Supplementary data file

Oligonucleotide-based CRISPR-Cas9 toolbox for efficient engineering of *Komagataella phaffii*

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List of Supplementary Tables

List of Supplementary Figures

Supplementary Table S1.Primers used in this study.

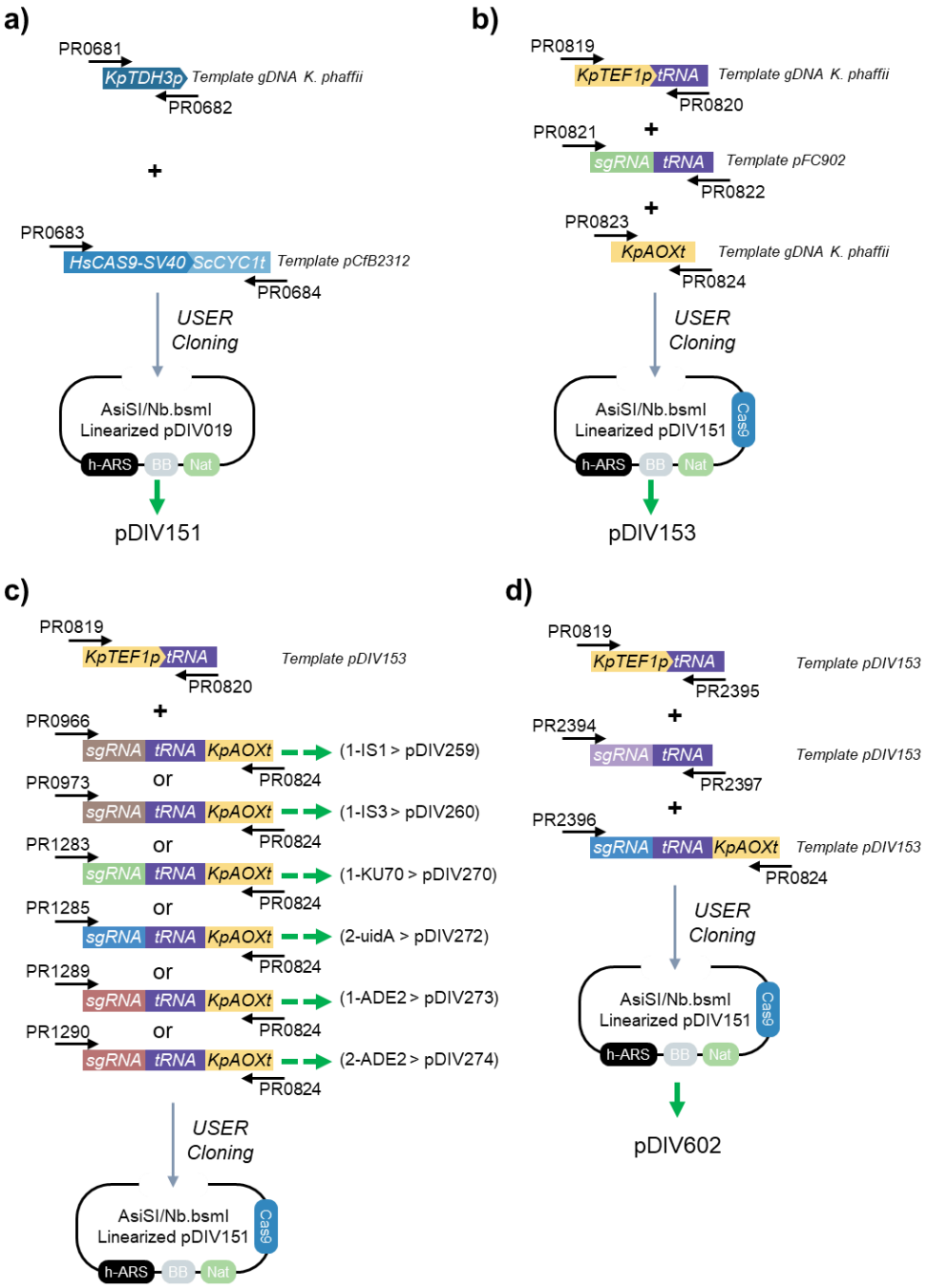
Name	Sequence	Purpose
PR_DIV0008	ATCTGTCAUATGTTACGTCCTGTAGAAACCCC	For construction of pDIV033 plasmid
PR_DIV0010	CGTGCGAUGCACACACCATAGCTTCAAAATG	For construction of pDIV033 plasmid
PR_DIV0011	ATGACAGAUTTTGTAATTAAAACTTAGATTAGATTGCT	For construction of pDIV033 plasmid
PR_DIV0105	CACGCGAUCTTCGAGCGTCCCAAAACC	For construction of pDIV033 plasmid
PR_DIV0120	ATCTGTCAUATGGTTAGTAAGGGGGAAGAGCT	For construction of pDIV479 plasmid
PR_DIV0681	CGTGCGAUCGCGTGCATTCTTTTTGTAGAAATGTCTTGGTGT	For construction of the basic Cas9-expressing vector
PR_DIV0682	ATTGTGUTTTGATAGTTGTTCAATTGATTGAA	For construction of the basic Cas9-expressing vector
PR_DIV0683	ACACAAUGGACAAGAAGTACTCCATTGG	For construction of the basic Cas9-expressing vector
PR_DIV0684	CACGCGAUGTACCGGCCGCAAATTAA	For construction of the basic Cas9-expressing vector
PR_DIV0810	CGCCAGATGTAATCACCGC	Primers for validation of KU70 Locus
PR_DIV0811	CATGGCGAAGACTCATAAGC	Primers for validation of KU70 Locus
PR_DIV0812	CTTCCCTGGCTTCCATGG	Primers for validation of KU70 Locus
PR_DIV0816	GGCATCCTACTGCTAATCCTTC	For validation of PEP4 locus
PR_DIV0817	GGCAATTGACATCGTAGTACC	For validation of PEP4 locus
PR_DIV0818	GCTCCTTAACATCCTCTCCATG	For validation of PEP4 locus
PR_DIV0819	CGTGCGAUTAACTCTGGCAACCAGTAACACG	For construction of the basic tRNA-based sgRNA expression cassette
PR_DIV0820	ATCCGTGAAUCGAACACGGGCCTCATCGATGGCAACGATGAA TTCTACCACTAGACCAATGATGCGTTGGCGAATAACTAAAATG TATGT	For construction of the basic tRNA-based sgRNA expression cassette
PR_DIV0821	ATTCACGGAUGATGCAACGACGACTGTTTGCGCTTAGTTTTAG AGCTAGAAATAGCAAGTTAAAATAAG	For construction of the basic tRNA-based sgRNA expression cassette
PR_DIV0822	ATCCTCTTGAUGCATCATCCGTGAATCGAAC	For construction of the basic tRNA-based sgRNA expression cassette
PR_DIV0823	aTCAAGAGGAUGTCAGAATGCCATTTGCC	For construction of the basic tRNA-based sgRNA expression cassette
PR_DIV0824	CACGCGAUTCTGTACTCTGAAGAGGAGTGGG	For construction of the basic tRNA-based sgRNA expression cassette
PR_DIV0961	GGTCTTAAUTAATGCCTCAGCTTCGAGCGTCCCAAAACC	For construction of pDIV479 plasmid
PR_DIV0966	ATTCACGGAUGATGCAGAAACAGTCCAACAGTAAGCGTTTTA GAGCTAGAAATAGCAAGTTAAAATAAG	For construction of the tRNA-based sgRNA expression cassette (IS1)
PR_DIV0967	GGGTTTAAUGGCTTGTCTGCCAATACTACT	For construction of GTSs with long homology (LH) sequences targeting integration site IS1
PR_DIV0968	GGACTTAAUAGTCGATTTTACGACTACCACA	For construction of GTSs with long homology (LH) sequences targeting integration site IS1
PR_DIV0969	GGCCGAAACCTCTCGTG	Validation primer for integration site IS1
PR_DIV0970	GGCATTAAUGGAAGCACATGGCCCTAC	For construction of GTSs with long homology (LH) sequences targeting integration site IS1
PR_DIV0971	GGTCTTAAUCCTGAATCAGCCTATGGGTC	For construction of GTSs with long homology (LH) sequences targeting integration site IS1
PR_DIV0972	CCTCCTGCTTCACTACCTC	Validation primer for integration site IS1
PR_DIV0973	ATTCACGGAUGATGCATGTTGATAATGGACCGTGGGGTTTTAG AGCTAGAAATAGCAAGTTAAAATAAG	For construction of the tRNA-based sgRNA expression cassette (IS3)
PR_DIV0974	GGGTTTAAUGCAGTGGTTGGAGGAAGAG	For construction of GTSs with long homology (LH) sequences targeting integration site IS3
PR_DIV0975	GGACTTAAUCGGAGGATTTTGATAGAAATTGGC	For construction of GTSs with long homology (LH) sequences targeting integration site IS3
PR_DIV0976	CAGTTGCAGCAACAACCTC	Validation primer for integration site IS3
PR_DIV0977	GGCATTAAUTCAACACCAGCAGCATTCA	For construction of GTSs with long homology (LH) sequences targeting integration site IS3

