# Impairment of infectious laryngotracheitis virus replication by deletion of the UL[-1] gene

Running title: UL[-1] is required for efficient ILTV replication

M. Nadimpalli[1,2], S.W.Lee[2,3], J.M. Devlin[2], J.R.Gilkerson[1] and C.A. Hartley[1],[2*]

[1]Centre for Equine Infectious Diseases, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria, 3010, Australia.

[2]Asia-Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria, 3010, Australia.

[3]College of Veterinary Medicine, Konkuk University, Seoul, Republic of Korea.

[*]Author for correspondence: Carol A Hartley, phone: +61 3 83447375, email: carolah@unimelb.edu.au

## Abstract

Infectious laryngotracheitis virus (ILTV) encodes several unique genes, including a pair of unique nuclear proteins UL0 and UL[-1] that are expressed during replication in cell culture. Although the UL0 gene has been shown to be dispensable for replication, the role of UL[-1] has not been elucidated. In this study a deletion mutant of ILTV lacking the UL[-1] gene was constructed using homologous recombination. The coding sequences of the gene were replaced with the gene for enhanced green fluorescent protein and the cytomegalovirus major immediate early promoter element. The progeny virus carrying the reporter gene was readily identified using fluorescent microscopy, but was unable to propagate in the permissive cells in the absence of wild type ILTV. Even after plaque purification and fluorescent associated cell sorting the recombinant virus deficient in UL[-1] gene could not be successfully isolated. Our findings suggest that the UL[-1] gene has an important role in ILTV replication.

## Introduction

Infectious laryngotracheitis (ILT) is an economically significant respiratory disease of chickens that is caused by infection with infectious laryngotracheitis virus (ILTV). The virus is taxonomically designated as *Gallid herpesvirus-1*, a member of the subfamily *Alphaherpesvirinae*, in the family *Herpesviridae* [1].

The genome of ILTV encodes for 80 predicted open reading frames [2]. Although many ILTV gene products have not been characterised directly, often their possible function can be inferred by their homology to genes in other alphaherpesviruses that have been characterised more comprehensively. Some ILTV genes have been used in vectored recombinant ILTV vaccines that use other viruses to express ILTV proteins [3,4]. The function of other ILTV genes has been studied directly, typically through the creation of defined ILTV mutants that lack the target gene [5-15]. The availability of suitable continuous cell lines, such as the chicken hepatoma LMH cell line [16], has facilitated the genetic manipulation of ILTV by homologous recombination [17]. The ILTV genome exhibits several unique features such as absence of a UL16 homologue, a large internal inversion within the unique long (UL) region that includes UL22 to UL44 genes, presence of iltovirus-specific genes (ORF A to ORF E) and translocation of UL47 to the UL region [8,12,18,19]. Apart from these, previous sequence analysis identified the presence of unique genes in the ILTV genome, such as UL0 and UL[-1] genes that are iltovirus-specific genes [20].

In a recent transcription profiling experiment, the transcription levels of ILTV genes were compared between wild type ILTV and an attenuated gG deletion mutant of ILTV. When compared to the expression in wild type virus, the expression of UL[-1] was reduced in the attenuated gG deficient ILTV *in vivo*, implying a role in virus pathogenicity [21]. The main objective of this study was to examine the role of UL[-1] by performing homologous recombination to delete the coding sequences of the UL[-1] gene and to subsequently characterise this deletion mutant.

## Materials and methods

### Virus and cells

The ILTV strain CSW-1 was used as the parent virus in this study [22]. Cultures of LMH cells were used for virus propagation and plaque assays [16]. Cell monolayers were cultivated in growth medium (GM) containing Dulbecco's Minimal Essential Medium (DMEM, Sigma), 10% (v/v) fetal calf serum (FCS, CSL) and 50 µg/ml ampicillin. Cells were incubated at 37°C in a humidified atmosphere of 5% (v/v) $CO_2$ in air.

