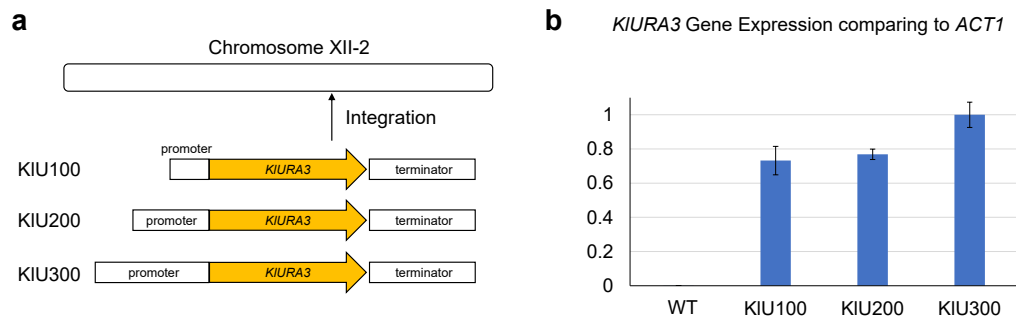


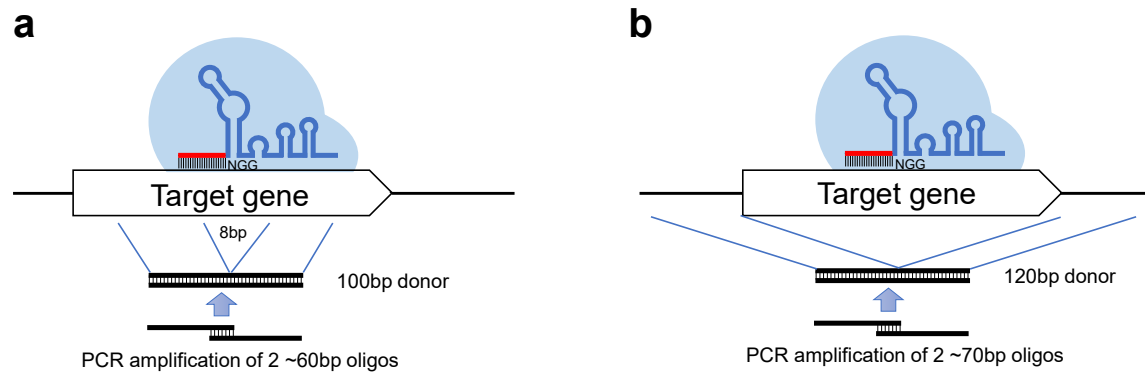
Supplementary Information

**A gRNA-tRNA Array for CRISPR-Cas9 Based Rapid
Multiplexed Genome Editing in *Saccharomyces cerevisiae***

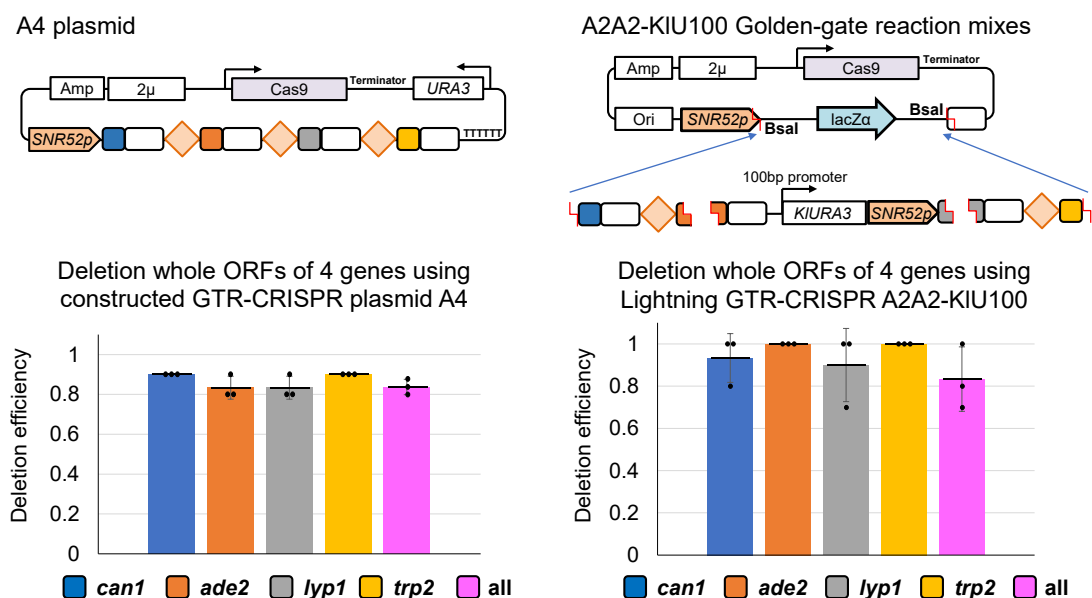
Zhang et al.



