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Troubleshooting the GFP-tagging gene knockout (GGKO) method for the *Leptosphaeria maculans* effectors *AvrLm6* and *AvrLm4-7*

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Lokalisera och avhjälpa fel i ”GFP-tagging gene knockout” (GGKO) metoden för *Leptosphaeria maculans* effektorerna *AvrLm6* och *AvrLm4-7*.

Troubleshooting the GFP-tagging gene knockout (GGKO) method for the *Leptosphaeria maculans* effectors *AvrLm6* and *AvrLm4-7*.

Keywords: *Leptosphaeria maculans*, *AvrLm6*, *AvrLm4-7*, GFP, GGKO

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Sammanfattning

Ett försök gjordes att GFP-markera *AvrLm6* och *AvrLm4-7* proteinerna via ”GFP-tagging gene knockout” (GGKO) vektor systemet som utvecklats av Saitoh et al. (2008). Syftet var att utröna om genprodukterna från dessa gener återfinns inne i värdväxten under infektionens gång. Inga pETHG-(target)KO vektorer genererades under projektets gång. Via analys av resultat från rutinmässiga kontroller i experimentet samt tester av vissa steg begränsades de potentiella kloningsproblemen till transformeringsteget med viss tvekan med avseende på om uppströmsflankerande regionen ligerades till pETHG eller ej. På grund av ett misstag användes fel vektor:insert förhållande i ligeringsreaktionen. De rekommenderade 1:1 – 1:5 förhållandena bör således användas. För att förhindra den potentiella upptagningen av tomma pETHG under transformeringen, kan pETHG behandlas två gånger med restriktionsenzymen därtill kan linjära vektorer separeras från obehandlade vektorer via gel extraktion. Det senare kan även utföras på ligeringsprodukten. Allmänna förbättringar innefattar: Sekvensera PCR produkten samt avlägsna potentiella restriktionsenzym inhibitorer, använda den maximala inkubationstiden för restriktionsenzym, utöka koloni screeningen, höja spektinomycin koncentrationen samt testa olika bakteriestammar för transformeringen.

Abstract

An attempt was made to GFP-tag the effector proteins of *AvrLm6* and *AvrLm4-7* using the GFP-tagging gene knockout method (GGKO) developed by Saitoh et al. (2008) in order to determine whether or not they are secreted. Successful pETHG-(target)KO vectors were not generated. The protocol was examined for potential errors. Fatal errors were pinpointed to the ligation reaction and the transformation required to generate and propagate the desired vector pETGH-(target)KO. The Downstreams Flanking Region inserts were evidently successfully ligated into the pETHG vector but for the Upstreams Flanking Region inserts the results were highly ambiguous. Due to a mistake, too high vector:insert ratios were used; altering them to the recommended 1:1 – 1:5 is hence expedient. It was reasoned that the bacteria possibly absorbed empty pETHG vectors instead of putative insert-carrying pETHG-(target)KO vectors during the transformation. The procedure could be improved by digesting pETHG twice prior to ligation as well by separating linearised and uncleaved pETHG by gel extraction. The latter could also be performed on the ligation product. Suggested general improvements include: Sequencing the PCR product and purifying it of potential restriction enzyme inhibitors, use the maximal incubation time for the restriction enzymes, expand the colony screening, increase the spectinomycin concentration and test different bacterial strains in the transformation.

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Appendix

Introduction

The commercially important plant oilseed rape (*Brassica napus*) is susceptible to the disease stem canker or blackleg (15) caused by the ascomycete *Leptosphaeria maculans* (http://pir.uniprot.org/taxonomy/5022). Different isolates of *L. maculans* are classified into one of three pathogenicity groups based on their interactions with the *B. napus* cultivars Wester, Quinta and Glacier; they are further subdivided into one of six 'races' based on the cultivar Jet Neuf. *L. maculans* is thus said to be a "species complex" (3). The fungi occurs world-wide (9) and its genome was recently published (13). The life cycle is complex with many regional and seasonal variations in regards to the length of the different stages. Prior to infection, the fungus is a sexually reproducing saprophyte living on plant debris. Ascospores and conidia are infectious and germinate in humid or wet conditions. As a pathogen, it endophytically colonises the host plant after entering it through stomata or wounds. Symptoms, in the form of necrotic lesions, arise during necrotrophic life stages that occur immediately after infection and up to 9 months later in the stem and pods. In seedlings or severe infections lesions may completely sever the stem. Seedlings are particularly susceptible to infection. The period from ascospores release to the development of lesions generally range from mid-spring to late autumn (15). Crop yield losses incurred by blackleg epidemics vary in severity. Documented losses of oilseed rape yield range from 100% (4,8) to between 30 – 50% to <10% (13).

Host interaction with *L. maculans* complies with the gene-for-gene concept (5). Resistance responses are elicited by direct or indirect interactions between resistance genes (*Rlm*) in *B. napus* and the corresponding avirulence genes (*AvrLm*) in the pathogen (3). The avirulence genes are named after the corresponding resistance gene and vice versa. Twelve *Rlm* genes (*Rlm1-9*; *LepR1-3*) (2,16) and nine *AvrLm* genes (*AvrLm1-9*) are known (3). These genes vary amongst cultivars and pathogen 'races' (3). Avirulence gene products, or effectors, are known or are expected to be secreted into the host plant tissue. Examples of fungal plant pathogen secreted effectors include the ToxA protein of *Pyrenophora tritici-repentis* that is secreted into the host cell and the Avr proteins of *Cladosporium fulvum* which are secreted into the apoplast (7). The localisation of effectors from *L. maculans* in *B. napus* is currently unknown. Effectors are characterised by their small size and ability to either facilitate infection (virulence factor/toxins) or elicit resistance responses (avirulence factor/elicitor) (7); the *AvrLm* genes have the latter function. In ascomycetes, they are usually cysteine-rich (12). Resistance responses in *B. napus* to *L. maculans* range from hypersensitivity responses to the development of necrotic, non-sporulating lesions (1).

AvrLm6 and *AvrLm4-7* were the second and third avirulence genes, respectively, to be genetically outlined in *L. maculans* (6,10). They are located in the gene clusters *AvrLm1-2-6* and *AvrLm3-4-7-9* which occur in AT-rich, gene-poor and recombination-deficient genomic regions. *AvrLm6* has an ORF and preprotein with predicted sizes of 435 bp and 144 aa including a 20 aa N-terminal signal peptide (6). For *AvrLm4-7* these are 432 bp, 143 aa and 21 aa (10). Both gene products are, as can be expected, cysteine rich; *AvrLm6* having six and *AvrLm4-7* eight residues. Their expression peaks seven days post infection after which it slowly decreases (measured in cotelydons; relative to β-tubulin). Neither gene has significant matches for homology in public DNA sequence databases nor do they share homology with other fungal avirulence genes or with each other. This is also a typical trait of fungal avirulence genes. *AvrLm6* corresponds to *Rlm6* and *AvrLm4-7*, uniquely, corresponds to both *Rlm4* and *Rlm7* (6,10).

