Supplementary Information

CRISPR assisted gRNA free one-step genome editing technique with no sequence limitations and improved targeting efficiency

Dongdong Zhao, Xu Feng, Xinna Zhu, Tao Wu, Xueli Zhang & Changhao Bi

Figure

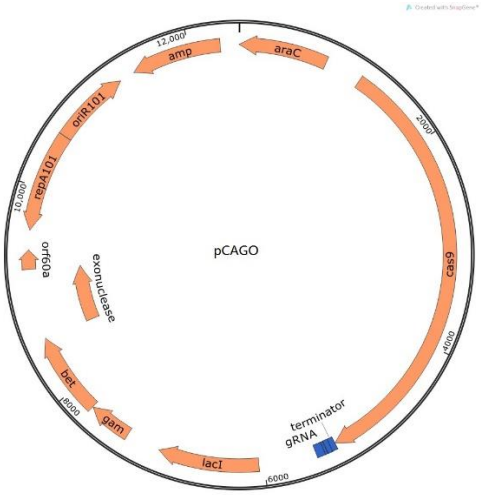


Figure S1. pCAGO map

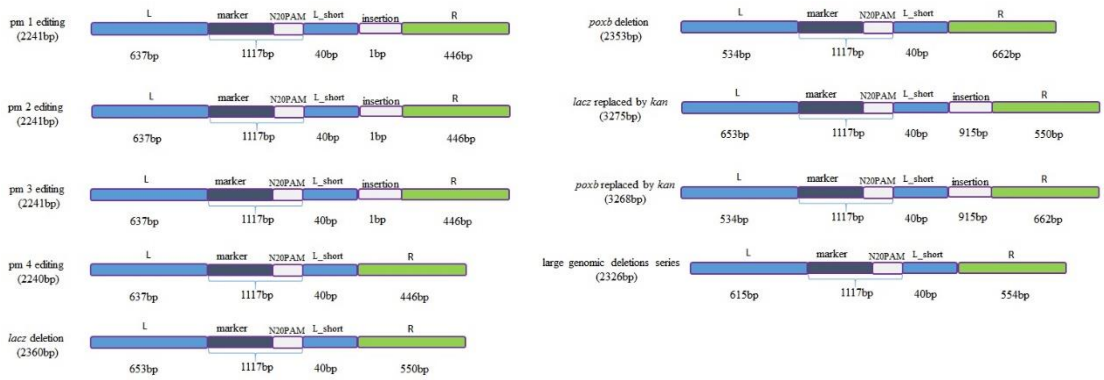


Figure S2. Editing cassettes with labeled by size

Table

Table S1. Primers for construction of pCAGO

Name	Sequence
(pkd46)_forward	CCAGGTCTCACCATGGATATTAATACTGAAACTGAGATCAAGC
(pkd46)_reverse	CCAGGTCTCAATTTTTTATAACCTCCTTAGAGCTCGAATTCCC
(cas9)_forward	CCAGGTCTCAAAATGGATAAGAAATACTCAATAGGCTTAGATATCGGC
(cas9)_(N20A)_reverse	CCAGGTCTCATTCGGTTCGATGGACTAGCTAAGATCTGACTCCATAACAGAGTACTCGCC
(N20B)_(gRNA)_forward	CCAGGTCTCACGAAGTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
(gRNA)_reverse	CCAGGTCTCAGGAGAGAACATACTGGCTAAATACGGAAGG
(ptrc)_forward	CCAGGTCTCACTCCATACGATATAAGTTGTAATTCTCATGTTTG
(ptrc)_reverse	CCAGGTCTCAATGGTATATCTCCTTGAATTCCATGGTC

Table S2. Primers used for analysis of chromosomal areas most similar to the slected N20PAM [a]

Primer Name	Sequence
N1G_F	GATTTGCCGCTGTCAAAC
N1G_R	ATCACCGCGAAAGTGAAG
N2G_F	GGCGGCATTCGTATCGAAGAC
N2G_R	TGGCCGATGGCATCAAAGG
N3G_F	CACCAGCATTGTCGGTTTAC
N3G_R	AAGCCGTGAAGGAGTGAAAG
N4G_F	CGCGTTAATCACGTCTCTATCG
N4G_R	TTTGATCTGGAGGCGCTAAAGG
N5G_F	TTCACGGAATGCGGCAACC
N5G_R	AGCGAACGAAGCGGAACTG
N6G_F	GGAACACCAGGCGATGATGTC
N6G_R	GCACCACCTGCTGTATGATCC
N7G_F	ATGGCAGGCCAGAATGAC
N7G_R	CTGCTGCTGCAACTTCAC
N8G_F	AAACCCGTCTGGTTCATACCC
N8G_R	GCCCTTCACCCAGAATATAAGC
N9G_F	GGATGATGTTGGCTTCAG
N9G_R	TACGGCTTCGATATCACC

[a] : Choromosomal areas most similar to selected N20PAM were predicted by Cas-OFFinder

Table S3. N20PAM similar areas [a] seqeunces and analysis result after Cas9/gRNA expression

number	similar DNA sequences	Positions[b]	Mismatches	Positive colonies[c]/Tested	
				pCAGO exp[d]	Pm4 exp[e]
1	cAGaCCAcCaAACCcAAGTgCGG	29456	6	10/10	10/10
2	TAGTtCtgCaAAaCGAAGTgGGG	608522	6	10/10	10/10
3	TgGcCaATCaAACaGAAaTATGG	507357	6	10/10	10/10

4	TAaaCCATCtAcCgGAAtTACGG	1067543	6	10/10	10/10
5	TtcgCCATtGAACtGAAcTAAGG	1398303	6	10/10	10/10
6	TgGTtCATCaAACCGcgGaAGGG	1580707	6	10/10	10/10
7	TAtTaCAcCGAtCCGAAGaATGG	2247074	5	10/10	10/10
8	gAGTCtATCtAtCtGAAcTATGG	3700191	6	10/10	10/10
9	TAtgtaATCaAACCGAAaTACGG	4619275	6	10/10	10/10

[a] : Choromosomal areas most similar to selected N20PAM were predicted by Cas-OFFinder

[b] : Genome positions of *Escherichia coli* (*K-12, MG1655*) predicted by Cas-OFFinder

[c] : Strains with wild type *E. coli* MG1655 seqeunce identified with PCR amplified and DNA sequencing

[d] : N20PAM Similar areas were PCR amplified and sequenced to check off-target possibility after expression pCAGO