### Generation of UL[-1] deficient ILTV (Δ[-1].eGFP+ve.ILTV)

The UL[-1] gene in ILTV CSW1 was replaced with the gene for enhanced green fluorescent protein (eGFP) under the cytomegalovirus (CMV) promoter [23], by the construction of a plasmid using pGEM-T (Promega) containing both the upstream and downstream regions of the UL[-1] gene, flanking the eGFP gene with a CMV promoter (eGFP.CMV) (Fig. 1A). The sizes of the upstream DNA sequences ([-1] up) and downstream DNA sequences ([-1] dw) were 858 bp and 799 bp respectively. Both of these regions and the eGFP.CMV coding sequence (1400 bp) were assembled using splice overlap extension (SOE) PCR [24]. The details of the primers used are listed in Table 1. This final construct ([-1]up-cGFPp-[-1]dw) was then ligated into the pGEM-T vector (Promega) to form p[-1]up-cGFPp-[-1]dw and the plasmids were transformed into *Escherichia coli* (*E. coli)* DH5α cells. The plasmid was then extracted from cultured *E. coli* transformants using a plasmid midi kit (Qiagen).

For co-transfection experiments 2 µg of CSW1 ILTV DNA extracted from LMH cells as previously described [25], 2 µg of linearised p[-1]up-cGFPp-[-1]dw and 2 µg of pRc-ICP4, immediate early trans-activator protein to increase viral infectivity [10] were used to transfect sub confluent monolayers of LMH cells grown in 6 well plates using XtremeGENE HP DNA (Roche) transfection reagent. The plates were incubated at 37 °C in a humidified atmosphere with 5% (v/v) $CO_2$ in air for 5 to 7 days until the cells showed cytopathic effects (CPE). The cells and supernatant were harvested and stored at -80 °C.

## Plaque purification of Δ[-1].eGFP+ve.ILTV and characterisation using PCR and DNA sequencing

The progeny virus from co-transfections was stored at -80°C prior to *in vitro* analysis. LMH cells were infected with the transfected cell lysate under an overlay medium containing 1% (w/v) methylcellulose. Using an inverted microscope, the required plaque phenotype (eGFP positive or negative) was carefully picked and maintained as separate samples for three rounds of plaque purification, as described previously [5]. After three rounds of plaque purification, the plaques resulting from purification of eGFP-expressing plaques continued to produce plaques both with and without fluorescence. Viral DNA was extracted from plaques generated using these plaque-purified viruses using the QIAamp Viral RNA mini kit (Qiagen), according to the manufacturer's instructions.

The viruses present in the different samples were characterised using PCR and DNA sequencing. The details of the primers used for *in vitro* characterisation are listed in Table 1. Nested PCRs were performed on the DNA extracted from eGFP-expressing plaques and plaques lacking eGFP expression. The first round of the PCR was designed to target the region of the ILTV genome across the entire recombination site. For this the primer pairs -1rF and -1rR were used. The amplified products of the first round PCR were used as templates for the second round PCR using a primer pair (-1rF and aR) specifically targeting the ILTV region upstream of the desired target gene and a sequence in the eGFP gene (Fig. 1B). The products were visualised on an agarose gel using SYBR safe buffer (Invitrogen), purified (Qiagen gel extraction kit) and sequenced using Big Dye Terminator v3.1 (Applied Biosystems).

## Fluorescent activated cell sorting (FACS) of Δ[-1].eGFP+ve.ILTV and characterisation using PCR

In order to further investigate whether Δ[-1].eGFP+ve.ILTV could be isolated and grown independently of wild type ILTV, FACS was applied to the infected cells. Briefly LMH cells were infected with lysate containing mixed populations of Δ[-1].eGFP+ve.ILTV and wild type ILTV and incubated until the formation of 100% CPE. The infected cells and supernatant were harvested and then washed twice with sterile phosphate buffered saline (PBS) using 233 × *g* for 5 min in an AllegraX-12R centrifuge (Beckman) to remove traces of growth medium. The final cell pellet was resuspended in sorting buffer (PBS containing 1 mM EDTA, 25 mM HEPES pH 7.0, 1% v/v FCS) and sorted using a Beckman Coulter Astrios Cell Sorter PC2 with a 100 µM nozzle tip (Flow Cytometry facility, Department of Microbiology and Immunology, The University of Melbourne). The cells were sorted into two populations; fluorescence-positive cells and fluorescence-negative cells. These populations of cells were collected in individual wells of 96-well trays at a rate of 1, 5, 10 and 50 cells per well and then the remaining cells (approximately 1ml) were sorted into larger (bulk) tubes. The cells in the 96 well plates were incubated at 37°C until the formation of CPE. The DNA of cells within the 96 well plates, and the bulk-sorted cells was extracted using QIAamp Viral RNA mini kit, according to the manufacturer's instructions, for PCR analyses.