Name	Sequence	Purpose
PR_DIV0978	GGTCTTAAUGTGCCTTTGGAGGCTGG	For construction of GTSs with long homology (LH) sequences targeting integration site IS3
PR_DIV0979	CGTGAGAGGCTGATAGCC	Validation primer for integration site IS3
PR_DIV1045	ATGACAGAUGTTGGCGAATAACTAAAATGTATGTAG	For construction of pDIV479 plasmid
PR_DIV1253	CCATATTAACATAACATGTATATAAACGTC	Standard primers for plasmid assembly validations and SANGER sequencing
PR_DIV1254	GAAACCATTATTATCATGACATTAACC	Standard primers for plasmid assembly validations and SANGER sequencing
PR_DIV1255	CGAAGTTATATTAAGGGTTGTCGAC	Standard primers for plasmid assembly validations and SANGER sequencing
PR_DIV1277	GGGTTTAAUTTCACCTACGGGTCTGACTACC	Primers for Construction of pDIV643 plasmid
PR_DIV1279	AAAGCATUGCGCACACACCATAGCTTCAAAATG	Primers for Construction of pDIV643 plasmid
PR_DIV1280	ACACCTUCGAGCGTCCCAAAACCTTC	Primers for Construction of pDIV643 plasmid
PR_DIV1283	ATTCACGGAUGATGCAAAGCAATACGACATCCACGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG	For construction of the basic tRNA-based sgRNA expression cassette (Ku70)
PR_DIV1285	ATTCACGGAUGATGCATACTTCAGAAGCGTTGGACCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG	For construction of the basic tRNA-based sgRNA expression cassette (uidA v2)
PR_DIV1286	CGACGAGCCAGCACTTTATAG	Validation primers for ADE2 locus
PR_DIV1287	GCCTCTTCCATCATAAGCCATAG	Validation primers for ADE2 locus
PR_DIV1288	GGAGGACAAATTTCTACCGAAC	Validation primers for ADE2 locus
PR_DIV1289	ATTCACGGAUGATGCAGCTGCGCAAGACCACATCGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG	For construction of the basic tRNA-based sgRNA expression cassette (ADE2 v1)
PR_DIV1290	ATTCACGGAUGATGCACAATGGAGACCGAAGTGTTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG	For construction of the basic tRNA-based sgRNA expression cassette (ADE2 v1)
PR_DIV1291	CTTTTCCAAGAATCGTAGAAACGATTAAAAAACTTCCAAACTCTCGAATTTAGTATTGTTTTTTAATAGATGTATATATAATAGTACACG	ss-GTS for ADE2 deletion
PR_DIV1292	TTTTTCACCTGCTAAGCACATTAATGCTGCGCAAGACCACATCtAgATCATTCAAAGATGAGGAGGCTATCGCCAAGTTAGCTGCCAAAT	ss-GTS for ADE2 mutation
PR_DIV1509	CGTAACAACAACTCACAGTCGATTGATTTGTAGACCTTTGTGGTAGTCGTAAAATCGACTGGTATTTGACAGGTTGGGGAG	For construction of GTS (60bp tails) integrating mVenus into IS1
PR_DIV1510	GAATGTTGTAAGAGGAGATTGGTTTTTAGGTGAGGAGAATGTAGGGCCATGTGCTTCCATCTTCGAGCGTCCCAAAACC	For construction of GTS (60bp tails) integrating mVenus into IS1
PR_DIV1511	GATCCAATGGGACAGGCTCCCCAAATTTATCAGCAACACGCCAATTTCTATCAAAATCCTGGTATTTGACAGGTTGGGGAG	For construction of GTS (60bp tails) integrating mVenus into IS3
PR_DIV1512	ATCACGGTGAGTCTCATATGAATAGTGCCCATTAGTGCGGACAGGTGAATGCTGCTGGTGCTTCGAGCGTCCCAAAACC	For construction of GTS (60bp tails) integrating mVenus into IS3
PR_DIV1517	GGGTTTAAUTAAGTCCTCAGCGGTATTTGACAGGTTGGGGAG	For construction of pDIV479 plasmid
PR_DIV2118	aGAAACAGUCCAACAGTAAGCGTTTTAG	Assembly of multiplex sgRNA targeting IS sites
PR_DIV2119	aCTGTTTCUGCATCaTCCGTGAAtCGAAC	Assembly of multiplex sgRNA targeting IS sites
PR_DIV2120	aTGTTGAUAATGGACCGTGGGG	Assembly of multiplex sgRNA targeting IS sites
PR_DIV2121	aTGTTGAUAATGGACCGTGGGG	Assembly of multiplex sgRNA targeting IS sites
PR_DIV2122	aCTGCCGCAUGAGATGAGATG	Assembly of multiplex sgRNA targeting IS sites
PR_DIV2123	aTGCGGCAGUTGCATCATCCGTGAATCGAAC	Assembly of multiplex sgRNA targeting IS sites
PR_DIV2124	aCTGGGGAGAUGCAGAGGTTTTAG	Assembly of multiplex sgRNA targeting IS sites
PR_DIV2125	aTCTCCCCAGUTTCtGCATCATCCGTGAATCGAAC	Assembly of multiplex sgRNA targeting IS sites
PR_DIV2268	ACATTCTAGAGTTCCATTTCTCAATTACTGATAATCAATTTAAAGGCAAGAATAAAAGTTGCTCAGCTGAACTTATTTGGTTACTTATCA	90 nt ss-GTS for PEP4 deletion
PR_DIV2269	CGGGCATAACTTTAGGGATG	For validation of of integration in IS sites
PR_DIV2394	AAAGTCCAUGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG	Assembly of multiplex sgRNA targeting PEP4 and uidA
PR_DIV2395	ATGGACTTUCCTGAACCATGCATCATCCGTGAATCGAAC	Assembly of multiplex sgRNA targeting PEP4 and uidA
PR_DIV2396	AGAAGCGTUGGACCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG	Assembly of multiplex sgRNA targeting PEP4 and uidA
PR_DIV2397	AACGCTTCUGAAGTATGCATCATCCGTGAATCGAAC	Assembly of multiplex sgRNA targeting PEP4

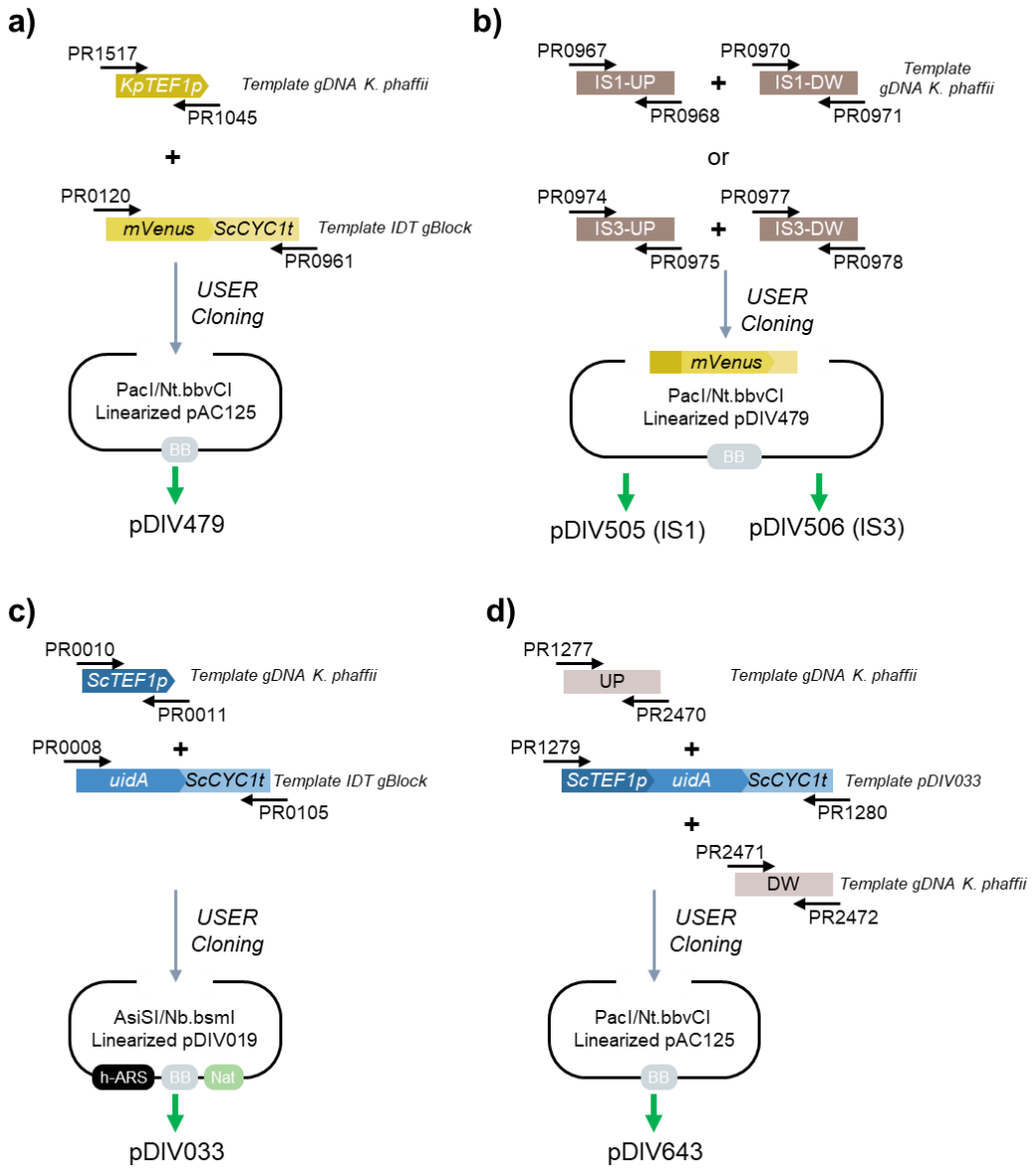
Name	Sequence	Purpose
		and uidA
PR_DIV2449	CGTGGAAGCAGCAACGAGG	Validation primes for restoring KU70 gene
PR_DIV2451	TGGCGATGATATCAAAGTTGG	Validation primes for restoring KU70 gene
PR_DIV2470	AATGCTTUGGATGTCGTATTGCTTGCTGAC	Primers for Construction of pDIV643 plasmid
PR_DIV2471	AAGGTGUCGGCTTCAGAAGGGAAATCT	Primers for Construction of pDIV643 plasmid
PR_DIV2472	GGTCTTAAUAGGACAGGTGAAGTAAACCCG	Primers for Construction of pDIV643 plasmid
PR_DIV2599	CTCTCTTGTAGGGGTCTCTACTGG	Validation primes for restoring KU70 gene
PR_DIV2660	CGTGGAAGCAGCAACGAGG	For construction of GTS to restore KU70
PR_DIV2661	TTTCCAAATTCAGGTCTTGTAACTC	For construction of GTS to restore KU70
PR_DIV3907	ATTTCTCAATTACTGATAATCAATTTAAAGGCAAGAATAAAAGTT GCTCAGCTGAACTTA	60 nt ss-GTS for PEP4 deletion
PR_DIV3908	TACTGATAATCAATTTAAAGGCAAGAATAAAAGTTGCTCA	40 nt ss-GTS for PEP4 deletion