[e] : N20PAM Similar areas were PCR amplified and sequenced to check off-target possibility after editing editing experiment pm4

Table S4 Primers for construction of editing cassettes and colony PCR identification

Primers	Sequence	Characteristics
N20PAM_Cm_F	TTCATGTGCAGCTCCATCACTGGAGCACCTCAAAAACACCA	Selection marker - N20PAM part amplification
N20PAM_Cm_R	CAACGTCATCTCGTTCTCCGCTTATTTGTTAACTGTTAATTGTCCTTACTTCGGTTCGATGGACTATTACGCCCCGC CCTGCCA	
Large_L500F	CCACGGCTGTGATTAGAAC	Large genome deletion
Large_ ReN20PAM2_L500R	CCAGGTCTC**ctgg**TACCCGCCGAAGAAATTCC	
ReN20PAM2BsaI_F	CCAGGTCTCA**ccag**TTCATGTGCAGCTCCATC	
ReN20PAM2_large_BsaI_R	CCAGGTCTCA**aggc**CAACGTCATCTCGTTCTC	
large_L10K _ BSAI_R500F	CCAGGTCTCA**gcct** acacgccgcgtgaacggcggtagatctcagaaaaacgctcGCGATTCGTTGAGTACAG	
large_L30K _ BSAI_R500F	CCAGGTCTCA**gcct** tatatgctgtgatgcaagaacagtttggtctgggcgtggaGCGATTCGTTGAGTACAG	
large_L70K _ BSAI_R500F	CCAGGTCTCA**gcct** ttacgatgaagcgaccgtccggcagtgttgatggcgtcaaGCGATTCGTTGAGTACAG	
large_L100K _ BSAI_R500F	CCAGGTCTCA**gcct** ccaaagcagaaatgccagctaattacctgattgtcggcagGCGATTCGTTGAGTACAG	
large_R500R	TGCGCTATCGAAAGTGAG	
Large_genome_R	TAGATGCGCCAGGATGCAG	
Large_genome_10k_F	ACCACCGGAAGGGTATACG	
Large_genome_30k_F	CGCGCCGATTCGTTTAGTTTC	
Large_genome_70kA_F	GAAGAGAACGTCGCATCTAC	
Large_genome_70kA_R	CGAAGATATTGCCCTCTCAC	
Large_genome_100kA_F	GCGCGTCAGCTGTGTATCAAC	
Large_genome_100kA_R	TGCCCTCTCACTTTCCATCGG	
Poxb_L500F	CCCTCCGTCAGATGAACTAAAC	*Poxb* editing
Poxb_L500BSAI_R	CCAGGTCTCA**CTGG**TAACACGACAACCGAAAC	
ReN20PAM2BSAI_F	CCAGGTCTCA**CCAG**TTCATGTGCAGCTCCATC	
ReN20PAM2_poxb_BSAI_R	CCAGGTCTCA**gcCT**GACAACCGAAACGCCACGGTTAAGCACCGCTTTGCGCATGCAACGTCATCTCGTTCTC	
Poxb_R500BSAI_F	CCAGGTCTCA**aGGC**ACTGGTCGGCGATATCAAG	
Poxb_R500R	CGACCACCACATCCACCAACAC	
kan_re_poxbfullF	CCAGGTCTCA**aggc**CGTCGGAATTGCCAGCTGGG	
kan_re_fullR	CCAGGTCTCA**agtc**TCAGAAGAACTCGTCAAGAAGGC	
poxb_kan_R500BSAI_F	CCAGGTCTCA**gact**ACTGGTCGGCGATATCAAG	

poxb_genome _F	CGCCTTATGCCCGATGATATTC	
poxb_genome _R	CCAGCACGCTGTTGTTAAAGAC	
lacz_L500F	CTTCCGGCTCGTATGTTG	
lacz_L500BSAI_R	CCAGGTCTCA**CTGG**GATAACTGCCGTCACTCC	
ReN20PAM2BSAI_F	CCAGGTCTCA**CCAG**TTCATGTGCAGCTCCATC	
ReN20PAM2_lacz_BSAI_R	CCAGGTCTCA**GCCT**CCGGCGCGTAAAAATGCGCTCAGGTCAAATTCAGACGGCACAACGTCATCTCGTTCTC	
lacz_R500BSAI_F	CCAGGTCTCA**AGGC**TGGCTTTCGCTACCTGGAGAG	
lacz_R500R	TCGCGTTCGGTTGCACTAC	*Lacz* editing
kan_re_laczfullF	CCAGGTCTCA**cgctC**GTCGGAATTGCCAGCTGGG	
kan_re_fullR	CCAGGTCTCA**agtc**TCAGAAGAACTCGTCAAGAAGGC	
lacz_kan_R500BSAI_F	CCAGGTCTCA**gact**GGCTTTCGCTACCTGGAGAG	
lacZ_genome_F	AAAACCCTGGCGTTACCCA	
lacZ_genome_R	CAGGCAGTTCAATCAACTGTTTACC	
ldhApm_L500F	TACTTACACATCCCGCCATCAGCAGG	
ldhApm_L500BSAI_R	CCAGGTCTCA**CTGG**TCCGTATCCAAGTGCAGCGGCG	
ReN20PAM2BSAI_F	CCAGGTCTCA**CCAG**TTCATGTGCAGCTCCATC	
ReN20PAM2_ldhA_BSAI_R	CCAGGTCTCA**TCCG**TATCCAAGTGCAGCGGCGCTGGAACTCGGTGTGGAGCAACGTCATCTCGTTCTC	
ReN20PAM2_ldhAS_BSAI_R	CCAGGTCTCATCCGCAACGTCATCTCGTTCTC	
ldhApm1_R500BSAI_F	CCAGGTCTCA**CGGA**Ccg aacg CCAGCAGACGCATACCAAAACC	CRISPR-tolerant and PAM-free
ldhApm2_R500BSAI_F	CCAGGTCTCA**CGGA**tcA aacg CCAGCAGACGCATACCAAAACC	regions editing
ldhApm3_R500BSAI_F	CCAGGTCTCA**CGGA**tcg Gacg CCAGCAGACGCATACCAAAACC	
ldhApm4_R500BSAI_F	CCAGGTCTCA**CGGA**tcgCCAGCAGACGCATACCAAAACC	
ldhApm_R500R	CAACAGGTGAACGAGTCCTTTGGC	
ldhA_genome_F	TTAGCGCACATCATACGGGTC	
ldhA_genome_R	GCGCCTACACTAAGCATAGTTG	
Poxb_L65F	ccgcgaatgtagtcactattgcgagctggtttccagcccggagcagatcccacaagtactggcgaTTCATGTGCAGCTCCATC	*Poxb* deletion using simple
Poxb_R40L40QR	CTTTTTCTTCCACCAATGGAAGCAATGCACGCAGAGTCGA**AATAGCTCTTGTGGGTGGGTTTCCTGGAAATA** **GCCGCTGC**CAACGTCATCTCGTTCTC	editing cassette construction strage