Supplementary Figure 2. Gene expression for *KIURA3* with different sizes of its promoter. **(a)** Graphic representations of integration of *KIURA3* with different sizes of its promoter. **(b)** Gene expression analysis of *KIURA3* comparing to *ACT1* by real-time PCR. The error bars indicate the standard deviation of all (n=4) biological replicates. Source data are provided as a Source Data file.



Supplementary Figure 3. Graphic representation of the PCR generation of donors for GTR-CRISPR system. Donors for repairing DSB were generated through PCR amplification of two oligonucleotide primers for (a) gene disruptions 100 bp and (b) whole ORFs deletions 120 bp. The Melting Temperatures (T_m) for primers are in the range of 52-60°C.



Supplementary Figure 4. GTR-CRISPR system and Lightning GTR-CRISPR system for simultaneous whole ORF deletions of 4 targets. Graphic representations and results of deleting the whole ORFs of 4 genes using constructed plasmid (GTR-CRISPR system) or Golden Gate reaction mix (Lightning GTR-CRISPR system). The data of bar charts represent mean averages of each gene or over-all disruption efficiencies. Each black dot represents the gene disruption efficiency of 10 colonies from each biological replicate, and the error bars indicate the standard deviation of all (n=3) biological replicates. Source data are provided as a Source Data file.



	WT	GTR1	GTR2	GTR3
<i>faa1Δ, faa4Δ, pox1Δ, are2Δ</i>	-	+	-	+
<i>pah1Δ, dpp1Δ, lpp1Δ, are1Δ</i>	-	-	+	+

Supplementary Figure 5. A picture shows that high levels of free fatty acids precipitated in flasks culture of GTR1 and GTR3 strains at 72 hours.

Supplementary Table 1. The primer design template for the Lightning GTR-CRISPR system (A2A2-KIU100)

PCR template	Primers	Primer Sequences
psgtRNA	Primer 1F	AAAGGTCTCA GATC NNNNNNNNNNNNNNNNNNNN GTTT TAGAGCTAGAAATAGCAAGTTA
	Primer 1R	AAAGGTCTCA RRRR RRRRRRRR TGC GCAAGCCCGGAATCGAACC GGG
pKIURA100	Primer 2F	AAAGGTCTCA NNNN NNNNNNNN GTTT TAGAGCTAGAAATAGCAAGTTAA
	Primer 2R	AAAGGTCTCA RRRR RRRRRRRR GATCATTATCTTCACTGCGG
psgtRNA	Primer 3F	AAAGGTCTCA NNNN NNNNNNNN GTTT TAGAGCTAGAAATAGCAAGTTAA
	Primer 3R	AAAGGTCTCT AAAC RRRRRRRRRRRRRRRRRRRR TGC GCAAGCCCGGAATCGAACC GGG

Note: N means the forward DNA sequence, R mean the reverse DNA sequence. The letters in red represent for the 1st gRNA target, greens for the 2nd target, yellows for the 3rd target, and blues for the 4th target. The letters highlighted in brown and blue are ligated to the pCas plasmid. And letters highlighted in green or yellow are ligated with each other with same color. For the second and third targets, the split of 20 base pairs does not need to be evenly distributed.

Supplementary Table 2. Time calculation (days) of different systems for multiplexed CRISPR/Cas9 based gene editing systems.

