTATGGGTTCG**TGGTCTGGACGAACTGTACGCTGAGTGGTCTGAG GTCGTGTCTACCAACTTCCGTGATGCATCTGGTCCAGCTATGA**CCGAGATCGGTGAACAG CCC**TGGGGTCGTGAGTTTGCACTGCGTGATCCAGCTGGTAACTGCGTGCATTTCGTCGCA GAAGAGCAGGACTAA
ZEO-gRNA1	CCAGTTCAAGTTACCTAAACAAATCAAA**agtcag**CTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTC**CTGACTGCTCGTGATGTTGC**GTTTTAGAGCTAGAAATAGCAAGTTAAAATA AGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGG TCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTC AAGAGGATGTCAGAATGCC
ZEO-gRNA2	CCAGTTCAAGTTACCTAAACAAATCAAA**tgccag**CTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTC**CTGGCATGGGTATGGGTTCG**GTTTTAGAGCTAGAAATAGCAAGTTAAAATA AGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGG TCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACTC AAGAGGATGTCAGAATGCC
ZEO-gRNA3	CCAGTTCAAGTTACCTAAACAAATCAAA**tctcgg**CTGATGAGTCCGTGAGGACGAAACGAG TAAGCTCGTC**CCGAGATCGGTGAACAGCCC**GTTTTAGAGCTAGAAATAGCAAGTTAAAAT AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATG GTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGACT CAAGAGGATGTCAGAATGCC

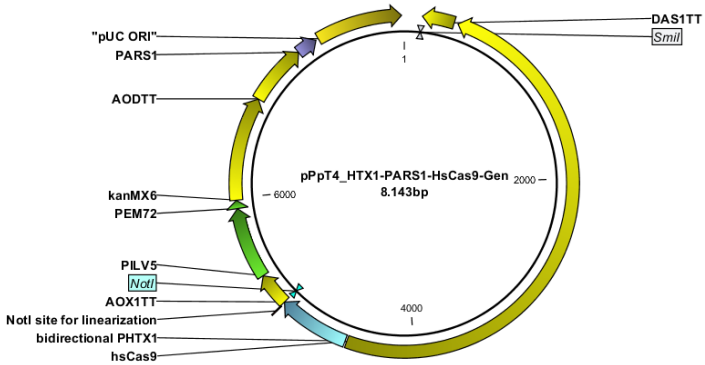
S2: Plasmids

Sequence files of the following plasmids are provided in genbank (.gb) format.

