# Marek's Disease Virus Pathogenesis

# and Latency

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## <u>DECLARATION</u>

I declare that all work included in this thesis has been composed by myself. Contributions to the work of this thesis by colleagues are fully acknowledged. No part of this work has been or will be submitted for any other degree or qualification.

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# ABSTRACT

Marek's Disease virus (MDV) is a highly contagious, widespread and persistent neoplastic $\alpha-$herpesvirus causing extensive lymphoblastic tumours in chickens. The virus is shed in feather dust and spread through inhalation. Vaccines are available to protect against the effects of MDV but not replication of the virus and subsequent contamination of the environment leading to flock exposure. Increased virulence in strains of MDV has been identified and currently available vaccines may not offer protection from the disease. Disease outbreaks result in economic losses as well as welfare issues. To break this cycle better methods of controlling MDV preventing both tumourogenesis and shedding of infectious virus must be developed. Targeting specific MDV genes key to maintaining latency and viral replication using siRNA could potentially be used as a control strategy. At the present time, many of the unique genes in MDV are largely uncharacterised.

15 uncharacterised open reading frames (ORFs) were screened for expression in a MDV latent infection model in a non-producer MDV transformed chicken lymphoblast cell line, RPL-1. Of these uncharacterised ORFs LORF1, LORF3, LORF11, LORF12, ANTISENSE, US2, MLTI, RLORF11, RLORF12, 23kDa and RLORF6 were expressed during latency. To investigate the effect of post-transcriptional knockdown of these ORF products two 25-mer siRNA oligonucleotides were designed for each gene, transfected into RPL-1 cells and analyzed using growth rate as an indicator of changed phenotype over a period of 120 hours post-transfection. RPL-1 cells transfected with a nonsense siRNA oligonucleotide were used as the control group. No significant changes in transfected cell growth over the controls were identified in LORF3, ANTISENSE, RLORF12, LORF1, LORF11 or MLTI. Increased RPL-1 cell growth was observed (adjusted p-value of 0.0094) in one of the two siRNA oligonucleotides specific for RLORF6 at 72 hours post-transfection. RLORF6 was further characterised using confocal microscopy techniques and was found to be localized in the nucleus but not the nucleolus of chicken embryo fibroblasts and chicken lymphoblastic cells.

# ACKNOWLEDGEMENTS

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# ABBREVIATIONS

| | |
|---|---|
| aa | Amino Acid |
| BAC | Bacterial artificial chromosome |
| bp | base pair |
| cDNA | Complimentary Deoxyribonucleic Acid |
| CEF | Chicken Embryo Fibroblasts |
| DABCO | 1, 4-diazabicyclo-[2.2.2] octane |
| DAI | DNA-dependent activator of interferon regulatory factors |
| DLR | Dual luciferase reporter assay |
| DNA | Deoxyribonucleic acid |
| dsRNA | Double-stranded RNA |
| EB Buffer | Elution buffer |
| EBV | Epstein-Barr virus or *Human Herpesvirus 4* |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FCS | Foetal Calf Serum |
| F2 | Second filial generation |
| gB | Viral glycoprotein B |
| gC | Viral glycoprotein C |
| gD | Viral glycoprotein D |
| gH | Viral glycoprotein H |
| gL | Viral glycoprotein L |
| GaHV3 | *Gallid Herpesvirus 3* or Marek's Disease Type 2 |
| HCF-1 | Herpes simplex virus-associated host cell factor 1 |
| HCMV | Human Cytomegalovirus or Human Herpesvirus5 |
| HSV-1 | Herpes Simplex Virus Type 1 |
| HSV-2 | Herpes Simplex Virus Type 2 |
| HVEM | Herpesvirus entry mediator |
| HVT | Herpesvirus of Turkeys or *Melagrid Herpesvirus 1* |
| IFN | Interferon |
| IL-2 | Interleukin 2 |
| $IR_L$ | Internal Repeat Long region of viral genome |
| IRs | Internal Repeat Short region of viral genome |
| KSHV | Karposi's Sarcoma-Associated Herpesvirus or *Human Herpesvirus 8* |
| LAT | Latency-associated non-coding RNA transcript |
| LB | Luria-Bertani |
| LTR | Left terminal repeat |
| MBP-1 | *Myc*-binding protein-1 |
| MD | Marek's Disease |
| MDV | Marek's Disease Virus Type 1 or *Gallid Herpesvirus 2* |
| MDV BAC | Marek's Disease Virus Bacterial Artificial Chromosome |
| Meq | Marek's EcoRI Q fragment protein |
| MHC I | Major Histocompatibility Complex class I |

| | |
|---|---|
| MHC II | Major Histocompatibility Complex class II |
| mRNA | Messenger RNA |
| miRNA | micro RNA |
| ND-10 | Cellular nuclear domain10 |
| NLR | Nucleotide-binding oligomerisation domain-like receptors |
| OBP | Origin Binding Protein |
| Oct-1 | POU domain protein Oct-1 |
| Orange G | 1-Phenylazo-2-naphthol-6, 8-disulfonic Acid Disodium Salt, 7 Hydroxy 8-(phenylazo)-1, 3-naphthalenedisulfonic Acid Sodium Salt |
| ORF | Open Reading Frame |
| $Ori_{lyt}$ | Origin of lytic replication |
| PAMPs | Pathogen-associated molecular patterns |
| PCR | Polymerase Chain Reaction |
| pp14 | Phosphoprotein 14 |
| pp24 | phosphoprotein 24 |
| pp38 | Phosphoprotein 38 |
| RISC | RNA-induced silencing complex |
| RLR | Cytoplasmic retinoic acid-inducible gene-I-like receptors |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RPMI | Roswell Park Memorial Institute medium |
| RTqPCR | Reverse Transcriptase Quantitative Polymerase Chain Reaction |
| RTR | Right terminal repeat |
| shRNA | Short Hairpin RNA |
| siRNA | Small interfering RNA |
| SPBS | Sterile Phosphate Buffered Saline |
| SDS | sodium dodecyl sulfate |
| TCTP | 23p growth- related transcriptionally-controlled tumour protein |
| TE Buffer | Tris-EDTA Buffer |
| TEMED | N, N, N', N' Tetrametylethylenediamine |
| TERT | Protein subunit with reverse transcriptase activity |
| TLR | Membrane-bound Toll-like receptors |
| TLR-3 | Human Toll-like receptor 3 |
| Triton X-100 | t-Octylphenoxypolyethoxyethanol |
| $TR_L$ | Terminal Repeat Long region of viral genome |
| TRs | Terminal Repeat Short region of viral genome |
| TWEEN 20 | Polyoxyethylenesorbitan Monolaurate |
| $U_L$ | Unique Long |
| Us | Unique Short |
| VIC | VP16 induced complex |
| VP16 | HSV homologue viral protein 16 |
| vTR | Viral telomerase RNA subunit |
| chTR | Chicken telomerase RNA subunit |
| VZV | Varicella-Zoster Virus |

# Chapter 1 - Introduction

## 1.1 *Herpesviridae*

The *Herpesviridae* family is one of the oldest and largest of the viruses dating back an estimated 400 million years (sirnaMcGeoch and Gatherer, 2004). Herpesvirus species have been described infecting a diverse range of species, including fish (Wolf and Darlington, 1971), cetaceans (Arbelo *et al.*, 2010), reptiles (Bicknese *et al.*, 2010; Clark and Karzon, 1972) and even bivalves (Farley *et al.*, 1972). Individual herpesviruses, however, typically have a very limited host range and many viruses infect only one host species. The herpesviruses are unique among viruses and share several distinctive factors which aid in defining them as a family (Pellett *et al.*, 2006). Firstly, the capsid contains double-stranded DNA encoding genes for nucleic acid metabolism, DNA synthesis and protein processing. Secondly, synthesis of viral DNA and capsid assembly occurs within the nucleus of the infected cell while the final assembly of the viron takes place in the cytoplasm. Thirdly, production of viral particles during lytic infection irreversibly damages the host cell resulting in its destruction. Lastly, herpesviruses are able to establish non-replicating latent infections with a later reversion to productive infection that facilitates virus survival from generation to generation even in small populations. In latent infections, the viral genome is maintained outside the host cell chromosome allowing persistent infection often for the life of the animal accompanied by continuous or sporadic viral shedding whenever there is a reversion to cytolytic infection, usually when the host is immunocompromised or under stress.

### 1.1.1 Taxonomic Classification

The order *Herpesvirales* encompasses all herpesviruses, but the vast majority of herpesviruses described to date are classified within the *Herpesviridae* family that is subdivided into three main subfamilies designated *Alphaherpesvirinae* (α), *Betaherpesvirinae* (β) and *Gammaherpesvirinae* (γ) based on viral particle architecture and biological properties. This was expanded with the addition of the *Alloherpesviridae* and *Malacoherpesviridae* families to include herpesviruses infecting fish, amphibians and bivalves as outlined in table 1.1 (Davison *et al.*, 2009; Pellett *et al.*, 2006). To date many recently described species of herpesviruses are still to be categorized. The assignment of the formal taxonomic classification for each species of *Herpesviridae* is designated by the *Herpesviridae* Study Group of the International Committee on Taxonomy of Viruses (http://www.ictvonline.org) and usually incorporates a reference to the definitive host species (Davison *et al.*, 2009). In addition to the formal name most species of viruses also have a designated acronym (for example HHV3 for *Human Herpesvirus 3*) and a common name (for example Varicella Zoster for *Human Herpesvirus 3*) both of which are often used to refer to the virus in the literature but have no formal taxonomic standing (Davison *et al.*, 2009).

The α-herpesviruses are currently divided into four genera plus some recently discovered unassigned chelonid viruses as shown in table 1.1 (Davison *et al.*, 2009). The *Simplexvirus* genus infects mammals and includes the well-studied Herpes Simplex Virus Type 1 and 2 (also known as or HSV-1/HSV-2 or *Human Herpesviruses 1 and 2*).

*Varicellovirus* also infects mammals and includes human Varicella Zoster Virus (VZV or *Human Herpesvirus 3*) and Pseudorabies virus (*Suid herpesvirus 1*) infecting pigs. The *Mardiviruses* described to date all infect avian species and include *Gallid herpesvirus 2* (also known as Marek's Disease Virus type 1 or MDV), *Gallid herpesvirus 3* (Marek's Disease Virus type 2 or GaHV3) and *Melagrid herpesvirus 1* (Herpesvirus of Turkeys or HVT). The *Iltoviruses* also infect birds and include Pacheco's disease virus (*Psittacid herpesvirus 1*) and Infectious Laryngotracheitis (*Gallid Herpesvirus 1*) the latter of which was formerly classified as a *Mardivirus* but was later found to be in a separate genus (Davison *et al.*, 2009; Hughes and Rivailler, 2007). Features common to most of the α-herpesviruses are a short reproductive cycle resulting in rapid spread in cell culture with very efficient destruction of infected cells and many species establish persistent infections in the dorsal root ganglia or other nerve cells (Pellett *et al.*, 2006).

β-herpesviruses also include four genera and some unassigned viruses all infecting mammals as shown in table 1.1 (Davison *et al.*, 2009). The C*ytomegaloviruses* infect primates and include Human Cytomegalovirus (*Human Herpesvirus 5*). *Muromegaloviruses* infect rodents and includes *Murid herpesvirus 1* (Mouse cytomegalovirus). *Roseolovirus* infects humans and includes *Human Herpesvirus 6* and *Human Herpesvirus 7*. *Proboscivirus* infects elephants and includes Elephant endotheliotropic herpesvirus (*Elephantid herpesvirus 1*). Properties that are common but not exclusive to β-herpesviruses include a slow reproductive cycle resulting in slow

growth *in vitro* and infected cells often becoming enlarged (cytomeglia). The β-herpesviruses establish latent infections in a variety of cells such as salivary glands, kidney and lymphoreticular cells (Pellett *et al.*, 2006).