CRISPR systems	Gene synthesis	Cas9 pre-transformation ^b	Helper plasmid ^b	gRNA plasmid construction ^c	Recovery after transformation	Yeast growth from plates	Total time spent
Plasmid-required systems							
HI-CRISPR ¹	3-5 ^a	-	-	3-4	2	2	10-13
CRISPRm ²	-	-	3-4	3-4	-	2	8-10
Csy4 based CRISPR ³	-	3	3-4	3-4	-	2	11-13
CasEMBLR ^{4, 5}	-	3	3-4	3-4	-	2	11-13
CRISPR by Mans and Rossum <i>et al</i> ⁶	-	3	-	3-4	-	2	8-9
GTR-CRISPR (This work)	-	-	-	3-4	1	2	6-7
Cloning-free systems							
CRISPR by Generoso <i>et al</i> ⁷	-	-	-	-	-	2	2
CAM ^{8, 9}	-	3	-	-	-	2	5
Lightning GTR-CRISPR (This work)	-	-	-	-	1	2	3

^agBlocks gene fragments (124-750bp) services provided from IDTDNA(US) for 2-4 days shipped, which is 3-5 days arrived.

^bcCalculation includes PCR, enzyme digestion and ligation, *E. coli* transformation and miniprep, and plasmid sequencing.

^bFor some methods, the helper plasmids have to be pre-built for construction the final gRNA plasmids.

Supplementary Note

DNA sequences for PCR amplification

psgtRNA

GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT
GGCACCAGAGTCGGTGC AAACAA GCGCAAGTGGTTTAGTGGTAAAATCCAACGTTGCCATC
GTGGGGCCCCCGGTTTCGATTCCGGGCTTGCGCA

Note: Sequence in yellow: gRNA scaffold; sequence in dark: linker; sequence in purple: tRNA^{Gly}.

pSNRtRNA

GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT
GGCACCAGAGTCGGTGC TTTT TTTATTTTTTGTCACTATTGTTATGTAAAATGCCACCTCTGA
CAGTATGGAACGCAAACCTTCTGTCTAGTGGATAACAGAATTTTTCTATGGCCAATTTA GGA
TCCACTAGT CTTTGAAAAGATAATGTATGATTATGCTTTCACCTCATATTTATACAGAAACT
TGATGTTTTCTTTCGAGTATATACAAGGTGATTACATGTACGTTTGAAGTACAACCTCTAGA
TTTTGTAGTGCCCTCTTGGGCTAGCGGTAAAGGTGCGCATTTTTTCACACCCTACAATGTTT
TGTTCAAAGATTTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCGCATGTTTCGGCGTT
CGAAACTTCTCCGCAGTGAAAGATAAATGATC TAGTTTTACAAGAAAACAAGCGCAAGT
GGTTTAGTGGTAAAATCCAACGTTGCCATCGTTGGGGCCCCCGGTTTCGATTCCGGGCTTGCG
CA

Note: Sequence in yellow: gRNA scaffold; sequence in green: *SNR52* terminator; sequence in blue: *SNR52* promoter; sequence in purple: tRNA^{Gly}.

pScURA

GTTT TAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT
GGCACCGAGTCGGTGCTTTT TTTATTTTTTGTCACTATTGTTATGTAAAATGCCACCTCTGA
CAGTATGGAACGCAAACCTTCTGTCTAGTGGATAACAGAATTTTCTATGGCCAATTTAGGA
TCCCGCGAACCCTGACAGAACAAAAACCTGCAGGAAACGAAGATAAATC ATGTCCG
AAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCCAAGCTATTTA
ATATCATGCACGAAAAGCAAACAAACTTGTGTGCTTCATTGGATGTTTCGTACCACCAAGG
AATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAACACATGTGGA
TATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAGGCATTATCCGCCAAG
TACAATTTTTTACTCTTCGAAGACAGAAAATTTGCTGACATTGGTAATACAGTCAAATTGC
AGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGAATGCACACGGTG
TGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCAGAAGAAGTAACAAAGGAA
CCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCCTATCTACTGGAGAAT
ATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTATCGGCTTTATTGC
TCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTATGACACCCGGTGT
GGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGTATAGAACCGTGGATGATGTCTG
TTCTACAGGATCTGACATTATTATTGTTGGAAGAGGACTATTGCAAAGGGAAGGGATGC
TAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGGAAGCATATTTGAGAAGATGCG
GCCAGCAAAACTAA AAAACTGTATTATAAGTAAATGCATGTATACTAAACTCACAAATTA
GAGCTTCAATTTAATTATATCAGTTATTACCCTGTGTCTAGT CTTTGAAAAGATAATGTAT
GATTATGCTTTCACTCATATTTATACAGAACTTGATGTTTTCTTTGAGTATATACAAGGT
GATTACATGTACGTTTGAAGTACAACCTCTAGATTTTGTAGTGCCCTCTTGGGCTAGCGGTA
AAGGTGCGCATTTTTTACACCCTACAATGTTCTGTTCAAAAGATTTTGGTCAAACGCTGT
AGAAGTGAAAGTTGGTGCATGTTTCGGCGTTTCGAACTTCTCCGCAGTGAAAGATAAA
TGATC TAGTTTTACAAAGAAAACAA GCGCAAGTGGTTTAGTGGTAAAATCCAACGTTGCC
ATCGTTGGGCCCCCGGTTTCGATTCCGGGCTTGCGCA

