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Construction and application of a built-in dual luciferase reporter for microRNA functional analysis

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ABSTRACT

Background: As key gene regulators, microRNAs post-transcriptionally modulate gene expression via binding to partially complementary sequence in the 3’ UTR of target mRNA. An accurate, rapid and quantitative tool for sensing and validation of miRNA targets is of crucial significance to decipher the functional implications of miRNAs in cellular pathways.
Results: Taking advantage of an improved restriction-free cloning method, we engineered a novel built-in dual luciferase reporter plasmid where *Firefly* and *Renilla* luciferase genes were assembled in a single plasmid named “pFila”. This design eliminates the transfection of a separate control plasmid and thus minimizes the time and labor required for miRNA-target sensing assays. pFila consistently produces *Firefly* and *Renilla* luciferase activities when transfected into human-, monkey- and mouse-derived mammalian cell systems. Moreover, pFila is capable of recapitulating the interaction of miR-16 and its known target CCNE1 in Hela cells. Additionally, pFila is shown to be a sensitive miR-biosensor by evaluating the inhibition efficiency of endogenous miRNA.
Conclusions: pFila would facilitate miRNA target identification and verification in a rapid and simplified manner. Also, pFila is a sensitive biosensor for active miRNA profiling *in vivo*.

Keywords: biosensor, luciferase, ligase-independent, miRNA, target

INTRODUCTION

MicroRNAs are key post-transcriptional regulators of gene expression in a variety of cellular events. They mediate translational repression, and sometimes destabilization, of target mRNAs by directing miRISC (<u>mi</u>cro<u>R</u>NA-<u>i</u>nduced <u>s</u>ilencing <u>c</u>omplex) to imperfect complementary sequences in 3’ UTR (Bartel, 2009). It has been predicted that more than 60% of human genes are putative targets of one or more miRNAs, while it is also suggested that an individual miRNA is capable of regulating multiple target mRNAs (Backes et al. 2010). Consequently, a major challenge in miRNA study is the experimental identification and validation of its functional target(s). Various reporter systems have been developed to probe the interaction between individual miRNA and its target (Lee et al. 2008), of which dual luciferase assay is widely adopted to achieve this end (Brennecke et al. 2005). The current dual luciferase assay encompasses two separate plasmids, one containing the region of interest and the other serving as internal control to normalize transfection variation (Robertson et al. 2010). A major drawback of this system is the tedious steps for preparing and transfecting control vector. A more convenient reporter is desired to simplify the conventional protocol.

In addition, there are several methods, including northern blotting, real-time PCR, microarray and deep sequencing, to quantify miRNAs (Willenbrock et al. 2009). However, these techniques simply output the homeostasis of endogenous miRNAs other than active molecules. Moreover, these approaches are either time-consuming and laborious or cost-ineffective. Therefore, a convenient and quantitative miRNA biosensor is desired to measure functional miRNA *in vivo*.

In this study, by taking advantage of ligase-free homologous recombination in *E. coli*, we engineered a novel reporter that integrated *Firefly* and *Renilla* luciferase genes (*Fluc* and *Rluc,* same below) in a single plasmid. Its expressivity and applicability were further examined to demonstrate that this novel reporter will facilitate the screening and sensing of miRNAs and their targets in a simplified and precise manner.

