Donor plasmids for phenotypically neutral chromosomal gene insertions in Enterobacteriaceae

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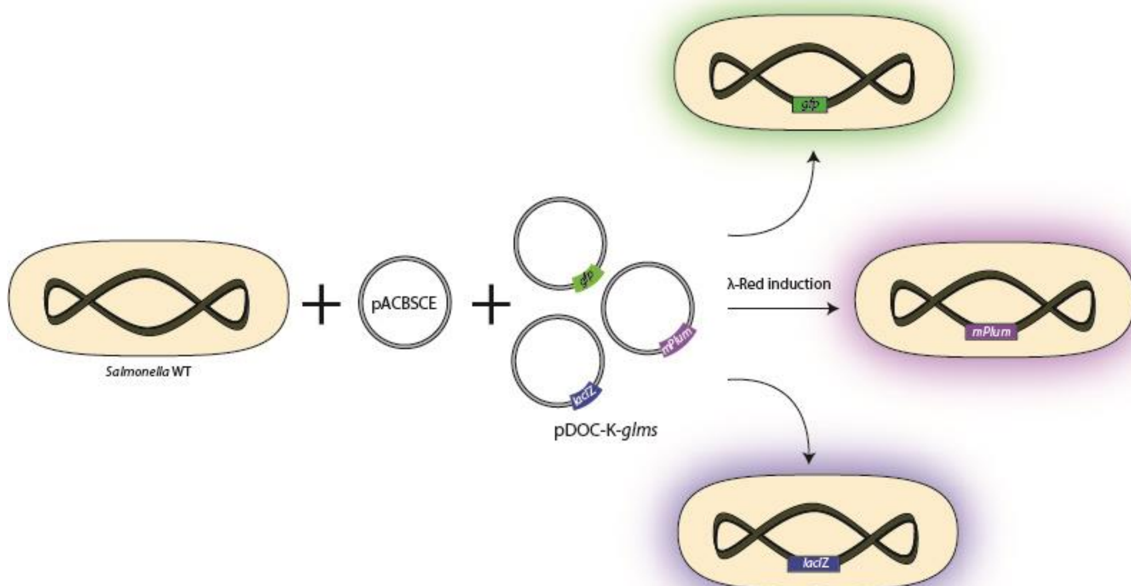
Abstract

Recombineering using bacteriophage lambda Red recombinase (λ-Red) uses homologous recombination to manipulate bacterial genomes and is commonly applied to disrupt genes to elucidate their function. This is often followed by introducing a wild-type copy of the gene on a plasmid to complement its function. This is often not however at a native copy number and introduction of a chromosomal version of a gene can be a desirable solution to provide wild-type copy expression levels of an allele in trans. Here, we present a simple methodology based on the lambda-red based 'gene doctoring' technique, where we developed tools used for chromosomal tagging in a conserved locus downstream of *glmS* and found no impact on a variety of important phenotypes. The tools described provide an easy, quick and inexpensive method of chromosomal modifications for the creation of a library of insertion mutants to study gene function.

Graphical abstract



34 **Introduction**

35 Despite having access to millions of bacterial genome sequences, many gene
36 functions remain unknown[1]. Several approaches have been adopted for modifying
37 the bacterial genome to characterise the role and function of predicted genes and
38 other elements [2, 3]. One of the most commonly used is `recombineering', using the
39 bacteriophage lambda-derived Red recombinase (λ-Red) [4] to mediate homologous
40 recombination with a linear double-stranded DNA fragment, directed by flanking
41 regions with homology to the target region [5, 6]. The original technique has been
42 refined in the 'gene doctoring' method [7] and proved applicable in various
43 *Enterobacteriaceae* including *Escherichia coli*, *Salmonella enterica* spp [8],
44 *Pseudomonas putida* [9] and *Klebsiella pneumoniae* [10].