The objective of this bachelor project was to determine whether the gene products of *AvrLm6* and *AvrLm4-7* are secreted into the plant during infection. To determine this, the GFP-tagging gene knockout (GGKO) vector system developed by Saitoh et al. (2008) was employed. Knowledge of where the avirulence gene products are secreted would allow the determination of which host proteins they interact with and further our understanding of the resistance response pathway in *B. napus*.

Materials and Methods

Fungal Isolate and Bacteria Strains

The French *L. maculans* isolate IBCN74 (alternative name, PHW1245) was used. All vectors were propagated in the *E. coli* strain DB3.1 and transformations were performed using DH5-α.

Table 1. Overview of flanking regions (UFR/DFR), full genes, pETHG vectors, primers, restriction enzymes, buffers and buffer dilution used in this project.

Gene	Size	Vector	Primers	Restriction Enzymes	Buffers (Dilution)
AvrLm6 (UFR):	360 bp	pETHG	UFR_*AvrLm6*_*Sal*I_Fw + UFR_*AvrLm6*_*Bam*HI_Rv	*Sal*I + *Bam*HI	Yellow Tango (2X)
AvrLm4-7 (UFR):	635 bp	pETHG - *AvrLm4-7*-DFR	UFR_*AvrLm4-7*_*Sal*I_Fw + UFR_*AvrLm4-7*_*Bam*HI_Rv	*Sal*I + *Bam*HI	Yellow Tango (2X)
AvrLm6F (UFR):	596 bp	pETHG	UFR_*AvrLm6*full_*Sal*I_Fw + UFR_*AvrLm6*full_*Bam*HI_Rv	*Sal*I + *Bam*HI	Yellow Tango (2X)
AvrLm4-7F (UFR):	493 bp	pETHG	UFR_*AvrLm4-7*full_*Sal*I_Fw + UFR_*AvrLm4-7*full_*Bam*HI_Rv	*Sal*I + *Bam*HI	Yellow Tango (2X)
AvrLm6 (DFR):	387 bp	pETHG	DFR_*AvrLm6*_*Cfr*42I_Fw + DFR_*AvrLm6*_*Kpn*I_Rv	*Cfr*42I + *Kpn*I	Buffer Blue (1X)
AvrLm6F (DFR):	473 bp	pETHG	DFR_ *AvrLm6*full_*Cfr*42I_Fw + DFR_*AvrLm6*full_*Kpn*I_Rv	*Cfr*42I + *Kpn*I	Buffer Blue (1X)
AvrLm4-7F (DFR):	443 bp	pETHG	DFR_ *AvrLm4-7*full_*Cfr*42I_Fw + DFR_*AvrLm4-7*full_*Kpn*I_Rv	*Cfr*42I + *Kpn*I	Buffer Blue (1X)

Genetic Material & Vectors

The UFRs, DFRs, full genes, primers, restriction enzymes and buffers used in this bachelor project are summarised in **Table 1**. For primer sequences and the location of restriction sites, see **Appendix (A)**. For UFR and DFR sequences and primer binding sites, see **Appendix (B)**. The UFRs, DFRs and full genes were previously cloned into pJET1.2 vectors and one DFR for *AvrLm4-7* was introduced into pETHG[1]

Stock Vector Preparation

Transformed stock DB3.1 *E. coli* bacteria containing either of the two pETHG vectors were cultivated in 5 ml liquid LB media (50 µg/µl spectinomycin) at 37°C for no more than 16 hr. The vectors were extracted and purified using the GeneJET™ Plasmid Miniprep Kit (Fermentas). Cells were harvested by centrifugation for 3 min; subsequent centrifugations were carried out at 11050 g. The kit was otherwise used according to the manufacturer's instructions.

PCR Amplification

The PCR mixtures (45 µl/reaction) contained: 5x Phusion Buffer (10µl) (Finnzymes), 0.22 mM dNTPs, 0.56 µM of each primer (see **Table 1**) and 1 u of Phusion DNA Polymerase (Finnzymes); deionised water composed the remaining mixture. For each reaction the pJET vector concentration ranged between circa 10 – 20 ng/µl. The cycling conditions were: 94°C for 2 min and 30 s; 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s; followed by 72°C for 7 min.

[1] Previously introduced by Hanneke Peele (supervisor).

Restriction Enzyme Treatment
The restriction digestion reaction mixtures (10 µl/reaction) contained: 10 u of either restriction enzyme (Fermentas), 10X reaction buffer (Fermentas) and deionised water (see **Table 1** for enzymes, buffers and buffer dilution). For each reaction the UFR and DFR concentration ranged between circa 85 – 120 ng/ul. The pETHG concentration was approx. 210 ng/ul. The reaction conditions were: 37°C for circa 16 hr followed by deactivation at 80°C for 20 min. Prior to ligation, pETHG were treated with 1 u of Shrimp Alkaline Phosphatase (SAP) for 30 min at 37°C followed by inactivation at 80°C for 15 min.

Ligation
For the ligation reaction, 2 µl of vector and 2 µl of UFRs or DFRs were used per reaction. The reaction mixtures contained (16 µl per reaction): 1 u of T4 DNA ligase, 10X Ligation Buffer for T4 DNA ligase and deionised water. The vector concentration was approx. 21 ng/ul. The UFR and DFR concentration ranged between circa 25 – 35 ng/ul. In the total reaction, the reaction buffer was diluted to 2X. The reaction conditions were: 22°C for approx. 1 hr followed by deactivation at 65°C for 10 min.

Transformation
100 µl of liquid competent DB3.1 or DH5-α bacterial solution was added to 10 µl of the ligation reaction mixtures and incubated on ice for 30 min. The mixture was subsequently heat-shocked at 42°C for 1 min (water-bath; mechanical mixing) and immediately returned to the ice for 2 min. 900 µl of SOC media (2 M MgCl2; 2 M Glucose) was added, followed by incubation at 37°C on shaker for 1 hr (150 – 200 rpm). This was followed by centrifugation at 5000 rpm for 5 min, resuspension of pellet in the remaining supernatant and cultivation on solid LB media (50 µg/µl spectinomycin) at 37°C for approx. 24 hr.