MATERIALS AND METHODS

DNA and RNA oligos

Unless stated elsewhere, all DNA and RNA oligos are presented as 5'→3' direction. Primers for amplifying *Fluc* gene were Pf, AAGGATCCAGGTGGCACTTTTCG **TGCGATCTGCATCTCAATTAG**; Pr, GAAAAATAAACAAATAGGGGTTCCGCGCAC **CTCACATGTTCTTTCCTGC** (sequence annealing to *Fluc* gene was shown in boldface; sequence complementary to insertion site on pRL-TK was underlined). 3' outermost primer P2R was CGAAAAGTGCCACCTGGATCCTT. Sequencing primers for pFila was SF, GATGCACCTG ATGAAATGGG; SR, AGGACAGGTG CCGGCAGCGC. For creation of *ApaI* site, see details in reference (Wang et al. 2009). RNA oligos were chemically synthesized and purified by Genepharma Co. Ltd., (Shanghai, P.R. China). Human miR16-1 was sense UAGCAGCACGUAAAUAUUGGCG and antisense CGCCAAUAUUUACGUGCUGCUA. Negative control for miRNA mimics was sense UUGUACUACACAAAAGUACUG and antisense CAGUACUUUUGUGUAGUACAA. siRNA against *Rluc* mRNA was sense GUAGCGCGGUGUAUUAUACdTdT and antisense GUAUAAUACACCGCGCUACdTdT. Methylated anti-miR-16-1 inhibitor CGC CAA UAU UUACGU GCU GCU A, scramble anti-miR control UUG UAC UAC ACA AAA GUA CUG.

Construction of plasmids

pFila was fabricated by bridging-PCR coupled with homologous recombination in bacteria. A duplex bridging PCR was conducted in a 50 µl mixture: Pf 4 µl (250 nM), Pr 2 µl (125 nM), P2R primer 2 µl (125 nM), pGL3-promoter plasmid 1 µl (5 ng), modified pRL-TK plasmid 1 µl (10 ng or 50 ng), 2 mM dNTP 5 µl, 25 mM $MgSO_4$ 2 µl, 10 x KOD buffer 5 µl, KOD plus 1 µl (1 unit), PCR-grade water 27 µl. The condition was: 95ºC 2 min, 30 cycles of (95ºC 15 sec, 55ºC 30 sec, 68ºC 6.5 min). The PCR products were digested with *DpnI* (Fermentas, Lithuania) at 37ºC for 2 hrs to destroy methylated plasmids while keeping the nascent DNA intact with the following reaction: PCR products, 26 µl; 10 x Tango buffer 3 µl; *Dpn I* 1 µl (1 unit). An aliquot of 5 µl digested PCR products were transformed into *E. coli* DH5α to generate recombinants that were subsequently sequenced to verify the integrity. Pf and Pr primer pair was used to amplify *Fluc* gene composed of SV40 promoter, *Fluc* coding region and SV40 late poly(A) signal. *Fluc* gene was designed to fuse into a modified pRL-TK plasmid downstream of 3'UTR of *Rluc* and upstream of beta-lactamase gene. For the sequence context of human CCNE1 3'UTR, see details in reference (Wang et al. 2009). The wild-type and mutated human CCNE1 target regions were sub-cloned into pFila with *Xba I* and *Apa I*.

Cell culture, transfection and dual luciferase assay

Human cervical carcinoma Hela cells, African green monkey kidney Vero cells and mouse myoblast C2C12 cells were maintained in high glucose DMEM (Invitrogen) supplemented with 10% fetal calf serum (Gibco) at 37ºC and 5% CO_2. 4 x 10^4 cells were seeded in a 24-well plate one day before transfection. For miRNA mimics and plasmid co-transfection, 1 µl 20 µM chemically synthesized miR16 mimics and 50 ng pFila (pFila-CCNE1-wildtype and pFila-CCNE1-mut1&2) or 50 ng pGL3-promoter (internal control) and pRL-ML plasmids (pRL-ML-CCNE1-wildtype and pRL-ML-CCNE1-mut1&2; 25 ng each) were mixed with 2 µl Lipofectamine2000 (Invitrogen) as transfection complex. For evaluation of endogenous miRNA inhibition with pFila, 20 nM 2'-O-methylated anti-human miR16-1 inhibitor was transfected into Hela cells by 0.5 µl Lipofectamine2000 (Invitrogen), RNAiMAX (Invitrogen), Sofast

(Sunmabio, China), Fugene (Roche), respectively. All transfections were performed in three independent experiments with each in triplicate. A DLR™ Assay (Promega) was adopted to measure luciferase activity in a Glomax luminometer essentially according to manufacturer's instruction.

Statistical analysis

Luciferase levels were reported as ratio over that observed in control transfections, where *Rluc* activities were normalized to *Fluc* activities. The data represented the mean ± S.D. of three independent experiments and were analyzed by Student's *t*-test. Differences below $p < 0.01$ were regarded as significant.