45 Complementation is a key step in validating gene function and single-copy
46 chromosomal insertions are often preferable to multiple plasmid-borne copies [11]. We
47 used the gene doctoring method and created tools to allow directed integration of
48 DNA to the chromosome. We demonstrate chromosomal integration of reporter
49 genes into a locus downstream of the *glmS* gene of *S.* Typhimurium (herein referred
50 to as the *glmS* site), where fitness impacts from disruption have been suggested to
51 be minimal [12]. The *glmS* site is the target locus of the Tn7 transposon-based system
52 that is widely used for chromosomal insertions in species including *S.* Typhimurium,
53 *E. coli*, [13] *Pseudomonas aeruginosa* and *Burkholderia mallei* [14]. We produced a
54 simplified plasmid into which a wide range of desired sequences can be cloned for
55 rapid insertion specifically at the *glmS* site. Our new plasmids are publicly available
56 (Table 1), and are expected to facilitate efficient, simple genome editing in a range of
57 bacterial species.

58 **Methods**

59 **Bacteria, plasmids and primers**

60 All plasmids and primers used in this study are detailed in table 1. *E. coli* DH10B and
61 NEB5α were used as hosts for vector construction. Reporter genes were inserted
62 into the chromosome of *Salmonella enterica* serovar Typhimurium ATCC 14028S.
63 Cells were made electrocompetent following the protocol detailed by Trampari et al.
64 [15]. Chemically competent cells were made following the $CaCl_2$ method of Sambrook
65 et al. [16] and transformed by heat-shock at 42 °C for 45 sec.

66 **Gene doctoring procedure**

67 HR1 and HR2 were amplified by PCR from *S*. Typhimurium using primers listed in
68 table 1, and added to the relevant MSCs through restriction ligation, using EcoRI and
69 KpnI for HR1, and XhoI and NheI for HR2, to make pDOC-K-*glmS*. *S*. Typhimurium
70 was transformed with the pDOC-K-*glmS* vector variant and the pACBSCE helper
71 plasmid [17] carrying the λ-Red genes. Successful recombinants were identified via
72 antibiotic selection. Chromosomal integration was induced following the gene
73 doctoring methodology outlined by Lee et al. [17]. Single colonies were screened for
74 chromosomal integration by PCR with primers annealing either side of the region to
75 be modified. Loss of gene doctoring plasmids was checked by patching colonies to
76 LB agar plates containing appropriate antibiotics and an index plate containing no
77 antibiotic. The kanamycin resistance cassette, flanked by Flp recombinase
78 recognition sites, was removed from WT::*lacIZ* by pCP20 as described by Datsenko
79 and Wanner [4].

80 **Whole genome and whole plasmid shotgun sequencing**

81 Genomic DNA was extracted using a FastDNA Spin Kit for faeces (MP Bio) and
82 plasmids were purified with a NucleoSpin Plasmid Miniprep kit (Macherey-Nagel).

83 Sequencing libraries were prepared with the Nextera XT DNA Library Prep kit
84 (Illumina) and quantified using the Quant-iT dsDNA high sensitivity assay kit.
85 Genome and plasmid samples were pooled and run on an Illumina NextSeq 500
86 using a mid-output sequencing kit for 150 bp paired-end reads. Sequences were
87 quality checked by FastQC v0.11.7 and trimmed with Trimmomatic v0.36 with a
88 minimum read length of 40 bp and a sliding window of 4 bp with average quality of
89 15. The reads were then mapped against the expected sequence for each sample in
90 Geneious Prime 2019 with the Geneious mapper and default settings.