Three different primer pairs (Table 1) were used for PCR amplification from the extracted DNA. One set of PCR primers was designed to differentiate the presence/absence of the eGFP gene in cells sorted based on their eGFP fluorescence. The other set of PCR primers were designed to amplify a sequence from the particular gene of interest (eGFP or UL[-1]) along with adjacent sequence in the ILTV genome (ILTV UL0 gene). More specifically, the [-1]rF/aR primer pair (detecting a region of the eGFP.CMV gene and also binding to and amplifying a portion of the ILTV UL0 gene, generating a 3 kbp amplicon) and the aF/aR primer pair (targeting only the eGFP region, generating a 1 kbp amplicon) were used to detect the eGFP gene in the UL[-1] deficient ILTV. To detect the UL[-1] region in the extracted DNA, the [-1]rF/[-1]qR primer pair (detecting part of the UL[-1] gene and also binding to and amplifying a portion of the ILTV UL0 gene, generating a 2 kbp amplicon) was used.

## Prediction of UL[-1] protein structure

The secondary structure of the UL[-1] protein sequence was predicted using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). This software uses the multiple protein database (PDB) structures and predicts the best model based on C-score. The C-score is a confidence score for estimating the quality of predicted models by I-TASSER, typically in the range from −5 to 2, where higher values signify a model with high confidence. TM-score is a recently proposed scale for measuring the structural similarity between two structures. A TM-score $> 0.5$ indicates a model of correct topology and a TM-score $< 0.17$ indicates a random similarity. Root mean square deviation (RMSD) is an average distance of all residue pairs in two structures [26-28].

## Results

### Plaque purification and subsequent characterisation of viruses using PCR and DNA sequencing

A recombinant virus was selected based on eGFP-expression in infected cells, and then underwent 3 rounds of plaque purification in an attempt to generate a homogeneous population of the recombinant virus. After three rounds of plaque purification, the resulting infected cells continued to show two different forms of CPE; (1) plaques containing only non-fluorescent cells (Fig. 2A) and, (2) plaques containing a mixed population of fluorescent and non-fluorescent cells (Fig. 2B).

The initial PCR, applied to DNA extracted from these different plaque types, was designed to amplify across the recombination region that contained either eGFP or UL[-1] genes using the primers [-1]rF and [-1]rR (Table 1). Using these primers, DNA from wild type ILTV would be expected to yield a 4827 bp product, while a 4627 bp product would result from Δ[-1].eGFP+ve.ILTV. Template DNA from the two plaque types generated products of approximately 4.8 kbp in length (Fig. 3A), which were sequenced to confirm the presence of recombination sequences on either end of the product, by using primers -1rF and -1rR respectively (data not shown). Since the difference in size between the products from Δ[-1].eGFP+ve.ILTV and wild type ILTV DNA was difficult to accurately identify, these PCR products were subjected to semi-nested PCRs that were designed to target either the eGFP region using the primer pair [-1]rF/aR, or the UL[-1] region using the primer pair [-1]rF/[-1]qR (Table 1). These PCR assays identified differences in the DNA extracted from the different plaque types. Semi-nested PCRs targeting the eGFP region amplified a product of the expected size for Δ[-1].eGFP+ve.ILTV (approximately 3 kbp) in samples corresponding to plaques containing the mixture of fluorescent and non-fluorescent cells. These primers did not amplify a product from plaques that contained only non-fluorescent cells (Fig. 3B). A second product from this nested PCR (approximately 1.7 kbp) was also amplified from these plaques and shown by sequence analysis to be contaminating vector sequence from the initial co-transfection. Semi nested PCRs targeting the UL[-1] region (Table 1) amplified a product of the expected size for the wild type virus (approximately 2 kbp) from all samples (Fig. 3C). The sequence analysis of the 3 kbp amplicon from the eGFP-targeted PCR showed the sequence expected after homologous recombination and replacement of the UL[-1] gene with eGFP gene and the CMV promoter (data not shown). Sequence analysis of the 2 kbp amplicon from the UL[-1] targeted PCR showed sequence identical to wild type DNA, and had no evidence of recombination in this region (data not shown). Despite a number of attempts to overcome the contaminating product in the semi-nested PCR assays, the negative control from the first round PCR continued to produce a band of approximately 1.3 kbp (Fig. 3). Sequence analysis determined that this amplicon was derived from non-specific binding of *Bradyrhizobium sp.*, which has been shown in other publications to be a common PCR contaminant [29].