Accession number

The sequence and annotation of pFila has been deposited in Genbank with accession number HQ425563. pFila is freely available upon request.

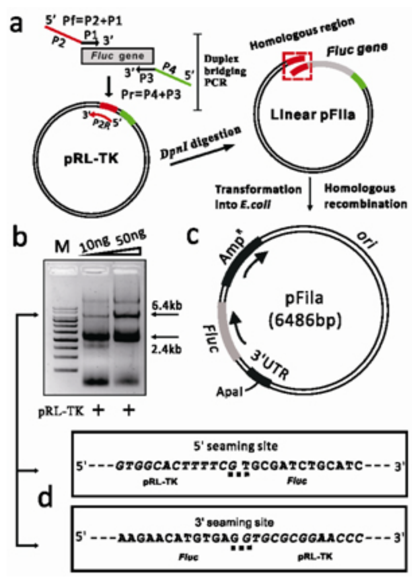

Fig. 1 Concept and engineering of pFila. (a) In this reaction, the primary process was the production of *Fluc* gene by Pf (Pf is composed of P1 and P2, which are complementary to upstream of *Fluc* gene and pRL-TK plasmid, respectively) and Pr (Pr is composed of P3 and P4, which are complementary to downstream of *Fluc* gene and pRL-TK plasmid, respectively) primers. In the secondary process, amplified *Fluc* gene bearing a deliberately designed region annealed with its homologous sequence in pRL-TK, thus generating a large linear fragment. In order to exponentially amplify the linear fragment, the outermost Pf and P2R (complementary to P2) primer pair initiated the ternary process to produce adequate fused fragment. (b) Duplex bridge PCR. The 2.4 kb fragment was *Fluc* gene, while the 6.4 kb fragment was the desired fusion product. M is 1 kb DNA ladder. (c) Map of pFila. The engineered pFila is 6486 bp in length. *XbaI* and *ApaI* restriction sites are located in the 3'UTR of *Rluc* to facilitate the cloning of miRNA target for reporter assay. (d) 5' and 3' seaming sites of *Fluc* into pRL-TK. For detailed information, please refer to Gene sequence 1.

RESULTS AND DISCUSSION

The complicated procedures of the current dual luciferase reporter assay for miRNA target screening prompted us to upgrade its practicality for simplified manipulations. Specifically, we aimed to integrate *Fluc and Rluc* genes in a single vector. As restriction sites were not available to sub-clone *Fluc* gene to pRL-TK plasmid with ligase-dependent method, we adopted an improved restriction-free gene fusion approach inspired by the principle of site-directed mutagenesis (Zheng et al. 2004). As shown in Figure 1a and Figure 1b, a duplex bridging PCR was carried out to produce the 6.4 kb linear fusion fragment with homologous sequences at both 5’ and 3’ ends. The PCR products were digested by *DpnI* and transformed into *E. coli* to achieve the circular plasmid based on homologous recombination. Sequencing of the recombinant (named pFila) revealed that *Fluc* gene had been successfully fused into pRL-TK plasmid at designed location (Figure 1c and Figure 1d; Gene sequence 1).

We then evaluated the expressivity of pFila in different mammalian systems. As presented in Figure 2a, luminescence of pFila was reported in a wide linear range when transfected into human-, mouse- and monkey-sourced cell lines at gradient amounts, indicating that pFila consistently produces luciferases *in vivo*. This also implies that the ordered assembly of *Fluc* and *Rluc* luciferase genes in pRL-TK plasmid does not interfere with their individual expression. Next, the applicability and reproducibility of pFila were examined by recapitulating the regulation of human miR16-1 and its known target CCNE1 (Wang et al. 2009). miR16-1 mimics down-regulated the *Rluc* activity fused with wild-type CCNE1 3’UTR but not a mutant 3’UTR (Figure 2b); the latter carried altered residues that were introduced in the miR16-1 “seed-pairing” recognition site (Figure 2b). This observation perfectly photocopied the result that was achieved by traditional dual reporter assay (Figure 2b), indicating that pFila as a more convenient reporter is fully applicable to miRNA functional analysis. Finally, we applied pFila carrying the wild-type and mutant 3’TUR of CCNE1 to assessing the blockage efficiency of endogenous miR16-1. 2’-O-methylated anti-miRNA-16-1 RNA oligo was transfected by four types of transfection reagents, *i.e.* Lipofectamine2000, RNAiMAX, Fugene, Sofast. Inhibition efficacy of endogenous miR16-1 varied, of which RNAiMAX achieved the most potent blocking effect. This assay implies that pFila is a sensitive miRNA biosensor to reflect the level of functional miRNAs. It also suggests that the choice of delivery method is an important determinant when conducting loss-of-function analysis of miRNAs.