Analysis
Eight colonies per transformant were selected and cultivated as described in "Stock Vector Preparation". The vector DNA was analysed for inserts by a restriction digestion following the protocol in "Restriction Enzyme Treatment".

Gel Electrophoresis
All PCR products, products of the restriction enzyme treatment and ligation reactions were analysed by gel electrophoresis at 90V for 30 min (PCR and restriction products) or 60 min (ligation products) on 1% agarose gels (10% GelRed). Empty pETHG vectors were used as controls for the analytical restriction digestion and ligation analysis. pETHG-AvrLm4-7-DFR was included as a control for the UFRs.

Sequencing
Sequencing was performed on vector material extracted from transformants at Macrogen Europe using M13F primers for UFRs and M13R primers for DFRs.

Results & Discussion

Cloning Strategy

The principle of GGKO is to enable visual determination of spatial gene expression via exchanging a chosen gene, or a part of it, with a green fluorescencent protein (GPF) encoding sequence whilst maintaining native expression (14). It is based on the vector pair pETHG and pCAMBIA-Bar-Rfa. The system uses the Upstreams Flanking Region (UFR) and the Downstreams Flanking Region (DFR) of the gene in question. These regions contain promotors, signal sequences and other motifs related to gene expression and gene product localisation. **Figure 1** provides a schematic overview of the system and its application in this bachelor project. In this project an attempt was made to produce two different constructs for each gene. In the first, the UFRs include the 5'UTR and a signal peptide sequence; for *AvrLm6*, the signal peptide is divided into two exons separated by an intron. The DFRs include the 3'UTR (11). In the second construct, the UFR was replaced by the full *AvrLm* gene and the DFR by a downstream sequence in the genome. The UFRs were provided with restriction sites for *Bam*HI and *Sal*I, and the DFRs with restriction sites for *Cfr*42I and *Kpn*I. The pETHG vector contained the same restriction sites. The inserts were cloned into pJET1.2 vectors, introduced and propagated in bacteria. PCR was then used to amplify the inserts.

Amplified inserts and pETHG are treated with the restriction enzymes and ligated together. The UFRs and DFRs are cloned into the pETHG vector in this way separately, both additions being followed by introduction and propagation in bacteria (see below). The resulting vector construct, pETHG-(target)KO, is used in LR Gateway recombination with pCAMBIA-Bar-Rfa which generates the final vector construct pCAMBIA-(target)KO. This involves homologous recombination between attL1 and attL2 in pETHG-(target)KO and attR1 and attR2 in pCAMBIA-Bar-Rfa. pCAMBIA-(target)KO is then introduced and propagated in bacteria. For propagation the *E. coli* strains DB3.1 (pETHG-(target)KO) and DH5-α (pCAMBIA-(target)KO) are used. After transformation bacteria are grown on solid media. Selection for positive transformants is based on antibiotic resistance. For pETHG-(target)KO specintomycin ($Spec^r$) is used and kanamycin (Km^r) for pCAMBIA-(target)KO. For the latter, ccdB acts as a positive marker by killing bacteria carrying empty vectors thus only allowing growth of bacteria where ccdB has been inactivated by insertion. ccdB is a lethal gene that prevents DNA replication by targeting DNA gyrase (http://openwetware.org/wiki/CcdB). Positive transformants are harvested, propagated in liquid media, analysed by restriction digestion and used for the subsequent steps.

pCAMBIA-(target)KO can then be introduced into the *Agrobacterium tumefaciens* strain C58 via calcium chloride transformation and subsequently used to transform *L. maculans*. In successful transformants, homologous recombination has occurred between the UFRs and DFRs of the vector and those in the genome of the wild-type fungi thereby replacing them and the sequence inbetween.

If successfully performed the result is a GFP tagged by the N-terminal signal peptide (first construct) or an effector protein GFP-tagged at the C-terminus (second construct). The knockout mutant is resistant against hygromycin (HPT). If the vector sequence is inserted at random into the genome the resulting ectotopic transformant will be resistant to both hygromycin and bialaphos (Bar). Mutants can thus be distinguished by cultivation on solid media containing hygromycin on one hand and hygromycin coupled with bialaphos on the other. mRNA analysis can also be used to determine if the correct gene is knocked-out. Finally, *Brassica napus* seedlings of both *L. maculans* resistant and susceptible cultivars are infected. Tissue samples are then analysed for GFP-activity in light and fluorescence microscopes.