### Cell sorting and subsequent characterisation of viruses using PCR

Cell sorting produced 96 well plates containing replicate wells with 1, 5, 10 and 50 cells per well of either fluorescence-positive sorted cells or fluorescence-negative sorted cells. Uninfected LMH cells were added to these wells, and after 3 days all wells showed CPE. No fluorescence was detected in the resulting CPE of either plate using fluorescence microscopy. Despite this finding, eGFP DNA was detected by PCR in the plate containing fluorescence-positive sorted cells, but not in the plate containing fluorescence-negative sorted cells. Sorted cells from both plates were PCR positive for UL[-1] DNA. In addition to the 96 well plates, infected cells were also sorted into larger samples, and each of these pooled samples of sorted cells (fluorescence-positive sorted cells and fluorescence-negative sorted cells) were positive for the presence of eGFP and UL[-1] DNA by PCR, as was the DNA extracted from the infected cells prior to sorting (data not shown). The results indicate fluorescent activated cell sorting was not able to isolate Δ[-1].eGFP+ve.ILTV, which may be a consequence of dual-infected cells (cells infected with both wild type and Δ[-1].eGFP+ve.ILTV). The detection of eGFP DNA in the large pooled sample of fluorescence-negative sorted cells may be due to the presence of a small number of cells that contained Δ[-1].eGFP+ve.ILTV but for which expression of eGFP was not present or below the threshold of detection by the cell sorter at the time of cell sorting.

## Prediction of UL[-1] protein structure

The *in vitro* characterisation of UL[-1] mutant by semi nested PCR and FACS analysis demonstrates the inability to isolate this mutant. This finding suggests the mutant is unable to replicate in the absence of wild type DNA and therefore suggests an important role for the UL[-1] gene in ILTV replication. In an attempt to identify the UL[-1] gene product, in the absence of significant nucleotide or amino acid homology with other known genes, the UL[-1] amino acid sequences were submitted to I-TASSER to determine whether any structural homologues could be identified. The UL[-1] protein had the highest C-score with one proposed model. The estimated accuracy of model 1 was: C-score: -1.60, TM-score: 0.36 ± 0.12 and RMSD: 15.3 ± 3.4 Å. The best model was used for structural similarity simulation and UL[-1] was found to have highest similarity with the mitochondrial ATP binding cassette (ABC) family of transporters.

## Discussion

The aim of this study was to characterise the role of UL[-1], a unique ILTV gene, by creating an ILTV deletion mutant lacking the UL[-1] gene. The transcription of this gene was shown to be down regulated in an attenuated gG deficient ILTV, when compared to wild type ILTV [21]. Although sequence and PCR analysis in our study showed that a recombinant ILTV deficient in the UL[-1] gene was generated by homologous recombination, the resultant Δ[-1].eGFP+ve.ILTV could not be isolated from wild type ILTV, even after three rounds of plaque purification. Although individual plaques of wild type ILTV could be re-isolated, this was not possible for the UL[-1] deficient ILTV mutant, even following fluorescent activated cell sorting.

The presence of eGFP positive cells suggest that Δ[-1].eGFP+ve.ILTV can enter and begin transcription and translation of the viral genome in the LMH cells, but was unable to establish infection independently, without wild type ILTV. Presumably this occurred because of the presence of UL[-1] gene product provided by the wild type ILTV, which was sufficient to enable replication of the UL[-1] deficient mutant. The inability of Δ[-1].eGFP+ve.ILTV to infect cells independent of wild type ILTV is consistent with a critical role for UL[-1] in viral replication. The finding that the UL[-1] deficient virus cannot grow independently of wild type ILTV is similar to results seen previously, in the construction and characterisation of a gI/gE deficient recombinant ILTV [5]. However, unlike the gI/gE mutant, we were unable to separate the recombinant DNA from wild type ILTV DNA.