In summary, we have successfully engineered a novel dual luciferase plasmid that incorporated *Fluc* and *Rluc* genes in a single vector, allowing the simultaneous expression of both luciferase genes. This improvement maintains comparable reproducibility but minimizes the time and labor required in conventional dual luciferase protocol. Furthermore, several lines of evidence were presented to demonstrate its application in miRNA functional analysis. These results indicate that pFila will find its wide application in the screening, identification and validation of miRNA with its potential mRNA targets.

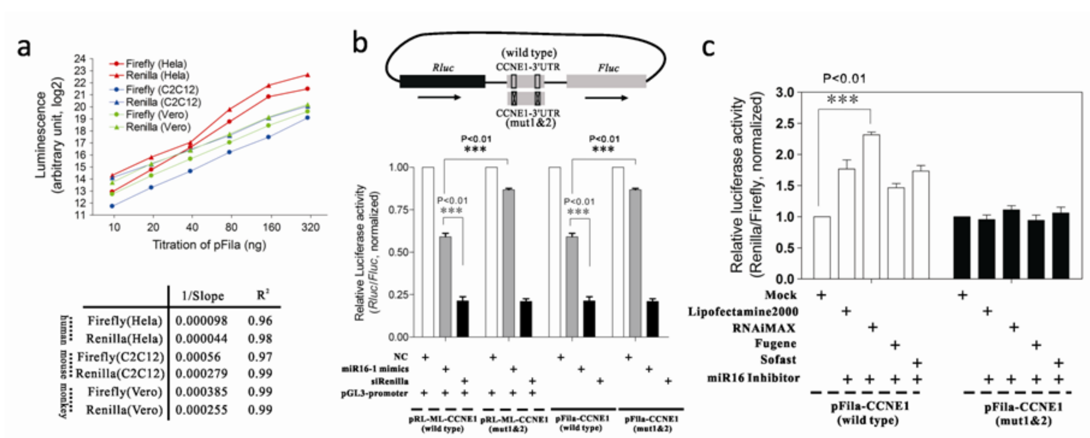

Fig. 2 Expressivity and application of pFila. (a) pFila consistently produces *Fluc* and *Rluc* activities in human-, mouse- and monkey-sourced mammalian cell lines. pFila was gradientlytransfected into the cells stated above and *Fluc* and *Rluc* luciferase levels were determined by a DLR^{TM} Assay (Promega). As shown in this graph, *Fluc* and *Rluc* activities were in a linear range for all the selected points. (b) pFila is capable of recapitulating the interaction of miR16 and its known target CCNE1. Wild-type and mutant CCNE1 3’UTRs were sub-cloned into pFila and co-transfected with miR16-1 mimics. Conventional dual luciferase reporter was conducted in parallel to compare their reproducibility. siRNA against *Rluc* serves as positive control. *Rluc* with mutant CCNE1 3’UTR was rescued in comparison with pFila-CCNE1-3’UTR-wildtype, indicating that CCNE1 is a direct target of miR16 as previously reported (Wang et al. 2009). (c) miR16-1 inhibitors were co-transfected with pFila-CCNE1-3’UTR-wildtype or mutant plasmids into Hela cells by different transfection reagents to block endogenous miR16-1. Inhibition efficacy varies with the selected agents, where RNAiMAX achieved the most potent inhibition. It implies that pFila is a sensitive biosensor for functional miRNA profiling.

