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Targeted gene disruption in *Francisella tularensis* by group II introns

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Abstract

Francisella tularensis is a highly infectious Gram-negative bacterium that is the causative agent of tularemia. Very little is known about the molecular mechanisms responsible for *F. tularensis* virulence, in part due to the paucity of genetic tools available for the study of *F. tularensis*. We have developed a gene knockout system for *F. tularensis* that utilizes retargeted mobile group II introns, or “targetrons”. These targetrons disrupt both single and duplicated target genes at high efficiency in three different *F. tularensis* subspecies. Here we describe in detail the targetron-based method for insertional mutagenesis of *F. tularensis* genes, which should facilitate a better understanding of *F. tularensis* pathogenesis. Group II introns can be adapted to inactivate genes in bacteria for which few genetic tools exist, thus providing a powerful tool to study the genetic basis of bacterial pathogenesis.

1. INTRODUCTION

Francisella tularensis causes the zoonotic disease tularemia, which has a high mortality rate in the pneumonic form [1]. Because of its high infectivity and ease of dissemination through aerosolization, the Centers for Disease Control and Prevention (CDC) has categorized *F. tularensis* as a potential biological weapon. There is currently no vaccine approved for human use in the U.S. against *F. tularensis*. The molecular mechanism(s) responsible for *F. tularensis* virulence are still not clear, primarily due to a lack of genetic tools amenable for use in the more virulent (in humans) subspecies *tularensis* and *holarctica* [2–4].

The *Francisella* Pathogenicity Island (FPI) is a cluster of seventeen genes that contribute to intramacrophage growth and virulence [5–7]. The FPI is duplicated in the highly virulent (for humans) subspecies *tularensis* and *holarctica*, while the low virulence (for humans) subsp. *novicida* contains a single FPI [8,9]. Currently, no biological function has been determined for any protein encoded within the FPI [8,9], and thus, a genetic system that inactivates duplicated FPI genes in highly virulent *F. tularensis* strains should afford insight into *F. tularensis* virulence.

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Targeted gene disruption in subspecies *novicida* has proven relatively easy [5,10–13], but relatively difficult in subspecies *tularensis* and *holarctica*. These virulent subspecies also require strict biocontainment conditions and have a limited number of approved antibiotic resistance markers, making targeted mutagenesis within these strains a more challenging task.

Recently, “targetron”, a gene knockout system that utilizes the *Lactococcus lactis* L1.LtrB group II intron, has been adapted for gene disruption in a growing number of bacteria (Table 1) [14–20]. The group II intron consists of a ribonucleoprotein (RNP) complex that basepairs with the DNA insertion site, then catalyzes insertion and reverse transcription of the intron RNA [14,21,22]. Because the insertion site is determined by base pairing between two loops of the RNA molecule (EBS1 and EBS2) and the target DNA, the intron can be retargeted to disrupt a specific gene by making EBS1 and EBS2 complimentary to the desired insertion site (Fig. 1A). The protein component of the RNP shows preferences for sequences flanking the insertion site, and thus a computer algorithm has been developed to identify preferred target sequences within any given DNA sequence [20]. Expression of the RNP with the retargeted RNA molecule in the appropriate host leads to specific insertional inactivation of the target gene.

One of the advantages of this type of targeted mutagenesis is the host-independent nature of the insertion event, i.e. the RNP does not rely upon host recombination machinery. Additionally, because of the high specificity toward the target site, the RNP (expressed from a plasmid) can be continuously expressed until the site is disrupted, at which time the RNP can be removed, making it a highly efficient gene inactivation technique for single and multiple identical genes. Finally, the intron insertion does not contain any antibiotic resistance genes, allowing for multiple insertions within the same host.

We have adapted the targetron system to inactivate genes in various *F. tularensis* subspecies [15]. This system should aid in the genetic dissection of *F. tularensis* virulence, especially the duplicated genes within the FPI. Here we describe in greater detail our method for the use of targetrons for insertion mutagenesis in *F. tularensis*, which was previously reported in a more abbreviated form [15].