The UL[-1] gene is unique to ILTV and generates a spliced mRNA transcript containing 501 codons [30]. The resultant 73 kDa protein is primarily detected in the nuclei of infected cells at later stages of replication, suggesting that the products of UL[-1] gene may be associated with host gene expression or with cleavage and encapsidation of viral DNA [30]. The positional homologue of ILTV UL[-1] ORF in HSV1 is the trans-activator protein infected cell protein-0 (ICP0) gene, which is conserved in other herpesviruses, including equine herpesvirus-1, bovine herpesvirus-1 and pseudorabies virus [31-34]. The genome of ILTV lacks the ICP0 gene, which is dispensable in other herpesviruses and no ICP0 homologues have been identified in other avian herpesviruses such as Marek's disease virus-1 and herpesvirus of turkeys [35,36]. Unlike the UL[-1] protein, the ICP0 protein has a conserved zinc binding motif and is expressed early in the infection [31,37]. The BLASTp analysis of the UL[-1] protein shows no amino acid similarity to homologues of ICP0 trans-activator family and instead the closest protein in the existing database is the UL0 protein of ILTV, consistent with previous reports [30]. It has been proposed that the UL0 and UL[-1] genes might have resulted from an ancient duplication of one gene, as indicated by their amino acid homology and common features, including mRNA structure and localization of the protein products [30]. The deletion of the ILTV UL0 gene showed that, unlike UL[-1] in this study, UL0 is dispensable for replication in cell culture. Whether the presence of the UL[-1] gene complements the missing function of UL0 in UL0 deletion mutants is not known. However if true, the results of the present study suggest that the reciprocal might not be true, since UL0 could not complement for the missing UL[-1] gene.

Further searches for homologues of UL[-1] used secondary protein structure prediction and homology based 3D modelling in the PDB library. Five predicted models were obtained for the UL[-1] protein. According to this model, the UL[-1] protein has the best available structural similarity with a mitochondrial ABC transporter and a predicted biological function, based on enzyme active sites and ligand binding sites, which relates to eukaryotic P-glycoprotein (Pgp), mitochondrial cytochrome-b complex and *E. coli* DNA polymerase III.

Since the predicted homologies generated by I-TASSER have low identities with the protein structures in the PDB library, the function of the UL[-1] gene at the amino acid level still remains inconclusive. Hence the function of UL[-1] in viral pathogenesis is unclear and requires further investigation. The multidrug resistance gene product, Pgp acts as a defense mechanism against toxins and is expressed in all animals including chickens, bacteria and fungi [38]. This protein belongs to the ATP binding cassette (ABC) family of transporters which are efflux pumps present on the plasma membrane of the cells and are involved in peptide transport [39]. Studies have reported the interactions of Pgp and enveloped viruses such as influenza, vesicular stomatitis virus (VSV) and human immunodeficiency virus-1 (HIV-1). These studies show that Pgp blocks the entry of these viruses through the plasma membranes of infected cells [40,41]. However, the presence of ABC efflux pumps in viruses has not been reported.

The ILTV gD gene has been shown to have an essential role in viral replication since a gD deficient virus could not be isolated on trans completing cells [15]. Further insight into functional aspects of the ILTV UL[-1] gene is difficult to obtain at this time, as the newly developed mutant is unable to infect cells without wild type ILTV. Future studies should focus on characterising the UL[-1] deficient virus on trans-complementing cell lines expressing the UL[-1] protein.- A bacterial artificial chromosome (BAC) technology has been applied to characterise the role of essential genes for several herpesviruses, including Marek’s disease virus-1 and herpesvirus of turkeys, viruses that have been successfully cloned as infectious BACs [42,43]. Such a robust and reliable technique needs to be developed in order to better understand the biology of ILTV and to perform more efficient genetic analysis.