The γ-herpesviruses contain four genera as well as some unclassified viruses all infecting mammals as outlined in table 1.1 (Davison *et al.*, 2009). *Lymphtocryptoviruses* infect primates and include Epstein - Barr virus (EBV or *Human Herpesvirus 4*). The *Rhadinovirus* genus infects mammals and includes Kaposi's Sarcoma-Associated Herpesvirus (KSHV or *Human Herpesvirus 8*) and Murine Gammaherpesvirus 68 (*Murid Herpesvirus 4*). *Macaviruses* infect mammals and include Malignant Catarrhal Fever virus (*Alcelaphine herpesvirus 1*) and Sheep-associated malignant catarrhal fever virus (*Ovine herpesvirus 2*). Percaviruses also infects mammals and include *Equine herpesvirus 2* and Badger herpesvirus (*Mustelid herpesvirus 1*). Common features of γ-herpesviruses include a particularly narrow host range often limited to the definitive host species, association with oncogenicity and replication in lymphoblastoid cells *in vitro* with a specificity for either T or B lymphocytes, although some viruses can also infect certain types of fibroblast and epithelial cell lines as well (Pellett *et al.*, 2006). MDV was originally classified as a γ-herpesvirus based on common biological properties, but was subsequently classified as an α-herpesvirus based on genome architecture (Buckmaster *et al.*, 1988; Camp *et al.*, 1991).

| Family | Subfamily | Genus | Host |
|---|---|---|---|
| *Herpesviridae* | *Alphaherpesvirinae* (α) | *Simplexvirus* | Mammals |
| | | *Varicellovirus* | Mammals |
| | | *Mardivirus* | Birds |
| | | *Iltovirus* | Birds |
| | | Unassigned | Chelonids |
| | **Betaherpesvirinae (β)** | *Cytomegalovirus* | Mammals |
| | | *Muromegalovirus* | Mammals |
| | | *Roseolovirus* | Mammals |
| | | *Proboscivirus* | Mammals |
| | | Unassigned | Mammals |
| | **Gammaherpesvirinae (χ)** | *Lymphtocryptovirus* | Mammals |
| | | *Rhadinovirus* | Mammals |
| | | *Macavirus* | Mammals |
| | | *Percavirus* | Mammals |
| | | Unassigned | Mammals |
| | **Unassigned** | Unassigned | Birds, Reptiles and Mammals |
| *Alloherpesviridae* | **None** | *Ictalurivirus* | Fish |
| | | Unassigned | Fish and Amphibians |
| *Malacoherpesviridae* | **None** | *Ostreavirus* | Oysters |

**Table 1.1:** Taxonomy and host range of the order *Herpesvirales* adapted from (Davison *et al.*, 2009).

### 1.1.2  Virus Particle Structure

The structure of a typical herpesvirus particle incorporates a core, icosahedral capsid, tegument and envelope as illustrated in figure 1.2.  Mature herpesvirus particles vary in size from 120 - 260 nm in size, making them one of the largest mature viral particles.  The core contains 124-230 kb of linear double-stranded viral DNA arranged in a torus or spool (Furlong *et al.*, 1972; Nazerian, 1974).  Herpesvirus capsids consist of a 100-110 nm diameter icosahedral arrangement of 162 capsomeres consisting of 150 hexons and 12 pentamers (Haarr and Skulstad, 1994; Nazerian and Burmester, 1968).  The capsid is surrounded by an amorphous protein-rich tegument and a lipid envelope containing external glycoprotein spikes that vary according to virus species.  The amorphous proteinacious tegument between the capsid and envelope contains many pre-synthesized proteins used during host cell infection.  The herpesvirus envelope has a trilaminar structure typical of phospholipid membranes and is composed of altered host cellular membranes containing variable numbers and amounts of glycoproteins depending on the viral species.  For a more comprehensive review of viral particle structure see Fields Virology (Pellett *et al.*, 2006).
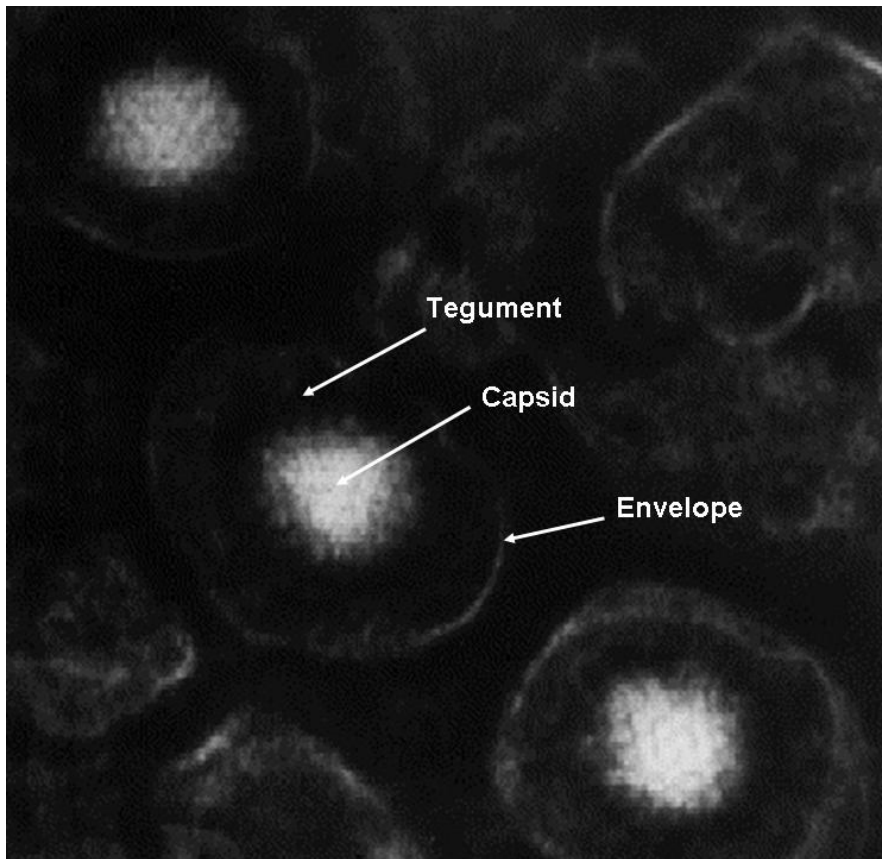
**Figure 1.2:** Electron micrograph of Varicella Zoster (HHV-3) virus particle illustrating the generalized Herpesviridae viral particle structure of an icosahedral capsid containing double stranded DNA, amorphous tegument and envelope with glycoprotein spikes. Photo adapted from image taken by Prof. Frank Fenner (John Curtin School of Medical Research, Australian National University, Canberra, Australia) downloaded from *ICTVdB - The Universal Virus Database*, version 4. (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/031.1.01.001.htm, accessed 10/10/10) (Büchen-Osmond, 2006)

### 1.1.3  Genome Arrangement

The herpesvirus genome encodes between 70-220 genes depending on the individual virus.  Genes are often designated by number and according to their location in the genome:  for example gene 30 located in the unique long region of the genome reading from L to R would be designated as UL30.  Genes encoding products required for viral replication and other essential functions are deemed fundamental core genes and are conserved in all herpesviruses (Alba *et al.*, 2001; Davison *et al.*, 2002; McGeoch *et al.*, 2006).  The 44 recognized core genes are listed according to function in table 1.3.  Each of the fundamental genes are located within one of 7 core blocks encoding between 2 and 12 genes whose order and polarity is conserved in all herpesviruses (Alba *et al.*, 2001; Pellett *et al.*, 2006).  Other genes are also conserved at the subfamily level.

| Function | Gene | HSV Homologue | Kinetic Gene Class |
|---|---|---|---|
| **Gene Regulation** | Multifunctional regulatory protein ICP27 | UL54 | $\alpha$, essential |
| **Nucleotide Metabolism** | Uracil DNA glycosylase | UL2 | $\beta$ or $\gamma_1$, non-essential |
| | dUTPase | UL50 | $\beta$, non-essential |
| | Ribonucleotide reductase, large subunit | UL39 | $\beta$, non-essential |
| **DNA Replication** | Helicase/primase complex | UL5 | $\beta$, essential |
| | (3 subunits) | UL8 | $\beta$, essential |
| | | UL52 | $\beta$, essential |
| | DNA polymerase | UL30 | $\beta$, essential |
| | ssDNA binding, ICP8 | UL29 | $\beta$, essential |
| | DNA polymerase processivity factor | UL42 | $\beta$, essential |
| **Virion Maturation** | Alkaline exonuclease | UL12 | $\beta$, non-essential |
| | Genome cleavage/packaging | UL28 | $\gamma$, essential |
| | | UL32 | $\gamma_2$, essential |
| | | UL33 | $\gamma_2$, essential |
| | Terminase/packaging | UL15a | $\gamma$, essential |
| | DNA packaging | UL25 | $\gamma_2$, essential |
| | Scaffold protease (cleaves to 2 proteins) | UL26 | $\gamma$, essential |
| | Scaffold | UL26.5 | $\gamma$, essential |
| | Capsid nuclear egress | UL31,UL34 | $\gamma$, essential |
| | Virion | UL16 | $\gamma$, essential |
| **Virion Capsid** | Major capsid protein | UL19 | $\gamma$, essential |
| | Minor capsid protein, portal protein | UL6 | $\gamma$, essential |
| | Capsid triplex | UL18 | $\gamma$, essential |
| | | UL38 | $\gamma_2$, essential |
| | Hexon tips | UL35 | $\gamma_2$, non-essential |
| **Virion Tegument** | Large tegument protein | UL36 | $\gamma_2$, essential |
| | Tegument protein | UL7 | $\gamma_1$, non-essential |
| | Protein kinase | UL13 | $\gamma$, non-essential |
| | Myristoylated | UL11 | $\gamma$, non-essential |
| | | UL14 | $\gamma_2$, non-essential |
| | | UL17 | $\gamma$, non-essential |
| | | UL37 | $\gamma_1$, essential |
| | | UL51 | $\gamma_1$, non-essential |
| **Viron Envelope** | Glycoprotein B | UL27 | $\gamma_1$, essential |
| | Glycoprotein H, VP22 | UL22 | $\gamma$, essential |
| | Glycoprotein L | UL1 | $\gamma$, essential |
| | Glycoprotein M | UL10 | $\gamma_2$, non-essential |
| | Glycoprotein N | UL49.5 | $\gamma_2$, essential |
| **Other** | Cell-to-cell fusion | UL24 | $\gamma_1$, non-essential |

**Table 1.3:** Conserved genes in alpha-, beta- and gammaherpesviruses grouped by general function HSV homologue and kinetic gene class ($\alpha$, $\beta$ or $\gamma$ genes & whether essential or non-essential for replication *in vitro*). Adapted from Fields Virology (Pellett *et al.*, 2006).

Genes unique to α-herpesviruses include those encoding glycoprotein D, Viral Protein 16 (VP16) and regulatory genes related to the HSV homologues of ICP0 and ICP4. In α-herpesviruses the HSV homologue of the conserved immediate early gene α4 (encoding ICP4) plays a key role in the regulation of early lytic gene expression by interacting with transcription factors allowing it to function in multiple roles as a repressor of viral mRNA synthesis as well as a transactivator (Liu *et al.*, 2010; Pellett *et al.*, 2006). Liu and colleagues reviewed the complex regulatory mechanism in HSV-1 where the activation of the viral mRNA synthesis cascade in lytic infection is initiated by accumulation of the ICP0 immediate early protein and is negatively regulated by ICP4 (Liu *et al.*, 2010). Effective gene expression of all β and γ genes therefore relies on ICP4 but there is some recent evidence that viral micro RNA (miRNA) also plays a role in ICP4 regulation (Li *et al.*, 2010; Waidner *et al.*, 2011). Unique genes to β-herpesvirinae include a block of 14 genes as well as genes associated with US22 and highly divergent immediate early genes associated with gene regulation. Genes conserved in γ-herpesvirinae encode unique proteins necessary for maintaining latency in dividing host cells.

Three additional genes appear to be ancestral to the α-herpesvirus HSV-1 but have been lost in certain other viral lineages (Davison *et al.*, 2002; McGeoch *et al.*, 2006). The origin binding protein (OBP), encoded by the HSV-1 homologue of UL9, is associated with DNA replication machinery and is conserved only within *α-herpesvirinae* and the

*Roseolovirus* of *β-herpesvirinae* but not within the rest of the β- or γ-herpesviruses

(Inoue *et al.*, 1994). The HSV-1 homologues of UL23 (encoding thymidine kinase) and

UL40 (encoding the small subunit of ribonucleotide reductase) are conserved only in *α-*

*herpesvirinae* and *γ-herpesvirinae* (Davison *et al.*, 2002). Genes unique to a particular

species are often associated with latency and transformation, an example being the Meq

gene in the α-herpesvirus MDV (Brown *et al.*, 2009), or immune modulation of the host

cell, examples being the numerous unique genes in the γ-herpesvirus KSHV many of

which demonstrate homology to host cellular immunomodulators (Rezaee *et al.*, 2006).