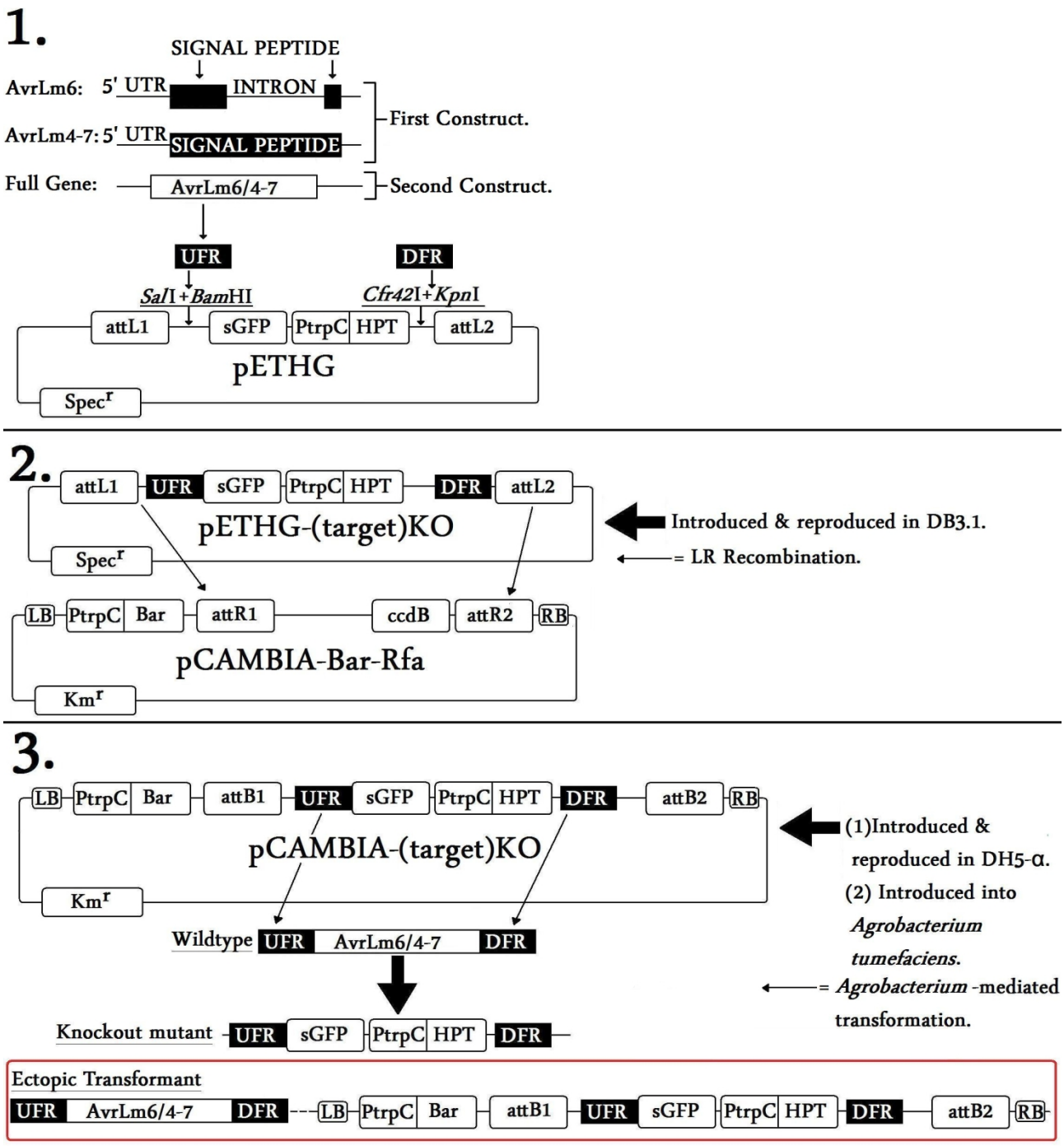

Figure 1. Overview of the GFP-tagging gene knockout (GGKO) vector system and its application in this bachelor project. (1): Restriction Reaction and Ligation (DFRs not shown due to their simplicity). (2): LR recombination between pETHG-(target)KO and the destination vector pCAMBIA-Bar-Rfa. (3): *Agrobacterium tumefaciens*-mediated transformation of *Leptosphaeria maculans*.

Overview

The objective of this bachelor project was to determine whether or not the gene products of the *L. maculans* avirulence genes *AvrLm6* and *AvrLm4-7* are secreted into the host plant during infection. No results to this effect were obtained as the inserts either failed to be cloned into the pETHG vectors or the pETHG-(target)KO vectors failed to be introduced into the bacteria. Hence no results will be presented. The basis of this discussion is instead a combination of results derived from routine laboratory control procedures on one hand and tests performed to determine the functionality of different steps on the other. The aim was to trouble-shoot the experimental protocol (see **Materials and Methods**).

PCR

In the initial PCR amplification, the most significant errors could be the absence of amplification and non-specific primer binding to the pJET1.2 vectors carrying the inserts. Gel analyses of the PCR products were performed routinely to determine whether or not the amplification reaction worked. The results of these for the UFRs and DFRs from the pJET1.2 vectors are presented in **Figure 2**. Bands of varying sizes are themselves evidence of amplification and of the absence of contamination, and furthermore the bands correspond to the known sizes of the inserts (see **Table 1**). The problem might be specificity. The inserts in the pJET1.2 vectors were successfully identified by sequencing using the respective primers (11). Hence the problem of poor specificity is also solved. Therefore mistakes in the PCR can be omitted from the list of possible errors. Of course, to be absolutely certain as to the identity of the product or to determine if any mutations have occurred sequencing the PCR product could still be performed. The specificity of the primers was tested on empty pETHG vectors using the same protocol as for the pJET1.2 vectors. The PCR produced non-specific products of sizes nearly or approximately corresponding to the sizes of *AvrLm4-7*, *AvrLm6F* and *AvrLm4-7-F* for the UFR and *AvrLm6*, *AvrLm6F* and *AvrLm4-7F* for the DFR (data not shown)[2]. Hence doubt as to their specificity for the pJET1.2 vectors is still warranted.

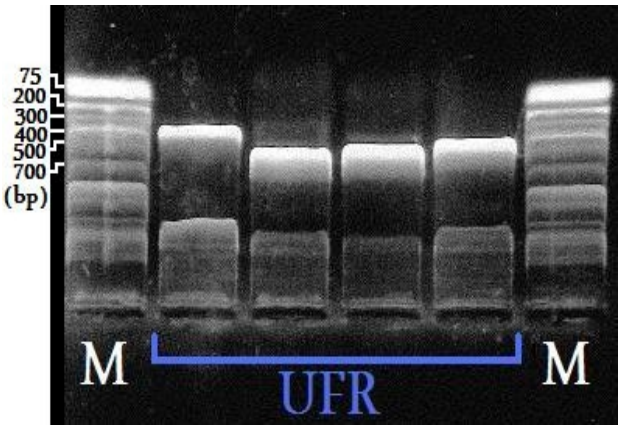

Figure 2.A. PCR amplification of UFRs analysed on a 1.0% agarose gel. From left to right*:* *AvrLm6*, *AvrLm4-7*, *AvrLm6F* and *AvrLm4-7F*. M denotes the size marker (GeneRuler™ 1 kb Plus DNA Ladder (Fermentas)).

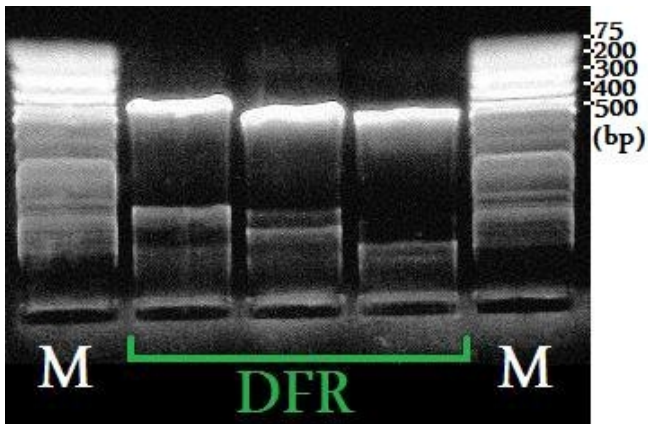

Figure 2.A. PCR amplification of DFRs analysed on a 1.0% agarose gel. From left to right: *AvrLm6*, *AvrLm6F* and *AvrLm4-7F*. M denotes the size marker (GeneRuler™ 1 kb Plus DNA Ladder (Fermentas)).