CAGO technique protocol

Step1: Editing cassette construction

(1) Amplification of modularized parts

The selection marker part with CRISPR/Cas9 recognition region (N20PAM) are PCR-amplified from plasmid pACYC184-M-crt with N20PAM sequence embedded in reverse primer, which is used as template for PCR amplification to add type IIS linkers. Primer pairs are designed as:
5'..TTCATGTGCAGCTCCATCACTGGAGCACCTCAAAAACACCA..3' and
5'..CAACGTCATCTCGTTCTCCGCTTATTTGTTAACTGTTAATTGTCCTTACTTCGGTTCGATGGACTATTACGCCCCGCCCTGCCA..3'.

For genome deletion, three modularized parts are used for editing cassette construction. The primers used for amplification of each part are in the following table.

Table S5. Primers designed for modularized parts amplification in genome deletion experiment

modularized parts	Primers design
left homology arm (L)	5'.. 18~22nt forward primer..3'
	5'..CCAGGTCTCACTGG-18~22nt reverse primer..3'
marker with N20PAM	5'..CCAGGTCTCACCAGTTCATGTGCAGCTCCATC..3'
	5'..CCAGGTCTCANNNN-40nt L_short-CAACGTCATCTCGTTCTC..3'[a]
right homology arm (R)	5'..CCAGGTCTCANNNN-18~22nt reverse primer..3'
	5'.. 18~22nt reverse primer ..3'

[a] :The 40nt L_short could be any DNA fragment upstream of L homologous arm as necessary.

[b] : "NNNN" was the 4 nt linkers between L_short and R, was alterable as needed.

For genome insertion and replacement, four modularized parts are used for editing cassette construction, the primers used for amplification of each part are in the following table.

Table S6. Primers designed for modularized parts amplification in genome insertion and replacement experiment

modularized parts	Primers design
left homology arm (L)	5'.. 18~22nt forward primer..3'
	5'..CCAGGTCTCACTGG-18~22nt reverse primer..3'
marker with N20PAM	5'..CCAGGTCTCACCAGTTCATGTGCAGCTCCATC..3'
	5'..CCAGGTCTCAGCCT-40nt L_short-CAACGTCATCTCGTTCTC..3'[a]
insertion	5'..CCAGGTCTCAAGGC-18~22nt forward primer..3'
	5'..CCAGGTCTCANNNN-18~22nt reverse primer..3'[b]
right homology arm (R)	5'..CCAGGTCTCANNNN-18~22nt reverse primer..3'
	5'.. 18~22nt reverse primer ..3'

[a] :The 40nt L_short could be any DNA frsgmrnt upstream of L homologous arm as necessary.

[b] : "NNNN" was the 4 nt linkers between insertion part and R, was alterable as needed.

(2) Modular parts assembly

Add around 100 ng of marker N20PAM part and equal molar amounts of the other assembly parts into a 15 μl total volume assembly reaction mixture:

marker N20PAM part (100 ng)

+ each additional assembly parts (to equal molar with marker with N20PAM part)

+ 1.5 µl 10X NEB T4 Buffer

+ 0.15 µl 100X BSA

+ 1 µl BsaI

+ 1 µl NEB T4 Ligase, 2 million cohesive end units / mL

+ dH20 to 15 µl

Perform the assembly reaction in a thermocycler with following condition:

3 min @ 37 C }

4 min @ 16 C } 25 cycles

5 min @ 50 C }

5 min @ 80 C } 1 cycle

Forward primer of L and reverse primer of R are used for the amplification from the assembly reaction to get large number of editing cassette.

For genome deletion, the editing cassette could also be constructed by PCR amplification without assembly. The left homology arm (L) is embedded in the forward primer with a length of about 65bp, the right homology arm (R) and L_short are embedded in the reverse primer with the length of 40bp for each. Two primers are designed as followed:

5'..65nt left homology arm-TTCATGTGCAGCTCCATC..3' and

5'..40nt reverse right homology arm-40nt reverse L_short-CAACGTCATCTCGTTCTC..3'. The PCR product is the editing cassette for genome editing.

Step2: Procedure for genome editing

MG1655 competent cells harboring pCAGO are prepared with IPTG induced λ-RED proteins. 50 µL competent cells are mixed with 400 ng editing cassette in a 2-mm Gene Pulser cuvette (Bio-Rad). After electroporation at 2.5 kV and suspended immediately in 1 ml of ice cold medium, cells are incubated for 2 hours at 30°C, and then plated on solid LB medium with ampicillin, chloramphenicol and 1% glucose.

For each editing experiment, five transformants are analyzed by colony PCR. A correct clone with inserted editing cassette is inoculated into LB media with ampicillin, IPTG, and L-arabinose for CRISPR/Cas9 system and λ-RED protein expression. After culturing for more than 6 hours, cells are plated on LB agar plates with ampicillin. Colonies are identified by colony PCR and the correct colonies are used for further verification by DNA sequencing.

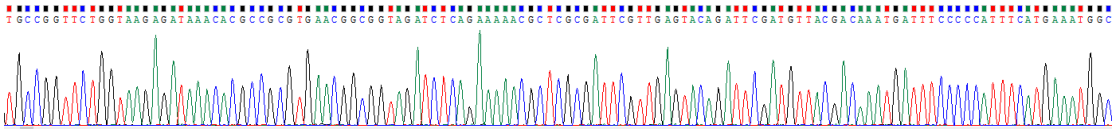
Step3: Plasmid curing and multiple rounds of genome editing

In edited strain, pCAGO is cured by growing overnight at 42°C. For consecutive editing, edited clone carrying pCAGO is used as parent strain for transformation of new editing DNA cassette.