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**Figure 1:** Targeted homologous recombination strategy used to create Δ[-1].eGFP+ve.ILTV. **(A)** The coding sequence of UL[-1] gene was replaced with eGFP.CMV to generate Δ[-1].eGFP+ve ILTV **(B)** Schematic representation of the different primer pairs used to examine the recombination region by nested PCR.

**Figure 2:** Photomicrographs of LMH monolayers after infection with progeny virus obtained from co-transfection experiments. After 3 rounds of plaque purification for plaques showing expression of fluorescence two different types of CPE could be observed. (**A)** Plaques containing only non-fluorescent cells. (**B)** Plaques that contain a mixed population of fluorescent and non-fluorescent cells. Monolayers were visualised using semi-dark field fluorescence microscopy at 100 × magnification.

**Figure 3:** PCR characterisation of viruses obtained from co-transfection experiments. DNA was extracted from different samples of CPE and used as a template in a series of PCRs. **(A)** PCR products from the first round of PCR using the primer pair [-1]rF and [-1]rR which target the entire recombination region. **(B)** PCR products from a semi-nested PCR utilising the amplicons from above and the primer pair [-1]rF and aR, targeting the GFP sequence. **(C)** PCR products from a semi-nested PCR utilising the amplicons from above and the primer pair [-1]rF and [-1]qR targeting UL[-1] sequences. Lanes are: **1** = DNA extracted from a plaque containing fluorescent and non-fluorescent cells (high proportion of fluorescent cells). **2** = DNA extracted from a plaque containing fluorescent and non-fluorescent cells (low proportion of fluorescent cells). **3** = DNA extracted from a plaque containing non-fluorescent cells only. **4** = DNA extracted from wild type ILTV (positive control). **5** = No template

control from first round of PCR. **6** = No template control for semi-nested PCRs. The Marker **(M)** is Hyperladder I (1 kbp, Bioline).

## Ethical compliance statement.

The authors have no conflict of interest to declare. This work in this project was not funded by a specific funding body or source of funds. This manuscript represents an accurate account of the work performed. This study represents original work that has not been submitted to any other journal for publication. No human or animal ethics approval was required for the completion of this study.

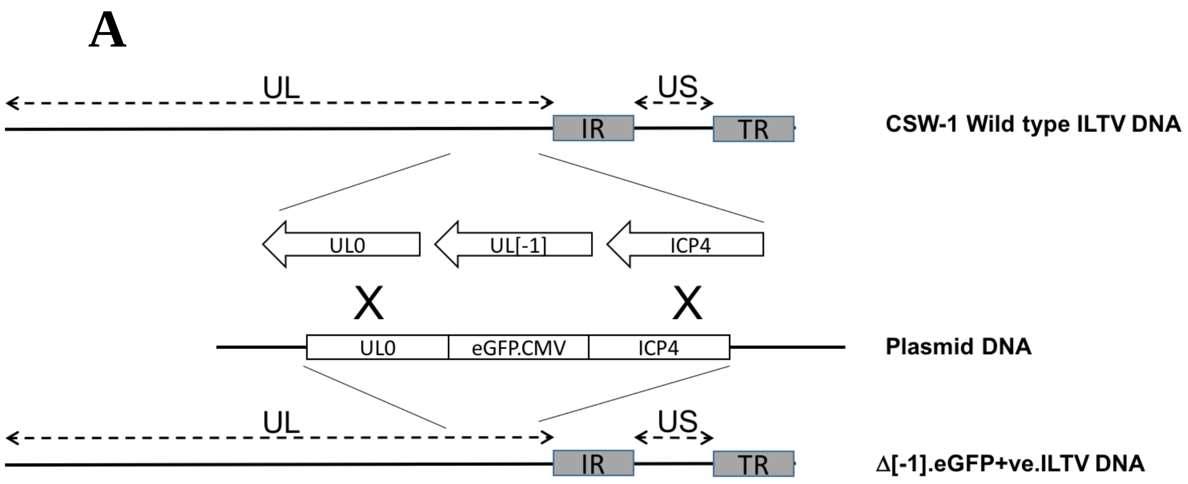
**Table 1.** Primers used for generation and *in vitro* characterisation of UL[-1] deficient ILTV