Overall genome arrangement in the herpesviruses can be categorized into six classes

designated A to F (Pellett *et al.*, 2006) and illustrated in figure 1.4. Some of these

classes can exist in more than one isomeric configuration depending on the repeat

segments of the gene. Group A, exemplified by *Human Herpesvirus 6*, contains a left

and right terminal repeat sequence. Group B is exemplified by Herpesvirus Saimiri that

has variable numerous repeats of the terminal sequences at both ends. Group C,

exemplified by EBV, contains 4 internal repeat domains denoted R1-R4. Group D,

exemplified by VZV, contains both internal and terminal repeat regions flanked by a

small unique region. The short component of Group D can invert relative to the long

creating two isomers. Group E, exemplified by MDV, has a more complex arrangement

with terminal repeat sequences at both ends consisting of **n** copies of sequence **a**

(denoted $\mathbf{a_n}$) followed by sequence **b** at one termini and the directly repeated sequence **a**

followed by sequence **c** at the opposite termini. The unique long ($U_L$) and unique short

($U_S$) regions of group E are also separated by inverted terminal repeat sequences $a_n'$, **b'**

and **c'** (inverted sequences denoted by primes), and thus exist as 4 equimolar isomers

due to both the long and short components being able to invert. Group F, exemplified

by the unassigned β-herpesvirus *Tupaia Herpesvirus 1* (Bahr and Darai, 2001), contains

non-identical terminal sequences that are not repeated either directly or inversely. These

genome arrangement classifications exist in addition to the subfamily classifications of

α, β and γ-herpesviruses and are not exclusive to any one subfamily.
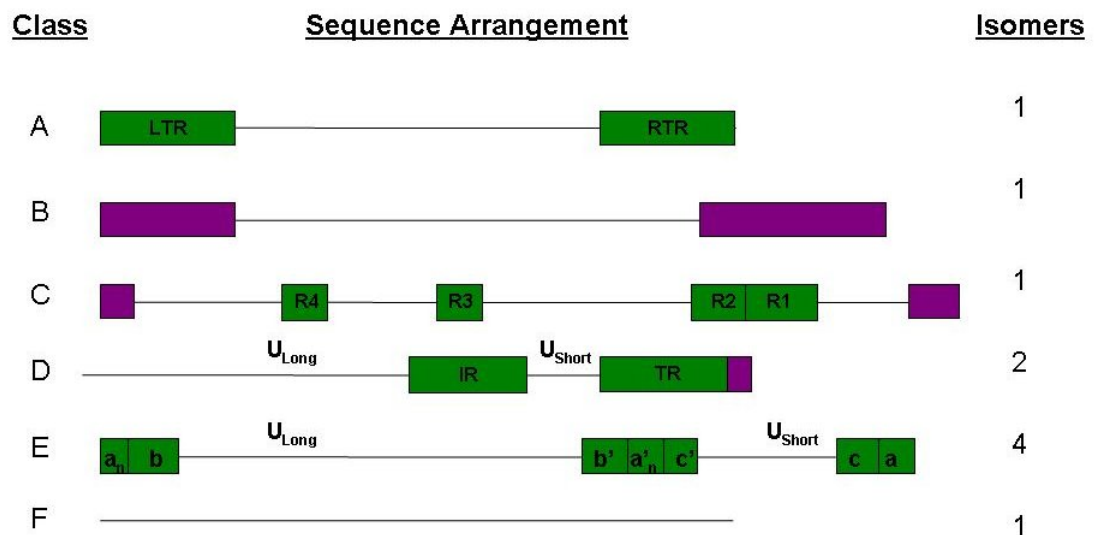


**Figure 1.4:** Schematic diagram depicting genomic arrangement of the six different classes of Herpesviridae adapted from Fields Virology (Pellett *et al.*, 2006). Unique or quasiunique regions are shown in **purple** and reiterated regions are shown in **green**. (LTR denotes Left Terminal Repeat, RTR denotes Right Terminal Repeat, R1-R4 denotes internal repeat domains, IR denotes internal repeats and TR denoted terminal repeats, $U_L$ denotes unique long regions and $U_S$ denotes unique short regions).

Functions of individual herpesvirus genes can also be classified according to their kinetic gene class designating the order of their sequential expression during the productive or lytic infection cascade (Pellett *et al.*, 2006) which will be discussed further in the following sections. Immediate-early genes ($\alpha$) are expressed first in the absence of viral protein synthesis and are followed by early genes ($\beta$). Lastly the $\gamma$ genes are expressed which can be sub-categorized into intermediate-late genes ($\gamma_1$) and true late genes ($\gamma_2$). It is important to note that these kinetic gene classes are not related to the subfamily designations of the same name. Additionally genes can be classified as essential or non-essential to viral replication *in vitro*.

### 1.1.4    Herpesvirus Life Cycle

The typical herpesvirus life cycle begins with viral entry into the host cell.
Complementary receptors on the viral envelope and the target host cell membrane
interact resulting in fusion allowing the viral capsid and tegument proteins to enter the
host cell cytoplasm.  Viral entry can occur either via fusion of the plasma membrane or
by endocytosis.  This section will focus mainly on the α-herpesviruses as it is beyond
the scope of this thesis to give a detailed review of the cellular mechanisms operating
during the life cycle for all herpesviruses.  For a general review of the herpesvirus life
cycle see Pellet *et al.* (2006).

In the α-herpesvirus HSV five viral glycoproteins (gB, gC, gD, gH and gL) have been
shown to be involved with viral entry through membrane fusion (reviewed in Akhtar and
Shukla, 2009).  Some viral entry can also occur by endocytosis through an alternative
mechanism (Akhtar and Shukla, 2009).   For membrane fusion gC is considered non-
essential for viral entry although it has been shown to enhance the efficiency of virus
binding (Shukla and Spear, 2001).  Initially, viral gB and gC bind with cellular heparin
sulphate proteoglycans bringing the two membranes into close apposition.  In HSV gD
then binds to one of its cellular receptors, such as nectin-1 and -2 and herpesvirus entry
mediator (HVEM), and undergoes a conformational change allowing it to form a multi-
protein fusion complex with gB, gD, gH and gL (Subramanian and Geraghty, 2007).
Membrane fusion is then initiated and the nucleocapsid and tegument proteins enter the
host cell cytoplasm.  The gD cellular receptors bound vary according to host cell type

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(Spear, 2004). The cellular entry mechanism dependent on gD binding is unique to the α-herpesviruses as viral entry occurs by other mechanisms such as pH dependent conformational changes of fusion proteins in other herpesviruses (Stampfer *et al.*, 2010). Considerable research has been done on the more detailed mechanisms of viral entry which are beyond the scope of this thesis, but for a review see (Akhtar and Shukla, 2009).

Once the tegument proteins and nuclear capsid have gained entry into the host cell cytoplasm the capsid containing viral DNA is transported to the nucleus where it is uncoated in order to initiate viral DNA synthesis. Most herpesviruses encode strong enhancers in their DNA which promote the transcription of the early viral genes (Pellett *et al.*, 2006). The α-herpesviruses are the exception and it is the virion tegument which contains essential transactivating proteins, one of the most important being the homologue of HSV VP16 encoded by ORF UL48, which promote the expression of immediate early viral genes.

VP16 interacts with the host cell proteins POU domain protein Oct-1 (Oct-1) and transcriptional co-activator herpes simplex virus-associated host cell factor 1 (HCF-1) to form the VP16 induced complex (VIC) which binds to the TAATGARAT (R = purine) sequence present in homologues of the HSV immediate early gene promoters (Preston *et al.*, 1988; Thompson *et al.*, 2009; Wilson *et al.*, 1997). HCF-1 is a conserved and complex cellular transcriptional co-regulator which interacts with numerous

transcription factor molecules to function as a bridge between transcription factors and chromatin modulation machinery (Mangone *et al.*, 2010; Narayanan *et al.*, 2005).  In HSV infection HCF-1 has been shown to be essential for mediation of immediate early viral gene transcription and has been associated with the cellular histone chaperone Asf1b, a protein regulating the progression of cellular DNA replication forks via chromatin reorganization (Narayanan *et al.*, 2005; Peng *et al.*, 2010).  The coupling of Asf1b to HCF-1 has been shown to promote viral DNA replication as well as its well-described functions as a transactivator (Peng *et al.*, 2010).  Interaction of the trans-activating cellular protein Oct-1 with VP16 and has been shown to be key to viral replication at low but not high multiplicities of viral infection (Kristie and Sharp, 1990; Nogueira *et al.*, 2004).  The HSV VP16 protein has been well characterised and a considerable amount of detail is known on its function and mechanism but is beyond the scope of this thesis.  For more detailed reviews see (Pellett *et al.*, 2006; Thompson *et al.*, 2009).

The viral capsid, containing condensed linear double-stranded DNA, is quickly transported from the cytoplasm to the host cell nuclear membrane via the microtubule and actin cytoskeleton (Dohner and Sodeik, 2005).  It is important to note that in α-herpesviruses transportation of the viral DNA occurs simultaneously with the tegument transactivating proteins, such as VP16, interacting with the host cellular receptors (Pellett *et al.*, 2006).  When the capsid reaches the nuclear membrane it docks with a nuclear pore and the virus is uncoated into the nucleoplasm in a polarized manner

leaving the empty capsid shell outside the nuclear membrane (Miyamoto and Morgan, 1971; Newcomb *et al.*, 2009).  In α-herpesviruses this polarization means that the HSV homologue of the α4 gene, which encodes the ICP4 protein, enters the nucleoplasm first (Newcomb *et al.*, 2009).  In HSV-1 the cleavage portal protein UL6 has been found to correlate with viral DNA release from the capsid suggesting its involvement in allowing the capsid to uncoat the viral genome (Newcomb and Brown, 2007).

Once the viral genome DNA has gained entry into the nucleus, it circularizes and initiates viral protein synthesis resulting in the remodelling of the host cell nucleus that enhances viral replication and blocks the host cell immune response.  Immediate early genes, such as the genes encoding ICP4, ICP22, ICP27 and ICP0, which contain promoters responsive to VP-16 are initially induced and expression occurs shortly after viral entry (apRhys *et al.*, 1989).  ICP4 is a moderate transactivator of viral mRNA synthesis on its own, but when it interacts with ICP0 it becomes a potent transactivator of viral mRNA synthesis (Liu *et al.*, 2010).  This interaction of ICP4 with ICP0 as well as several other genes is key to overcoming intrinsic cellular resistance to viral infections and initiating viral mRNA replication (Gu and Roizman, 2007; Hagglund and Roizman, 2004; Hancock *et al.*, 2010).  Kalamvoki and Roizman showed that ICP0 enhanced viral DNA synthesis and gene expression by playing a key role in the disruption of  the compartmentalization of the host cell nucleus which is a key mechanism for intrinsic cellular defence against viral infections  (Kalamvoki and Roizman, 2010).  The complex mechanism for compartmentalization disruption

involves the recruitment of cellular cyclin D3 by ICP0 to cellular nuclear domain10

(ND-10) structures, such as ATRX and hDaxx which are vital to maintaining

compartmentalization of cells (Kalamvoki and Roizman, 2010; Lukashchuk and Everett,

2010). The binding of ND-10 structures by cellular cyclin D3 activates cyclin

dependent kinase 4 which has been associated with reactivation of latent HSV (Schang

*et al.*, 2002). After nuclear remodelling has been completed gene expression is then able

to proceed with all viral gene transcription carried out by host RNA Polymerase II

(Costanzo *et al.*, 1977).


Once initiated, the gene expression sequence during a typical herpesvirus cytolytic

infection is tightly regulated according to kinetic gene class with immediate early ($\alpha$),

early ($\beta$), intermediate ($\gamma_1$) or late ($\gamma_2$) genes expressed in a well-ordered cascade

illustrated in figure 1.5. In $\alpha$-herpesviruses immediate early proteins such as ICP4

encoded by $\alpha$ genes are expressed under the control of pre-synthesized tegument viral

protein transactivators. Immediate early proteins in turn promote expression of early

proteins encoded by $\beta$ genes such as UL29 encoding ICP8. The immediate early protein

ICP0 contains multiple domains and plays its role in regulating the switch between $\alpha$

to $\beta$ gene transcription by binding in the G/C rich C-terminal domain resulting in

dissociating histone deactylase from the CoREST/REST repressor complex  (Gu and

Roizman, 2007). ICP4 mediates viral activation and suppression by forming a

transcriptional complex on the  promoters of various genes allowing the sequential

expression of genes (Sampath and Deluca, 2008).  Expression of the $\gamma_1$ and $\gamma_2$ genes,

encoding proteins such as ICP5, gB, gC and gD, are expressed after the $\beta$ genes and are

enhanced by viral protein synthesis.  A more detailed review of late gene expression can

be found in (Boehmer and Nimonkar, 2003; Pellett *et al.*, 2006).

Assembly of the viral particle takes place following viral protein synthesis  where the

nucleocapsid containing double-stranded DNA is assembled within the nucleus and is

transported to the cytoplasm by primary envelopment at the inner nuclear membrane and

de-envelopment at the outer nuclear membrane (Kelly *et al.*, 2009).  The inner tegument

is added in the cytoplasm and the viral particle is transported via kinesin-dependent

microtubule transport to the Golgi apparatus where the outer tegument is added and

secondary envelopment takes place.  The enveloped virus is then transported to the cell

membrane and released by exocytosis.