91 **Confirming fitness neutrality of the *glmS* insertion site**

92 Growth kinetics were determined by measuring optical density (OD) at 600 nm at 15-
93 minute intervals across 20 hours using a FLUOstar Omega plate reader (BMG
94 Labtech). Competition assays were undertaken by growing strains normalised by OD
95 in LB broth incubated at 37 °C for 24 hours and plating the culture on LB agar
96 supplemented with IPTG and Xgal after 0 and 24 hours of coculture. Both assays
97 consisted of a minimum of 5 technical replicates of 2 biological replicates. Biofilm
98 assays were undertaken following the protocols described in Baugh et al. [18]. The
99 *Galleria mellonella* infection model was used to determine relative pathogenicity
100 following the protocol outlined in Bender et al. [19] with 10 larvae per condition.
101 Preliminary experiments with wild type *S*. Typhimurium determined that the lethal
102 dose required to kill all larvae in 24 hours was approximately 1.5×10^4 CFU per
103 inoculum.

104 **Results**

105 **Constructing a vector for rapid chromosomal integrations**

106 We modified pDOC-K [17] to include the necessary homologous regions to target
107 insertions to the Tn7 recognition site downstream of *glmS*. The integration vector
108 pDOC-K-*glmS* was generated by cloning homologous regions to the *glmS* site (HR1
109 and HR2) into the first (MCS1) and second (MCS2) multiple cloning sites of pDOC-K,
110 respectively (Figure 1). Successful recombinants were confirmed through PCR and
111 Sanger sequencing. The creation of the pDOC-K-*glmS* vector reduces the number of
112 genetic manipulations required, compared to pDOC-K, for the insertion of any
113 sequence to the *glm*S site of *S.* Typhimurium. Insertions at this conserved site are
114 possible in many other Gram-negative bacteria that are amenable to Tn7-based
115 insertion protocols, only requiring species-specific modifications to the homologous
116 regions of pDOC-K-*glmS* [13].
117 A third multiple cloning site (MSC3) was added to pDOC-K-*glmS* to easily facilitate
118 ligation of genes of interest into this plasmid for chromosomal integration (Figure 1a).
119 The additional nucleotides for MCS3 were included on the forward primer used for
120 the amplification of HR2 (HR2_For (MCS3), Table 1). MCS3, containing XhoI, NdeI,
121 SmaI, NotI and HindIII restriction sites, allows chromosomal manipulations to remain
122 accessible for most molecular biology laboratories with commonly available reagents
123 and basic knowledge of restriction ligation. Additionally, the homologous regions can
124 be changed through restriction ligation to allow a relatively easy, inexpensive and
125 quick method of directed insertion into any desired locus. These regions could be
126 produced as chemically synthesised DNA fragments or as PCR products.

127 **Integrating reporter genes into the *Salmonella* genome**

128 The pDOC-K-*glmS* vector was used to integrate three reporter genes into the
129 chromosome of *S*. Typhimurium to create strains with easily-identifiable phenotypic
130 markers. These included elements of the *lac* operon, consisting of the *lac* repressor
131 *lacI* and β-galactosidase *lacZ*, and two fluorescent proteins, enhanced green
132 fluorescent protein (eGFP) and mPlum. Fluorescent markers were integrated into the
133 chromosome under the control of the strong, constitutive promoter of *acpP*, which
134 exhibits high levels of transcription in *Salmonella* [20]. Reporter genes were amplified
135 by PCR and ligated blunt-end in the SmaI position of MCS3 in pDOC-K-*glmS*. Blunt-
136 end ligation allows transgenes to be integrated in a forward or reverse orientation,
137 with the forward orientation observed for the *lacIZ* and *mplum* genes, and both
138 orientations observed for the *egfp* gene (Table 1). Successful introduction of *egfp*
139 and *mplum* was observed through green and purple fluorescence, respectively. The
140 orientation of the *egfp* gene in the vector affected fluorescence, with increased
141 fluorescence seen when the *egfp* gene was transcribed in the same direction as the
142 upstream kanamycin resistance cassette, but orientation did not seem to affect
143 fluorescence when *egfp* was chromosomally located. Successful integration of the
144 *lac* operon into the vector and the chromosome was confirmed through whole
145 genome sequencing, which revealed chromosomal insertion at the desired site only,
146 with no non-specific insertions observed. It was also confirmed by blue/white
147 screening of colonies growing on agar supplemented with X-gal and IPTG. The
148 kanamycin resistance cassette can be removed from the chromosome following
149 insertion using flippase-based antibiotic cassette removal, and this could be modified
150 for scar-free genome modifications if necessary [21]. This is a highly efficient method
151 that works consistently independently of the nature of the target. Kanamycin cassette