Sequencing data

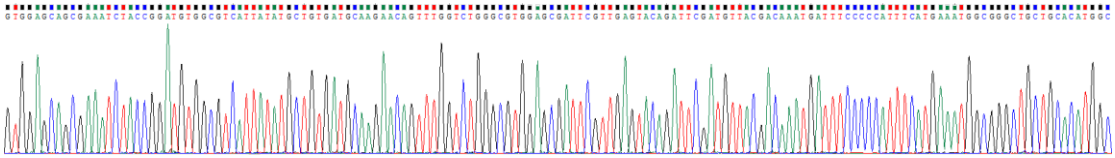
Deletion of 9.53kb:

TGCCGGTTCTGGTAAGAGATAAACACGCCGCGTGAACGGCGGTAGATCTCAGAAAAACGCTCGCGA
TTCGTTGAGTACAGATTCGATGTTACGACAAATGATTTCCCCCATTTCATGAAATGGC



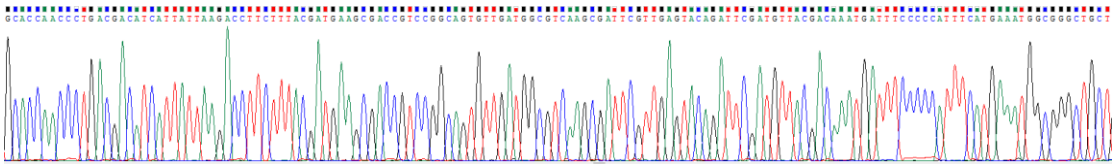
Deletion of 30.08kb:

GTGGAGCAGCGAAATCTACCGGATGTGGCGTCATTATATGCTGTGATGCAAGAACAGTTTGGTCTG
GGCGTGGAGCGATTCGTTGAGTACAGATTCGATGTTACGACAAATGATTTCCCCCATTTCATGAAAT
GGCGGGCTGCTGCACATGGC



Deletion of 70.04kb:

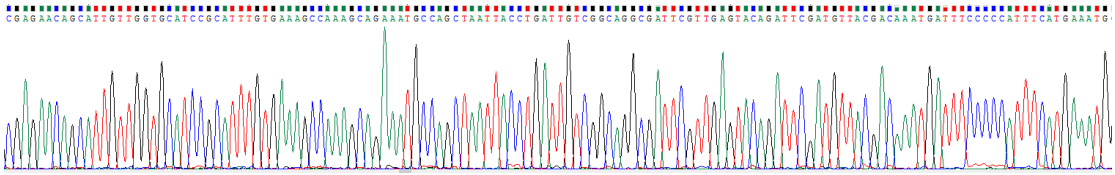
GCACCAACCCTGACGACATCATTATTAAGACCTTCTTTACGATGAAGCGACCGTCCGGCAGTGTTGAT
GGCGTCAAGCGATTCGTTGAGTACAGATTCGATGTTACGACAAATGATTTCCCCCATTTCATGAAATG
GCGGGCTGCT



Deletion of 99.99kb:

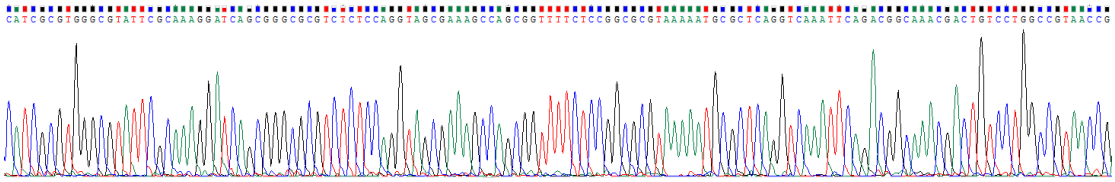
CGAGAACAGCATTGTTGGTGCATCCGCATTTGTGAAAGCCAAAGCAGAAATGCCAGCTAATTACCTG
ATTGTCGGCAGGCGATTCGTTGAGTACAGATTCGATGTTACGACAAATGATTTCCCCCATTTCATGAA

ATG



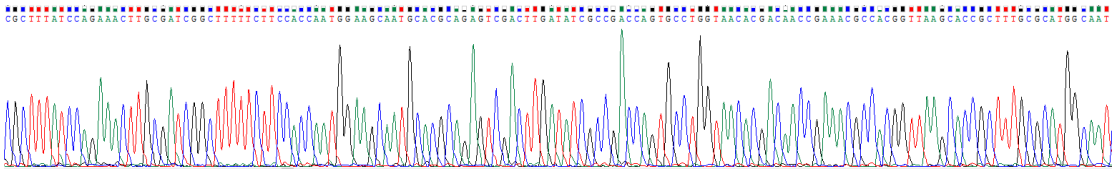
***Lacz* deletion:** 1018bp was deleted from the *lacz* gene

CATCGCGTGGGCGTATTCGCAAAGGATCAGCGGGCGCGTCTCTCCAGGTAGCGAAAGCCAGCGGTT
TTCTCCGGCGCGTAAAAATGCGCTCAGGTCAAATTCAGACGGCAAACGACTGTCCTGGCCGTAACCG



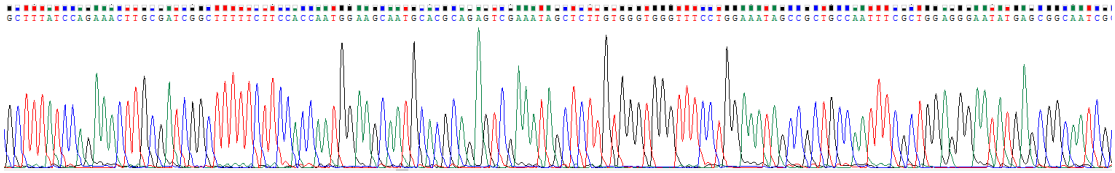
***Poxb* deletion:** 443 bp was deleted from the *poxb* gene

CGCTTTATCCAGAAACTTGCGATCGGCTTTTTCTTCCACCAATGGAAGCAATGCACGCAGAGTCGACT
TGATATCGCCGACCAGTGCCTGGTAACACGACAACCGAAACGCCACGGTTAAGCACCGCTTTGCGCA
TGGCAAT



***Poxb* deletion using simple editing cassette construction strage:** 587bp was deleted from the *poxb* gene

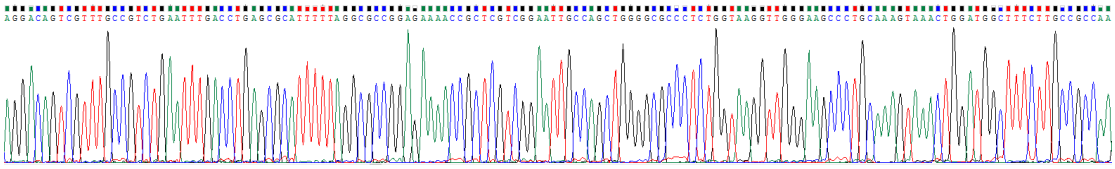
GCTTTATCCAGAAACTTGCGATCGGCTTTTTCTTCCACCAATGGAAGCAATGCACGCAGAGTCGAAAT
AGCTCTTGTGGGTGGGTTTCCTGGAAATAGCCGCTGCCAATTTCGCTGGAGGGAATATGAGCGGCA
ATCG