Latency occurs when the virus enters a non-replicating suspended state with limited

gene expression at the beginning of the life cycle as illustrated in figure 1.5.  The latency

associated transcript (LAT), which overlaps the $\alpha 0$ gene but is transcribed in the

opposite direction and encodes several spliced RNA species but no proteins, was

originally regarded as the only viral gene expressed during HSV latency (Pellett *et al.*,

2006).  miRNAs have since been described mapping to the LAT transcripts in both

HSV-1 and HSV-2 which silence ICP0 expression (Tang *et al.*, 2009; Umbach *et al.*,

2008; Umbach *et al.*, 2009; Umbach *et al.*, 2010).  Tang and colleagues also

demonstrated the negative regulation of the LAT encoded miRNAs by the ICP4 viral transactivator (Tang *et al.*, 2009). These findings all suggest that miRNA plays a key role in the regulation of HSV latency and will be explored further in the next section.
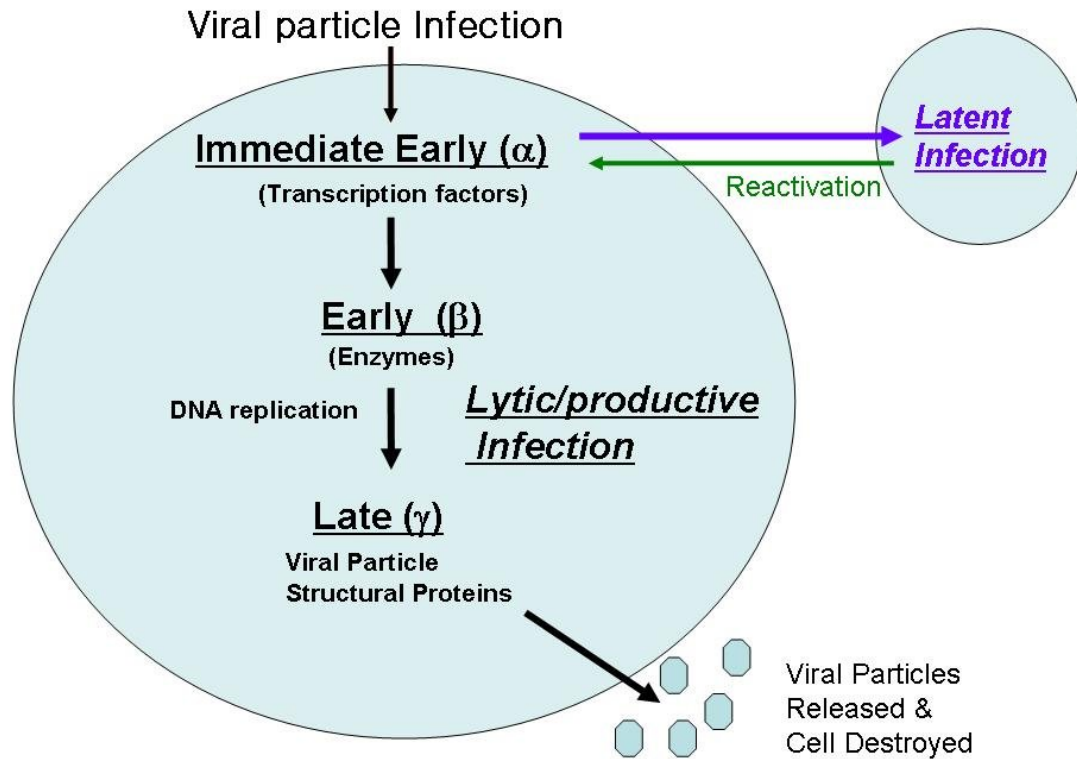


**Figure 1.5:** Schematic diagram showing a simplified herpesvirus lytic and latent life cycle. Adapted from a diagram kindly provided by Dr. Robert Dalziel, The Roslin Institute, University of Edinburgh, UK

Latency can revert to lytic infection when triggered by stress in the host which is influenced by modulation of the host immune system (Pellett *et al.*, 2006). Simplified versions of herpesvirus infection stipulate multi-cellular organisms rigidly exist in one of three states: true latency in which all the infected cells are latent, partial latency in which certain infected cells revert to lytic infection producing viral particles either

asymptomatically or symptomatically in the host organism, or true lytic infection where all infected cells are actively producing viral particles and there is shedding of infectious virus (Pellett *et al.*, 2006). It is becoming increasingly apparent that latent infection exists in a much more complex and dynamic state than previously thought mediated principally by host γ-interferon expression (Carr *et al.*, 2009; Decman *et al.*, 2005). Decman and colleagues demonstrated that γ-interferon expressed by CD8 T cells can block HSV-1 reactivation from latency and inhibit ICP0 and gC promoter activity suggesting a balance between latency and productive infection controlled by the host immune system (Decman *et al.*, 2005). This model is consistent with the clinical features of herpesvirus reactivation following immunocompromise of the host that would cause decreased levels of γ-interferon hence allowing reactivation.

### 1.1.5 Herpesvirus MicroRNAs

The discovery of integrated developmental genes for non-coding RNA also known as miRNAs in the soil nematode *C. elegans* and the pathway for its processing in the cell (Lee *et al.*, 1993) led to the discovery of RNA interference as a key regulatory mechanism in virtually all metazoan organisms. Cellular miRNAs play a vital role in post-transcriptional gene expression and have been found in a huge array of tissues and species encompassing almost every somatic cell in every metazoan eukaryote examined as well as viruses most notably *herpesviridae* (Pfeffer *et al.*, 2004; Sullivan and Ganem, 2005). Since their initial discovery the list of known miRNAs continues to expand with the current version of the miRbase database (version 16) (http://microrna.sanger.ac.uk/ accessed 28/12/10) containing 15,172 miRNAs, 235 of which are viral miRNAs (Griffiths-Jones *et al.*, 2008). Herpesviruses account for the vast majority (over 97%) of the viral miRNAs described to date. Sequence analysis has shown a high degree of miRNA sequence conservation within viral species but almost no conservation between viral species (Walz *et al.*, 2010).

miRNA precursors can be over 3,000 nucleotides long and are transcribed by RNA polymerase II in the nucleus as capped and polyadenylated long primary transcripts which are both temporally and spatially regulated (Cai *et al.*, 2004). These are processed by the nuclear enzyme Drosha into short hairpin miRNA precursors of ~70 nucleotides. These pre-miRNAs are transported by Exportin-5 from the nucleus where they are processed by the endoribonuclease enzyme Dicer in the cytoplasm to yield

transient ~22 nucleotide RNA duplexes from which the single strands are preferentially

loaded into the RNA-induced silencing complex (RISC). The short RNA strands are the

mature miRNA complex which bind to the 3' untranslated regions of mRNA (Lee *et al.*,

1993). When there is exact pairing between the miRNA and the corresponding mRNA,

then transcript degradation occurs and there is translational gene silencing. When there

is imperfect pairing, then there is translational interference of the specific gene without

transcript degradation (Heidersbach *et al.*, 2006). Certain miRNAs have also been

shown to up-regulate translation by binding with other 3' untranslated region binding

complexes (Lee *et al.*, 2010a; Vasudevan *et al.*, 2007).

The discovery of viral sequences encoding five miRNA in the γ–herpesvirus EBV

infected Burkett's Lymphoma cells (Pfeffer *et al.*, 2004) precipitated the discovery that

viral miRNA could modulate the expression of host genes. Since this initial discovery

over 220 herpesvirus miRNAs have been described and miRNAs have been found in

every herpesvirus examined with the exception of VZV (Boss *et al.*, 2009; Umbach *et

al.*, 2009). The α and γ-herpesviruses miRNAs described to date have been located in

blocks whereas the β-herpesvirus miRNA appears to be more diffuse and associated

with individual genes (Boss *et al.*, 2009). Viral miRNAs are conserved in their position

but not in their sequence, and homology between viral miRNAs exists only in isolated

cases (Walz *et al.*, 2010). A review by Grey and colleagues (2008) outlined four general

mechanisms by which miRNA could affect viral replication: firstly by viral miRNA

post-transcriptional targeting of viral mRNA expression, secondly by viral miRNA

post-transcriptional targeting of cellular mRNA expression, thirdly by host or virus miRNA direct cleavage of viral mRNA or fourthly viral miRNA direct cleavage of cellular mRNA (Grey *et al.*, 2008). Numerous examples of the first two mechanisms have been documented (Galardi *et al.*, 2007; Tang *et al.*, 2008; Tang *et al.*, 2009; Umbach *et al.*, 2008; Yao *et al.*, 2009a), but to date there have been no examples of direct cleavage of viral or cellular mRNA by viral miRNA. The biology of herpesviruses facilitates viral miRNA as an effective method for post-transcriptional gene targeting which could account for the vast majority of viral miRNAs identified to date being herpesviruses. Nuclear expression in the host cell of the herpesvirus genome and its associated miRNAs aids the targeting of mRNA as cleavage of the primary miRNA transcript by the nuclear enzyme Drosha into its active form and mRNA synthesis both take place in the nucleus allowing them to interact (Cullen, 2006).

Among the α-herpesviruses many of the miRNAs in HSV1 and HSV2 have been mapped to a region of latency-associated non-coding RNA transcripts (LAT) (Boss *et al.*, 2009). In HSV-1 these LAT miRNAs overlap in an antisense fashion with the lytic genes encoding ICP0, ICP4 and ICP34.5 (a viral neurovirulence factor) (Boss *et al.*, 2009; Liu *et al.*, 2010; Tang *et al.*, 2008; Tang *et al.*, 2009; Umbach *et al.*, 2008). In HSV-1 the LAT acts as a precursor miRNA and its processed miRNAs have been shown to down-regulate ICP0 and ICP4 expression (Umbach *et al.*, 2008). Five miRNAs have also been identified in HSV-2 mapping to the LAT located antisense to ICP0 (Umbach *et al.*, 2009; Umbach *et al.*, 2010). In both HSV-1 and HSV-2 miRNAs were also found

to reduce the expression of the key neurovirulence factor encoded by ICP34.5 and therefore may influence the outcome of viral infection in the peripheral nervous system, the site of latency (Tang *et al.*, 2008). Several miRNAs have also been described in MDV, HVT and GaHV3 which will be reviewed later. In summary, herpesvirus miRNA plays a crucial role in modulating latency and transactivation and appears to be conserved within species, but not between different genera suggesting miRNA is under strong evolutionary pressure to adapt to the host environment (Boss *et al.*, 2009; Gottwein and Cullen, 2008; Walz *et al.*, 2010).

## 1.2 Marek's Disease Virus (*Gallid Herpesvirus 2*)

Marek's Disease (MD) affects domestic poultry worldwide and is caused by the highly contagious cell-associated oncogenic α-herpesvirus *Gallid Herpesvirus 2*, which is also known as MDV. Chickens (*Gallus gallus*) are the main target species of MD, but it has been described in a milder form in other species such as quail, turkeys and geese (Nair *et al.*, 2008). MDV causes rapidly fatal and widespread lymphoproliferative tumours in susceptible chickens inflicting large losses on the global poultry industry every year, although with the widespread use of a vaccine since the 1970's the losses are no longer as substantial (Calnek and Witter, 1997; Witter and Schat, 2003). Estimates of the cost of MDV to the poultry industry have been as high as 2 billion US dollars per year (Calnek and Witter, 1997; Nair *et al.*, 2008).

### 1.2.1 History

MD was originally described clinically by Joseph Marek, a Hungarian veterinary pathologist, in 1907 as a polyneuritis in chickens (Marek, 1907). The disease did not bear his name, though, until almost 40 years after its description when it was differentiated from another clinically similar retroviral disease, Avian Leucosis (Davison and Nair, 2004). The causative organism of Marek's disease, an α-herpesvirus, was independently isolated in both the UK and USA (Churchill and Biggs, 1967; Nazerian *et al.*, 1968). Around this time MDV evolved from its original form which caused mild polyneuropathy to causing aggressive visceral lymphoid T cell tumours with associated high mortality (Witter, 1997). Despite the successful introduction of an attenuated vaccine based on the virulent HPRS-16 strain in 1969 (Churchill *et al.*, 1969) MDV continued to evolve and multiple strains were identified which could overcome vaccine protection (Davison and Nair, 2004). Several alternate vaccines were developed, including the highly effective CVI988 Rispens vaccine strain of MDV (Rispens *et al.*, 1972a; Rispens *et al.*, 1972b), but these vaccines were not effective against all virulent MDV strains. This development led to increased interest and research on new MDV control methods and vaccines that will be discussed later in the chapter.

### 1.2.2 Taxonomy & Strain Variation

The Marek's Disease-like viruses are included in the Mardivirus genus that only infects avian hosts (Table 1.6). *Gallid Herpesvirus 2* or MDV contains all virulent strains as well as some attenuated strains such as CVI988 Rispens. *Gallid Herpesvirus 3* and

*Melagrid Herpesvirus 1* are both mildly pathogenic and elicit antigenic cross-reactivity to *Gallid Herpesvirus 2* which has been exploited in vaccines. The widely used vaccine strain Herpesvirus of Turkeys 3 (HVT-3) is a strain of *Melagrid Herpesvirus 1* (Davison and Nair, 2004). MDV has been further classified according to strain pathogenicity into the categories: mild (m), acute (v), very virulent (vv), and very virulent plus (vv+), each representing a substantial increase in pathogenicity measured by the severity of symptoms and the ability of the virus to cross the blood brain barrier during lytic infection (Osterrieder *et al.*, 2006; Witter, 1997; Witter *et al.*, 2005).