Restriction Reaction

The lack of product from the putative pETHG-(target)KO vectors in the restriction digestion analysis could be a result of either or both instances of non-functional restriction enzymes and non-specific restriction digestion. Whether or not these errors should be considered was tested on the pJET1.2 vectors as well as pETHG-*AvrLm4-7*-DFR using the restriction protocol in **Materials and Methods** adding modified incubation times of 1 hr (both vectors), 8 hr and 16 hr (only pETHG-*AvrLm4-7*-DFR). The results of these tests are presented in **Figure 3**. The restriction product bands are clearly visible for the *Cfr*42I and *Kpn*I enzyme pair whilst those of *Bam*HI and *Sal*I are more diffuse but still clearly discernible. The sizes of the bands correlate with the expected sizes of the UFRs and DFRs (see **Table 1**). The presence of bands of the correct sizes suggests that the restriction enzymes worked and the absence of superfluous bands that they maintained specificity for both vectors. However, restriction reaction with an incubation time of 1 hr on pETHG-*AvrLm4-7*-DFR vector did not produce any bands. The 8 hr and 16 hr treatments did not produce different results. It might therefore be reasonable to assume that even the empty pETHG vector requires longer incubation times at least for *Cfr*42I and *Kpn*I. The very weak bands for the products for *Bam*HI and *Sal*I suggest poor enzyme efficiency. One reason could be that the buffer was

[2] The original protocol included Taq PCR analysis of transformant colonies, this was discontinued and replaced the presented restriction digestion procedure for this reason.

improperly diluted to 1X, which gives *Sal*I an efficiency of 0 – 20% (100% for *Bam*HI). Another is that they exhibited the lowest possible efficiency of 50% in the 2X buffer dilution. To increase the amount of digested UFR inserts, it is possible to either use the longest possible incubation time (16 hr) or to perform several reactions simultaneously and pool the result. Another potential problem, which was not tested for here, is that some component of the PCR reaction mixture inhibits the enzymes. Adding a PCR purification step should be considered just to preclude that possibility. Another potential issue is a lack of structural support, in the form of nucleotides upstream of the restriction site, for the restriction enzymes. If none is present the enzyme will be unable to bind to the DNA. Two to four nucleotides should suffice according to one primer design guide (http://www.iechs.org/staff/Andrews/files/BTEC%202010/CHAPTER_6_-_Primer_Design.pdf). In the primers used in this project, three nucleotides were upstream of the restriction sites and hence a lack of support should not be an issue, see **Appendix (A)**.

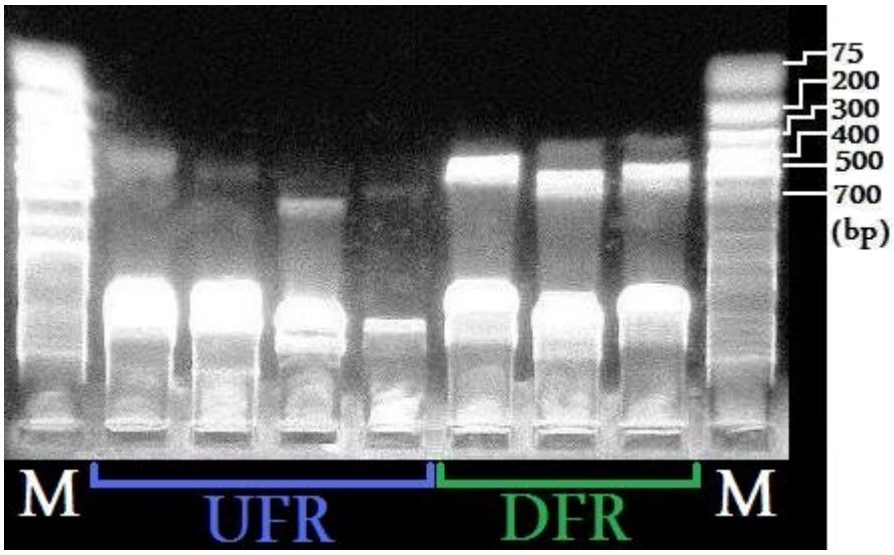

Figure 3.A. The digested products of the pJET1.2 vectors analysed on a 1.0% agarose gel. From left to right: *AvrLm6* (UFR), *AvrLm4-7F* (UFR), *AvrLm4-7* (UFR), *AvrLm6* (UFR), *AvrLm6* (DFR), *AvrLm6F* (DFR) and *AvrLm4-7F* (DFR). M denotes the size marker (GeneRuler™ 1 kb Plus DNA Ladder (Fermentas)).

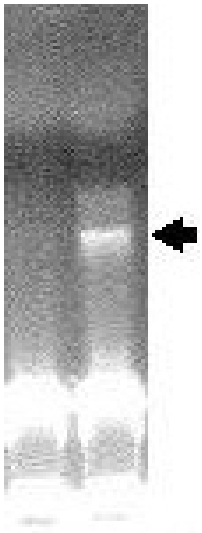

Figure 3. B. The digested product of pETHG-*AvrLm4-7*-DFR analysed on a 1.0% agarose gel (excised from a larger gel picture).

Ligation

For the ligation reaction the most significant potential error is the absence of ligation due to a non-active ligase enzyme. Using the protocol in **Materials and Methods**, the ligation reaction was tested on restriction enzyme treated UFRs, DFRs and vectors. The results for the DFRs are presented in **Figure 4**. As is evident, the bands in the lanes of the putative pETHG-(target)KO vectors are visibly larger than the empty pETHG vector. Although all three bands are approximately of the same size despite differing insert-sizes is explained by the fact that the size-difference between the inserts is very small being at the largest circa 86 bp. Hence the ligase enzyme appears to have been active. For the UFRs the situation is more ambiguous. For these inserts the test was performed a total of three times and each product analysed twice on 1% agarose gels without any results indicating the presence of vector at all in the ligation solutions. In other instances the gel electrophoresis result was unreadable putatively due to unequal distribution of agarose in the gel. Hence the success or failure of the UFR ligation cannot be ascertained and remains unclear. Another important point is the amount of vector and molar ratio of insert. The T4 DNA ligase (Fermentas) activity is optimal at a vector concentration of 1 – 5 ng/µl (adding up to 20 – 100 ng in a 20 µl solution) and a molar ratio of 1:1 to 1:5 which for a vector of circa 4 kb and inserts with a size varying from circa 0.4 to 0.65 kb adds up to 2 – 10 ng or 3.25 – 16.25 ng of insert (http://www.promega.com/techserv/tools/biomath/calc06.htm). In a 20 µl solution this is a concentration of 0.1 – 0.5 ng/µl or 0.16 – 0.8 ng/µl. Due to mistaking the amount of vector given in

the protocol for a concentration of ng/µl, the total amount of insert used in this project were much too high. The intention was to have a ratio of circa 1:1. **Table 2** presents the actual molar ratios used in this project. Yet this seems to have scarcely mattered in the case of the DFRs, which were evidently cloned into the pETHG vectors. For the UFRs, nothing can be said given that all the analyses failed. Regardless, another alteration to the protocol would be to dilute the PCR products so as to obtain the amounts recommended in the Fermentas protocol as well as to test different molar ratios to different vector concentrations to determine if any one gives a better result.