2. Description of method

2.1 Overview

The overall method for insertion mutagenesis utilizing the Targetron gene knockout system is outlined in Fig. 2. In this system, a ribonucleoprotein (RNP) complex is targeted to disrupt specific gene(s). The RNP consists of the L1.LtrB RNA molecule, which is a group II intron, and the LtrA protein. The RNP complex recognizes specific DNA sequences by base-pairing between exon binding site 1 (EBS1), EBS2, and delta (δ) of the intron RNA with intron binding site 1 (IBS1), IBS2, and δ' within the target gene, along with binding of LtrA to flanking sites (Fig. 1A). L1.LtrB RNA reverse splices into the insertion site, and LtrA then reverse transcribes the inserted RNA. The intron is altered to inactivate specific genes by first identifying potential LtrA-binding sites within the desired target gene, and then modifying EBS1 and EBS2 within the RNA molecule to be complimentary to the target DNA sequence. We detail the specific methods for targetron inactivation of *F. tularensis* genes below, making note when these methods deviate from targetron systems in other bacterial species.

In order to adapt this system to *F. tularensis*, several attributes needed to be incorporated into the *F. tularensis*-specific targetron plasmid pKEK1140 (Fig. 1B) [15]. These attributes will also be important when adapting the targetron system to any other bacterial species. First, a plasmid backbone was used that had an origin of replication active in *E. coli* (for cloning purposes), and an origin of replication active in *F. tularensis* (for continued expression of the

targetron in *F. tularensis*). Second, the kanamycin resistance marker, appropriate for use within select agent forms of *F. tularensis*, was optimized for expression by providing a *F. tularensis* promoter. Third, the targetron (Ll.LtrB and LtrA) was also optimized for expression by providing a *F. tularensis* promoter. Fourth, a *lacZα* fragment is present within the Ll.LtrB intron at the site replaced during retargeting, to facilitate blue-white screening for correct clones. Finally, the plasmid was made conditionally replicative by the introduction of a temperature sensitive mutation in the *F. tularensis* origin of replication; this facilitates removal of the targetron plasmid after the target gene has been inactivated. The inclusion of any counter-selectable marker (e.g. *sacB*, *rpsL*, etc.) effective in the particular bacterial species would be sufficient for removal of the targetron plasmid.

2.2 Step-by-step protocol

Selecting potential target sites for gene disruption—To identify potential targetron insertion sites within a specific gene, an algorithm is used at the Sigma-Aldrich Targetron website (www.sigmaaldrich.com/targetron). This algorithm searches for potential LtrA recognition sites within a sequence, and then provides the sequence of the appropriate oligonucleotides to retarget Ll.LtrB. To access the Targetron algorithm, an access code must be purchased from Sigma-Aldrich. The sequence of the gene of interest is entered into the website window, along with the access code. The website then provides a list of potential insertion sites in the sense (s) or anti-sense (a) DNA target strand with corresponding scores, E-values, and primer designs. Three primer sequences are given for each potential site, referred to as IBS, EBS1d, and EBS2; these three primers, along with the EBS universal primer, are used in a splicing-by-overlap extension PCR, as described below. The primer sequences for IBS and EBS1d include restriction sites that facilitate insertion into the targetron plasmids pACD4 and pACD4K-C; however we altered one of these restriction sites in the *F. tularensis* targetron plasmid pKEK1104, and thus a *XhoI* restriction site must be introduced into the IBS primer in place of the *HindIII* site for cloning into pKEK1140.

Either sense (s) or anti-sense (a) insertion sites are effective targets [16,19,23], but potential insertion sites nearest to the start codon are preferred. The higher the score and the lower the E-value, the more likely the insertion will take place, according to the design of the algorithm [20]. The RNP shows a preference for T at −23, G at −21, A at −20, and T at +5, relative to the insertion site (Fig. 1A) thus potential sites should be chosen with this consideration. These RNP nucleotide preferences, along with other proprietary design considerations that maximize intron functionality, are incorporated into the Targetron algorithm (Sigma-Aldrich). We recommend a BLASTN search of the entire target genome (if available) to ensure the uniqueness of the DNA target region. If the targetron is intended to inactivate the same gene in multiple subspecies, potential insertion sites should be chosen that are identical across subspecies; we have routinely chosen potential insertion sites that are identical in *F. tularensis* subsp. *tularensis*, *holarctica*, and *novicida*. Finally, we recommend the selection of two potential insertion sites for each target gene, since targetrons can vary in efficiency [16, 19,23].