Some studies refer to an alternate system of classification with *Gallid Herpesvirus 2* as MDV type 1, *Gallid Herpesvirus 3* as MDV type 2 and *Melagrid Herpesvirus 1* as Herpesvirus of Turkeys (Table 1.6). These different classifications can cause confusion as MDV type 2 and *Gallid Herpesvirus 2* have similar names but denote two different viruses. For the purpose of this study *Gallid herpesvirus 2* or MDV type 1 will be designated as MDV, *Gallid herpesvirus 3* will be designated as GaHV3 and *Melagrid herpesvirus 1* will be designated as HVT.

| Genus Mardivirus (Marek's disease-like viruses) | Alternate Nomenclature | Comments & Selected Species |
|---|---|---|
| **Columbid Herpesvirus 1** | Pigeon Herpesvirus | |
| **Gallid Herpesvirus 2** | Marek's disease virus Type 1 | Most Pathogenic strains, but also includes apathogenic CVI988 Rispens vaccine strain |
| **Gallid Herpesvirus 3** | Marek's disease virus Type 2 | Low pathogenicity or attenuated strains |
| **Melagrid Herpesvirus 1** | Turkey Herpesvirus | Low Pathogenicity, includes HVT3 vaccine strain |

**Table 1.6:** Taxonomic structure of the Mardivirus genus showing the alternate nomenclature equivalents and example strains (Davison *et al.*, 2009).

### 1.2.3 Genome Structure and Gene Function

The MDV genome consists of approximately 180,000 base pairs (bp) with a 44% G/C composition encoding over 103 functional genes (Tulman *et al.*, 2000). The genomes of several strains of MDV have been completely sequenced (Kingham *et al.*, 2001; Spatz *et al.*, 2007a; Spatz *et al.*, 2007b; Spatz and Rue, 2008; Tulman *et al.*, 2000) and with the increased availability of high throughput sequencing the genome of many more strains should become available in the future.

The overall genome structure of MDV can be categorized as class E and consists of a Unique Long ($U_L$) and Unique Short ($U_S$) region flanked by respective inverted repeat sequences designated Internal/Terminal Repeat Short ($IR_S$/$TR_S$) and Internal/Terminal Repeat Long ($IR_L$/$TR_L$) (Roizman and Sears, 1996; Spatz and Silva, 2007a). Most of the genes specific to MDV are located in the $TR_L$ and $IR_L$ regions of the genome. The $U_L$ and $U_S$ regions of the genome are generally conserved in arrangement but not sequence with other $\alpha$-herpesviruses (Davison and Nair, 2004; Li *et al.*, 1994). The MDV

genome arrangement is very similar to HSV-1 and -2 and contains an origin of lytic

replication (Ori$_{lyt}$) located in the IR$_L$ and TR$_L$ flanking the U$_L$ region between the

phosphoprotein 38 (pp38 or ORF 73.0) and phosphoprotein 14 (pp14 or ORF 75.0)

genes (Camp *et al.*, 1991; Parcells *et al.*, 2003). The genes adjacent to the Ori$_{lyt}$ can be

grouped into those associated with latency and transformation (Meq, IL8 & pp14) and

those associated with early lytic infection (pp38, pp24 & *Bam*HI-H encoded protein)

which are expressed in an antisense fashion respective to each other (Parcells *et al.*,

2003). Pathogenic MDV contains a number of unique genes many of which are not well

characterized. Of the unique genes studied, some of the most notable are the Meq and

IL-8 genes which have both been associated with oncogenesis (Kingham *et al.*, 2001).

Genes associated with lytic replication or latency are usually expressed separately, with

the exception of 23kDa, Meq and RLORF5 which Heidari and colleagues found to be

expressed in both lytic and latent infections (Heidari *et al.*, 2008; Parcells *et al.*, 2003).


To date 14 unique viral miRNAs have been found in MDV located in the regions

flanking the Meq oncogene and within the LAT (Burnside *et al.*, 2006; Burnside *et al.*,

2008; Griffiths-Jones *et al.*, 2008; Morgan *et al.*, 2008; Yao *et al.*, 2009b). Eighteen

miRNAs in two clusters have been identified in apathogenic GaHV3 and seventeen

miRNAs in HVT but none of these appear to be conserved with MDV (Griffiths-Jones *et
al.*, 2008; Yao *et al.*, 2009b; Yao *et al.*, 2007). MDV miRNAs appear highly conserved

between different strains of the virus despite the identification of sequence variations in

the Meq gene between different MDV strains (Morgan *et al.*, 2008). One of the MDV

viral miRNAs described in the region of the Meq gene was mdv1-miR-M4 which has homology with the chicken miRNAs miR-155, a cellular miRNA playing a role in the function of the immune system (Morgan *et al.*, 2008; Yao *et al.*, 2007). Yao and colleagues demonstrated a decrease of miR-155 coupled with an increase of MDV-encoded miRNAs in the MDV-transformed cell line MSB-1 compared to normal lymphocytes which supported evidence for modulation of the host immune response by mdv1-miR-M4 viral miRNA (Yao *et al.*, 2009a). In summary, miRNAs in MDV appear to play an important role in the control of latency and pathogenesis through several mechanisms but further research is needed to characterise their function.

### 1.2.4 Life Cycle & Biology

Electron microscopy of MDV reveals a typical herpesvirus particle consisting of a 95-100 mµ capsid with 162 capsomeres and a 150-170 mµ enveloped viral particle (Nazerian and Burmester, 1968). The DNA in MDV is wound around a central torus or spool shape connecting the two inner poles of the capsid, similar to that described for other herpesviruses (Nazerian, 1974). MDV viral particles are very robust and have been documented to survive for up to a year in many environments (Calnek and Witter, 1997), making decontamination of an infected poultry house very difficult.

The life cycle of MDV in the chicken, (Davison and Nair, 2004), starts with an infected bird shedding mature viral particles from its feather follicle epithelium which mixes with feather dander to create an aerosol of dust and viral particles contaminating the

surrounding area.  The infectious viral particles are inhaled by uninfected susceptible

birds and infection occurs when the virus enters pulmonary B cells and to a lesser extent

macrophages in the air sacs (Baaten *et al.*, 2009).  These cells migrate via the lymphatic

system to the lymph nodes and bursa of Fabricius where the virus enters a short cytolytic

phase causing inflammation and lymphocytolysis and viral particles can be detected in

the lymphatic tissue (Baigent *et al*., 2005b).  A primary immunosuppression due to the

depletion of lymphocytes has been documented at this point in the life cycle (Calnek *et

al*., 1984).  Initial cytolytic infection occurs in B cells causing inflammation in the

lymphoid tissue, particularly in the bursa of Fabricius, from 7 days onward and induces

the activation and subsequent infection of CD4 T cells where the virus establishes a

latent infection (Davison and Nair, 2004).  Some CD8 T cells also become infected

during the initial lytic replication, but CD4 cells prevail as the predominant virus-

infected cells during the subsequent latent infection (Islam *et al.*, 2002; Morimura *et al.*,

1996).  After the initial 6 to 7 day cytolytic phase the virus switches to latency primarily

in CD4 T cells with limited expression of viral or tumour antigens even though the viral

genome persists.  The latently infected T cells are able to migrate to the visceral organs

and peripheral nerves throughout the body via the bloodstream.  From 14 to 21 days

post-infection the latently infected T cells in the viscera and nerves can undergo

neoplastic transformation and can proliferate to form gross lymphomas in susceptible

birds.  These lesions result in the symptoms and eventual mortality of MD.

A second immunosuppression is a significant feature of MDV infection and occurs in

late cytolytic infection due to lymphocyte depletion from cytolysis of thymocytes

anywhere from day 3 to 35 post infection (Islam *et al.*, 2002). This immunosuppression is most profound in very virulent MDV strains, but has been documented to some extent in birds vaccinated with apathogenic HVT-3 (Islam *et al.*, 2002). From 10 days post-infection onward, MDV undergoes productive replication in the feather follicle epithelium that results in high levels of viral antigen and infectious viral particles. These particles are shed in feather dust throughout the life of an infected bird which continues the viral life cycle. This life cycle is summarized in figure 1.7.

Clinical manifestations of MD can be classified into three major areas: lymphomas of visceral organs, neurological signs and immune suppression. Mortality resulting from widespread lymphomas can occur from 2 weeks post-infection in aggressive MDV strains. In the low pathogenicity strains a polyneuritis, particularly in the sciatic nerves, has been noted with the associated ascending paralysis, but mortality is generally low. In the most pathogenic strains aggressive lymphoid tumours, eye tumours, arthrosclerosis and profound immunosuppression have all been documented (Davison and Nair, 2004; Witter, 1997; Witter and Schat, 2003). In addition to the mortality caused by MDV-induced lymphomas it is apparent that the immune suppression caused by subclinical MDV also impacts infected birds by causing increased susceptibility to other infections such as infectious bursal disease, *E.coli* and the response to vaccine administration for other conditions (Islam *et al.*, 2002). In summary, the complex life cycle of MDV induces cell-mediated immunity in the host that can lead to mortality and subclinical immune suppression.
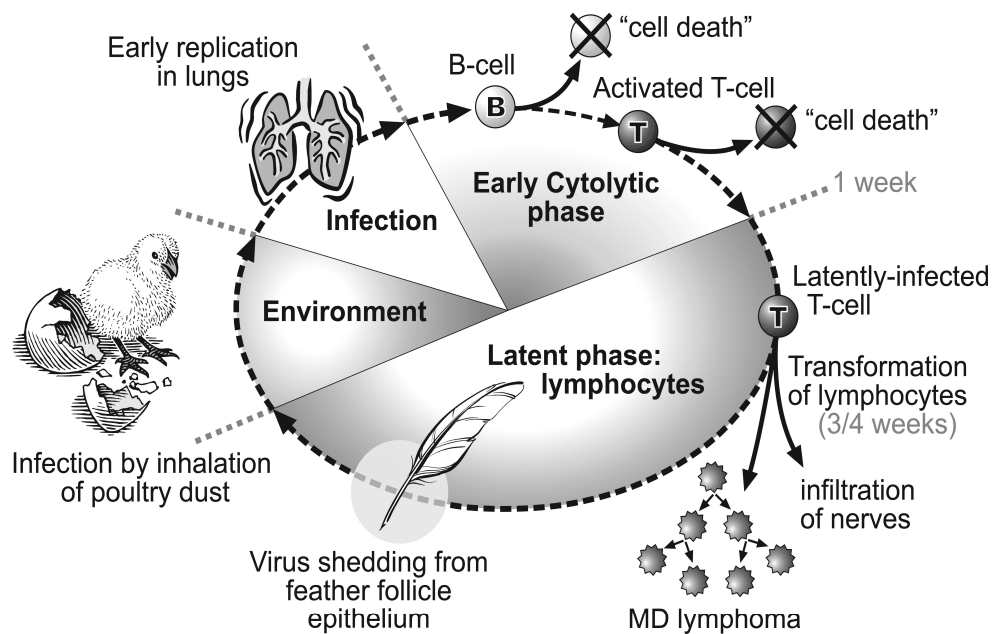
**Figure 1.7:** Life cycle of Marek's disease in chickens. Diagram kindly provided by Dr. Venugopal Nair.

### 1.2.5 MDV Tumourigenesis

The rapid formation of aggressive T-cell tumours during MDV lytic infection is one of the hallmarks of the disease and is directly linked to mortality and virulence factors. Several genes and their encoded proteins as well as both viral and cellular miRNAs have been associated with tumourigenesis in MDV. Uncharacterized genes may also play a role in tumourigenesis.

The RLORF7 gene encodes the polymorphic 339 aa Marek's EcoRI Q fragment protein, hence its designation as Meq. The MDV genome contains two copies of Meq transcribed R to L in the TRL and L to R in the IRL regions flanking the unique long region of the genome (Spatz *et al.*, 2007b). Meq overlaps the RLORF6 gene in the same orientation and 23kDa as well as genes encoding two hypothetical proteins MDV ORF 5.2 and 5.3 in the opposite orientation as illustrated in figure 1.8, hence the designation "Meq loci" describing this cluster of genes (Spatz *et al.*, 2007b). In addition to wild-type Meq several variants, differing in their biological and physical properties, have been described to date: L-Meq (containing a 60 aa insertion between residues 190 and 191 mainly in attenuated and low pathogenicity strains), S-Meq (containing a 41 aa deletion between residues 190 and 191), VS-Meq (containing a 92 aa deletion between residues 174 and 175), and ΔMeq (containing only 98 aa from the N-terminal region of Meq and a 30 aa frame-shifted distinct C-terminus) (Chang *et al.*, 2002a; Chang *et al.*, 2002b; Deng *et al.*, 2010; Okada *et al.*, 2007). Meq is considered unique to MDV as no

homologues have been found in any other species of herpesvirus (Kingham *et al.*, 2001;

Lupiani *et al.*, 2004; Petherbridge *et al.*, 2004).
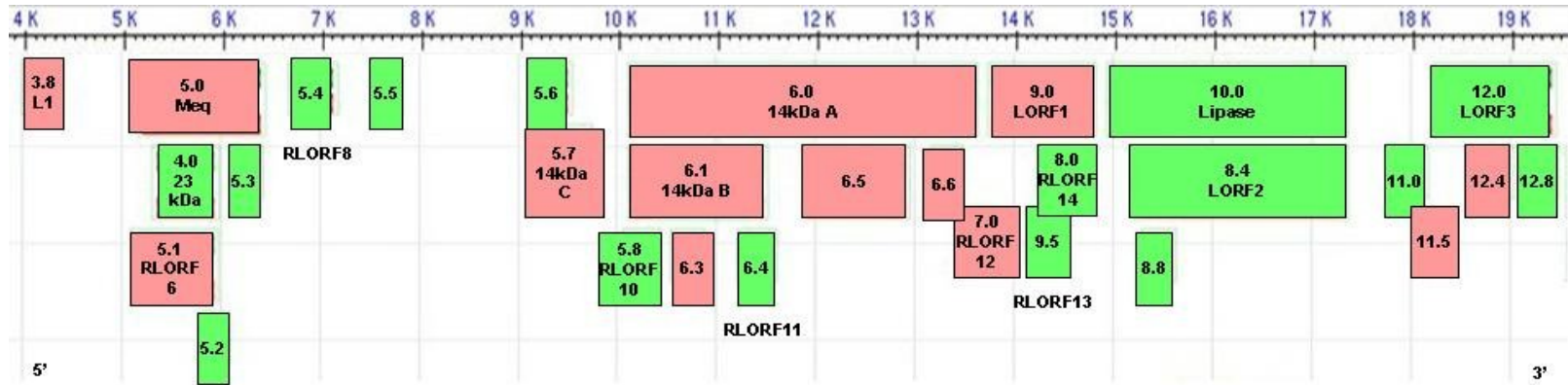
**Figure 1.8:** Diagram of the relative location of the potential target genes located in the IR$_L$ region of the MDV genome including the "Meq loci" illustrating the overlapping regions of the Meq, RLORF6 and 23kDa genes. Genes depicted in green are translated from L to R and genes depicted in red translated from R to L. Diagram adapted from the CVI988 Rispens MDV genome (Spatz *et al.*, 2007b) published in the GenBank (Benson *et al.*, 2008) graphic interface.