Table 2. The actual molar ratio used during the ligation reaction (calculated with http://www.promega.com/techserv/tools/biomath/calc06.htm).

Flanking region/Full gene	Molar ratio to vector
AvrLm6 (UFR):	11.8
AvrLm4-7 (UFR):	6.8
AvrLm6F (UFR):	7.3
AvrLm4-7F (UFR):	9.1
AvrLm6 (DFR):	17.4
AvrLm4-7 (DFR):	13.9
AvrLm6F (DFR):	14.1

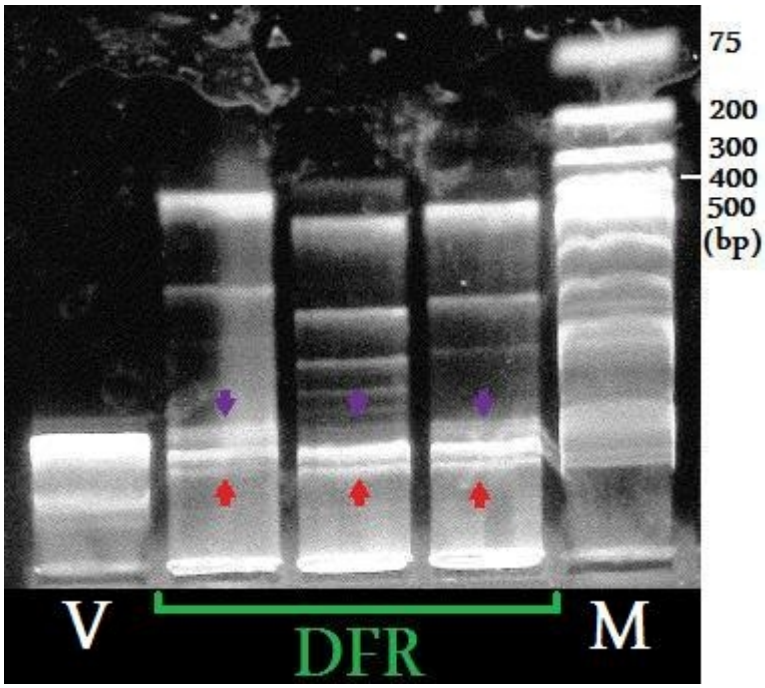

Figure 4.A. The ligation product for the DFRs analysed on a 1.0% agarose gel. From left to right: *AvrLm6*, *AvrLm6F* and *AvrLm4-7F*. M denotes the size marker (GeneRuler™ kb Plus DNA Ladder (Fermentas)).V denotes control (empty pETHG). Red arrows denote putative pETHG-(target)KO vectors. Purple arrows denote putative empty vectors.

Transformation

The potential error of foremost concern regarding the transformation is that the bacteria fail to absorb the vectors. Considering the growth media selectant spectinomycin and resistance to that antibiotic expressed by any bacteria carrying pETHG or its derivative, the presence of colonies on one hand and the extraction of the empty pETHG vectors from these colonies is sufficient evidence to prove the transformation's success.

In each and every transformation during the course of this bachelor project colonies were present for all transformants (data not shown). Nevertheless, all analyses – both the earlier Taq PCR colony analysis and the restriction digestion – conducted on putative pETHG-(target)KO vectors extracted and purified from the transformants were negative. Assuming, solely for the point of this discussion, that pETHG-(target)KO vectors were indeed present in the ligation mixture the error relate rather to the absorption of still empty vectors. Empty vectors may still be present in the ligation mixture due to either incomplete cleavage of the vectors or failed SAP treatment and subsequent self-ligation. As empty vectors are more readily absorbed by bacteria in relation to vectors carrying inserts, although it is debatable if the relatively minute size difference between pETHG and pETHG-(target)KO should cause any problems, colonies resulting from a transformation using a mixed vector solution are more likely to contain empty vectors. Bands that putatively correspond to empty vectors are visible above the pETHG-(target)KO-DFR vector bands in **Figure 4**. The solution closest at hand to this problem is to ensure cleavage of all empty pETHG vectors during the restriction reaction. However, the restriction reactions were incubated for the maximum time of 16 hr recommended by the manufacturer; incubation times longer than this risk losing or reducing enzyme specificity. An alternative to a longer reaction time is a "double-reaction" protocol, whereby the product of an initial restriction reaction is purified with a PCR purification kit and subsequently treated with restriction enzymes a second time. Larger volumes of

pETHG solution would be required to compensate for the loss of vectors during purification. The drawback is the time required. A far less time-consuming alternative available is to perform a gel extraction of the vector DNA. The GeneJET™ Gel Extraction Kit (Fermentas) was tested on both linear and circular pETHG vectors with subsequent successful transformation albeit with reduced colony yield compared to transformations using the vectors directly taken from the same stock solution (data not shown). The same gel procedure could also be used to separate ligated vectors from any remaining empty vectors. Due to the reduced transformation efficiency working with higher volumes and concentrations are likely necessary. Another improvement to the protocol would be to expand the colony screening from eight to as many colonies as possible depending on the number of available colonies. In order to reduce the number of colonies with innate spectinomycin resistance (i.e. "background growth"), the antibiotic concentration could be increased from 50 µg/µl to any level that is found not to be toxic to the pETHG carrying bacteria.

An hypothetical error source is the loss of inserts from successfully introduced pETGH-(target)KO vectors during either of the two cultivation steps subsequent to the transformation. One possible solution should that error need to be considered would be to use other suitable bacterial strains.

Summary

The solutions and alterations suggested in the discussion above are summarised below. Thus:

- ✓ Sequence PCR products from the pJET1.2 vectors.
- ✓ Purify the PCR product to ensure that no component inhibits the restriction enzymes.
- ✓ Use the longest possible incubation time for *Bam*HI and *Sal*I or perform several reactions simultaneously and pool the resulting product.
- ✓ Test different amounts of vector and molar ratios of inserts in the ligation to ascertain if any amount or ratio yields a better result.
- ✓ Perform a "double reaction" on the pETHG to ensure that all vectors are cleaved.
- ✓ Alternatively, cleaved pETHG can be separated from circular vectors by gel extraction. The same can be done for ligated vectors.
- ✓ Expand colony screening from eight to as many colonies as possible.
- ✓ Increase the spectinomycin concentration.
- ✓ If all the above fail, switching to other bacterial strains should be considered.