Splicing-by-overlap-extension PCR—Once the potential target sites have been chosen, the corresponding IBS, EBS1d, and EBS2 oligonucleotide primers are synthesized (with the substitution of a *XhoI* restriction in place of *HindIII* in the IBS primer if using the *F. tularensis*-specific targetron plasmid pKEK1140, as mentioned above) by any commercial oligonucleotide synthesis source. A splicing-by-overlap-extension (SOE) PCR reaction [1, 24] is performed in the following manner. A four-primer master mix is first made with 2 μl IBS primer (100 μM), 2 μl EBS1d primer (100 μM), 2 μl EBS2 primer (20 μM), and 2 μl EBS universal primer (20 μM; Table 2; Sigma-Aldrich) added to 12 μl deionized H₂O. The PCR reaction is then composed of 12 μl deionized H₂O, 1 μl four-primer master mix, 1 μl intron

PCR template (Sigma Aldrich), and 25 µl JumpStart REDTaq Ready Mix (Sigma-Aldrich). The PCR reaction consists of a denaturation step of 94°C for 30 sec, followed by 30 cycles of 94°C 15 sec, 55°C 30 sec, and 72°C 30 sec, and then a final extension of 72°C for 2 minutes.

Construction of retargeted targetron plasmid—For construction of the *F. tularensis*-specific targetrons, the 350 bp PCR product from the SOE reaction described above is then digested with *Xho*I and *Bsr*GI, gel purified (Qiagen), and ligated into pKEK1140 (Fig. 1B) digested and purified similarly. For the ligation reaction mix, a proportion of 3:1 of SOE fragment to vector was used, along with 1 µl of T4 DNA Ligase (NEB) and 2 µl 10X ligase buffer, brought up to a total volume of 20 µl with deionized H₂O and incubated at 16°C for at least 12 h. Electrocompetent *E. coli* strain DH5α [25] was transformed via electroporation (200Ω, 25µF, 2.5 kV; BIO-RAD Gene Pulser II) with the ligation mixture, then added to 1 ml Luria-Bertani (LB) medium and incubated at 37°C for 1 h on a roller drum, and finally plated on LB agar with 40 µg ml⁻¹ 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal) and 50 µg ml⁻¹ kanamycin (Kan) and incubated at 37°C overnight. The *lacZα* fragment within L1.LtrB in pKEK1140 results in β-galactosidase activity and blue colonies in transformed DH5α cells, unless it is replaced with the 350 bp retargeted-intron product, which will then result in white colonies in transformed DH5α cells. It should be noted that all commercially available targetron vectors (Sigma-Aldrich) are retargeted using *Hind*III/*Bsr*GI fragments, rather than the *Xho*I/*Bsr*GI fragments used to retarget pKEK1140. Additionally, some commercially available targetron vectors (pACD4, pACD4K-C) contain *lacZα* while others (the *Staphylococcus aureus* targetron plasmid pNL9164, and the *Clostridium perfringens* targetron plasmid pJIR750ai) do not, and thus blue-white screening cannot be performed with these plasmids.

DNA preparations from white colonies are prepared according to standard protocols (e.g. Maniatis [26]), and then screened for the presence of the 350 bp retargeted portion of L1.LtrB via restriction enzyme digestion followed by gel electrophoresis. We have found *Bgl*II to be a useful enzyme to screen the resulting colonies. *Bgl*II digestion of the unmodified pKEK1140 results in three fragments of 3975, 2752, and 1694 bp, whereas replacement of the *lacZα* fragment with the retargeted SOE fragment results in three fragments of 3561, 2752, and 1694 bp. All potential retargeted targetron plasmids should be confirmed by sequencing; EBS universal, IBS, and EBS1d primers used to create the SOE fragment are all appropriate primers for sequencing reactions.

Transformation of *F. tularensis*—Once the retargeted *F. tularensis*-specific targetron plasmid has been confirmed to be correct by sequencing, *F. tularensis* is transformed with this plasmid, either by electroporation or by cryotransformation. Both techniques are used to introduce DNA into *F. tularensis* at approximately equal frequencies, and thus both are outlined here.