Meq has been well-characterised and is widely regarded as the major oncoprotein in MDV with high expression documented in both MDV induced T cell tumours and MDV transformed cell lines (Jones *et al.*, 1992; Osterrieder *et al.*, 2006). Studies utilizing siRNA techniques to analyze the transforming potential of Meq in DF-1 cells transfected with a Meq overexpression plasmid demonstrated that Meq alone could act on host cell targets to change growth rate and morphology even in the absence of intact MDV (Levy *et al.*, 2005). Meq has also been shown to maintain latency by blocking apoptosis in latently infected T cells and transactivating latent gene expression as well as being essential for tumour induction (Lupiani *et al.*, 2004; Parcells *et al.*, 2003). Studies demonstrating that the deletion of the Meq coding region in pathogenic MDV resulted in attenuation of the virus while preserving its antigenic properties as a vaccine also provided convincing evidence of the association between Meq and oncogenesis (Lee *et al.*, 2010b; Silva *et al.*, 2010). The structure of Meq contains three distinct regions: a DNA-binding domain, a basic region-leucine zipper (bZIP) domain with homology to the Jun/Fos family of transcriptional activators, and a proline-rich transactivation domain at the carboxy terminus (Anobile *et al.*, 2006; Jones *et al.*, 1992). As with other Jun/Fos proteins the bZIP domain of Meq can form homodimers with itself and heterodimers with other cellular proteins such as c-Jun and v-Jun which are transcribing factors for activating protein 1 complex (AP-1) playing a key role in regulating normal cellular events as well as neoplastic transformation (Hess *et al.*, 2004; Levy *et al.*, 2005). *In vivo* studies have shown that both homodimers and heterodimers of the Meq bZIP domain are required for transformation of lymphocytes (Suchodolski *et al.*, 2009).

Recombinant virus studies have also shown that homodimerisation of Meq is a requirement for MDV virulence (Brown *et al.*, 2009). Meq also has been shown to interact with other non-bZIP cellular proteins regulating the cell cycle such as p53 (a tumour suppressor), C-terminal binding protein-1 (a highly conserved cellular transcriptional co-repressor regulating cell development, proliferation and apoptosis) and heat shock protein 70 (a molecular chaperone protein associated with viral oncogenesis) (Brown *et al.*, 2006; Deng *et al.*, 2010; Zhao *et al.*, 2009). Meq was shown to bind directly with the p53 protein resulting in the inhibition of cellular p53-mediated transcriptional activity and apoptosis playing a key role in MDV transformation (Deng *et al.*, 2010). Tumours caused by very virulent strains of MDV were found to express high levels of viral miRNAs, particularly mdv1-miR-M4 associated with the Meq gene, despite equivocal levels of Meq transcription suggesting the association of viral miRNA with increased pathogenicity (Morgan *et al.*, 2008). Meq has been the subject of intensive research and considerably more detail has been described regarding its mechanisms which are beyond the scope of this thesis but for a recent review see (Deng *et al.*, 2010).

Subversion of cellular telomerase associated with control of the normal cell cycle by viral homologues has been proposed as a mechanism for oncogenesis in several viruses (Katzenellenbogen *et al.*, 2010; Kaufer *et al.*, 2010). MDV encodes two copies of a viral telomerase RNA subunit (vTR) with an 88% homology to the chicken telomerase RNA subunit (chTR) which is thought to play a role in tumourigenesis in MDV (Fragnet

*et al.*, 2003).  The TR complexes with a protein subunit with reverse transcriptase

activity (TERT) to form the telomerase ribonucleoprotein complex which compensates

for the progressive telomere shortening that occurs during mitosis and has been

implicated in immortalizing cells and tumourigenesis (Artandi, 2006).  More recent

studies have confirmed the findings of an earlier study (Lounis *et al.*, 2004) showing that

the promoter for vTR is up to 3-fold more efficient at binding TERT than the chTR

promoter implying that vTR may compete with chTR for binding of TERT which would

facilitate the efficient formation of tumours by MDV (Chbab *et al.*, 2010).


Manipulation of cellular miRNA by oncogenic herpesviruses has also been proposed as

a mechanism for tumourogenesis.  In a study by Burnside and colleagues deep

sequencing was used to compare miRNA sequences between MDV-infected and

uninfected chicken embryo fibroblasts (CEFs) which revealed a cluster on chicken

chromosome 1 expressing high levels of cellular miR-221 and miR-222 (Burnside *et al.*,

2008).  Galardi and colleagues also demonstrated increased levels of cellular miRNAs

miR-221 and miR-222 coupled with a decrease in the cyclin-dependent kinase inhibitor

of the cell cycle p27$^{kip1}$ *in vitro* in aggressive prostate carcinoma cells compared with

two slow-growing prostate carcinoma cell lines (Galardi *et al.*, 2007).  This down-

regulation of p27$^{kip1}$ coupled with increased expression of miR-221 and miR-222

increased proliferation in human prostate carcinoma cells (Galardi *et al.*, 2007), a

finding which was mirrored in the MDV transformed lymphoblastoid cell line MSB-1

(Lambeth *et al.*, 2009a) suggesting a similar cellular mechanism.  In separate studies Xu

and colleagues have also demonstrated the consistent down regulation of host cell

miRNA gga-MiR-26a in chicken lymphocytes transformed by 3 different avian

oncogenic viruses, including MDV, all with distinct transformation mechanisms

compared to normal lymphocytes (Xu *et al.*, 2010).  This study also went on to show

that miR-26a directly targets chicken interleukin 2 (IL-2) which plays a major role in T

cell development, differentiation and homeostasis by a complex and tightly regulated

mechanism (Xu *et al.*, 2010).  Disruption of IL-2 has previously been linked with

leukaemia, autoimmune disease and viral infection, including MDV (Gesbert *et al.*,

1998; Kaiser *et al.*, 2003).  Additionally miR-26a is down regulated in several human

cancers suggesting that its mechanism is not unique to MDV (Kota *et al.*, 2009; Visone

*et al.*, 2007).  These findings led to the conclusion that down-regulation of miR-26a in

MDV and other oncogenic avian viruses can cause an up-regulation of IL-2 leading to

increased T cell proliferation and contributing to tumourigenesis, but the specific viral

genes eliciting these changes were not identified (Xu *et al.*, 2010).

The above interactions all demonstrate that MDV is adept at exploiting complex existing

host cell miRNA mechanisms controlling cell proliferation and homeostasis to influence

the development and progression of tumourigenesis during its life cycle.

### 1.2.6  Diagnosis of MDV

Diagnosis of pathogenic MDV in the field can often be challenging due to the similarity

of the clinical signs of MDV with Lymphoid Leukosis.  There is also the ubiquitous

presence of attenuated vaccine strains of MDV in most commercial poultry environments that can complicate identification of pathogenic MDV strains. Several serological methods for identification of pathogenic MDV can be utilized in conjunction with gross pathology for diagnostic investigation of flock outbreaks. Classical or mild MDV infection presents clinically as an ascending paralysis and post-mortem examination reveals an oedematous enlargement of the peripheral nerves, often in conjunction with small grey lymphomas in the visceral organs (Nair *et al.*, 2008). Acute MDV infection can present clinically as sudden death or paralysis and on post-mortem examination the peripheral nerves are grossly enlarged in conjunction with extensive and diffuse lymphatomatous involvement of the visceral organs, and occasionally skin and eyes. Atrophy of the thymus (source of T cells) and bursa of Fabricius (source of B cells) are common post-mortem findings in acute MDV infection due to lymphocyte depletion (Nair *et al.*, 2008).

Laboratory confirmation of post-mortem findings is commonly undertaken using a Polymerase Chain Reaction (PCR) (Becker *et al.*, 1992; Nair *et al.*, 2008; Silva, 1992) or a Reverse Transcriptase quantitative Polymerase Chain Reaction (RTqPCR) (Abdul-Careem *et al.*, 2006; Baigent *et al.*, 2005a; Gimeno *et al.*, 2005; Islam *et al.*, 2006a; Islam *et al.*, 2004) both using primers homologous to unique regions of pathogenic MDV strains such as the Meq gene. Feather tips are most often used for MDV diagnosis and surveillance as they have a high viral load, are easy to collect, relatively stable and require no specialized equipment for sampling (Davison *et al.*, 1986b). A nested PCR

assay can also be used to differentiate between Meq (present in pathogenic MDV) and L-Meq (containing a ~180 bp insert in the CVI988 vaccine strain MDV) allowing a precise differentiation between pathogenic and vaccine strains of MDV (Murata *et al.*, 2007).   Other methods for MDV detection include agar gel precipitation (Chubb and Churchill, 1968) and enzyme-linked immunosorbent assay (ELISA) for MDV antigen (Davison *et al.*, 1986a; Scholten *et al.*, 1990) and antibody (Cheng *et al.*, 1984).  The amount of MDV DNA present in the blood of an infected animal has been found to directly correlate to the amount of gross lesions of MDV present at post-mortem and has been proposed as a method for early detection and monitoring of MDV infection (Gimeno *et al.*, 2008; Islam *et al.*, 2006b; Islam *et al.*, 2002)

### 1.2.7  Control of MDV – Vaccination & Viral Vectors

The first vaccine for MDV was developed in 1969 by attenuation by multiple passages in tissue culture of the virulent HPRS-16 strain and was subsequently put into widespread use (Churchill *et al.*, 1969).  The vaccine campaign was successful in preventing MDV induced lymphomas, but since the vaccine was a live replicating virus vaccinated birds became infected and shed MDV hence it did not produce sterilizing immunity (Davison and Nair, 2004).  Despite the success of vaccination, MDV continued to adapt into more virulent forms that were able to break through vaccine protection.  A major emergence of vaccine resistance and increased virulence of MDV has been documented approximately every 20 years since the 1960's (Nair, 2005; Osterrieder *et al.*, 2006; Witter, 1997).  Other live vaccines introduced to control

breakthrough MDV include the apathogenic SB-1 strain of GaHV3 (Schat and Calnek, 1978a; Schat and Calnek, 1978b), apathogenic strain 3 of HVT (HVT-3) (Witter *et al.*, 1970; Witter *et al.*, 1976) and the attenuated MDV strain CVI988 (Rispens *et al.*, 1972a; Rispens *et al.*, 1972b).  Several bivalent vaccines containing both an apathogenic GaHV3 (either strain SB-1 or 301B-1) plus HVT-3 were introduced to give enhanced protection as the two strains used together were found to be synergistic (Davison and Nair, 2004).  Marek's disease is currently controlled using a combination of vaccination and biosecurity measures in most commercial poultry flocks, but breakthrough cases continue to be reported.  Currently the most virulent strains of MDV are only responsive to the Rispens or CVI988 strain of vaccine (Davison and Nair, 2004).

All MDV vaccines in current use consist of whole-cell cultures containing live virus which are individually injected into each bird. Most commercial poultry are vaccinated either at day old or *in ovo* one to two days before hatching with a live whole cell MDV vaccine.  These vaccines have a limited shelf life and require a continuous -80ºC cold chain for storage from the manufacturer to end user and are stable at room temperature for less than one hour once reconstituted.  This poses inherent difficulties in delivering effective vaccine protection, particularly in hot climates or where the infrastructure may be less developed.  Vaccine misuse rather than failure most likely plays a role in many outbreaks, but it is not always immediately apparent when improper handling of the vaccine renders it ineffective.  If the current trend of increasing vaccine resistance and increased virulence continues then currently available vaccines may not be effective in

protecting from challenge by future strains of MDV, which would have significant economic and food security implications for the global poultry industry (Witter, 1997).