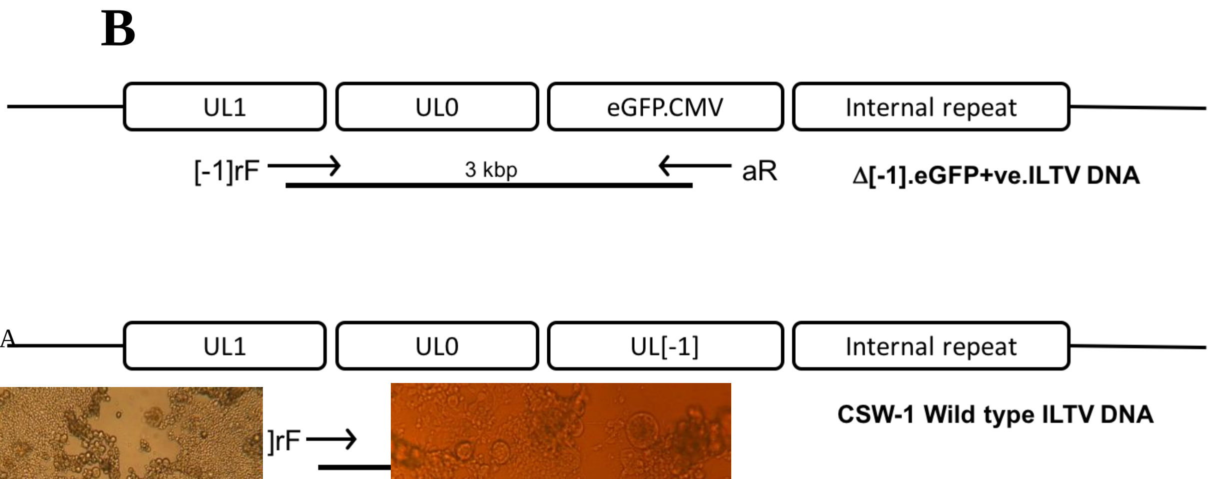
| **ne** | **Orientation** | **Primer sequence 5'-3'** | **Target sequence[b]** | **Purpos** |
|---|---|---|---|---|
| **a)** | Forward | TATCCGCTAGGGTGATTCGT | 109019-109038 | Generati<br>Δ[-1].eGFP- |
| | Reverse | *atgaccccgtaattgattacta*CGAATGTTTATTATTTTGGCGCATA | eGFP, 109876-109852 | |
| | Forward | *tatgcgccaaaataataaacattcg*TAGTAATCAATTACGGGGTCAT | 109852-109876, eGFP | |
| | Reverse | *ggcccgttcgttgaaat*TTATCTAGATCCGGTGGAT | 111522-111538, eGFP | |
| | Forward | *atccaccggatctagataa*ATTTCAACGAACGGGCC | eGFP, 111538-111522 | |
| **b)** | Reverse | AGCCCTAGCCCCCACTATAC | 112320-112301 | |
| | Forward | ACCTAGGTCTCCGTTCACGA | 108079-108098 | *In vitro* chara<br>for neste |
| | Reverse | TAGACGCTGCAGGAAAAGCA | 112906-112887 | |
| | Forward | CTGTGGGGCCCAATTCTTCT | 109970-109989 | |
| | Reverse | AGCGCGGATGAAGGAGAAAA | 110103-110084 | |
| | Forward | AACGCCAATAGGGACTTTCC | eGFP.CMV | |
| | Reverse | TGCTCAGGTAGTGGTTGTCG | eGFP.CMV | |

[a] Primers used for SOE PCR are italicised and are represented in both upper and lower case lettering. The lower case sequence in a primer is complementary to the upper case sequence of the next and is designed to anneal different PCR templates together.

[b] Indicates the target sequence for each primer. Primer positions that bind to ILTV are based on the target sequences of CSW-1 ILTV strain used as reference with Genbank accession number JX646899.

Figure 1





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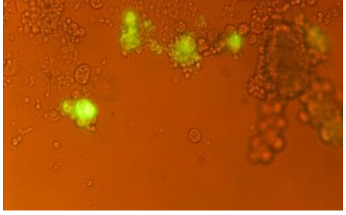

B

Figure 2

Figure 3