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Appendix A – Primer Sequences

Legends

- ✓ Higlighted Yellow: Restriction site of *Sal*I.
- ✓ Highlighted Green: Restriction site of *BamH*I.
- ✓ Highlighted Red: Restriction site of *Cfr42*I.
- ✓ Highlighted Blue: Restriction site of *Kpn*I.

UFR_*AvrLm6*:
UFR_*AvrLm6*_*Sal*I_Fw: AAGGTCGAC GCCCTCCTAGCACTAAACAA
UFR_*AvrLm6*_*Bam*HI_Rv: AAGGGATCC GCTCTCGCAGGCGCACAACA

UFR_*AvrLm4-7*:
UFR_*AvrLm4-7*_*Sal*I_Fw: AAGGTCGAC AAATCCAAGCGCTATCTCTA
UFR_*AvrLm4-7*_*Bam*HI_Rv: AAGGGATCC GCGAATTTCTCCAGATAT

UFR *AvrLm6* full:
UFR_*AvrLm6*full_*Sal*I_Fw: AAGGTCGAC ATGGTGATTTACCTACCCCT
UFR_*AvrLm6*full_*Bam*HI_Rv: AAGGGATCC TTGGATTTGTCCTTCCCAGT

UFR *AvrLm4-7* full:
UFR_*AvrLm4-7*full_*Sal*I_Fw: AAGGTCGAC ATGCCACTATCCCTCGAGAT
UFR_*AvrLm4-7*full_*Bam*HI_Rv: AAGGGATCC GTCGCAACCACGAGTCCTTG

DFR_*AvrLm6*:
DFR_*AvrLm6*_*Cfr*42I_Fw: AAGCCGCGG GCCTTGGCTGTATTGCTACC
DFR_*AvrLm6*_*Kpn*I_Rv: AAGGGTACC GGGTAGAGAAGATTAGCTTC

DFR_*AvrLm4-7*:
DFR_*AvrLm4-7*_*Cfr*42I_Fw: AAGCCGCGG TTCTTGCTACCGTCTTTGTT
DFR_*AvrLm4-7*_*Kpn*I_Rv: AAGGGTACC CTTAGGAAGGAGTTTTAGTATAGG

DFR *AvrLm6* full:
DFR_ *AvrLm6*full_*Cfr*42I_Fw: AAGCCGCGG TAGATTTAGCGGAGAACGTC
DFR_*AvrLm6*full_*Kpn*I_Rv: AAGGGTACC ACCACAAATTATCGACGAAC

DFR *AvrLm4-7* full:
DFR_ *AvrLm4-7*full_*Cfr*42I_Fw: AAGCCGCGG AACTACAAATTTTCGCATAGG
DFR_*AvrLm4-7full*_*Kpn*I_Rv: AAGGGTACC GTATATAGGAGTGCGCGTCT

Appendix B – UFRs and DFRs

Legends

- ✓ Highlighted Yellow: Forward Primer Binding Site.
- ✓ Highlighted Green: Reverse Primer Binding Site.
- ✓ Underlined: Signal Peptide Sequence.

AvrLm6

UFR:

TTACAATCTAAAGTTAAATAAGCCCTCTTGTGTTAATTATTCTCGGTCAAATAGATACA
TTAGGTCTCACAATCTAAAGTTAAATAAGCCCTCCTATGTTAATTGTTCTCGGTTAAAT
AGATACATTAGGTCTTACAATCTAAAGTTAAATAAGCCCTCCTGTGTTAATTATTCTCG
GTCAAATAGATACATTAGGTCTCACAATCTAAAGTTAAATAAGCCCTCCTATGTTAATT
ATTCTCGGTCAAATAGATACATTAGGTCTCACAATCTAAAGTTAAATAAGCCCTCCTAT
GTCAATTGTTCTCGGTTAAATAGATACATTAGGTCTCACAATCTAAAGTTAAATAAGCC
CTCCTAGCACTAAACAAGCTAGTAGTAACCTGAACTAATCTAAATTATTTCTAGCTTTA
AACTTTCATCGCATTTAATCTTCAATTTGTCTGTTCAAGTTATGGATATATATTAAACTT
GATCCCACATATTCACTTCACATTCTTAACAATCTATAAACAATCTTCGCACGTTCCAA
AGCCCTATTTATAACCTAGCATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGA
ATAGCGTACGTGTCTCTCTTAACGCTACACTAAACGCACTTTTAAACCCAATCTAGGAC
TACCACGATTAGTCAGCCTCATTTGTTGTGCGCCTGCGAGAGC

DFR:

GCCTTGGCTGTATTGCTACCACTACGGTTCAAAACTGGTTTACAGTTGCCATATTTATCT
AAAGTAGACTAAGAAGTAGCTTAGGTAGAAGAAGTAAACCTAAATAGGGTTTACTATA
CTATGTATTCTACTTTTACTTCTACCTCTATTAATCTACTAGTTTCCTACTAACTATTTAA
GTATTTTACCCCTCCTTTCTTTAATCTTCTTTACCACCTCTATTACCACCTCTACCACTAC
CTCTACCACCACCTCTATCACCACCACCACCTAAACAACCTCTATTATCACCACCTCCT
ATTAGTAAAAATATTTAAACTTATTATTATGTTATATATGCGTATAGCATGAAGCTAAT
CTTCTCTACCC

Full Gene UFR:

ATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGAATAGCGTACGTGTCTCTCTTA
ACGCTACACTAAACGCACTTTTAAACCCAATCTAGGACTACCACGATTAGTCAGCCTCA
TTTGTTGTGCGCCTGCGAGAGCGGTAGACGTGATGGAGTTGACGATACCCGAACTTTG
AAAGTTGTCAAAGGCACTGGAGGCAGATTTGTTTTTAGTATGTTACTAGATACAACCTA
TCCTAAAATTTGCTAACACTCTAAGGCAGCAGATATTGGACAAAAGCCGAAGGCGCTC
CGCACGAAGGCAACTATGTACTAGATCTGCTTCCTTTAATATATTCACTTTTTAACGCG
TTCTAGGCTCATGCTATAAACGGCACTATTACGAAAAAAGGGACTAACATCCAAGCCC
ATGACGATGGTCTCATCGGCGGAGAGGAGATGAACAGTTTATGTCCTGAACATTCCAC
TTGCTTCTCGCCTAATCTTAAGGCCAAATCGACCCACTCCTGCGGTCCAGACGGGAAAT
ATGGGTGCGTCAGCGCTTGGTTATCTGTTAACTGGGAAGGACAAATCCAA