Supplementary table S2. Plasmids used this study.

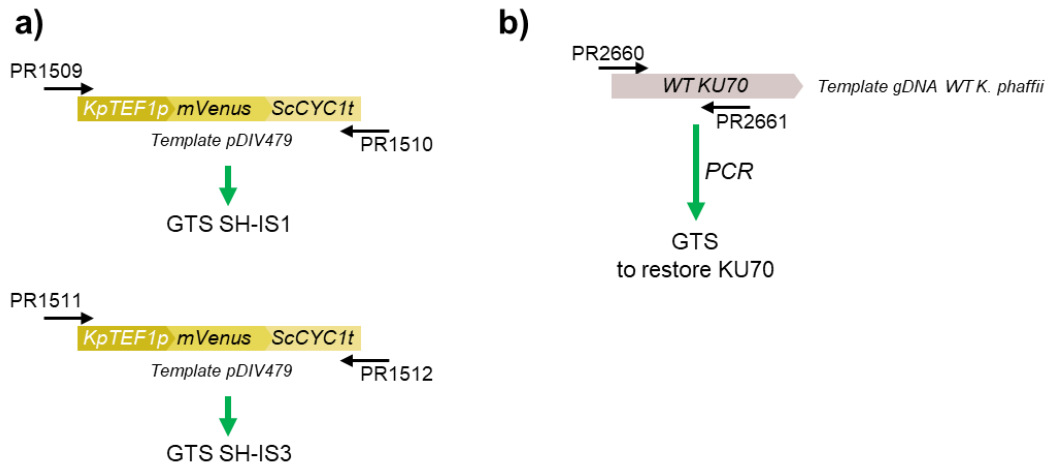
Name	Parent	Purpose	Source
pAC125	---	Backbone vector with USER cloning cassette (PacI/Nt.BbvCI) AmpR marker	Hansen et al. 2011
pCfB2312	---	Expresses Cas9 cassette. Template for PCR for cloning pDIV151.	Stovicek et al 2015
pDIV019	---	Multi-species compatible vector with USER cassette (AsiSI/Nb.BsmI) and NatMX marker	Strucko et al. 2021
pDIV033	pDIV019	A template for the uidA cassette amplification and positive control for X-Gluc screen.	This study
pDIV151	pDIV019	Cas9 expressing vector harboring USER cloning cassette (AsiSI/Nb.BsmI)	This study
pDIV153	pDIV151	Generic Cas9-sgRNA vector to be used as a template for new plasmid cloning	This study
pDIV259	pDIV151	Cas9-sgRNA vector targeting IS1 locus	This study
pDIV260	pDIV151	Cas9-sgRNA vector targeting IS3 locus	This study
pDIV270	pDIV151	Cas9-sgRNA vector targeting KU70 gene	This study
pDIV272	pDIV151	Cas9-sgRNA vector targeting uidA gene locus 2	This study
pDIV273	pDIV151	Cas9-sgRNA vector targeting ADE2 gene at locus 1	This study
pDIV274	pDIV151	Cas9-sgRNA vector targeting ADE2 gene at locus 2	This study
pDIV479	pAC125	Basic plasmid expressing mVenus cassette flanked with USER cloning sites	This study
pDIV505	pDIV479	Integrative plasmid ~1000 bp overhangs into Kp IS1	This study
pDIV506	pDIV479	Integrative plasmid ~800 bp overhangs into Kp IS3	This study
pDIV507	pDIV479	Integrative plasmid ~1000 bp overhangs into Kp IS5	This study
pDIV508	pDIV479	Integrative plasmid ~1000 bp overhangs into Kp IS7	This study
pDIV519	pDIV151	Cas9-sgRNA vector targeting IS1 and IS3 loci	This study
pDIV520	pDIV151	Cas9-sgRNA vector targeting IS1, IS3 and IS7 loci	This study
pDIV521	pDIV151	Cas9-sgRNA vector targeting IS1, IS3, IS7 and IS5 loci	This study
pDIV581	pDIV151	Cas9-sgRNA vector targeting PEP4 gene	This study
pDIV602	pDIV151	Multiplex Cas9-sgRNA vector targeting PEP4 and uidA genes	This study
pDIV643	pAC125	Integrative plasmid ~ 500 bp overhangs for disruption of KU70 gene via uidA cassette.	This study



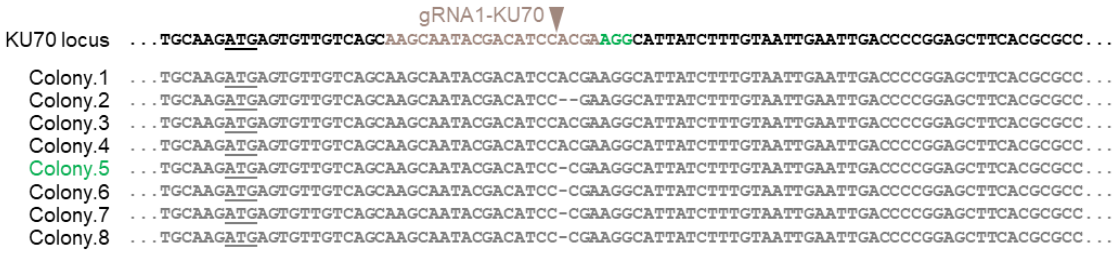
Supplementary Figure S1. USER cloning workflow for assembly of Cas9-sgRNA vectors used in this study. Cloning procedure for assembly of Cas9 expressing vectors **a)** pDIV151, **b)** pDIV153, **c)** pDIV259, pDIV260, pDIV270, pDIV272, pDIV273 and pDIV274, and **d)** pDIV602. Thin black arrows (annotated by "PR####") represent primers used for PCR amplification. Colored text boxes show DNA fragments containing functional parts. Thick green arrows indicate the name of a final construct in each specific case.