For electroporation of *F. tularensis*, the bacteria should first be grown in TSAP media (tryptic soy broth 40 g/L with 0.1% cysteine, 25µg/ml ferrous sulfate, 25 µg/ml sodium pyruvate, and 25 µg/ml sodium metabisulfite) overnight (16–19 hr) at 37°C, then 1 ml of this culture is added to 4 ml of TSAP and cells are grown to mid-log phase at 37°C (0.3–0.6 OD₆₀₀). 1 ml of mid-log cells are then centrifuged 1 min at 12,000 rpm in tabletop centrifuge, the supernatant is removed, and the cell pellet resuspended in 500 µl 0.5 M sucrose. This step is repeated twice, with the cell pellet first being resuspended in 500 µl 0.5 M sucrose, and then in 50 µl 0.5 M sucrose. At least 1 µg of targetron plasmid is then added to the cells, and they are placed in 0.2 cm electrode gap cuvette and electroporated (600Ω, 25µF, 2.5 kV). The cells are then inoculated into TSAP and incubated at 30°C 1 h, then plated onto TSAP containing 50 µg/ml kanamycin and incubated at 30°C up to 5 days. Note that it is important to now grow cells at 30°C instead of 37°C, due to the temperature-sensitive nature of the targetron plasmid. The various *F.*

tularensis subspecies exhibit different growth rates in the laboratory, thus the longer time periods are necessary to obtain colonies with e.g. *F. tularensis* subsp. *holarctica* LVS.

For cryotransformation, we grow *F. tularensis* on TSAP agar overnight at 37°C, then scrape cells off the plate with a sterile inoculating loop and resuspend in 500 µl 0.2 M RbCl. Cells are centrifuged in a tabletop centrifuge for 1 min at 12,000 rpm at 4°C, the supernatant is removed, and pellet resuspended in 25 µl 0.2 M RbCl. It is important that the cells are highly concentrated at this step, and this is achieved most easily by resuspending plate-grown bacteria in a small volume of liquid as outlined here; we strive for a final OD₆₀₀ of >20. 2 µg of targetron plasmid and 25 µl of Transformation Buffer (15.8 g NaCl/L, 6.0 g/L MgSO₄, 2.94 g/L CaCl₂, 6.05 Tris base, 250 µl/5ml BD BBL Isovitalax Enrichment, pH 6.8) are added to the 25 µl of *F. tularensis* cells and incubated at room temperature 10 min. The mixture is then immersed in liquid nitrogen for 5 min, and thawed at room temperature for 5 min. The cells are then placed on a sterile 0.2 micron filter on a TSAP agar plate, and incubated 5–6 hr at 30°C. Finally, the filter is removed and placed in 125 µl 0.2 M RbCl and vortexed to resuspend the cells, then the liquid is plated on TSAP agar containing 50 µg/ml kanamycin. Plates are incubated at 30°C (due to the temperature-sensitive plasmid) up to 5 days. It should be noted that 0.2 M KCl can be used in place of 0.2 M RbCl in the protocol above, but we have observed higher efficiency of cryotransformation using RbCl.

Identifying and isolating the intron insertion mutant—Once the transformant colonies grow, targetron insertions can be identified through colony PCR and/or by PCR on chromosomal DNA isolated from transformant colonies, using combinations of gene-specific and intron-specific primers. In our experience, targetron insertions exist at low frequency within the initial transformant colony, and further purification of colonies by plate streaking is necessary to isolate “pure” mutant. The presence of a targetron insertion is determined using the intron-specific primer EBS universal combined with a gene specific primer, while the presence of the mutated gene versus the wild-type gene is determined using gene-specific primers that amplify across the insertion site. If the targetron inserts in the “sense” strand of the gene, the EBS universal primer is paired with a primer specific to the 5′ end of the target gene, whereas if the targetron inserts in the “antisense” strand, the EBS universal primer is paired with a primer specific to the 3′ end of the target gene. To perform colony PCR, we typically perform the PCR reactions in the following manner. The reaction mixture contains 36 µl deionized H₂O, 5 µl 10X KOD buffer, 2 µl (25 mM) MgCl₂, 2 µl (2 mM) dNTPs, 2 µl Primer 1 (25 pmol/µl), 2 µl Primer 2 (25 pmol/µl), and 0.5 µl KOD DNA polymerase (Novagen). We then apply a sterile pipette tip directly to a colony, and then dip the pipette tip into the reaction tube. The PCR reaction is carried out by 2 min preheat at 98°C, followed by 30 cycles of 15 s at 98°C, 15 s at 55°C, and 1 min/kb at 72°C. To perform PCR on isolated chromosomal DNA from the colonies, the chromosomal DNA is first isolated with Easy-DNA Kit (Invitrogen), and then utilized in PCR as outlined above. In our experience, screening ten primary transformant colonies by PCR with an intron-specific primer paired with a gene-specific primer, we find all ten colonies will yield a positive signal, indicating targetron insertion in all ten colonies.