Note: Sequence in yellow: gRNA scaffold; sequence in green: *SNR52* terminator; sequence in red:

URA3 from *S. cerevisiae* (the sequences around it without color are its endogenous 50bp promoter and terminator); sequence in blue: *SNR52* promoter; sequence in purple: tRNA^{Gly}.

pKIURA300

GTTT TAGAGCTAGAAA TAGCAAGTTAAAA TAAGGCTAGTCCGTTATCAACTTGAAAAAGT
GGCACCGAGTCCGGTGCTTTT TTTCTTTTTTGTCCACCACCAAAATGTCAATTTTTTGGCCAT
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CCTCGGTACATAAATATATGTGATTCTGGGTAGAAGATCGGTCTGCATTGGATGGTGGTAA
CGCATTTTTTTTACACACATTACTTGCCTCGAGCATCAAATGGTGGTTATTCGTGGATCTATA
TCACGTGATTTGCTTAAGAATTGTCGTTTCATGGTGACACTTTTAGCTTTGACATGATTAAG
CTCATCTCAATTGATGTTATCTAAAGTCATTTCAACTATCTAAGATGTGGTTGTGATTGGG
CCATTTTGTGAAAGCCAGTACGCCAGCGTCAATACACTCCCGTCAATTAGTTGCACC ATGT
CCACAAAATCATATACCAGTAGAGCTGAGACTCATGCAAGTCCGGTTGCATCGAAACTTT
TACGTTTAAATGGATGAAAAGAAGACCAATTTGTGTGCTTCTCTTGACGTTTCGTTTCGACTGA
TGAGCTATTGAAACTTGTTGAAACGTTGGGTCCATACATTTGCCTTTTGAAAACACACGTT
GATATCTTGGATGATTTCAAGTTATGAGGGTACTGTCGTTCCATTGAAAGCATTGGCAGAGA
AATACAAGTTCTTGATATTTGAGGACAGAAAATTCGCCGATATCGGTAACACAGTCAAAT
TACAATATACATCGGGCGTTTACCGTATCGCAGAATGGTCTGATATCACCAACGCCACG
GGGTTACTGGTGCTGGTATTGTTGCTGGCTTGAAACAAGGTGCGCAAGAGGTACCCAAAG
AACCAAGGGGATTATTGATGCTTGCTGAATTGTCTTCCAAGGGTTCTCTAGCACACGGTGA
ATATACTAAGGGTACCGTTGATATTGCAAAGAGTGATAAAGATTTTCGTTATTGGGTTTCAT
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GTAGGTTTAGACGACAAAGGCGATGCATTGGGTCAGCAGTACAGAACCCTCGACGAAGTT
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CCTAAGGTTGAAGGTGAAAGATACAGAAATGCTGGATGGGAAGCGTACCAAAAGAGAAT
CAGCGCTCCCCATTAA TTATACAGGAACTTAATAGAACAAATCACATATTTAATCTAATA
GCCACCTGCATTGGCACGGTGCAACACTACTTCAACTTCATCTTACAAAAAGATCACGTGA
TCTGTTGTATTGAACTGAAAATTTTTTGTGTTGCTTCTCTCTCTCTCTCTTTTCATTATGTGAGA
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GTGCGCATTTTTTTCACACCCTACAATGTTCTGTTCAAAAGATTTTGGTCAAACGCTGTAGA
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TCTAGTTTTTACAAGAAAACAA GCGCAAGTGGTTTAGTGGTAAAATCCAACGTTGCCATC
GTTGGGCCCCCGGTTTCGATTCCGGGCTTGCGCA