***Lacz* replaced by *kan*:** 1018bp of *lacz* was replaced by 915 bp fragment derived from the *kan* gene

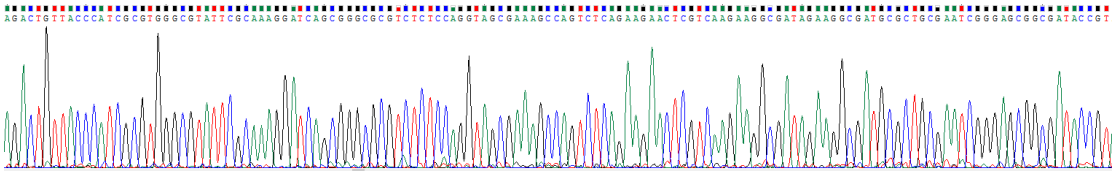
Forward

AGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTAGGCGCCGGAGAAAACCGCTCGTC
GGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTT
CTTGCCGCCAA



Reverse

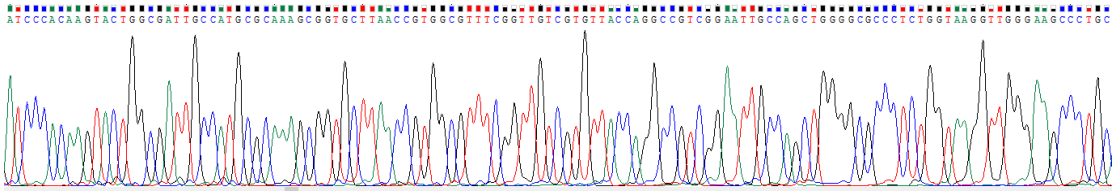
AGACTGTTACCCATCGCGTGGGCGTATTCGCAAAGGATCAGCGGGCGCGTCTCTCCAGGTAGCGAA
AGCCAGTCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGC
GATACCGT



Poxb replaced by *kan*: 443 bp of *poxb* was replaced by 915 bp fragment derived from the *kan* gene

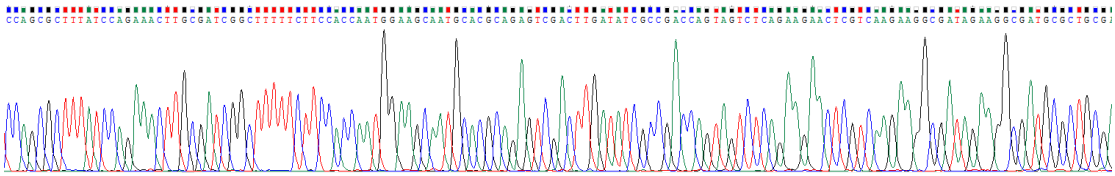
Forward

ATCCCACAAGTACTGGCGATTGCCATGCGCAAAGCGGTGCTTAACCGTGGCGTTTCGGTTGTCGTGT
TACCAGGCCGTCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGC



Reverse

CCAGCGCTTTATCCAGAAACTTGCGATCGGCTTTTTCTTCCACCAATGGAAGCAATGCACGCAGAGTC
GACTTGATATCGCCGACCAGTAGTCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCT
GCG



pCAGO Sequence

>

CACAACCGGCACGGAACTCGCTCGGGCTGGCCCCGGTGCATTTTTTAAATACCCG
CGAGAAATAGAGTTGATCGTCAAAACCAACATTGCGACCGACGGTGGCGATAGG
CATCCGGGTGGTGCTCAAAAGCAGCTTCGCCTGGCTGATACGTTGGTCCTCGCGC
CAGCTTAAGACGCTAATCCCTAACTGCTGGCGGAAAAGATGTGACAGACGCGAC
GGCGACAAGCAAACATGCTGTGCGACGCTGGCGATATCAAAATTGCTGTCTGCCA
GGTGATCGCTGATGTACTGACAAGCCTCGCGTACCCGATTATCCATCGGTGGATG
GAGCGACTCGTTAATCGCTTCCATGCGCCGCAGTAACAATTGCTCAAGCAGATTT
ATCGCCAGCAGCTCCGAATAGCGCCCTTCCCCTTGCCCGGCGTTAATGATTTGCC
CAAACAGGTCGCTGAAATGCGGCTGGTGCGCTTCATCCGGGCGAAAGAACCCCG
TATTGGCAAATATTGACGGCCAGTTAAGCCATTCATGCCAGTAGGCGCGCGGACG
AAAGTAAACCCACTGGTGATACCATTCGCGAGCCTCCGGATGACGACCGTAGTG
ATGAATCTCTCCTGGCGGGAACAGCAAAATATCACCCGGTCGGCAAACAAATTCT
CGTCCCTGATTTTTCACCACCCCCTGACCGCGAATGGTGAGATTGAGAATATAAC
CTTTCATTCCCAGCGGTCGGTCGATAAAAAAATCGAGATAACCGTTGGCCTCAAT
CGGCGTTAAACCCGCCACCAGATGGGCATTAAACGAGTATCCCGGCAGCAGGGG
ATCATTTTGCGCTTCAGCCATACTTTTCATACTCCCGCCATTCAGAGAAGAAACCA
ATTGTCCATATTGCATCAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCTCG
CTAACCAAACCGGTAACCCCGCTTATTAAAAGCATTCTGTAACAAAGCGGGACCA
AAGCCATGACAAAAACGCGTAACAAAAGTGTCTATAATCACGGCAGAAAAGTCC
ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCCATA
AGATTAGCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATA
CCCGTTTTTTTGGGAATTCGAGCTCTAAGGAGGTTATAAAAAATGGATAAGAAAT
ACTCAATAGGCTTAGATATCGGCACAAATAGCGTCGGATGGGCGGTGATCACTG
ATGAATATAAGGTTCCGTCTAAAAAGTTCAAGGTTCTGGGAAATACAGACCGCCA
CAGTATCAAAAAAAATCTTATAGGGGCTCTTTTATTTGACAGTGGAGAGACAGCG
GAAGCGACTCGTCTCAAACGGACAGCTCGTAGAAGGTATACACGTCGGAAGAAT
CGTATTTGTTATCTACAGGAGATTTTTTCAAATGAGATGGCGAAAGTAGATGATA
GTTTCTTTCATCGACTTGAAGAGTCTTTTTTGGTGGAAGAAGACAAGAAGCATGA
ACGTCATCCTATTTTTGGAAATATAGTAGATGAAGTTGCTTATCATGAGAAATAT
CCAACTATCTATCATCTGCGAAAAAAATTGGTAGATTCTACTGATAAAGCGGATT
TGCGCTTAATCTATTTGGCCTTAGCGCATATGATTAAGTTTCGTGGTCATTTTTTG
ATTGAGGGAGATTTAAATCCTGATAATAGTGATGTGGACAAACTATTTATCCAGT
TGGTACAAACCTACAATCAATTATTTGAAGAAAACCCTATTAACGCAAGTGGAGT
AGATGCTAAAGCGATTCTTTCTGCACGATTGAGTAAATCAAGACGATTAGAAAAT
CTCATTGCTCAGCTCCCCGGTGAGAAGAAAAATGGCTTATTTGGGAATCTCATTG
CTTTGTCATTGGGTTTGACCCCTAATTTTAAATCAAATTTTGATTTGGCAGAAGAT