Once colonies with a targetron insertion are identified, these are further purified by plate streaking (TSAP with 50 µg/ml kanamycin, 30°C), and testing individual colonies via PCR with gene-specific primers. Primers that amplify across the insertion site will reveal whether the population only contains the mutated gene, or contains both wild-type and mutant genes. In a mixed population, two bands will be visualized through gel electrophoresis, one the size of the wild-type amplicon (amplified in the control parent strain) and another 915 bp larger than the wild-type amplicon [15]. The relative intensity of these two bands indicates the prevalence of the targetron insertion within the population, and further plate-streaking serves to increase the prevalence of the targetron insertion, until a pure population with only mutated

gene is detected. In our experience with multiple *F. tularensis*-specific targetrons, one to three cycles of plate streaking are required to isolate pure mutant colonies; at each stage we routinely screen at least ten isolated colonies. Alternatively, colonies can be grown in liquid culture rather than on plates, and then dilutions of the culture plated to achieve individual colonies, which can then be screened for the targetron insertion, as detailed above; we have found this method to be equally efficient at identifying and purifying mutant colonies. The targetron insertions should be verified by sequencing the PCR fragment generated with gene-specific primers. When two identical targets exist within the host strain (e.g. the duplicated FPI genes), the isolation of pure mutant (i.e. with both genes inactivated) takes longer than when only a single target gene is present.

Once PCR with gene-specific primers indicates that a colony is a pure population of insertion mutant, the targetron plasmid is then removed by plate-streaking the colony on TSAP (no kanamycin) with incubation at 37°–40°C. Individual colonies are then patched on TSAP with 50 µg/ml kanamycin to identify the kanamycin sensitive colonies, which have lost the targetron plasmid. We routinely utilize 37°C rather than 40°C, to minimize any negative effects that may result from higher temperature incubation, and find that the frequency of plasmid loss can be detected in approximately 1 in 50 colonies following overnight growth. Incubation at 40°C leads to a more dramatic loss of plasmid, with most colonies displaying a Kan^s phenotype following overnight growth. Loss of the plasmid can be confirmed by a PCR screen using KanprobeF and KanprobeR primers (Table 2) that are specific for the targetron plasmid and produce a 365-bp PCR product. Either colony PCR or PCR utilizing isolated DNA can be used for this step, as described above.

Southern hybridization—Southern hybridization should be performed on the chromosomal DNA from the isolated mutant strain, to confirm that only a single targetron insertion exists in the genome of the mutant strain. The probe consists of an intron-specific 378-bp PCR product amplified with oligonucleotides IntronDn and IntronUp (Table 2). This probe can be generated from the targetron plasmid used to mutagenize the strain, or it can be amplified from the chromosome of the mutant strain itself, using the PCR guidelines outlined above. The targetron probe is then labeled with horseradish peroxidase (HRP) utilizing Amersham ECL Direct Nucleic Acid Labelling And Detection Systems (GE Healthcare), according to the manufacturer's instructions.

We perform digestions of chromosomal DNA with *Nde*I, which has proven a useful enzyme because it has sufficient sites within the *F. tularensis* genome to yield reasonable size fragments (typically between 1–6 kbp), yet it does not digest within the targetron insertion. The digested chromosomal DNA (~10 µg/well) is then separated by gel electrophoresis using a 0.8% agarose gel (TAE buffer). The Southern blot protocol is then performed as specified in the manufacturer's instructions (Amersham ECL Detection Systems, GE Healthcare), utilizing VacuGene vacuum blotting system (GE Healthcare) for transfer onto 0.45 µ nitrocellulose membrane (GE Water & Process Technologies), followed by UV cross-linking with UV Stratalinker (model 1800, Stratagene). Hybridization with the 378 bp targetron probe and subsequent washes were performed at 42°C (without urea) in an Isotemp Hybridization Incubator, and the membrane was subsequently subjected to autoradiographic detection, as per manufacturer's directions.