Gene sequence 1. Sequencing of the seaming sites: sequence annealing to Fluc gene was shown in boldface; sequence complementary to insertion site on pRL-TK was underlined.

Seq.1 (sequencing primer SF: GATGCACCTGATGAAATGGG)

TTTTTAAATCCAATTCGGTTGAACGAGTTCTCAAAAATGAACAATAATTCTAGATTCCGAGATATCGGTAATGGGC
CCTAGAGCGGCCGCTTCGAGCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGA
AAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTA
ACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCT
CTACAAATGTGGTAAAATCGATAAGGATCCAGGTGGCACTTTTCG**TGCGATCTGCATCTCAATTAG**TCAGCAACCA
TAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATCGCTGACT
AATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTT
GGAGGCCTAGGCTTTTGCAAAAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGACGCCAAAAACAT
AAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGA
TACGCCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGAGTACTTCG
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AAAAATTTTGAACGTGCAAAAAAAGCTCCCAATCATCCAAAAAATTATTATCATGGATTCTAAAACGGATTACCAGG
GGATTCAGTCGATGTACACGTCGTCACATCTCATCTACCTTCCCGGTTTATGATACGATTTTGTGCAAGAGTCTTC
GATAGGACAAGACAATTGCACTGATCATGACCTCTCTGATCTACTGGACTGCTAGGTGCGACCTTGTCTCATAGAC
TGCCTGGCCGGTAGAAATTCTCGCC

Seq.2 (sequencing primer SR: AGGACAGGTG CCGGCAGCGC)

CCTGGAAATAAGAGGTGAGGTGATCATCAGGTAATGGGGTACTGTCGACGATACCCAATGCTATCAGGAGGCACGT
TATTCAGCGATCTGTCTATTCGGTCATCATAGTTGCTGACTCCCGTCGGTAGATAACTACGATACGGAGGCTACAA
TCTGCCCAGTGCTGCATGATACGCGAGACCACGCTCACCGGCTCAGATTATCAGCATAACAGCAGCGAGGGCCGAG
CGCAGAGTGTCTGCACTTATCCGCTCCATCCAGTCTATAATGTGCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAA
TAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGC
TCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTC
CGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGT*/
CATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGA
CCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTG
GAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGC
ACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCA
AAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATC
AGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCAC**CTC**
ACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCG
CCGCAGCCGAACGACCGAGCGCAGCGAGTCAGAGCGAGTGGGG

Seq.3 pFila sequence

Base pair:	6486bp
HSV TK promoter	7-759
Chimeric intron	826-962
T7 RNA polymerase Promoter (-17 to +2)	1006-1024
T7 RNA polymerase transcription initiation site	1023
Rluc reporter gene	1034-1969
XbaI restriction site	1971-1976
ApaI restriction site	1995-2000
SV40 late polyadenylation signal(upstream)	2039-2240
SV40 promoter	2279-2481
Fluc reporter gene	2511-4163
SV40 late polyadenylation signal(downstream)	4195-4416
Beta-lactamase (AmpR)	4800-5660
pBR322 plasmid replication origin	5802-6445