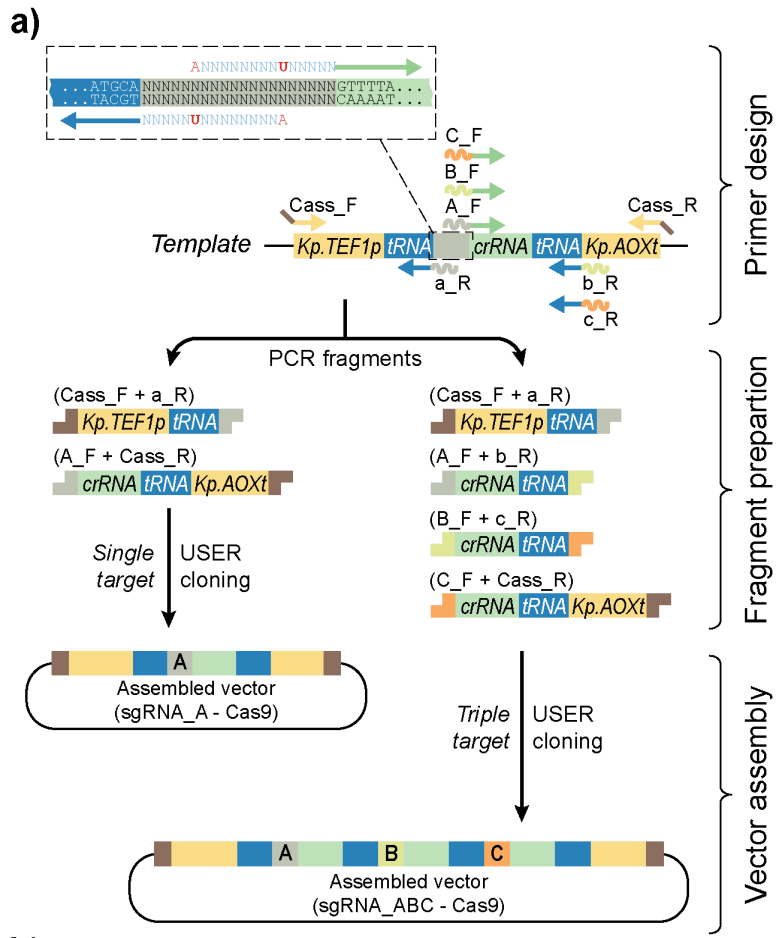
Supplementary Figure S2. USER cloning workflow for assembly of vectors harboring GTSs used in this study. Cloning procedure for assembly of **a)** pDIV479 plasmid, **b)** pDIV505 and pDIV506 plasmids, **c)** pDIV033, and **d)** pDIV643 plasmid. Thin black arrows (annotated by "PR####") represent primers used for PCR amplification. Colored text boxes show DNA fragments containing functional parts. Thick green arrows indicate the name of a final construct in each specific case.



Supplementary Figure S3. PCR-based for assembly of linear GTSs used in this study. Amplification of PCR fragments **a)** GTS-SH-IS1 and GTS-SH-IS3 and **b)** GTS to restore KU70 gene. Thin black arrows (annotated by "PR####") represent primers used for PCR amplification. Colored text boxes show DNA fragments containing functional parts. Thick green arrows indicate the name of a final construct in each specific case.



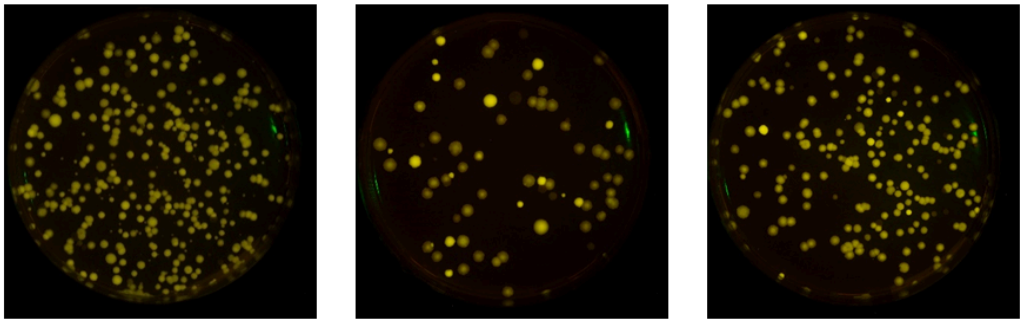
Supplementary Figure S4. An excerpt of Sanger sequencing results of *KU70* locus. Eight random colonies were selected from transformants by pDIV270 plasmid (sgRNA1-KU70). Light brown text indicates 20 nt guiding sequence, green denotes for PAM sequence. Underlined text indicated a start codon of the *KU70* gene.



b)

Cass_F	CGTGCGAUTAACTCTGGCAACCAGTAACACG
x_R	NNNNNNNNTGCATCATCCGTGAATCGAAC
X_F	NNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
Cass_R	CACGCGAUTCTGTACTCTGAAGAGGAGTGGG

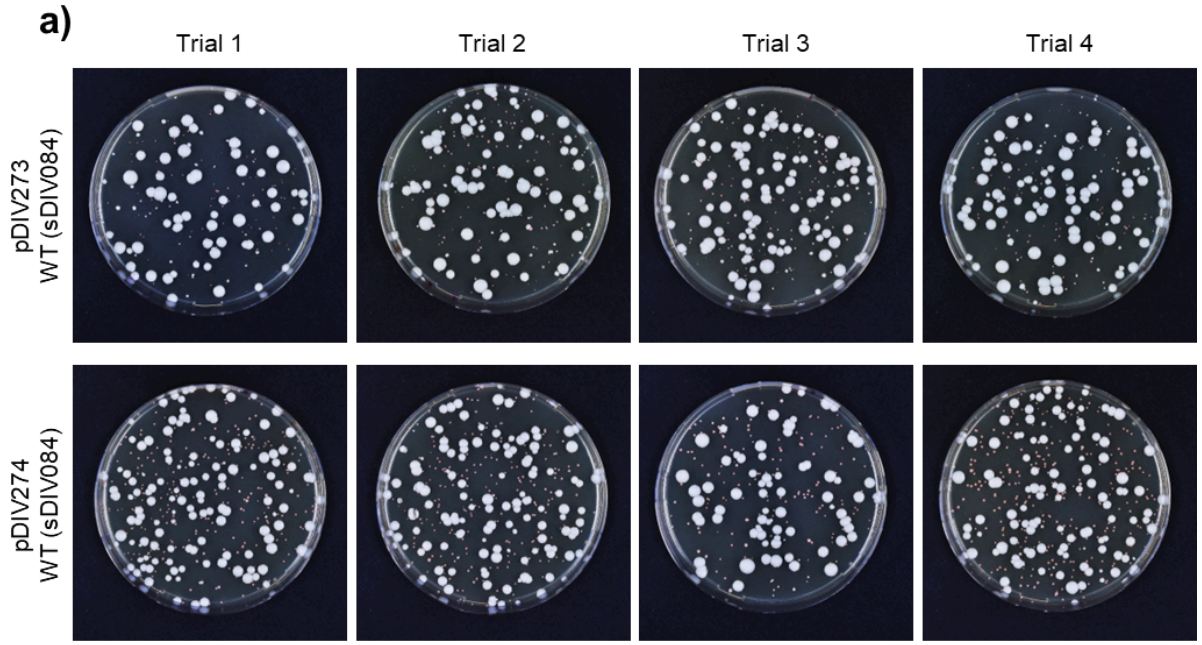
c)



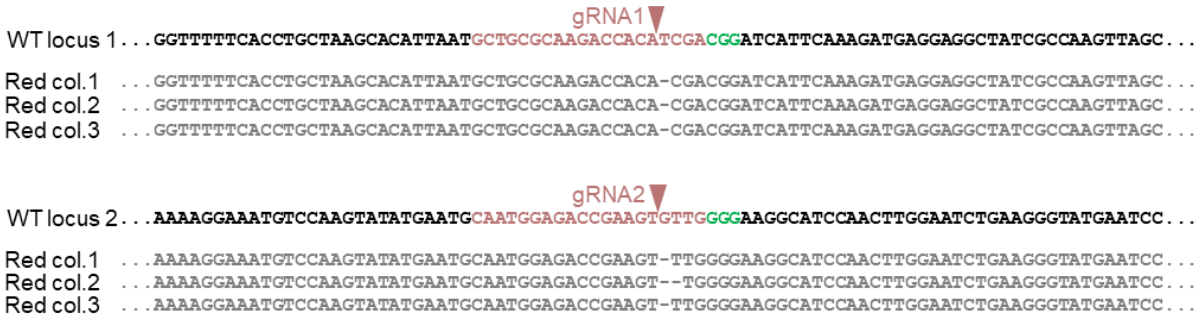
Fluorescent colonies, %	~ 95	~ 93	~ 94
PCR validated col. (correct/total)	4/5	4/5	3/5

Supplementary Figure S5. A generalized protocol for Cas9-sgRNA vector assembly using USER cloning and multiplex experimental test. **a)** Schematic depiction of a workflow for sgRNA-Cas9 vector assembly. The workflow can be split into three steps:

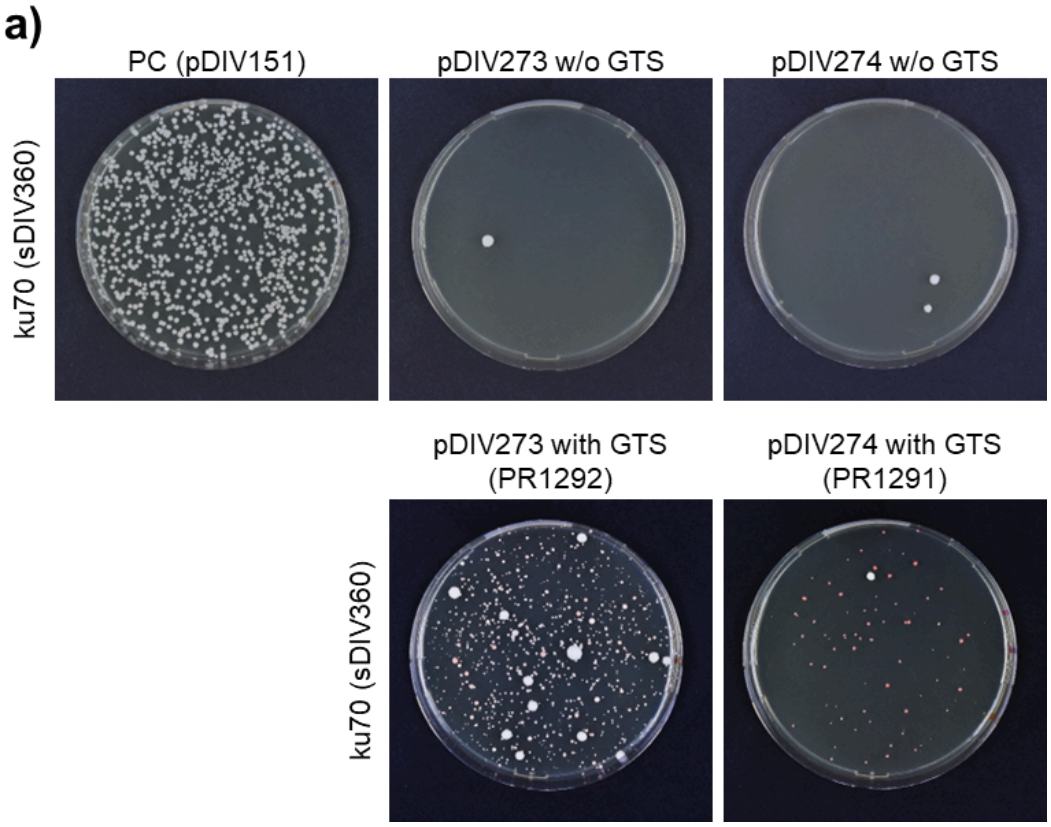
1-Primer design, 2-Fragment preparation, and 3-Vector assembly. In the step1, for each new sgRNA two primers (thick blue and green arrows) equipped with uracil containing tails are designed (depicted in the top left box). The tails are designed so that upon USER assembly it will encode a target specific 20nt guiding sequence (depicted as colorful wave lines and annotated with lowercase letters “a, b, c” for the reverse “R” primers, and capital letters “A, B, C” for forward “F” primers. In the step2, specific uracil tails containing fragments are generated by PCR using primers depicted in parenthesis. In case of a single sgRNA that would guide to the target “A”, two PCR fragments are generated and assembled into a Cas9 expressing vector (left side of the figure). In case of multiple sgRNAs that would target (in this example three targets) “A, B and C”, four fragments are generated and cloned into a Cas9 expressing vector (right side of the figure). Using the same logic, vectors expressing two, four or more sgRNAs can be assembled **b)** Standard primer sequences for sgRNA cassette cloning. N - represents a USER tail that would result in a unique targeting sequence upon assembly. **c)** As a proof-of-concept, we first tested whether our CRISPR method allows for multiplexing. To explore this possibility, we designed four GTSs containing plasmids pDIV505, pDIV506, pDIV507 and pDIV508 expressing the mVenus gene flanked by 1000 bp up- and downstream targeting sequences of four integration sites IS1, IS3, IS5, and IS7, respectively. In parallel, we made three CRISPR vectors producing sgRNAs targeting two, three and four different ISs: pDIV519 (IS1 + IS3), pDIV520 (IS1 + IS3 +IS7), and pDIV521 (IS1+IS3+IS5+IS7). We then performed three co-transformation experiments with the appropriate combinations of GTSs and CRISPR vectors. In all three cases, transformants displaying varying intensities of fluorescence were obtained indicating that colonies with increasing mVenus gene copy were achieved as a result of multiplexing. This view was confirmed by performing diagnostic PCR reactions of five colonies from each of the three experiments as the majority of the transformants analyzed contained the maximum number of two, three, or four mVenus gene copies expected from the experiment. We note that for each experiment, formation of the highly fluorescent transformants is not the most frequent event; and for mutations that do not cause a visible phenotype, additional screening will therefore be necessary to identify the transformants containing all desired gene alterations. On the other hand, if the goal is to delete many genes, our results indicate that transformants containing all possible deletions can be easily selected if the deleted genes are replaced with a visible marker.



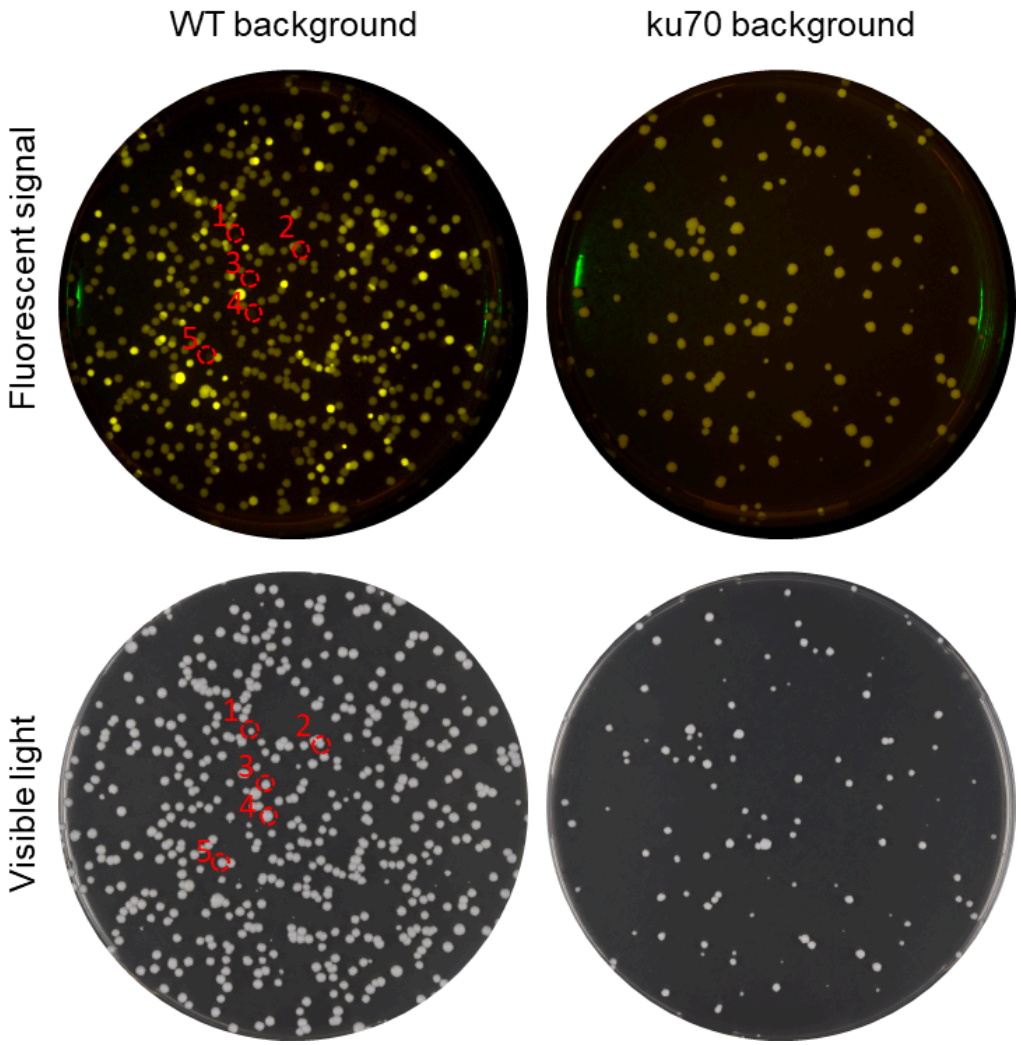
Supplementary Figure S6. Images of Transformation plates of *ADE2* experiment. Top row, four replicates transformed with pDIV273 plasmid, bottom row, with pDIV274 plasmid. Note, the significantly decreased fitness represented by significantly smaller size of red cells as compared to the white ones. Image contrast was enhanced for better color representation.



Supplementary Figure S7. An excerpt of Sanger sequencing results of ADE2 locus. Three red colonies resulting from transformants by pDIV273 (sgRNA1) plasmid (top panel) and from transformants by pDIV274 (sgRNA2) plasmid (bottom panel) were sequenced and aligned to the wild-type loci of *ADE2*. Light red text indicates 20 nt guiding sequences, green- PAM sequences.

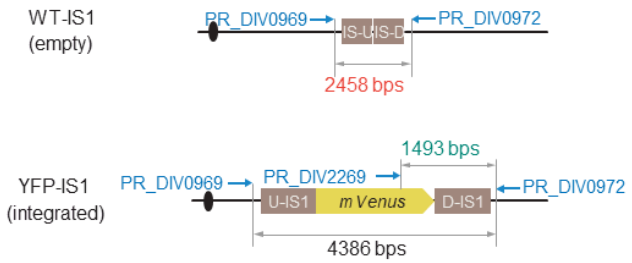


Supplementary Figure S8. Images of agar plates representing a TAPE experiment. A TAPE is used to evaluate how efficient a given sgRNA guides a CRISPR nuclease to the desired target site. The TAPE experiment compares the transformation efficiencies of two different experiments. First, it compares the efficiencies obtained with CRISPR plasmid encoding the sgRNA to a corresponding plasmid that does not. A relatively low number of transformants obtained with the sgRNA coding CRISPR plasmid indicates efficient cleavage as most transformants die in the absence of a repair template. Second, it compares the efficiencies obtained with the sgRNA coding CRISPR vector in the absence and presence of a relevant repair template. If a relatively high transformation efficiency is obtained in the presence of a repair template, the cause of death in the absence of the repair template is most likely due to a break in the target sequences. Moreover, the experiment shows that this break is useful for genetic engineering by gene targeting. Each plate represents equimolar amounts of plasmid DNA transformed. Note, only few colonies of *ku70* strain survive when sgRNA-Cas9 plasmids are transformed without repair templates (GTS).