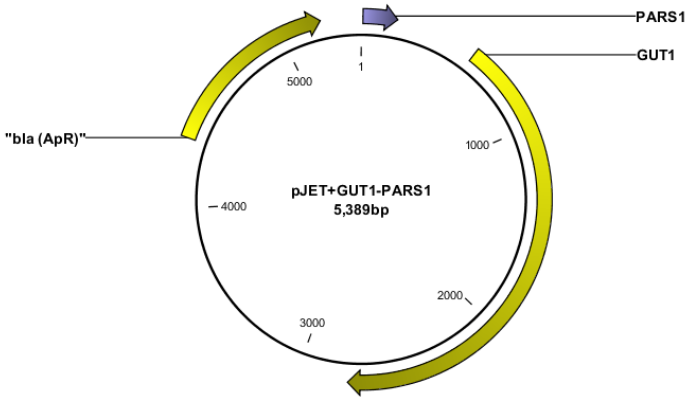
pPpT4_pHTX1-hf-HsCas9-GUT1-gRNA1



pPpT4_HTX1-PARS1-HsCas9-Gen



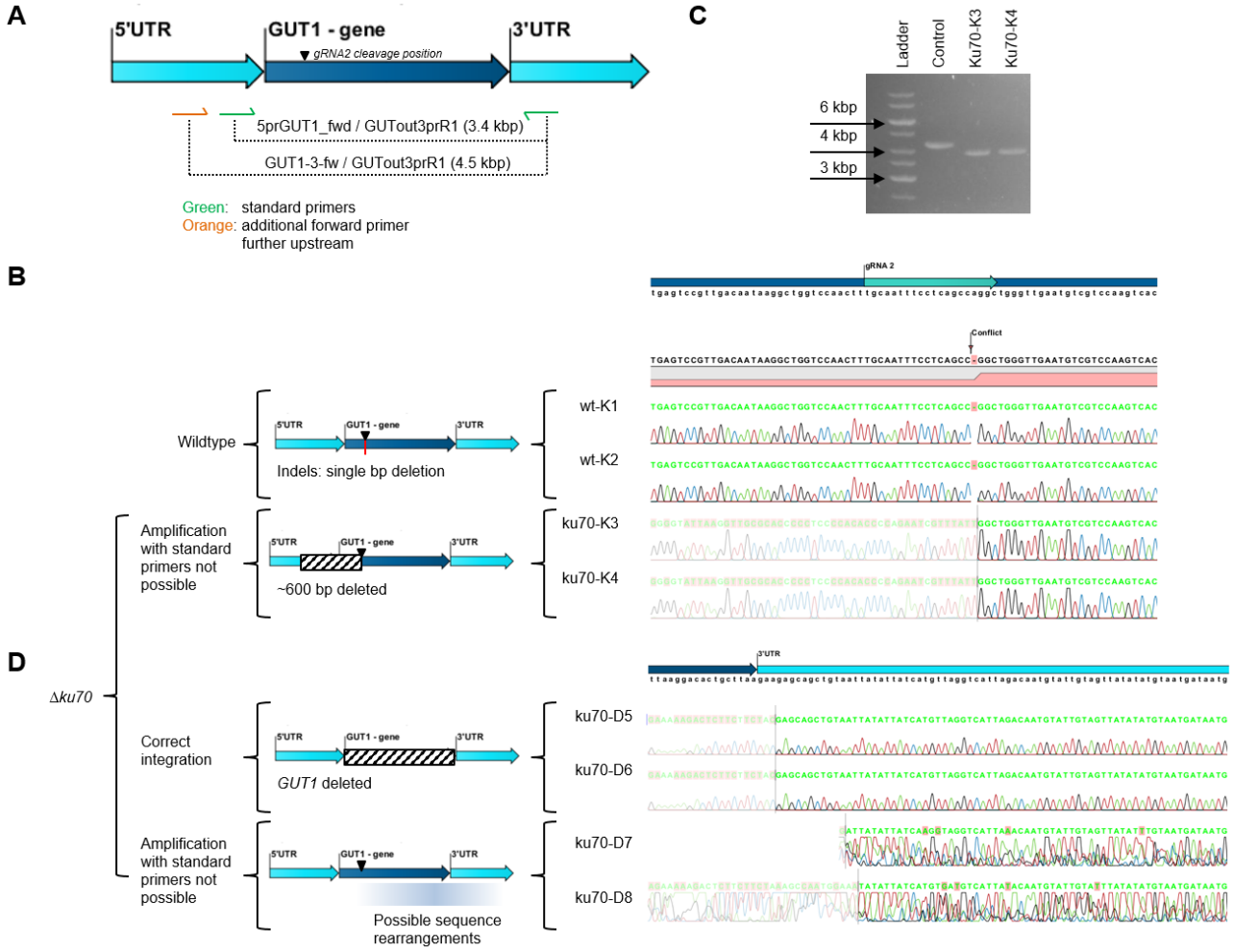
pJet-GUT1-PARS1



S3: Sequencing of the *GUT1* locus upon CRISPR/Cas9 cleavage in *P. pastoris Δku70*

When solely a CRISPR/Cas9 plasmid was transformed to target the *GUT1* gene (no donor), sequencing of *P. pastoris Δku70, Δgut1* CRISPR/Cas9 transformants resulted in ~600 bp deletions upstream of the Cas9 cleavage site (Figure S3B,C). Compared to the *P. pastoris Δku70* transformants, *P. pastoris* wildtype *Δgut1* transformants bore short insertions or deletion mutations (indels, Weninger et al., (2016)) (Figure S3B).

The co-transformation of CRISPR/Cas9 plasmids and donor DNA, and sequencing of *P. pastoris Δku70 Δgut1* transformants resulted either in the complete removal of the *GUT1* gene or sequencing reads, which could only be aligned partially, containing mixed sequences. These mixed reads may indicate chromosomal rearrangements or translocations. An example is shown in Figure S3D.



S 3: Sequencing of the *GUT1* locus of *P. pastoris Δku70* transformants. A. Scheme of the *GUT1* locus and primer pairs used for PCR amplification/sequencing. **B.** Sequencing of the *GUT1* locus of *P. pastoris* wildtype and *Δku70* transformants (transformed with CRISPR/Cas9 plasmids to target *GUT1* [without donor DNA]). NHEJ-mediated repair in *P. pastoris* wildtype resulted in short indel mutations (wt-K1 and wt-K2). *P. pastoris Δku70 Δgut1* transformants ku70-K3 and ku70-K4 bore large deletion mutation (approximately 600 bp). The region upstream of the Cas9 cleavage site was deleted. **C.** Also the PCR products (primers GUT1-3-fw and GUTout3prR1) of ku70-K3 and ku70-K4 were ca. 0.6 kbp reduced in size compared to the wildtype control, in line with the deletions observed in the sequencing. **D.** Sequencing of the *GUT1* locus of *P. pastoris Δku70* transformants, which were co-transformed with CRISPR/Cas9 plasmids to target *GUT1* and donor DNA. The sequence of the 3' end of *GUT1* and 3'UTR aligned to various sequencing reads is shown. ku70-D5 and ku70-D6: the integration of the donor fragment for the removal for the *GUT1* CDS was obtained; the shaded part of the sequence corresponds to the 5'UTR of *GUT1*. Ku70-D7 and ku70-D8: If no donor was integrated mixed reads were obtained, which may indicate sequential rearrangements.