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  1 AGATCTAAAT GAGTCTTCGG ACCTCGCGGG GGCCGCTTAA GCGGTGGTTA
 51 GGGTTTGTCT GACGCGGGGG GAGGGGGAAG GAACGAAACA CTCTCATTCG
101 GAGGCGGCTC GGGGTTTGGT CTTGGTGGCC ACGGGCACGC AGAAGAGCGC
151 CGCGATCCTC TTAAGCACCC CCCCGCCCTC CGTGGAGGCG GGGGTTTGGT
201 CGGCGGGTGG TAACTGGCGG GCCGCTGACT CGGGCGGGTC GCGCGCCCCA
251 GAGTGTGACC TTTTCGGTCT GCTCGCAGAC CCCCGGGCGG CGCCGCCGCG
301 GCGGCGACGG GCTCGCTGGG TCCTAGGCTC CATGGGGACC GTATACGTGG
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401 TGCGGGACGA GCCGGGTCAC GCGGCTGACG CGGAGCGTCC GTTGGGCGAC
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 451 AAACACCAGG ACGGGGCACA GGTACACTAT CTTGTCACCC GGAGGCGCGA
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 551 CGTTGCTCGC GTTTGCTGGC GGTGTCCCCG GAAGAAATAT ATTTGCATGT
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 801 CAGAAGTTGG TCGTGAGGCA CTGGGCAGGT AAGTATCAAG GTTACAAGAC
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 901 TTGCGTTTCT GATAGGCACC TATTGGTCTT ACTGACATCC ACTTTGCCTT
 951 TCTCTCCACA GGTGTCCACT CCCAGTTCAA TTACAGCTCT TAAGGCTAGA
1001 GTACTTAATA CGACTCACTA TAGGCTAGCC ACCATGACTT CGAAAGTTTA
1051 TGATCCAGAA CAAAGGAAAC GGATGATAAC TGGTCCGCAG TGGTGGGCCA
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5301 AATGAAGCCA TACCAAACGA CGAGCGTGAC ACCACGATGC CTGTAGCAAT
5351 GGCAACAACG TTGCGCAAAC TATTAACTGG CGAACTACTT ACTCTAGCTT
5401 CCCGGCAACA ATTAATAGAC TGGATGGAGG CGGATAAAGT TGCAGGACCA
5451 CTTCTGCGCT CGGCCCTTCC GGCTGGCTGG TTTATTGCTG ATAAATCTGG
5501 AGCCGGTGAG CGTGGGTCTC GCGGTATCAT TGCAGCACTG GGGCCAGATG
5551 GTAAGCCCTC CCGTATCGTA GTTATCTACA CGACGGGGAG TCAGGCAACT
5601 ATGGATGAAC GAAATAGACA GATCGCTGAG ATAGGTGCCT CACTGATTAA
5651 GCATTGGTAA CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT
5701 TAAAACTTCA TTTTTAATTT AAAAGGATCT AGGTGAAGAT CCTTTTTGAT
5751 AATCTCATGA CCAAAATCCC TTAACGTGAG TTTTCGTTCC ACTGAGCGTC
5801 AGACCCCGTA GAAAAGATCA AAGGATCTTC TTGAGATCCT TTTTTTCTGC
5851 GCGTAATCTG CTGCTTGCAA ACAAAAAAAC CACCGCTACC AGCGGTGGTT
5901 TGTTTGCCGG ATCAAGAGCT ACCAACTCTT TTTCCGAAGG TAACTGGCTT
5951 CAGCAGAGCG CAGATACCAA ATACTGTTCT TCTAGTGTAG CCGTAGTTAG
6001 GCCACCACTT CAAGAACTCT GTAGCACCGC CTACATACCT CGCTCTGCTA
6051 ATCCTGTTAC CAGTGGCTGC TGCCAGTGGC GATAAGTCGT GTCTTACCGG
6101 GTTGGACTCA AGACGATAGT TACCGGATAA GGCGCAGCGG TCGGGCTGAA
6151 CGGGGGGTTC GTGCACACAG CCCAGCTTGG AGCGAACGAC CTACACCGAA
6201 CTGAGATACC TACAGCGTGA GCTATGAGAA AGCGCCACGC TTCCCGAAGG
6251 GAGAAAGGCG GACAGGTATC CGGTAAGCGG CAGGGTCGGA ACAGGAGAGC
6301 GCACGAGGGA GCTTCCAGGG GGAAACGCCT GGTATCTTTA TAGTCCTGTC
6351 GGGTTTCGCC ACCTCTGACT TGAGCGTCGA TTTTTGTGAT GCTCGTCAGG
6401 GGGGCGGAGC CTATGGAAAA ACGCCAGCAA CGCGGCCTTT TTACGGTTCC
6451 TGGCCTTTTG CTGGCCTTTT GCTCACATGG CTCGAC
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