152 removal has been achieved for all the targets described in this study with eight out of
153 the eight colonies checked for each target, having lost the antibiotic marker, after
154 flippase treatment.
155 The fitness neutrality of the *glmS* insertion site has been previously suggested by
156 existing publications on Tn7 transposition [12-14]. We thoroughly investigated the
157 fitness of *S*. Typhimurium with the chromosomally integrated *lac* operon (WT::*lacIZ*)
158 compared to the wild type, and found no difference in biofilm formation (Figure 1b),
159 growth kinetics (Figure 1c), competitive fitness (Figure 1d), pathogenicity (Figure 1e),
160 membrane permeability, antibiotic susceptibility and motility (data not shown)
161 between the parent strain and that carrying the insertion adjacent to *glmS*. Similarly,
162 introduction of diverse targets downstream of the *glmS* locus had no measurable
163 impact on fitness (data not shown). These included genes encoding the
164 transcriptional regulator -RamR, the membrane protein EnvZ and the cyclic-di GMP
165 regulating protein YjcC [15].
166 **Conclusion**
167 Gene doctoring is a convenient and efficient variation of recombineering in any strain
168 that is not amenable to transformation by the linear double-stranded DNA required
169 by other methods. We used this approach to develop a useful tool for chromosomal
170 integrations that is easy-to-use and has no discernible fitness costs, the pDOC-K-
171 *glmS* vector. Its application is not limited to chromosomal tagging but can also be
172 used for the complementation of a range of different proteins, replacements as well
173 as introduction of point mutations. In our recent work on biofilm evolution, we used
174 this system to introduce a series of different genes back to *Salmonella* mutants and
175 successfully complemented phenotypes of interest, with no observed fitness cost [15].
176 We encourage further modification and sharing of gene doctoring plasmids based on

177 our constructs to broaden the available toolkit for gene doctoring of diverse targets
178 and facilitate efficient genetic engineering in multiple bacterial species.

179 **Author Contribution**

180 ERH, ET, NT and MAW wrote and edited the manuscript. ET and NT designed and
181 created the vectors described in this study. ERH and GJW performed phenotypic
182 assays.