Full Gene DFR:

TAGATTTAGCGGAGAACGTCGTTTATGCCGCTGAATTTGGGTGCCTAGCATAGACTGTA
CTTGTGAAGAGCTCAGCTTGCTAGATAAGTACTTAGCGTGCTTGGTCGTTACCTATCAT
ACTCTACGCATCCAGAAGACTAGCCATTGTCTTGTGTATAGTGTAGTAAGGGACATTTC
CACCCTCAAGCTAGCCCGTCTCAAGTGCGGCGAACCCAACTCTAGGTAGCCTAACAGG
TATCTAACGTTGAATCTAGCGGCTTTATTATTTGCCTAGCATTTTCGCGAGTTCCTTAAT
TATGTAGATAGAACTTTGACGAAAAGTGCTAATTAACGCAAGGTCGCGTAGCATTGAA
CCTAAGCAGTCAAGAAATGACCCTAGATGTAAGATCGAAACGGAAAGGTTAGTGCAG
CCTATCCACTGTTAGCCTGTGCTCCGTTCGTCGATAATTTGTGGT

AvrLm4-7

UFR:

GTATCTAGCAACCTTATGTATCTAGTAAGCTTATGTATCTAGTAAGCTTATGTATCTAG
TAAGCTTACTATCGCATACCAAACATTAGGCAAAAGTAAACCCTATTACCCTACCCTAA
TAAGTTAATATATATGGTGGGATATTAATTAGATATTAATATTGCGATACTTACTTATC
TAGTAACCTTATGTATGTAAGCTTACTTAATAGCAACTAATATTCTTCTAGGTTATAATT
AGCTTAATATCTATGTTATAAGCCTTACTTTACCTATATTAGTTAGTAACCTAGACCAA
CCATATCTATATTTACGTGTGCGTAGTAGATAAGTAAGCTTACTAGAGTTAATGCCTAT
TTTTGTAACACTATACTATACTTATTTGCTACACTAGATTATACTAAACTAGGTTAAAA
TTAATTTGCATATACCAATTAAGTCCTATAAATCCAAGCGCTATCTCTATAATTATAAT
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TACTACCTTGTTAATATTAGATATTGCTAAAATAAGCATGTAAGTTATATCTAATCTTA
CAAGATAAGTAGCGCTAATAGGGAAAGCTACTCTACATGGAAGCTTACTAGATAAGCA
ATCTTACTAGATAGGAGAACCCTACTAGATAAGTAAGCTTGCTAGATAAGTAATCTTA
CTAGATAGAAGAACCCTGCTAGATAGGTAAGCTTACTTGATAAGTAATCCTACTAGAT
AGGAGCACCCTACTAGTAAGAATAATTATTACATAGAGAAGTTCTATATAATGAGGAT
ATAGAACAAAGATATTAAGACACAAGTTACAACGACAAGCTTATTTAACAATCAAGTT
GTTTACTCCTATTTTTGTTATATCTAACTTACTTATTAATATCTTTACTTCCACCAAGTAT
AAACCCTTTGACAGTTAACAACATGCCACTATCCCTCGAGATAATCTTAACGCTACTCG
CTCTCTCTATCCCTACAATTACAGCTTGTAGAGAGGCCTCAATATCTGGAGAAATTCGC

DFR:

TTCTTGCTACCGTCTTTGTTAGCGGTTGATCCATCAGCCTAACTTCCTTGACCCACGCTT
ATTTTAAAAGATATAGATAGTAATAGCAATAGTCTACATACTTAATCTTTTAGTATTTT
TACTCTAATATTGTGCCTTGGCATGGTGGGTCTGGTTGAAAATAAGGTCTAGCCTAAGT
TAGAATAGGTTGAAACTTGATTTGGGTGGGATAATGGTATAAAGAAGGGGAGGATCAT
AGGAAAGAATGTTAGGCAACTCAACACTTAATTCTCCAAGATATCTTATTAATGGAAA
TTTCTATCCCTTAATCTATTATTTAATCTAGTTTGTTTATCATAAGATACCCTATACTAA
GACTTGCCCCTTACCCTTCTCCTTGTTTAAATGGATTACTCTCCTTAACAGTTCACTATA
GCTCCCAATACTAATCCCTATACCTATGCGCTTATTATAACTCTAAAGTTACCTTTTAGT
AGCAAATCTACCGCCTAAATTAGCGCTACTATAGGTATTTTACTACACAATATTAACTT
AATCTATAGTAGAGCCTACTATTATAGCTTTAAGCAGAATTTACCTATACTAAAACTCC
TTCCTAAG

Full Gene UFR:

ATGCCACTATCCCTCGAGATAATCTTAACGCTACTCGCTCTCTCTATCCCTACAATTAC
AGCTTGTAGAGAGGCCTCAATATCTGGAGAAATTCGCTATCCTCAAGGCACTTGTCCCA
CAAAGACTGAAGCTTTGAATGATTGTAACAAAGTAACGAAGGGCTTAATTGACTTTAG
TCAATCGCATCAACGTGCCTGGGGTATAGATATGACGGCCAAAGTCCAATGTGCGCCC
TGCATAACTACCGACCCTTGGGATGTAGTTCTTTGCACTTGCAAGATCACGGCGCATAG
ATATCGAGAATTCGTTCCCAAAATTCCCTATAGCAGCTTTAGCTCAGCACCTGGAGTTA
TATTTGGCCAGGAGACTGGTTTAGACCATGACCCTGAATGGGTTGTTAACGTAAGATTA
AATATTCTACTAGGAACCTAAGACTAACTACAAATAGATGAAAGCAAGGACTCGTGGT
TGCGAC

Full Gene DFR:

AACTACAAATTTTCGCATAGGCTATAGGCTTAGGTTAGGTTTAGGTTACATAGGCTATA
GGCTTAGGGTAGGGTTAGGTTGCATAGACCTTAGGCTTAGGTTAGGTCTTACGTAAGTA
GGTTAGGGTTAGGGTTAGGTTACACAGGCTATAGGCTTATGTTAGGTATAGGGTCTTAC
GTAATCTACTTATAGTATATATATAATAGGCATGTATCTTAGGTATCTATTATAGGTGT
AACAATAAGGTAGAATTGCCTGCCTATTATTCTATGCCAAGTTAGGCCTAATAAAGGC

CCGACTCACTAATATATATAGAGAGCAGCTCGCCTATAGTTAGGATAGGCATGTTCCTC
TTATCCTTTTCTACAAGACCTATAAGTATAGGAATCTTATCCTTTTCTATATAGACGCGC
ACTCCTATATAC

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