GCTAAATTACAGCTTTCAAAAGATACTTACGATGATGATTTAGATAATTTATTGG
CGCAAATTGGAGATCAATATGCTGATTTGTTTTTGGCAGCTAAGAATTTATCAGA
TGCTATTTTACTTTCAGATATCCTAAGAGTAAATACTGAAATAACTAAGGCTCCC
CTATCAGCTTCAATGATTAAACGCTACGATGAACATCATCAAGACTTGACTCTTTT
AAAAGCTTTAGTTCGACAACAACTTCCAGAAAAGTATAAAGAAATCTTTTTTGAT
CAATCAAAAAACGGATATGCAGGTTATATTGATGGGGGAGCTAGCCAAGAAGAA
TTTTATAAATTTATCAAACCAATTTTAGAAAAAATGGATGGTACTGAGGAATTAT
TGGTGAAACTAAATCGTGAAGATTTGCTGCGCAAGCAACGGACCTTTGACAACG
GCTCTATTCCCCATCAAATTCACTTGGGTGAGCTGCATGCTATTTTGAGAAGACA
AGAAGACTTTTATCCATTTTTAAAAGACAATCGTGAGAAGATTGAAAAAATCTTG
ACTTTTCGAATTCCTTATTATGTTGGTCCATTGGCGCGTGGCAATAGTCGTTTTGC
ATGGATGACTCGGAAGTCTGAAGAAACAATTACCCCATGGAATTTTGAAGAAGTT
GTCGATAAAGGTGCTTCAGCTCAATCATTTATTGAACGCATGACAAACTTTGATA
AAAATCTTCCAAATGAAAAAGTACTACCAAAACATAGTTTGCTTTATGAGTATTT
TACGGTTTATAACGAATTGACAAAGGTCAAATATGTTACTGAAGGAATGCGAAA
ACCAGCATTTCTTTCAGGTGAACAGAAGAAAGCCATTGTTGATTTACTCTTCAAA
ACAAATCGAAAAGTAACCGTTAAGCAATTAAAAGAAGATTATTTCAAAAAAATA
GAATGTTTTGATAGTGTTGAAATTTCAGGAGTTGAAGATAGATTTAATGCTTCATT
AGGTACCTACCATGATTTGCTAAAAATTATTAAAGATAAAGATTTTTTGGATAAT
GAAGAAAATGAAGATATCTTAGAGGATATTGTTTTAACATTGACCTTATTTGAAG
ATAGGGAGATGATTGAGGAAAGACTTAAAACATATGCTCACCTCTTTGATGATAA
GGTGATGAAACAGCTTAAACGTCGCCGTTATACTGGTTGGGGACGTTTGTCTCGA
AAATTGATTAATGGTATTAGGGATAAGCAATCTGGCAAAACAATATTAGATTTTT
TGAAATCAGATGGTTTTGCCAATCGCAATTTTATGCAGCTGATCCATGATGATAG
TTTGACATTTAAAGAAGACATTCAAAAAGCACAAGTGTCTGGACAAGGCGATAG
TTTACATGAACATATTGCAAATTTAGCTGGTAGCCCTGCTATTAAAAAAGGTATT
TTACAGACTGTAAAAGTTGTTGATGAATTGGTCAAAGTAATGGGGCGGCATAAGC
CAGAAAATATCGTTATTGAAATGGCACGTGAAAATCAGACAACTCAAAAGGGCC
AGAAAAATTCGCGAGAGCGTATGAAACGAATCGAAGAAGGTATCAAAGAATTAG
GAAGTCAGATTCTTAAAGAGCATCCTGTTGAAAATACTCAATTGCAAAATGAAAA
GCTCTATCTCTATTATCTCCAAAATGGAAGAGACATGTATGTGGACCAAGAATTA
GATATTAATCGTTTAAGTGATTATGATGTCGATCACATTGTTCCACAAAGTTTCCT
TAAAGACGATTCAATAGACAATAAGGTCTTAACGCGTTCTGATAAAAATCGTGGT
AAATCGGATAACGTTCCAAGTGAAGAAGTAGTCAAAAAGATGAAAAACTATTGG
AGACAACTTCTAAACGCCAAGTTAATCACTCAACGTAAGTTTGATAATTTAACGA
AAGCTGAACGTGGAGGTTTGAGTGAACTTGATAAAGCTGGTTTTATCAAACGCCA
ATTGGTTGAAACTCGCCAAATCACTAAGCATGTGGCACAAATTTTGGATAGTCGC
ATGAATACTAAATACGATGAAAATGATAAACTTATTCGAGAGGTTAAAGTGATTA
CCTTAAAATCTAAATTAGTTTCTGACTTCCGAAAAGATTTCCAATTCTATAAAGTA