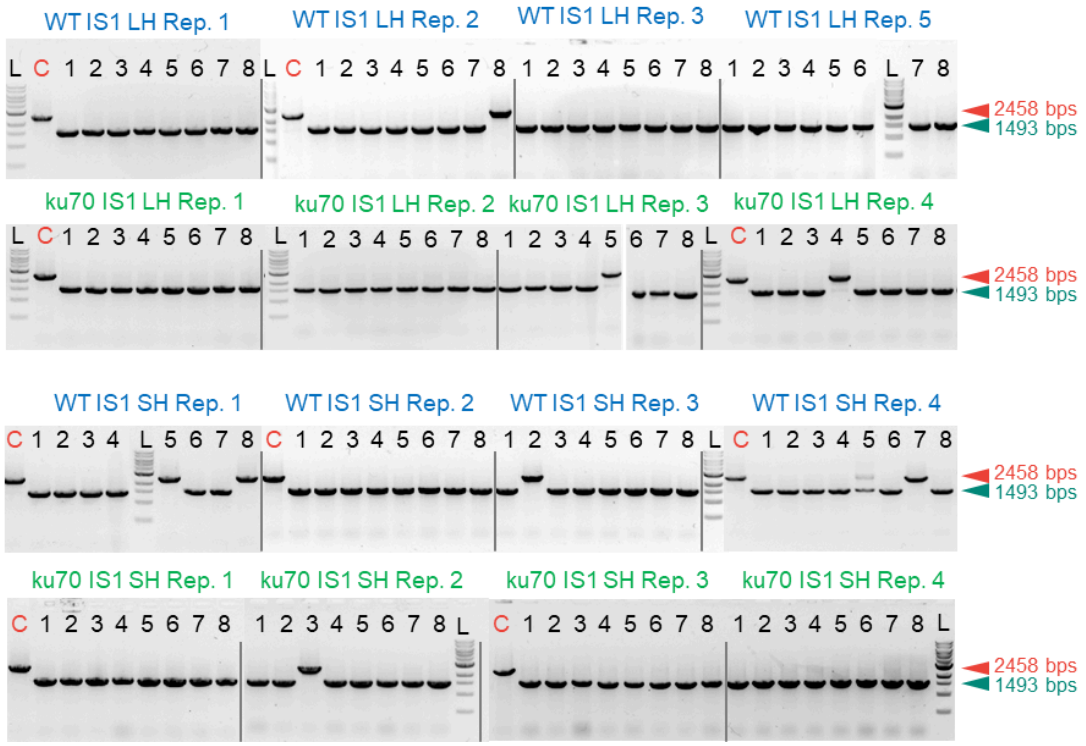


Supplementary Figure S9. Images of agar plates with yellow fluorescent colonies. Top row, fluorescent signal, bottom row, images under visible light. The full plate pictures representing the snippets from Figure 2 in the main text. Red dashed circles show the location of five non-fluorescent colonies (top) that are visible under normal light (bottom). Left, a typical transformation (GTS-mVenus + sgRNA-Cas9 plasmid) plate image when mVenus expression cassette is transformed into WT (NHEJ proficient) strain. Right, a typical transformation (GTS-mVenus + sgRNA-Cas9 plasmid) plate image when mVenus expression cassette is transformed into ku70 (NHEJ deficient) strain.

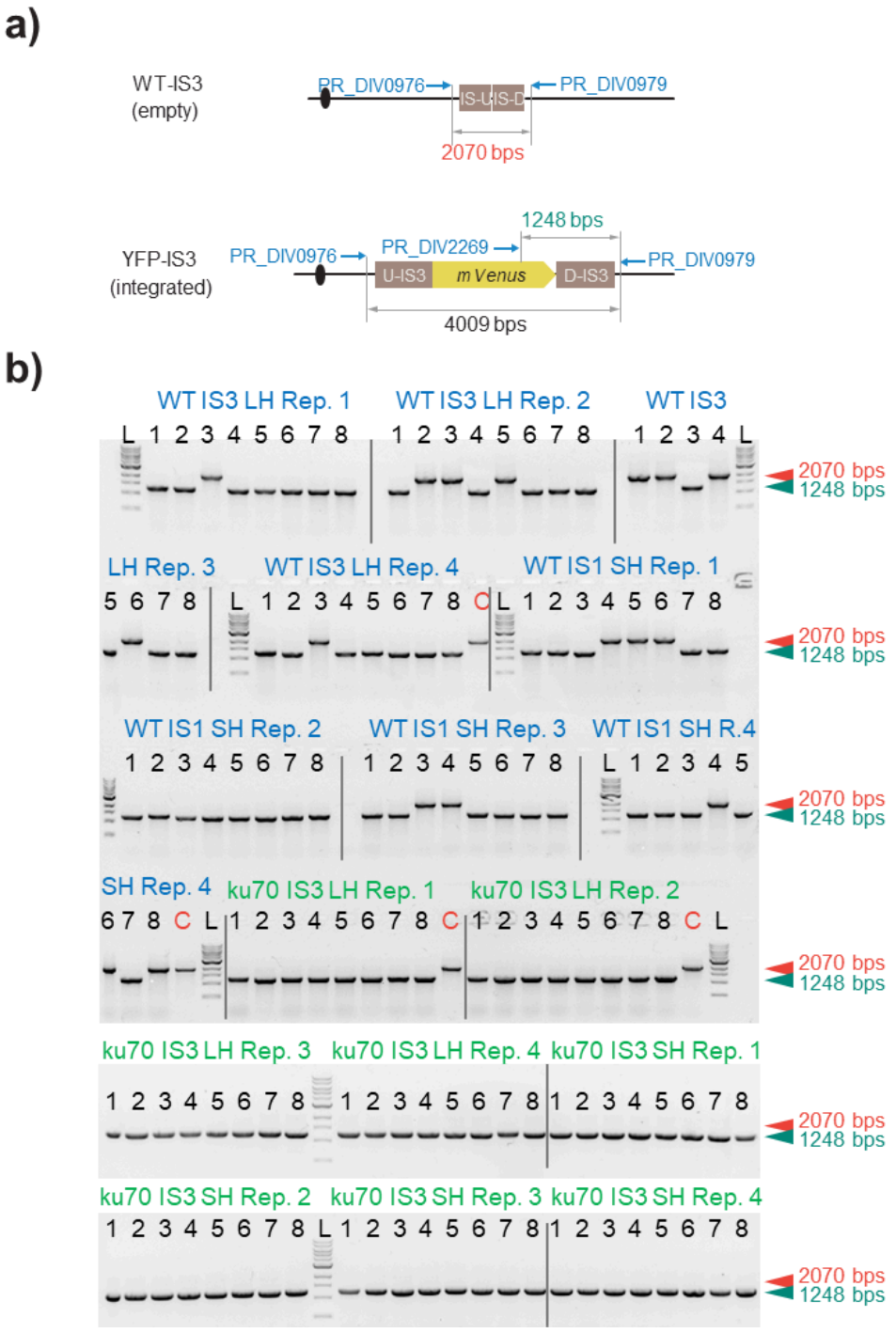
a)