MDV vaccines, while protective to the bird, do not prevent viral replication and shedding.  The implication of this is that vaccinated birds can act as a reservoir of infection in densely populated poultry housing, particularly in the broiler sector, allowing MDV to develop increased virulence without killing the host during the relatively short growing period thus precipitating the emergence of increasingly virulent strains (Nair, 2005).  Gandon and colleagues proposed the hypothesis that a partially effective vaccine can exert high pressure for evolutionary adaptation towards virulence in a wild-type virus resulting in an immunized population able to harbour more virulent pathogens than a susceptible population (Gandon *et al*., 2001).  This hypothesis was subsequently proven in models for mouse malaria (Mackinnon and Read, 2004) and *Bordetella pertusis* (Van Boven *et al*., 2005).   It is tempting to speculate that this could be the case in MDV as well, but it is important to note that no study has proven that vaccination without sterilizing immunity coupled with intensive poultry husbandry fuels the shift towards increased MDV virulence.  Sterilizing immunity is defined as the protection from MDV infection and shedding as well as the symptoms of the disease.

The technique of incorporating the MDV viral genome into a bacterial artificial chromosome (BAC) has assisted the study of MDV *in vitro* by eliminating the necessity of directly working with infectious virus and facilitating manipulation of the viral

genome. To date most sequenced MDV strains have been developed into BACs (Petherbridge *et al.*, 2004; Petherbridge *et al.*, 2003; Spatz *et al.*, 2007a; Spatz *et al.*, 2007b). BAC construction entails cloning the BAC cassette, encoding the bacterial replication machinery, into the viral genome necessitating the disruption of a viral gene at the insertion site. In MDV the BAC insertion site is in the US2/US3 region of the genome that is destroyed (Petherbridge *et al.*, 2004). MDV BACs can produce lytic infections with the inserted bacterial replication genes intact, however a method was developed for self-excision of the bacterial replication genes and the subsequent restoration of the missing US2 gene to yield reconstituted virus (Zhao *et al.*, 2008). The reconstituted MDV virus produced infections that were greatly increased in pathogenicity and replication when compared to the BAC parent strain (Zhao *et al.*, 2008). This development is significant as BACs facilitate manipulation of the viral genome and making recombinant MDV strains, but the replication kinetics of the resulting virus *in vivo* were often poor. The reconstitution of intact virus from modified MDV BACs enables the production of engineered MDV viral vectors and vaccines as well as facilitating the archiving of viral strains without the risk of mutagenesis from repeated passages in tissue culture.

Using BAC techniques a vaccine coupled with a recombinant viral vector targeting MDV was developed based on reconstituted virus from a HVT-3 BAC containing an insertion expressing shRNA targeting the MDV viral genes gB which plays a key role in viral entry and UL29 which plays an essential role in viral DNA replication (Lambeth *et*

*al.*, 2009b). The recombinant vector acts not only to stimulate immunity against MDV with the HVT-3 vaccine in the conventional manner, but also protects through the shRNA insertions targeting gB and UL29 which would be predicted to reduce MDV infection rates and shedding. Therefore, it would seem that this virus could act both as a vaccine and as a viral vector. Early trials demonstrated protection from MDV challenge in vaccinated birds equal to HVT-3 alone coupled with some reduction of MDV viraemia in early infection compared with HVT-3 vaccinated birds (Lambeth *et al.*, 2009b).

Recombinant MDV vaccines have also been developed from pathogenic strains of MDV with the Meq protein coding sequence deleted using BAC techniques which have proved to be sufficient to attenuate the virus (Lee *et al.*, 2008; Silva *et al.*, 2010). Use of the Meq deletion recombinant viruses as vaccines showed they protected against virulent MDV challenge in susceptible chickens more effectively than the CVI988 Rispens vaccine strain, which has long been regarded as the most effective vaccine available (Lee *et al.*, 2010b; Silva *et al.*, 2010). Separate trials of a Meq deleted vaccine administered to day old chicks gave limited protection from a very virulent strain of MDV (rMD5) challenge at 5 and 12 days post-vaccination (Li *et al.*, 2010). These Meq deletion recombinant vaccines show potential over conventional MDV vaccines, but they still have the disadvantage of utilizing a live replicating virus vaccine. Viral vectors targeting MDV replication with siRNA (Lambeth *et al.*, 2009b) are a promising new development, but they also do not deliver sterilising immunity to MDV in their

present form due to their insertion in replicating HVT-3.  Without sterilizing immunity

to MDV it is likely the cycle of circulating virus in vaccinated birds will continue to fuel

the shift towards increased virulence.


### 1.2.8    Control of MDV - Resistance

Genetics undoubtedly play a role in the outcome of MDV infection and several naturally

occurring haplotypes have been associated with MDV resistance or susceptibility.  Of

the haplotypes identified to date none are capable of completely preventing MDV

infection, only of reducing the effects of MDV- induced mortality by varying degrees.

Resistance to MDV infection has been studied since the 1930's and has been identified

in several lines of chickens with distinct haplotypes (Bacon *et al.*, 2000; Bumstead and

Kaufman, 2004; Davison and Nair, 2004; Heifetz *et al.*, 2007).


Resistance to MDV is undoubtedly a polygenic trait but the chicken major

histocompatibility complex (MHC) locus has demonstrated the strongest single

association with MDV resistance or susceptibility.  The MDV resistance and

susceptibility observed in certain lines of chickens designated B19 and B21 appears to

be linked to 2 distinct haplotypes located in the 3'-untranslated region of the chicken

BG1 allele associated with MHC class I (MHC I) expression (Goto *et al.*, 2009;

Hepkema *et al.*, 1993; Schat *et al.*, 1994).  Comparison of the spleen proteome of the

MDV-resistant B21 chickens and the susceptible B19 chickens during MDV infection

undertaken by Thanthrige-Don and colleagues identified differences in protein

expression at several time points post-infection (Thanthrige-Don *et al.*, 2010).  Resistant

B21 chickens showed increased expression in early infection of two predicted products

of the peroxiredoxin-1 and peroxiredoxin-4 genes encoding components of natural killer

cell enhancing factor which functions as an antioxidant and is associated with the

regulation of apoptosis (Han *et al.*, 2005; Thanthrige-Don *et al.*, 2010).  Nitrous oxide

(NO) has been shown to increase the expression of peroxiredoxin-1 (Abbas *et al.*, 2008)

and high levels of NO have been found in B21 chicken tissues (Xing and Schat, 2000)

suggesting a possible mechanism for the observation of increased levels of

peroxiredoxins.  In the same study susceptible B19 chickens were found to upregulate

heat shock protein 90 (HSP90) by 4-fold compared to B21 chickens in early infection

(Thanthrige-Don *et al.*, 2010).  HSP90 has been associated with up-regulation of cell

surface expression of MHC I (Kunisawa and Shastri, 2006) as well as other immune

functions (Javid *et al.*, 2007; Srivastava, 2002).  In another study MHC I expression

levels were correlated directly with the MHC-linked MDV susceptibility in B19 and

B21 chickens.  Susceptible B19 chickens expressed high levels of MHC I and resistant

B21 chickens expressed low levels of  MHC I (Kaufman and Salomonsen, 1997).  Levy

and colleagues demonstrated *in vitro* that pathogenic MDV infection specifically down-

regulated MHC I in CEFs and this effect could be reversed with native chicken

interferon which increased MHC I expression (Heller *et al.*, 1997; Levy *et al.*, 2003).

Jarosinski and colleagues went on to show that in MDV infection there is a decrease of

cellular surface MHC I in response to the homologue of HSV UL49.5 which has also

been shown to cause an MHC I decrease in VZV (Jarosinski *et al.*, 2010).  One proposed

mechanism for B19 haplotype susceptibility is high levels of MHC I expression associated with HSP90 allowing for improved replication kinetics in the infecting virus (Davison and Nair, 2004).

Other genetic loci have also been implicated in MDV resistance and over 15 quantitative trait loci regions have been identified in lines of MDV resistant and susceptible chickens which may reveal more details of the mechanisms of MDV resistance when studied (Bumstead and Kaufman, 2004; Davison and Nair, 2004; Heifetz *et al.*, 2007; McElroy *et al.*, 2005). Baaten and colleagues demonstrated *in vivo* that line 6 MDV-resistant chickens, previously associated with decreased early viral replication (Bumstead and Kaufman, 2004), had delayed viral replication and earlier onset of γ-IFN production following respiratory MDV infection compared to susceptible line 7 chickens suggesting that early viral replication was important in determining the outcome of MDV infection (Baaten *et al.*, 2009). Line 6 and 7 chickens have the same BG1 allele haplotypes, unlike lines B19 and B21, suggesting that the differences in MDV resistance are due to a different allele (Davison and Nair, 2004). Although the mechanisms of the naturally occurring MDV resistance yield valuable insights into MDV pathogenesis, the existing lines of MDV-resistant chickens do not appear to offer sufficient protection against lymphoma-induced mortality at present to justify stopping vaccination. With the increasing understanding of the mechanisms of MDV infection and pathogenesis it may be possible in the future to engineer lines of MDV resistant chickens carrying small interfering RNA (siRNA) sequences targeting selected viral genes.

### 1.2.9 Introduction to RNA Interference Techniques for Gene Silencing in Chickens

RNAi is a term used to describe post-translational suppression of protein expression either through miRNA described previously or siRNAs which involves introducing synthetic ~22 nucleotide RNA sequences into cells to suppress targeted genes. Introduction of long double-stranded RNA (dsRNA) into most non-mammalian cells results in degradation of the targeted homologous messenger RNA (mRNA) transcripts, causing a net decrease in gene-specific protein expression and effective sequence-specific gene silencing (Heidersbach *et al.*, 2006). In mammalian cells, however, introduction of long dsRNA activates the innate immune system responsible for recognizing the molecular signatures of pathogen-associated molecular patterns (PAMPs) which plays a key role in microbial immunity as well as the recognition of self and non-self (Heidersbach *et al.*, 2006; Kawai and Akira, 2009; Samuel-Abraham and Leonard, 2010). Innate immune responses produce inflammation through the production of cytokines and interferon mediated by phagocytes such as macrophages and dendritic cells (Kawai and Akira, 2009). Innate immunity receptors have been grouped into three families which are: membrane-bound Toll-like receptors (TLRs), cytoplasmic retinoic acid-inducible gene-I-like receptors (RLRs) and nucleotide-binding oligomerisation domain-like receptors (NLRs) (Kawai and Akira, 2009; Yokota *et al.*, 2010). Additionally a DNA-dependent activator of interferon regulatory factors (DAI) which senses DNA has been recently described (Yokota *et al.*, 2010). The TLRs detect PAMPs on the cell membrane or in the lumen of intracellular vesicles while the RLRs and NLRs detect intracellular PAMPs (Kawai and Akira, 2009). RLRs belong to the

RNA helicases enzyme family that specifically binds double-stranded RNA in the cytoplasm and are key to recognition of viral or exogenous RNA (Yoneyama and Fujita, 2008). The activation of the TLR, RLR or NLR signalling pathways trigger a cascade resulting in the induction of type 1 interferon and inflammatory cytokines (Yokota *et al.*, 2010). An example of these interactions in herpesviruses is the association of immunity to HSV-1 with Human Toll-like receptor 3 (TLR-3) dependent induction of α-IFN, β-IFN and λ-IFN (Zhang *et al.*, 2007). The innate immune response is well studied and a great deal more detail on the specific molecular mechanisms are available, but beyond the scope of this study. For reviews see (Kawai and Akira, 2009; Van de Walle *et al.*, 2008; Yokota *et al.*, 2010). When exogenous dsRNA is introduced into the mammalian cell, the innate immune response triggers a universal gene silencing in the cell and apoptosis (Heidersbach *et al.*, 2006; Samuel-Abraham and Leonard, 2010). This was a problem in applying RNAi to mammalian cells until it was demonstrated that short (~21 bp) dsRNA sequences, rather than the long dsRNA, introduced into cells could inactivate specific genes with minimal triggering of the innate immune system (Elbashir *et al.*, 2001). This discovery enabled the use of RNAi based tools to manipulate gene expression in mammalian systems that subsequently revolutionized the field of functional genomics. Several methods have been developed to effectively deliver siRNA into cells using plasmids, electroporation and lentiviral-based systems (Maurisse *et al.*, 2010; Stewart *et al.*, 2003).