CGTGAGATTAACAATTACCATCATGCCCATGATGCGTATCTAAATGCCGTCGTTG
GAACTGCTTTGATTAAGAAATATCCAAAACTTGAATCGGAGTTTGTCTATGGTGA
TTATAAAGTTTATGATGTTCGTAAAATGATTGCTAAGTCTGAGCAAGAAATAGGC
AAAGCAACCGCAAAATATTTCTTTTACTCTAATATCATGAACTTCTTCAAAACAG
AAATTACACTTGCAAATGGAGAGATTCGCAAACGCCCTCTAATCGAAACTAATGG
GGAAACTGGAGAAATTGTCTGGGATAAAGGGCGAGATTTTGCCACAGTGCGCAA
AGTATTGTCCATGCCCCAAGTCAATATTGTCAAGAAAACAGAAGTACAGACAGG
CGGATTCTCCAAGGAGTCAATTTTACCAAAAAGAAATTCGGACAAGCTTATTGCT
CGTAAAAAAGACTGGGATCCAAAAAAATATGGTGGTTTTGATAGTCCAACGGTA
GCTTATTCAGTCCTAGTGGTTGCTAAGGTGGAAAAAGGGAAATCGAAGAAGTTA
AAATCCGTTAAAGAGTTACTAGGGATCACAATTATGGAAAGAAGTTCCTTTGAAA
AAAATCCGATTGACTTTTTAGAAGCTAAAGGATATAAGGAAGTTAAAAAAGACT
TAATCATTAAACTACCTAAATATAGTCTTTTTGAGTTAGAAAACGGTCGTAAACG
GATGCTGGCTAGTGCCGGAGAATTACAAAAAGGAAATGAGCTGGCTCTGCCAAG
CAAATATGTGAATTTTTTATATTTAGCTAGTCATTATGAAAAGTTGAAGGGTAGT
CCAGAAGATAACGAACAAAAACAATTGTTTGTGGAGCAGCATAAGCATTATTTA
GATGAGATTATTGAGCAAATCAGTGAATTTTCTAAGCGTGTTATTTTAGCAGATG
CCAATTTAGATAAAGTTCTTAGTGCATATAACAAACATAGAGACAAACCAATACG
TGAACAAGCAGAAAATATTATTCATTTATTTACGTTGACGAATCTTGGAGCTCCC
GCTGCTTTTAAATATTTTGATACAACAATTGATCGTAAACGATATACGTCTACAA
AAGAAGTTTTAGATGCCACTCTTATCCATCAATCCATCACTGGTCTTTATGAAACA
CGCATTGATTTGAGTCAGCTAGGAGGTGACTGAGCATCAAATAAAACGAAAGGC
TCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTCTAGGTTTATACATAGGCGAGT
ACTCTGTTATGGAGTCAGATCTTAGCTAGTCCATCGAACCGAAGTAGTTTTAGAG
CTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGC
ACCGAGTCGGTGCTTAGCATCCAAACTCGAGTAAGGATCTCCAGGCATCAAATAA
AACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGT
GAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTT
ATACCTAGGGCGTTCGGCTGCGGCTCTACTTTTGTTTGTTAGTCTTGATGCTTCAC
TGATAGATACAAGAGCCATAAGAACCTCAGATCCTTCCGTATTTAGCCAGTATGT
TCTCTCCATACGATATAAGTTGTAATTCTCATGTTTGACAGCTTATCATCGTTAAT
TAACTAGTCATATGGGCATGCATTTACGTTGACACCATCGAATGGTGCAAAACCT
TTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATG
TGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGAC
CGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAA
AGTGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACA
ACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTG
CACGCGCCGTCGCAAATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTG
CCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTCGAAGCCTGTAAAGCGG

CGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCT
GGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTA
TTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGA
CGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGC
GCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGG
CATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGAC
TGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCG
TTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGC
CATTACCGAGTCCGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATACGAC
GATACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCACCATCAAACAGGATT
TTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCA
GGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCAC
CCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATG
CAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAAT
TAATGTGAGTTAGCGCGAATTGATCTGGTTTGACAGCTTATCATCGACTGCACGG
TGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCTGTGCA
GGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATA
ATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAATGAGCTGTT
GACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTT
CACACAGGAAACAGACCATGGAATTCAAGGAGATATACCATGGATATTAATACT
GAAACTGAGATCAAGCAAAAGCATTCACTAACCCCCTTTCCTGTTTTCCTAATCA
GCCCGGCATTTCGCGGGCGATATTTTCACAGCTATTTCAGGAGTTCAGCCATGAA
CGCTTATTACATTCAGGATCGTCTTGAGGCTCAGAGCTGGGCGCGTCACTACCAG
CAGCTCGCCCGTGAAGAGAAAGAGGCAGAACTGGCAGACGACATGGAAAAAGG
CCTGCCCCAGCACCTGTTTGAATCGCTATGCATCGATCATTTGCAACGCCACGGG
GCCAGCAAAAAATCCATTACCCGTGCGTTTGATGACGATGTTGAGTTTCAGGAGC
GCATGGCAGAACACATCCGGTACATGGTTGAAACCATTGCTCACCACCAGGTTGA
TATTGATTCAGAGGTATAAAACGAATGAGTACTGCACTCGCAACGCTGGCTGGGA
AGCTGGCTGAACGTGTCGGCATGGATTCTGTCGACCCACAGGAACTGATCACCAC
TCTTCGCCAGACGGCATTTAAAGGTGATGCCAGCGATGCGCAGTTCATCGCATTA
CTGATCGTTGCCAACCAGTACGGCCTTAATCCGTGGACGAAAGAAATTTACGCCT
TTCCTGATAAGCAGAATGGCATCGTTCCGGTGGTGGGCGTTGATGGCTGGTCCCG
CATCATCAATGAAAACCAGCAGTTTGATGGCATGGACTTTGAGCAGGACAATGA
ATCCTGTACATGCCGGATTTACCGCAAGGACCGTAATCATCCGATCTGCGTTACC
GAATGGATGGATGAATGCCGCCGCGAACCATTCAAAACTCGCGAAGGCAGAGAA
ATCACGGGGCCGTGGCAGTCGCATCCCAAACGGATGTTACGTCATAAAGCCATG
ATTCAGTGTGCCCGTCTGGCCTTCGGATTTGCTGGTATCTATGACAAGGATGAAG
CCGAGCGCATTGTCGAAAATACTGCATACACTGCAGAACGTCAGCCGGAACGCG
ACATCACTCCGGTTAACGATGAAACCATGCAGGAGATTAACACTCTGCTGATCGC