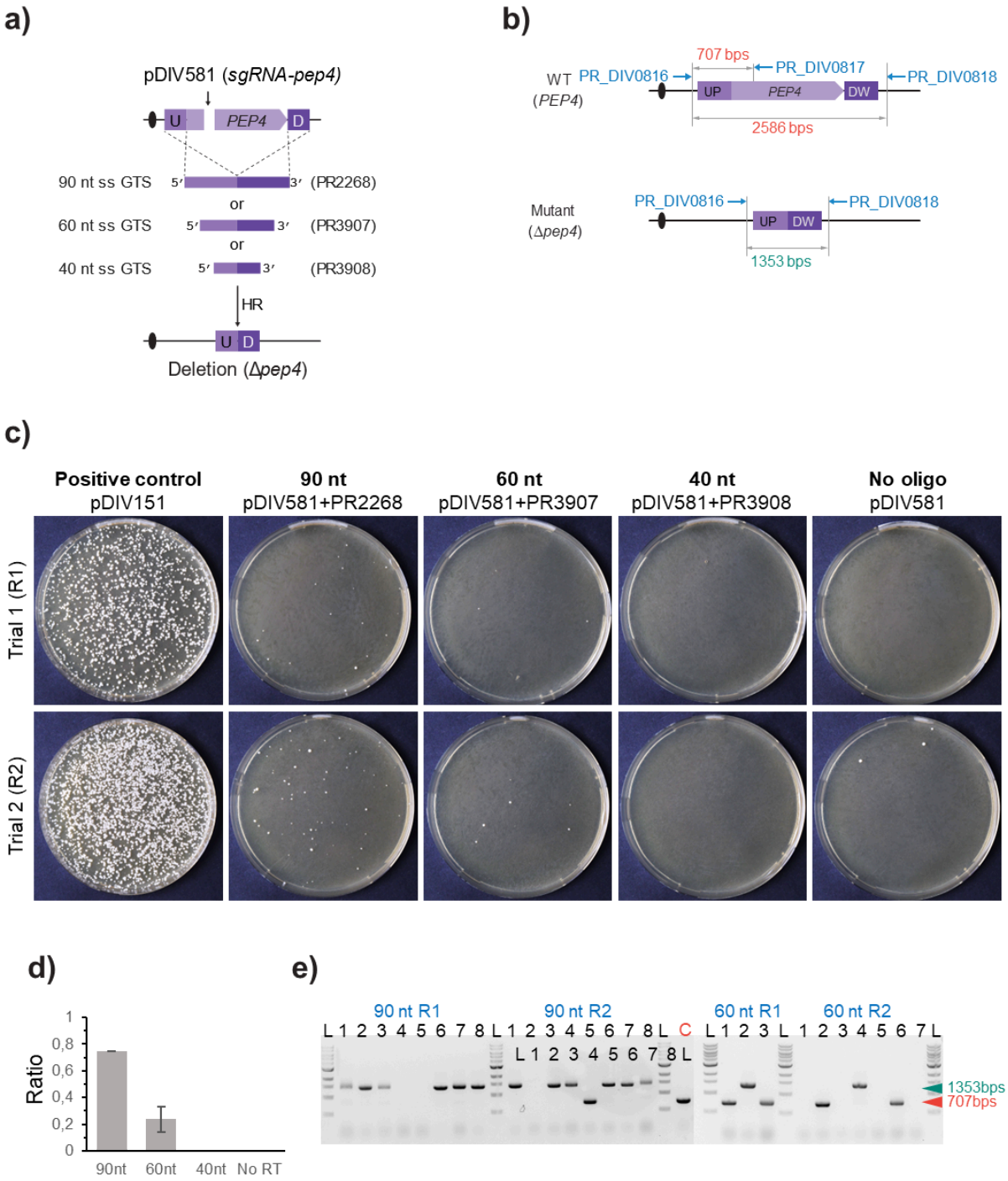
b)



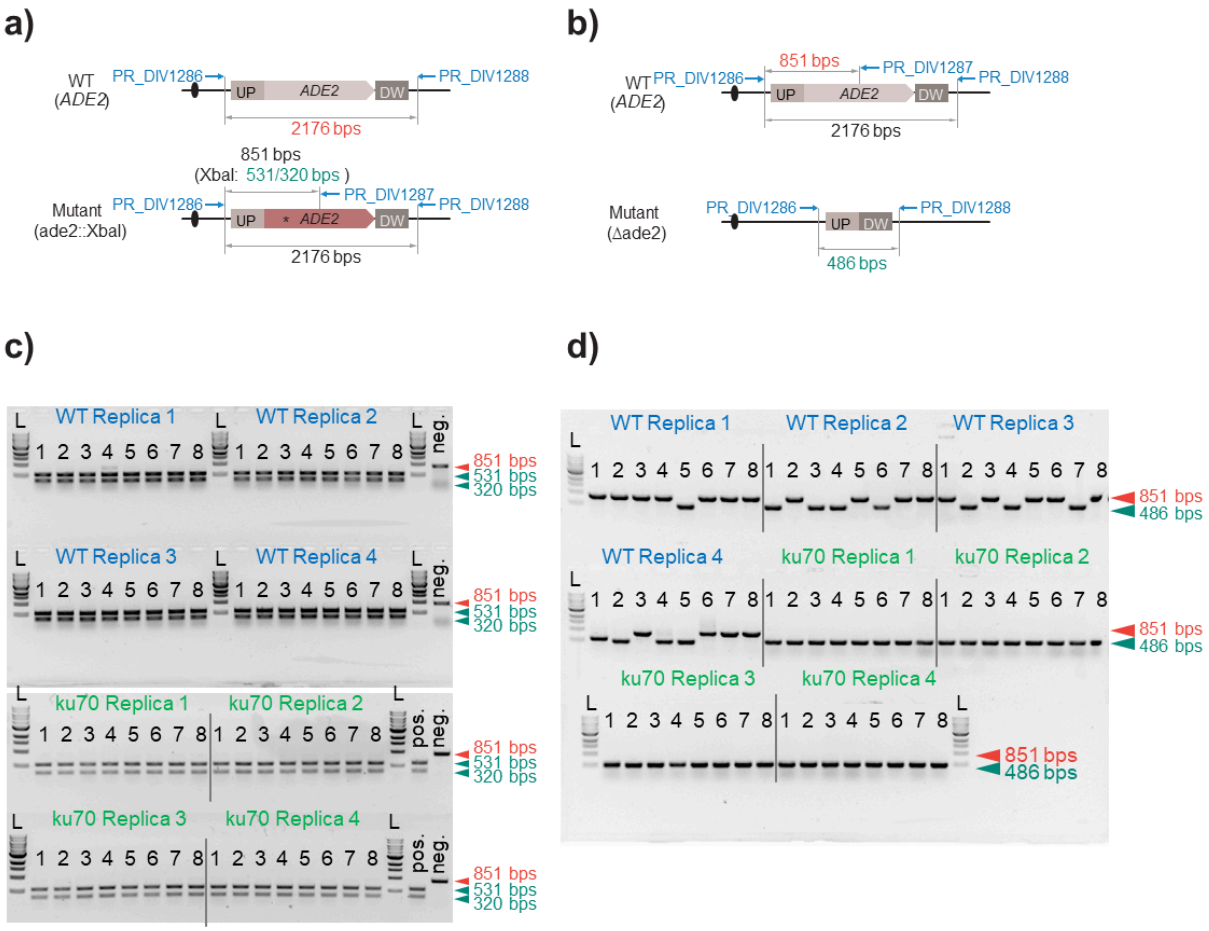
Supplementary Figure S10. Validation of integrated mVenus cassette into integration site (IS1). **a)** Schematic depiction of the IS1 locus, top wild-type (WT) and bottom, modified (YFP-IS1). Blue arrows indicate primer binding positions, and their names (blue text). Red text indicates expected band size if the locus is "empty" and green text - when mVenus is correctly integrated in the IS1 site. **b)** A composite image of agarose gels showing colony PCR results for IS1 locus. Blue text, bands obtained in NHEJ proficient (WT) background strain, and green text in NHEJ deficient (ku70) strain. Four biological replicates (Rep.) were tested in each strain background. L - 1 kb ladder (NEB), C - untransformed strain was used as a control.



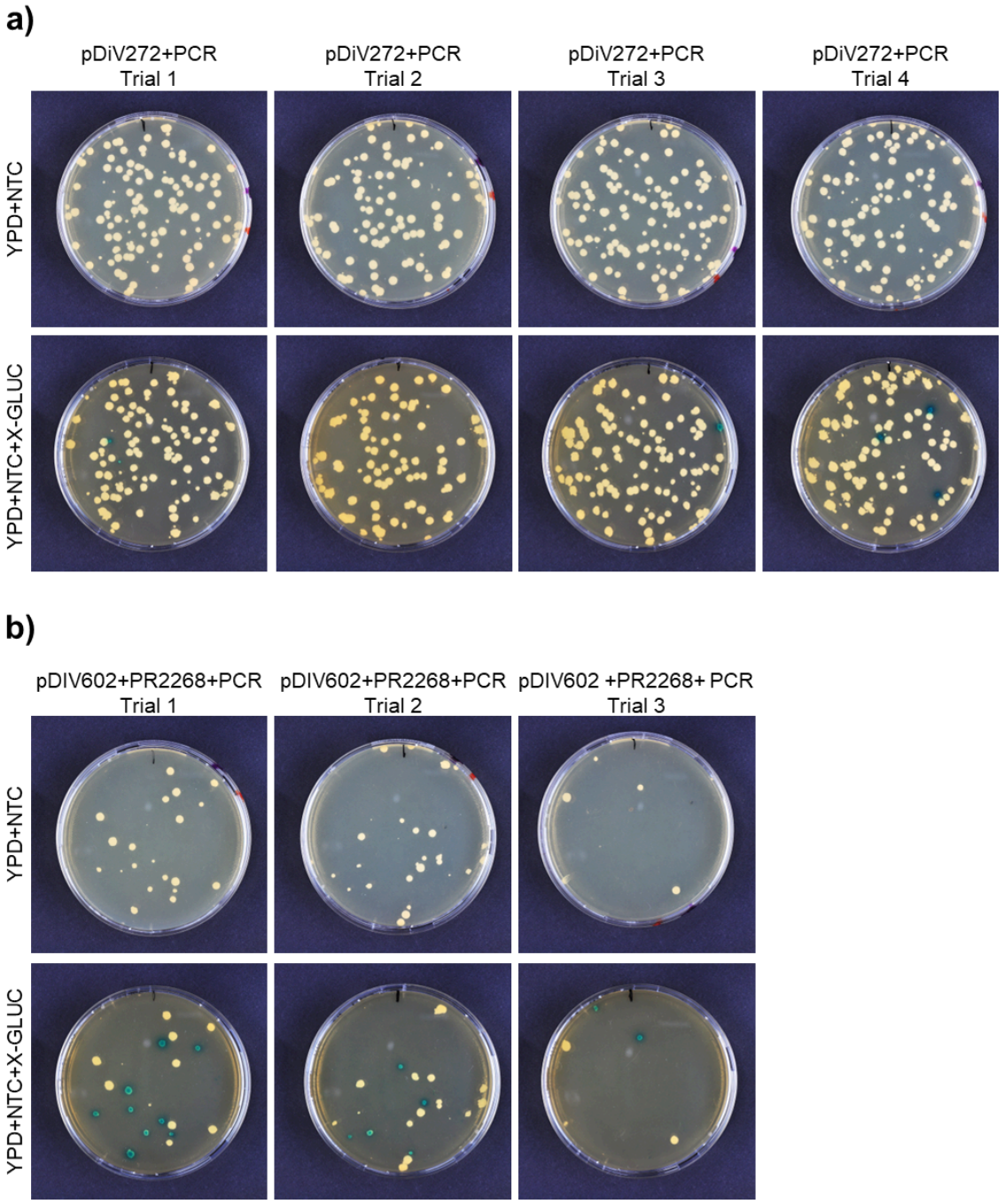
Supplementary Figure S11. Validation of integrated mVenus cassette into integration site (IS3). **a)** Schematic depiction of the IS3 locus, top wild-type (WT) and bottom, modified (YFP-IS3). Blue arrows indicate primer binding positions, and their names (blue text). Red text indicates expected band size if the locus is "empty" and green text - when mVenus is correctly integrated in the IS3 site. **b)** A composite image of agarose gels showing colony PCR results for IS3 locus. Blue text, bands obtained in NHEJ proficient (WT) background strain, and green text in NHEJ deficient (ku70) strain. Four biological replicates (Rep.) were tested in each strain background. L - 1 kb ladder (NEB), C - untransformed strain was used as a control.