183 **Conflict of interest**

184 The authors declare that there are no conflicts of interest.

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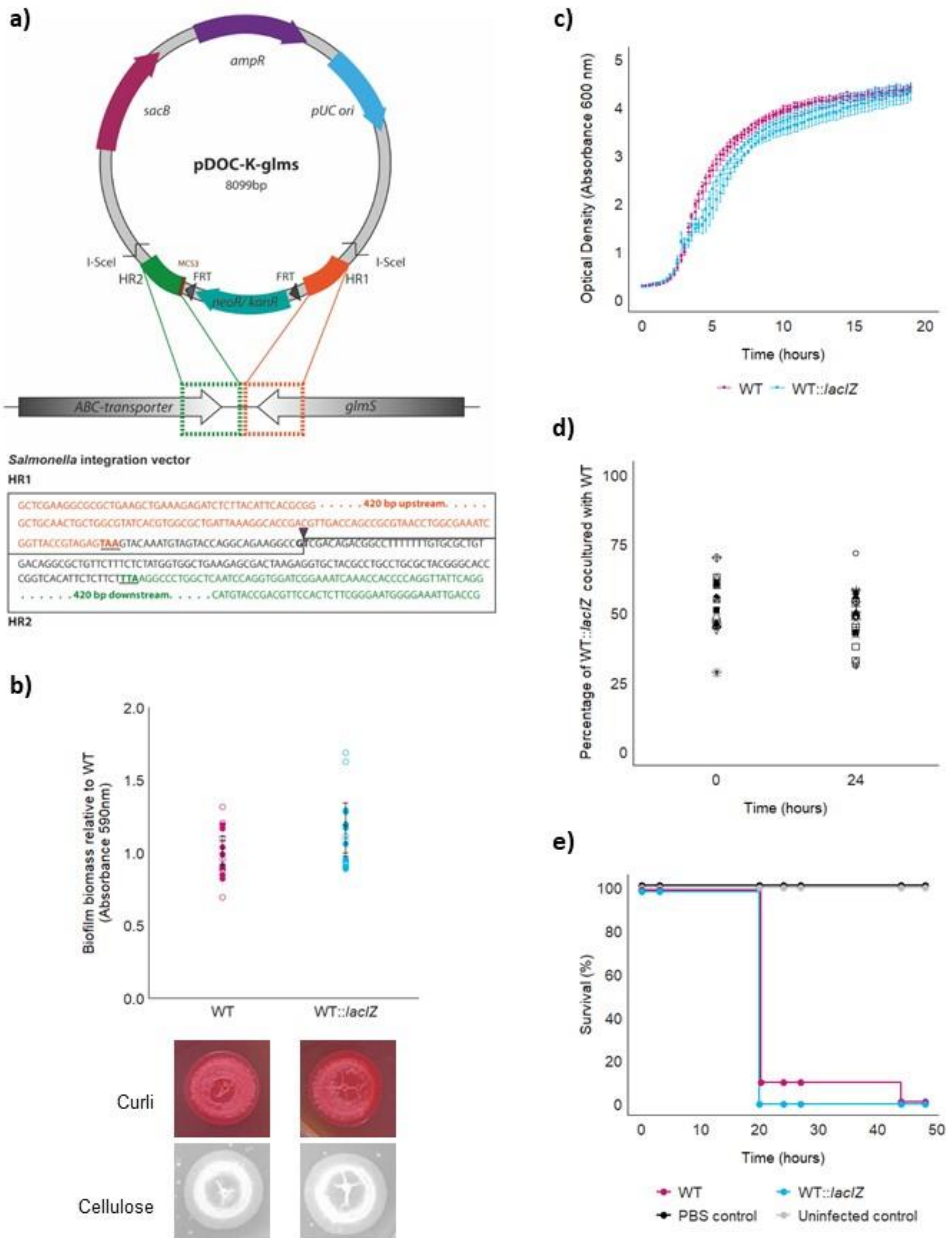
276

277 **Figure 1**



278

279 Error! Reference source not found.**Figure 1. a: Vector map of pDOC-K-*glmS*.** HR1

280 (highlighted in orange) is a 420bp region homologous to the end and the

281 downstream part of *glmS* (Tn7 recognition site-indicated with an arrow), cloned in the
282 MCS1 of the pDOC-K vector. HR2 (highlighted in green) starts at the Tn7 recognition
283 site and expands 420bp downstream. MCS3 is shown integrated alongside HR2. **b:**
284 **Comparison of biofilm formation** by wild type Salmonella Typhimurium and
285 WT::*lacIZ*, determined by biofilm biomass and biofilm matrix composition of curli and
286 cellulose. Points distinguish two biological replicates, each with 8 technical
287 replicates. Error bars show 95% confidence intervals. **c: Growth kinetics** of
288 WT::*lacIZ* compared to wild type *S*. Typhimurium over 20 hours. Points represent the
289 mean of each biological replicate, of which there were 2, both with 5 technical
290 replicates. Error bars show 95% confidence intervals. **d: Competition assay**
291 between wild type *Salmonella* Typhimurium and the strain with chromosomally
292 integrated *lacIZ* (WT::*lacIZ*). Points represent the percentage of WT::*lacIZ* present
293 after 0 and 24 hours of co-culture, in 2 biological replicates with a minimum of 9
294 technical replicates. **e: Pathogenicity** of WT::*lacIZ* compared to wild type
295 *Salmonella* Typhimurium in a *Galleria mellonella* infection model.