CCTGGATAAAACATGGGATGACGACTTATTGCCGCTCTGTTCCCAGATATTTCGC
CGCGACATTCGTGCATCGTCAGAACTGACACAGGCCGAAGCAGTAAAAGCTCTT
GGATTCCTGAAACAGAAAGCCGCAGAGCAGAAGGTGGCAGCATGACACCGGACA
TTATCCTGCAGCGTACCGGGATCGATGTGAGAGCTGTCGAACAGGGGGATGATG
CGTGGCACAAATTACGGCTCGGCGTCATCACCGCTTCAGAAGTTCACAACGTGAT
AGCAAAACCCCGCTCCGGAAAGAAGTGGCCTGACATGAAAATGTCCTACTTCCA
CACCCTGCTTGCTGAGGTTTGCACCGGTGTGGCTCCGGAAGTTAACGCTAAAGCA
CTGGCCTGGGGAAAACAGTACGAGAACGACGCCAGAACCCTGTTTGAATTCACTT
CCGGCGTGAATGTTACTGAATCCCCGATCATCTATCGCGACGAAAGTATGCGTAC
CGCCTGCTCTCCCGATGGTTTATGCAGTGACGGCAACGGCCTTGAACTGAAATGC
CCGTTTACCTCCCGGGATTTCATGAAGTTCCGGCTCGGTGGTTTCGAGGCCATAA
AGTCAGCTTACATGGCCCAGGTGCAGTACAGCATGTGGGTGACGCGAAAAAATG
CCTGGTACTTTGCCAACTATGACCCGCGTATGAAGCGTGAAGGCCTGCATTATGT
CGTGATTGAGCGGGATGAAAAGTACATGGCGAGTTTTGACGAGATCGTGCCGGA
GTTCATCGAAAAAATGGACGAGGCACTGGCTGAAATTGGTTTTGTATTTGGGGAG
CAATGGCGATGACGCATCCTCACGATAATATCCGGGTAGGCGCAATCACTTTCGT
CTACTCCGTTACAAAGCGAGGCTGGGTATTTCCCGGCCTTTCTGTTATCCGAAATC
CACTGAAAGCACAGCGGCTGGCTGAGGAGATAAATAATAAACGAGGGGCTGTAT
GCACAAAGCATCTTCTGTTGAGTTAAGAACGAGTATCGAGATGGCACATAGCCTT
GCTCAAATTGGAATCAGGTTTGTGCCAATACCAGTAGAAACAGACGAAGAATCC
ATGGGTATGGACAGTTTTCCCTTTGATATGTAACGGTGAACAGTTGTTCTACTTTT
GTTTGTTAGTCTTGATGCTTCACTGATAGATACAAGAGCCATAAGAACCTCAGAT
CCTTCCGTATTTAGCCAGTATGTTCTCTAGTGTGGTTCGTTGTTTTTGCGTGAGCC
ATGAGAACGAACCATTGAGATCATACTTACTTTGCATGTCACTCAAAAATTTTGC
CTCAAAACTGGTGAGCTGAATTTTTGCAGTTAAAGCATCGTGTAGTGTTTTTCTTA
GTCCGTTACGTAGGTAGGAATCTGATGTAATGGTTGTTGGTATTTTGTCACCATTC
ATTTTTATCTGGTTGTTCTCAAGTTCGGTTACGAGATCCATTTGTCTATCTAGTTCA
ACTTGGAAAATCAACGTATCAGTCGGGCGGCCTCGCTTATCAACCACCAATTTCA
TATTGCTGTAAGTGTTTAAATCTTTACTTATTGGTTTCAAAACCCATTGGTTAAGC
CTTTTAAACTCATGGTAGTTATTTTCAAGCATTAACATGAACTTAAATTCATCAAG
GCTAATCTCTATATTTGCCTTGTGAGTTTTCTTTTGTGTTAGTTCTTTTAATAACCA
CTCATAAATCCTCATAGAGTATTTGTTTTCAAAAGACTTAACATGTTCCAGATTAT
ATTTTATGAATTTTTTTAACTGGAAAAGATAAGGCAATATCTCTTCACTAAAAACT
AATTCTAATTTTTCGCTTGAGAACTTGGCATAGTTTGTCCACTGGAAAATCTCAAA
GCCTTTAACCAAAGGATTCCTGATTTCCACAGTTCTCGTCATCAGCTCTCTGGTTG
CTTTAGCTAATACACCATAAGCATTTTCCCTACTGATGTTCATCATCTGAGCGTAT
TGGTTATAAGTGAACGATACCGTCCGTTCTTTCCTTGTAGGGTTTTCAATCGTGGG
GTTGAGTAGTGCCACACAGCATAAAATTAGCTTGGTTTCATGCTCCGTTAAGTCA
TAGCGACTAATCGCTAGTTCATTTGCTTTGAAAACAACTAATTCAGACATACATC

TCAATTGGTCTAGGTGATTTTAATCACTATACCAATTGAGATGGGCTAGTCAATG
ATAATTACTAGTCCTTTTCCTTTGAGTTGTGGGTATCTGTAAATTCTGCTAGACCT
TTGCTGGAAAACTTGTAAATTCTGCTAGACCCTCTGTAAATTCCGCTAGACCTTTG
TGTGTTTTTTTTGTTTATATTCAAGTGGTTATAATTTATAGAATAAAGAAAGAATA
AAAAAAGATAAAAAGAATAGATCCCAGCCCTGTGTATAACTCACTACTTTAGTCA
GTTCCGCAGTATTACAAAAGGATGTCGCAAACGCTGTTTGCTCCTCTACAAAACA
GACCTTAAAACCCTAAAGGCTTAAGTAGCACCCTCGCAAGCTCGGTTGCGGCCGC
AATCGGGCAAATCGCTGAATATTCCTTTTGTCTCCGACCATCAGGCACCTGAGTC
GCTGTCTTTTTCGTGACATTCAGTTCGCTGCGCTCACGGCTCTGGCAGTGAATGGG
GGTAAATGGCACTACAGGCGCCTTTTATGGATTCATGCAAGGAAACTACCCATAA
TACAAGAAAAGCCCGTCACGGGCTTCTCAGGGCGTTTTATGGCGGGTCTGCTATG
TGGTGCTATCTGACTTTTTGCTGTTCAGCAGTTCCTGCCCTCTGATTTTCCAGTCTG
ACCACTTCGGATTATCCCGTGACAGGTCATTCAGACTGGCTAATGCACCCAGTAA
GGCAGCGGTATCATCAACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTA
AGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAAT
TAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACA
GTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCA
TCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTAC
CATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGA
TTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGC
AACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGT
AGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGG
TGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGG
CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTC
CGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGC
ACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTG
AGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTG
CCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTC
ATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGA
GATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACT
TTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAG
GGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATT
ATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATT
TAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCT
GCATCGATTTATTATGACAACTTGACGGCTACATCATTCACTTTTTCTT