3. Concluding remarks

Once retargeted, the Targetron group II intron system shows amazing specificity for only the target gene of interest, making it a useful tool for targeted inactivation of specific genes. We consider one of the major advantages of the targetron system to be the relative host-independence of the group II introns for their function. The critical aspect for function is the

expression of the RNP complex within the host strain; once expressed, the RNP will then home into the target sequence and inactivate it, without the involvement of host recombination machinery. This is especially useful in bacterial species for which recombination occurs at a low frequency and for which genetic manipulation systems are rudimentary. Targeted gene knockouts in *F. tularensis* are still relatively cumbersome, especially in the more virulent subspecies, which appear to have a lower frequency of recombination, and thus the targetron system has proved to be a valuable technique in these strains. Moreover, the capacity for the targetron to inactivate all sequences that it recognizes is especially useful in the study of the duplicated FPI genes in the more virulent *F. tularensis* subspecies. The list of genes which we have successfully inactivated in various *F. tularensis* subspecies, including the highly virulent subspecies *tularensis*, continues to grow, and currently includes *blaB*, *iglC1* and *iglC2*, *iglD1* and *iglD2*, *mglA*, *recA*, *uvrA*, and *pilE4*. Moreover, since the inserted targetron does not encode antibiotic resistance markers, it facilitates the construction of strains with multiple targetron insertions, and we have constructed several *F. tularensis* strains with multiple targetron insertions in different genes (e.g. *iglC1* and *iglC2* and *recA*, etc.).

The key to adapting this system to other bacterial species is ensuring the expression of the RNP complex through the use of a suitable expression vector with a promoter that functions in the host. This facilitates continuous expression of the RNP, which will eventually inactivate its target sequence. The introduction of a counterselectable marker into the plasmid (e.g. temperature sensitivity) is advantageous because the RNP can then be removed once the target gene is inactivated. To date, the targetron system has been adapted to function in at least ten different bacterial species (Table 1). The targetron system is likely to facilitate genetic studies in many additional bacterial species for which genetic techniques are not fully developed, just as it has facilitated targeted mutagenesis aimed at deciphering *F. tularensis* virulence.

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Figure 1.

A) Schematic of RNP interaction with target site. Example shows the target site within the *F. tularensis* *blaB* gene. LtrA has preferences for basepairs shown at -23, -21, -20, and +5 in relation to the insertion site (designated with Δ), while the EBS2 and EBS1- δ loops of the LtrB RNA basepair with the target sequences shown. The targetron algorithm identifies potential recognition sites within genes based on the LtrA preferences, while the SOE PCR with specific oligonucleotides alters EBS2 and EBS1 within LtrB to specifically basepair to the target sequence, resulting in specific insertion of the intron into the target gene. **B).** Map of the *F. tularensis* targetron plasmid pKEK1140, with the features outlined in the text.

Figure 2.

Flow chart for targetron inactivation of *F. tularensis* genes.

Table 1

List of bacteria genetically manipulated by group II introns

Bacterium	Reference
<i>Escherichia coli</i>	[14,20]
<i>Shigella flexneri</i>	[14]
<i>Salmonella enterica</i>	[14]
<i>Lactococcus lactis</i>	[16]
<i>Clostridium perfringens</i>	[17]
<i>Staphylococcus aureus</i>	[18]
<i>Clostridium acetobutylicum</i>	[19]
<i>Pseudomonas aeruginosa</i>	[23]
<i>Agrobacterium tumefaciens</i>	[23]
<i>Francisella tularensis</i>	[15]
<i>Proteus mirabilis</i>	[27]
<i>Azospirillum brasilense</i>	[28]

Table 2

Oligonucleotide primers used in this study

Primer	Sequence 5'-3'
IntronDn	TAGGAGAACCTATGGGAACGAAACG
IntronUp	ACGCGTCGCCACGTAATAAATA
EBSUniversal	CGAAATTAGAACTTGCGTTCAGTAAAC
KanprobeF	TGCATGGTTACTCACCCTGC
KanprobeR	TACAACCTATTAATTCCCCTCG