296 **Table 1: Plasmids and primers used in this study**

Tool	Source/Sequence	Description
pDOC-K	[17]	Gene doctoring plasmid modified in this study.
pDOC-K-*glmS*	This study (Addgene ID 158058)	Donor plasmid for chromosomal integrations of any sequence at a locus downstream of the *glm*S gene in *S.* Typhimurium (and possibly other *Enterobacteriaceae*), using kanamycin resistance as a selectable marker.
pDOC-K-*glmS*-GFPfor	This study (Addgene ID 158059)	Vector based on pDOC-K-*glmS*, used for insertion of *egfp* and the constitutive *acpP* promoter into the *S.* Typhimurium chromosome. *Gfp* is in the forward orientation giving strong fluorescence.

pDOC-K-*glmS*-GFPrev	This study (Addgene ID 158060)	Vector based on pDOC-K-*glmS*, used for insertion of *egfp* and the constitutive *acpP* promoter into the *S.* Typhimurium chromosome. *Gfp* is in the reverse orientation giving weak fluorescence.
pDOC-K-*glmS*-*lacIZ*	This study (Addgene ID 158061)	Vector based on pDOC-K-*glmS*, used for insertion of the *lacI/Z* inhibitor/reporter system into the *S.* Typhimurium chromosome. Expression is controlled by the IPTG-inducible *lac* promoter.
pDOC-K-*glmS*-mPlum	This study (Addgene ID 158062)	Vector based on pDOC-K-*glmS*, used for insertion of mPlum and the constitutive *acpP* promoter into the *S.* Typhimurium chromosome. mPlum is in the forward orientation giving strong fluorescence.

pACBSCE	[17]	Helper plasmid containing λ-Red genes for recombination.
pZEP08	[22]	Template for the amplification of *egfp* fluorescent marker.
mPlum-pBAD	[23] (Addgene ID 54564)	Template for the amplification of mPlum fluorescent marker.
*gfp*_For (acpP)	TGCTCATGGATCC**GTTGCAAATTTTTCAACATTTTATACACT**GATTTAAGAAGGAGATATACATATGAGTAA	Primers for the amplification of *gfp* from pZEP08, with the *acpP* promoter sequence (in bold) included on the forward primer.
*gfp*_Rev	TGCTCATGGTACCTTATTTGTAGAGCTCATCCAT	
mplum_For (acpP)	**GTTGCAAATTTTTCAACATTTTATACACT**ACGAAAACCATCGCGAAAGCGAGTTTTGGATTTAAGAAGGAGATATACATATGGTGAGCAAGGGCGAGGAG	Primers for the amplification of mPlum from mPlum-pBAD, with the *acpP* promoter sequence (in bold) included on the forward primer.
mplum_Rev	TTAGGCGCCGGTGGAGTGG	
HR1_For	TACGTGAATTCGCTCGAAGGCGCGCTGAAG	Primers for the amplification of

HR1_Rev	ACGTAGGTACCCGGCCTTCTGCCTGGTACTACATTTG	homologous region 1 from *S*. Typhimurium.
HR2_For (MCS3)	TACG**TCTCGAGCATATGCCCGGGGCGGCCGCAAGCTT**TCGACAGACGGCCTTTTTTTG	Primers for the amplification of homologous region 2 from *S*. Typhimurium, and the introduction of MCS3 (in bold).
HR2_Rev	GCATAGCTAGCCGGTCAATTTCCCCATTCCC	
lacIZ_For	AGATCCCTCAATAGCGGCCGCACCATCGAATGGCGCA	Primers for the amplification of *lacI* and *lacZ* from *E. coli* MG1655.
lacIZ_Rev	CCCAAGCTTCTCGAGTTATTTTTGACACCAGACCAACTGGTAATGGTAGCGACCGGCGCT	

297