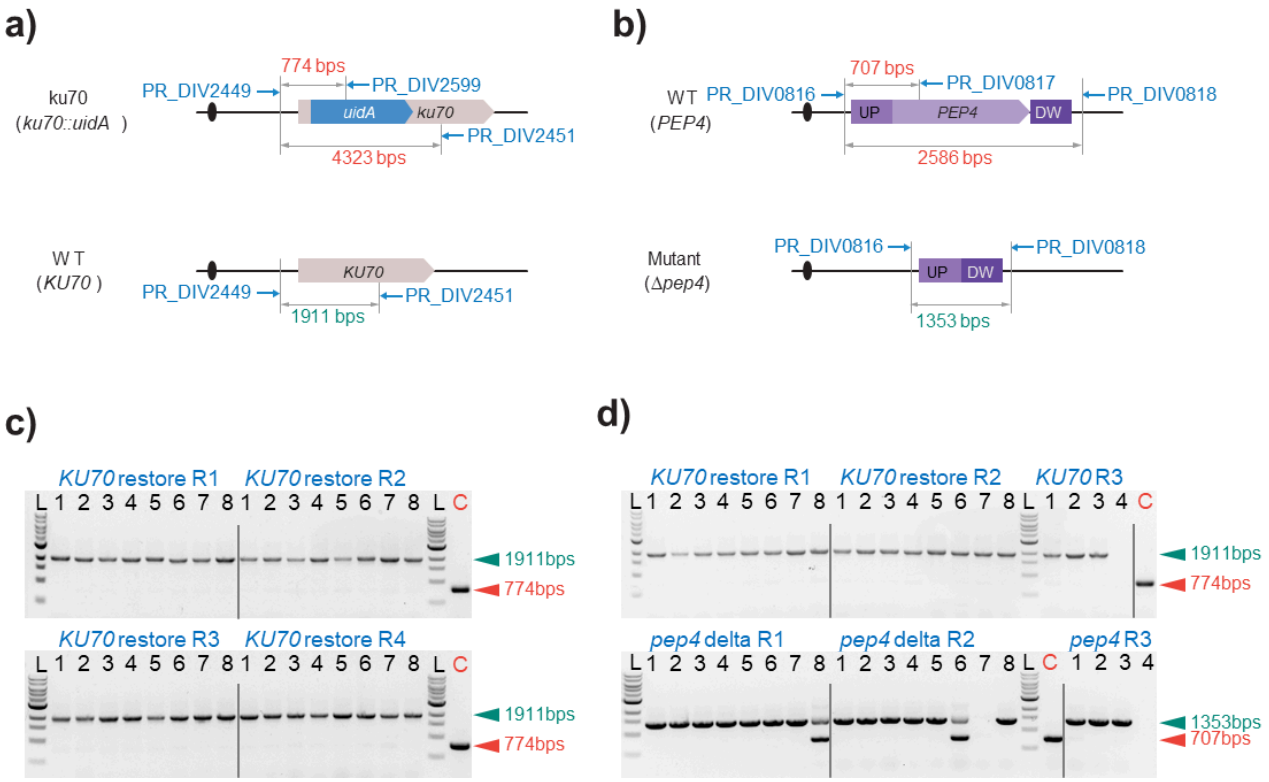
Supplementary Figure S12. Single-stranded oligonucleotide size test for PEP4 gene deletion. **a)** Schematic depiction of *PEP4* gene-mutagenesis using a short single-stranded GTSs for full deletion. U - upstream and D - downstream.. **b)** Schematic depiction of the *PEP4* locus validation by PCR, top wild-type (WT) and bottom, deleted (Δpep4). Blue arrows indicate primer binding positions, and their names (blue text). Red text indicates expected band size if the locus is wild-type "*PEP4*". Green text shows band size if the *PEP4* gene was deleted. **c)** Transformation plates of the PEP4 gene deletion experiment usig varying size oligonucleotide GTSs. **d)** Results of *PEP4* locus mutation efficiencies using short oligonucleotides as GTS. The bars show the ratio of colonies with the expected GTS directed mutation vs. total number of tested colonies for the corresponding GTS in *ku70* (NHEJ deficient) strains. Error bars represent standard deviation based on two biological replicates.. **e)** A composite image of agarose gels showing colony PCR results for *PEP4* gene deletion via ss-GTS. Two biological replicates (R#) were tested in each strain background. L - 1 kb ladder (NEB), C - unaltered strain with unaltered PEP4 locus used as a control.



Supplementary Figure S13. Validation of modified ADE2 locus using short single stranded DNA oligonucleotides as GTS. **a)** Schematic depiction of the *ADE2* locus, top wild-type (WT) and bottom, modified (ade2::XbaI). Blue arrows indicate primer binding positions, and their names (blue text). Red text indicates expected band size if the locus is "*ADE2*". Green text shows sizes of two bands resulting after a PCR fragrant is digested with XbaI enzyme if ADE2 locus was correctly mutated. **b)** Schematic depiction of the *ADE2* locus, top wild-type (WT) and bottom, deleted (Δade2). Blue arrows indicate primer binding positions, and their names (blue text). Red text indicates expected band size if the locus is wild-type "*ADE2*". Green text shows band size if the *ADE2* gene was deleted. **c)** A composite image of agarose gels showing colony PCR results for *ADE2* locus mutation via ss-GTS. PCRs were treated with XbaI enzyme prior loading on gel. Blue text, bands obtained in NHEJ proficient (WT) background strain, and green text in NHEJ deficient (ku70) strain. Four biological replicates (Replica) were tested in each strain background. L - 1 kb ladder (NEB), pos. - positive control, neg- negative control. **d)** A composite image of agarose gels showing colony PCR results for *ADE2* locus deletion via ss-GTS. Blue text, bands obtained in NHEJ proficient (WT) background strain, and green text in NHEJ deficient (ku70) strain. Four biological replicates (Replica) were tested in each strain background. L - 1 kb ladder (NEB), pos. - positive control, neg- negative control.



Supplementary Figure S14. Images of agar plates of *ku70* reversion and *PEP4* deletion experiment. **a)** Transformation plates (top row) and corresponding replica plates (bottom row) of the *ku70* reversion. **b)** Transformation plates (top row) and corresponding replica plates (bottom row) of the multiplex experiment for *PEP4* deletion and *ku70* reversion



Supplementary Figure S15. Validation of modified *KU70* locus *PEP4* gene deletion. **a)** Schematic depiction of the *KU70* locus, top modified (*ku70::uidA*) and bottom, wild-type (*KU70*). Blue arrows indicate primer binding positions, and their names (blue text). Red text indicates expected band size if the locus is "*ku70::uidA*". Green text shows the size of a PCR band when KU70 was restored. **b)** Schematic depiction of the *PEP4* locus, top wild-type (WT) and bottom, deleted (Δpep4). Blue arrows indicate primer binding positions, and their names (blue text). Red text indicates expected band size if the locus is wild-type "*PEP4*". Green text shows band size if the *PEP4* gene was deleted. **c)** A composite image of agarose gels showing colony PCR results for *KU70* locus. Four biological replicates (R#) were tested in each strain background. L - 1 kb ladder (NEB), C - untransformed sDIV291 strain was used as a control. **d)** A composite image of agarose gels showing colony PCR results for simultaneous recovery of *KU70* (top Gel) and *PEP4* (bottom Gel) gene deletion via ss-GTS. Three biological replicates (R#) were tested in each strain background. L - 1 kb ladder (NEB), C - untransformed sDIV291 strain was used as a control.