Note: Sequence in yellow: gRNA scaffold; sequence in dark green: *SUF17* terminator; sequence in red: *URA3* from *Kluyveromyces lactis* (the sequences around it without color are its endogenous 300 bp promoter and terminator); sequence in blue: *SNR52* promoter; sequence in purple: tRNA^{Gly}.

pKIURA200

GTTT TAGAGCTAGAAA TAGCAAGTTAAAA TAAGGCTAGTCCGTTATCAACTTGAAAAAGT
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CCGTGGTTATTCGTGGATCTATATCACGTGATTTGCTTAAGAATTGTCGTTTCATGGTGACA
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CCGTCAATTAGTTGCACC ATGTCCACAAAATCATATACCAGTAGAGCTGAGACTCATGCA
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TTGCCTTTTGAAAACACACGTTGATATCTTGGATGATTTTCAGTTATGAGGGTACTGTCGTT
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GCCCTCTTGGGCTAGCGGTAAAGGTGCGCATTTTTTCACACCCTACAATGTTCTGTTCAAA
AGATTTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCGCATGTTTCGGCGTTTCGAAACTT
CTCCGCAGTGAAAGATAAATGATC TAGTTTTTACAAGAAAACAA GCGCAAGTGGTTAGT
GGTAAAATCCAACGTTGCCATCGTTGGGCCCGGTTTCGATTCCGGGCTTGCGCA

Note: Sequence in yellow: gRNA scaffold; sequence in dark green: *SUF17* terminator; sequence in red: *URA3* from *Kluyveromyces lactis* (the sequences around it without color are its endogenous 200 bp promoter and terminator); sequence in blue: *SNR52* promoter; sequence in purple: tRNA^{Gly}.

pKIURA100

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CAGTAGAGCTGAGACTCATGCAAGTCCGGTTGCATCGAAACTTTTACGTTTAATGGATGA
AAAGAAGACCAATTTGTGTGCTTCTCTTGACGTTTCGTTTCGACTGATGAGCTATTGAAACTT
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ACCCTACAATGTTCTGTTCAAAAGATTTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCG
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AACAA GCGCAAGTGTTTTAGTGGTAAAATCCAACGTTGCCATCGTTGGGCCCCCGGTTTCG
ATTCCGGGCTTGCGCA

Note: Sequence in yellow: gRNA scaffold; sequence in dark green: *SUF17* terminator; sequence in red: *URA3* from *Kluyveromyces lactis* (the sequences around it without color are its endogenous 100 bp promoter and terminator); sequence in blue: *SNR52* promoter; sequence in purple: tRNA^{Gly}.

pSCLEU

GTTT TAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT
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CCAACTGTGGGAATACTCAGGTATCGTAAGATGCAAGAGTTCGAATCTCTTAGCAACCAT
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ATGACTGGAAATTTTTTGTAAATTTT CAGAGGTGCGCTGACGCATATACCTTTTTTCAACTGA
AAAATTGGGAGAAAAAGGAAAGGTGAGAGCGCCGGAACCGGCTTTTCATATAGAATAGA
GAAGCGTTCATGACTAAATGCTTGCATCACAATACTTGAAGTTGACAATATTATTTAAGGA
CCTATTGTTTTTTCCAATAGGTGGTTAGCAATCGTCTTACTTTCTAACTTTTCTTACCTTTTA
CATTT CAGCAATATATATATATATATTTTCAAGGATATACCATTCTA ATGTCTGCCCTAAG
AAGATCGTCGTTTTGCCAGGTGACCACGTTGGTCAAGAAATCACAGCCGAAGCCATTAAG
GTTCTTAAAGCTATTTCTGATGTTTCGTTCCAATGTCAAGTTCGATTTCGAAAAATCATTTAAT
TGGTGGTGCTGCTATCGATGCTACAGGTGTTCCACTTCAGATGAGGCGCTGGAAGCCTCC
AAGAAGGCTGATGCCGTTTTGTTAGGTGCTGTGGGTGGTCCTAAATGGGGTACCGGTAGT
GTTAGACCTGAACAAGGTTTACTAAAAATCCGTAAAGAAGTTCAATTGTACGCCAACTTA
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AAAGGAAGACGATGGTGATGGTGTGCTTGGGATAGTGAACAATACACCGTTCCAGAAGT
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GAAACCATCAAGAACGAATTCCCTACATTGAAGGTTCAACATCAATTGATTGATTCTGCCG
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GTTCAAAAGATTTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCGCATGTTTCGGCGTTC
GAAACTTCTCCGCAGTGAAAGATAAATGATC TAGTTTTACAAGAAAACAA GCGCAAGTG
GTTTAGTGGTAAAATCCAACGTTGCCATCGTTGGGCCCCCGGTTTCGATTCCGGGCTTGCGC
A

Note: Sequence in yellow: gRNA scaffold; sequence in dark green: *SUF17* terminator; sequence in red: *LEU2* from *S. cerevisiae* (the sequences around it without color are its endogenous 408bp promoter and terminator); sequence in blue: *SNR52* promoter; sequence in purple: tRNA^{Gly}.

Full Plasmid Sequences for pCas vector:

LOCUS Exported 8713 bp ds-DNA circular SYN 16-AUG-2018

DEFINITION synthetic circular DNA

ACCESSION .

VERSION .

KEYWORDS pCas

SOURCE synthetic DNA construct

ORGANISM recombinant plasmid

REFERENCE 1 (bases 1 to 8713)

AUTHORS Yueping Zhang

FEATURES Location/Qualifiers

source	1..8713
	/organism="recombinant plasmid"
	/mol_type="other DNA"
rep_origin	2..1344
	/label=2u ori
	/note="yeast 2u plasmid origin of replication"
promoter	1371..1475
	/gene="bla"
	/label=AmpR promoter
CDS	1476..2336
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	/product="beta-lactamase"
	/label=AmpR
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	/translation="MSIQHFRVALIPFFAAFLPVF AHPETLVKVKDAEDQLGARVGYI ELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRRIHYSQNDLVEYS PVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTIGGPKELTAFLHNMGDHVTRLDRW EPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQQIDWMEADKVAGPLLRSA LPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGAS LIKHW"
rep_origin	2507..3095
	/direction=RIGHT
	/label=ori
	/note="high-copy-number ColE1/pMB1/pBR322/pUC origin of replication"
promoter	3108..3376
	/label=SNR52 promoter
	/note="promoter for the S. cerevisiae small nucleolar RNA gene SNR52"
CDS	complement(3384..3680)

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 QRRDWENPGVTQLNRLAAHPPFASWRNSEEARTDRPSQQLRSLNGEWDAPCSG"

promoter complement(3724..3754)
 /label=lac promoter
 /note="promoter for the E. coli lac operon"

misc_RNA 3836..3907
 /label=gRNA scaffold
 /note="guide RNA scaffold for the Streptococcus pyogenes
 CRISPR/Cas9 system"

terminator 3936..4123
 /gene="S. cerevisiae ADH1"
 /label=ADH1 terminator
 /note="transcription terminator for the S. cerevisiae
 alcohol dehydrogenase 1 (ADH1) gene"

CDS complement(4133..4153)
 /codon_start=1
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 antigen"
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 /translation="PKKKRKV"

CDS complement(4157..8257)
 /codon_start=1
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 pyogenes Type II CRISPR/Cas system"
 /label=iCas9
 /note="generates RNA-guided double strand breaks in DNA"
 /translation="DKKYSIGLDIGTNSVGWAVITDEYKVPSSKKFKVLGNTDRHSIKKN
 LIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRLLEESF
 LVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLV DSTYKADLR LIYLALAHMIKF
 RGHFLIEGDLNPDNSDVKLFIQLVQTYNQLFEENPINASGVDAKILSARLSKSRRL
 NLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKD TYDDDLNLLAQI
 GDQYADLFLAAKNLSDAILSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQ
 QLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLR
 KQRTFDNGSITHQIHLGELHAILRRQEDFY PFLKDNREKIEKILTFRIPYYVGPLARGN
 SRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVL PKHSLLEYF
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 VEISGVEDRFNASLGTYHDLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERL
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ORIGIN

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