**1.3  Investigation of Selected Uncharacterised MDV Genes**

Unique viral genes often have key roles in regulating latency and transformation in herpesviruses as each virus adapts to its particular host (Pellett *et al.*, 2006).  MDV encodes at least 103 proteins some of which are unique either to MDV or the *Mardivirus* genus, and apart from some well-defined examples, such as Meq and pp38, are not well-characterised (Tulman *et al.*, 2000).   The second portion of this thesis focuses on work done to investigate the function of genes in MDV whose function was poorly defined. With the exception of US2, a conserved gene in all herpesviruses encoding a tegument protein of unknown function, all the genes investigated were unique to the *Mardivirus* genus or only to MDV.  The following synopsis consists of a brief background and literature search for candidate genes.  As many of the genes have been referred to in the literature by several different names, attempts have been made to standardize the designations for each gene.  The nomenclature used to refer to the MDV ORF numbers corresponds to the GenBank database (Benson *et al*., 2008).  Genes that do not have a common designation will be referred to by their ORF number.  In order to simplify the following gene descriptions genes transcribed from the 5' termini of the sense strand will be designated left to right (L to R) and genes transcribed from the complimentary strand will be designated right to left (R to L).  A diagram of the repeat region of the MDV genome is illustrated in figure 1.8 and gene information is summarised in table 1.9 for all the genes investigated.

| Designation | Unique to MDV | MDV ORF | Location and Orientation in MDV Genome | Overlapping Genes |
|---|---|---|---|---|
| RLORF6 | Y | 5.1 | IRL (R to L) | Meq (sense) and |
|  |  | 77.5 | TRL (L to R) | 23kDa (compliment), ORF5.2 (compliment), ORF5.3 (compliment) |
| 23kDa | Y | 4.0 | IRL (L to R) | ORF5.2 (sense) and |
|  |  | 77.0 | TRL (R to L) | RLORF6 (compliment), Meq (compliment) |
| RLORF5a / L1 | Y | 3.8 | IRL (R to L) | None |
|  |  | 78.1 | TRL (L to R) |  |
| RLORF11 | Y | 6.4 | IRL (L to R) | Spliced gene encoding 14kDa lytic protein A & B (compliment) |
|  |  | 75.4 | TRL (R to L) |  |
| MLTI | Y | 6.5 | IRL (R to L) | Spliced gene encoding 14kDa lytic protein A (sense) |
|  |  | 75.2 | TRL (L to R) |  |
| RLORF12 | Y | 7.0 | IRL (R to L) | Spliced gene encoding 14kDa lytic protein A (sense) and LORF1 |
|  |  | 74.0 | TRL (L to R) | (sense) in IRL, No overlap in TRL |
| LORF1 | Y | 9.0 | TRL / UL (R to L) | RLORF12 (sense) and |
|  |  |  |  | RLORF13 (compliment), RLORF14* (compliment) |
| LORF3 | Y | 12.0 | UL (L to R) | ORF12.8 (sense)and |
|  |  |  |  | ORF11.5 (compliment), ORF 12.4 (compliment) |
| MNFH | Y | 49.1 | UL (L to R) | HSV homologue of UL36 (MDV ORF 49.0) (compliment) |
| LORF6 | Y | 49.5 | UL (L to R) | HSV homologue of UL36 (MDV ORF 49.0) (compliment) |
| LORF8 | Y | 57.8 | UL (R to L) | HSV homologue of cell fusion protein UL45 (MDV ORF 58.0) (compliment). |
| LORF11 | Y | 72.0 | UL (R to L) | ORF71.8, ORF72.2, ORF72.4 (all compliment) |
| LORF12 | Y | 72.8 | UL (R to L) | ORF72.6 (compliment) |
| ANTISENSE** | Y | 83.0 | IRS (L to R) | $\alpha$4 gene encoding ICP4 (compliment) |
|  |  | 101.0 | TRS (R to L) |  |
| US2*** | N | 91.0 | US (R to L) | ORF91.5 (compliment) |

**Table 1.9:** Summary of gene information for uncharacterized MDV genes investigated. Genes transcribed on the same strand as overlapping genes have no designation and genes transcribed in the opposite orientation designated 'compliment'. Note: *RLORF14 encodes Early 24kDa phosphoprotein, pp24, ** ANTISENSE denotes antisense to ICP4 RNA protein encoding gene, ***US2 denotes HSV-1 US2-like protein encoding gene. Table compiled from information contained in GenBank genome of MDV strain CVI988 and CU-2 (Benson *et al.*, 2008; Spatz *et al.*, 2007b; Spatz and Rue, 2008).

### 1.3.1   The 'Meq Loci' Genes

The well-characterised Meq is located in the repeat region of the genome and is unique

to pathogenic MDV (section 1.2.5).  However, four unique and uncharacterised repeated

genes (RLORF6, 23kDa, MDV ORF5.2, MDV ORF5.3) are also located in the 'Meq

loci' as shown in figure 1.8.  Two of these genes, RLORF6 (MDV ORF 5.1 & 77.5) and

23kDa (MDV ORF 4.0 & 77.0) were selected for screening in this study.  Frequent

deletions and mutations have been identified in Meq (Spatz *et al.*, 2007a; Spatz *et al.*,

2007b) which usually leads to a corresponding change in the gene sequence and encoded

protein composition of other 'Meq loci' genes.  Screening for MDV-chicken protein

interactions with a yeast 2-hybrid assay followed up by an *in vitro* binding assay

revealed no matches for RLORF6, but the 23kDa ORF (MDV strain Md5) demonstrated

binding with chicken glycolytic enzyme α-enolase (encoded by the cellular ENO1 gene)

(Niikura *et al.*, 2004).  α-enolase is a ubiquitous protein playing an essential part in the

glycolytic pathway and is found in the cytoplasm of all prokaryotic and eukaryotic cells.

Subramanian and Miller demonstrated that α-enolase RNA (ENO1) contains an

additional translation initiation site encoding *Myc*-binding protein 1 (MBP-1) which

allows two different translation products to be formed from a single α-enolase mRNA

(Subramanian and Miller, 2000).  This led to the suggestion that post-translational

interference with the α-enolase mRNA could potentially cause changes in expression of

both α-enolase and MBP-1.  MBP-1 binds to the *c-myc* promoter which down-regulates

*c-myc* expression (Ray and Miller, 1991).  *C-myc* is a DNA-binding protooncogene

important for cell growth and differentiation (Marcu *et al.*, 1992) and has been shown to

be over-expressed in several malignancies (Cole, 1986). High levels of ENO1 specific antibody have also been described as features of infections and autoimmune diseases (Terrier *et al.*, 2006). It is difficult to predict whether these findings have any significance, but it is tempting to speculate that 23kDa's association with the ENO-1 gene and its proximity to other oncogenic viral genes such as Meq could predict an association with tumourigenesis, however there is currently no evidence to support this. Heidari and colleagues demonstrated that 23kD, along with Meq and RLORF5, were expressed in both the cytolytic and latent phases of infection (Heidari *et al.*, 2008). 23kDa has been shown to localise to the nucleus but there was no reference in the literature as to where RLORF6 localised or if it was expressed during latent and lytic infection.

### 1.3.2 Repeat Long Regions of the Genome

Many of the unique genes in MDV are located in the $IR_L$ and $TR_L$ regions of the genome which contain many key genes in addition to Meq that are associated with transformation and latency such as pp24, pp38 and IL-8. There are many unique genes in this region which have not been well-characterised. Five uncharacterised genes from this region (RLORF5a, RLORF11, MLTI, RLORF12, and LORF1) were selected for these studies and are detailed in figure 1.8 and table 1.9.

The RLORF5a gene (MDV ORF 3.8 & 78.1), previously designated L1, is unique to MDV and encodes a repeated gene product (Jarosinski *et al.*, 2005; Kingham *et al.*,

2001).  Schat and colleagues demonstrated that RLORF5a was not necessary for *in vitro* or *in vivo* lytic replication or the establishment of latency in MD lymphoblastoid chicken cell lines, but was expressed in latency in their experimental model (Schat *et al.*, 1998). Jarosinski and colleagues compared the growth kinetics of the reconstituted virus from a RLORF5a deletion mutant MDV strain Rb1b BAC to the parental pRb1b reconstituted virus and found no significant changes *in vitro* or *in vivo* (Jarosinski *et al.*, 2005).  These findings led to the conclusion that RLORF5a did not play any obvious role in attenuation of virulent MDV.  Differences in RLORF5a expression during latent infection between different cell lines were noted in earlier studies (Schat *et al.*, 1998) suggesting that RLORF5a expression could vary depending on the stage of the virus life cycle.   It was subsequently found that the MDV miRNAs mdv-miR-M1 and -M11 were embedded within the RLORF5a transcript (Yao *et al.*, 2008).  The significance of these findings is unknown but it is tempting to speculate that the role of RLORF5a could be more complex than previously thought if mdv-miR-M1 and –M11 plays a role in gene regulation, however there is no supporting evidence at this time.  RLORF5a was included in this study because no studies had been done examining the effect of removal of the gene product using siRNA on a non-producer MDV lymphoblastic cell line.  It was hypothesized that the removal of the RLORF5a gene product from an established non-producer MDV cell line and analysis for phenotypic changes would help confirm whether RLORF5a had any measurable effect in latent infections.

Niikura and colleagues screened for MDV-chicken protein interactions as described

previously in RLORF11 (MDV ORF 6.4 & 75.4), RLORF12 (MDV ORF 7.0 & 74.0)

and LORF1 (MDV ORF 9.0). Interactions were only found between RLORF12 and 23p

growth- related transcriptionally-controlled tumour protein (TCTP) encoded by the

chicken gene TPT1 (Niikura *et al.*, 2004). TCTP is a highly conserved protein with a

housekeeping function that has been identified in multiple tissues in virtually all

eukaryotic organisms and has been associated with many vital cellular functions

(Bommer and Thiele, 2004). Studies have shown TCTP over-expression inhibits Na, K-

ATPase cell plasma membrane activity in a dose-dependent manner (Jung *et al*., 2004).

The Na K-ATPase membrane protein plays a vital role in the cell maintaining the

homeostasis of cells. TCTP over-expression has been linked with smooth muscle

vasoconstriction leading to systemic hypertension (Kim *et al.*, 2008) and with formation

of cataracts in mice and in human lens cells *in vitro* (Kim *et al.*, 2009). Cataracts have

been described as a feature in the pathology of highly virulent MDV infection (Dukes

and Pettit, 1983; Pandiri *et al.*, 2008). It is tempting to speculate as to whether the

pathology of some of the more acute symptoms of MDV such as cataracts and brain

oedema seen in virulent and very virulent+ strains could be associated with changes to

TCP0 function and the subsequent disruption of Na/K balance in the cell, but currently

there is no evidence to support this.


Sequence differences between attenuated and pathogenic virus strains were used as one

indicator of the suitability of a unique gene for study. MLTI (ORF6.5 & 75.2), first

described in the CVI988 strain of MDV (Spatz *et al.*, 2007b), encodes a 328 aa gene

product in the attenuated CVI988 Rispens strain of MDV, but only a 64 aa gene product

in pathogenic strains of MDV.  Sequencing of the strain CVI988 MDV genome revealed

that RLORF12 encoded a truncated 64 aa protein for one of the repeats (ORF74.0)

relative to the oncogenic strains containing a 115 aa protein in both repeated sequences

(Spatz *et al.*, 2007b).  Even though LORF1 was not a repeated gene it was included in

this section as its position overlaps the $TR_L$ and $U_L$ region.  No sequence variation

between strains was identified in the LORF1 or RLORF11 genes (Spatz *et al.*, 2007b).

No functional information, other than the gene description detailed in table 1.9, was

available for the RLORF11, MLTI and LORF1 genes.


### 1.3.3  Other Regions of the Genome

Several other genes of interest (LORF3, LORF11, MNFH, ANTISENSE, US2, LORF6,

LORF8 and LORF12) were located in regions of the genome out with those described

above and identified for inclusion in this study.


LORF3 (MDV ORF 12.0) is located in the $U_L$ region of the MDV genome which is

associated with conserved HSV-1 homologues encoding intermediate and late genes

such as the genes encoding the homologue of HSV-1 UL1-like protein and HSV-1 UL2-

like protein adjacent to LORF3.  A LORF3 homologue of unknown function has been

described in Duck Enteritis Virus, an unassigned α-herpesvirus affecting waterfowl (Li

*et al.*, 2009; Liu *et al.*, 2009), but not in other avian α-herpesviruses outside the

*Mardivirus* genera such as Gallid Herpesvirus-1 or Psittacid Herpesvirus 1 (Thureen and Keeler, 2006). The functions of LORF3 as well as the three genes overlapping LORF3 (figure 1.8 and table 1.9) have not been described (Spatz *et al.*, 2007b).

LORF11 (MDV ORF 72.0) is located in the $U_L$ region adjacent to the gene encoding pp38 which is associated with latency and tumourigenesis (Spatz *et al.*, 2007b). Homologous genes to LORF11 have been identified only in strain HPRS-24 GaHV3 and serotype 3 HVT and BLAST searches of LORF11 showed matches only within the genus *Mardiviruses* (Lee *et al.*, 2007). Lee and colleagues generated a LORF 11 deletion mutant MDV virus based on the pathogenic Md5 strain (designated rMd5Δ LORF11). Similar growth characteristics *in vitro* were found when comparing lytic infection by pathogenic rMd5 virus to deletion mutant virus rMd5ΔLORF11 in primary duck embryo fibroblasts *in vitro* (Lee *et al.*, 2007). *In vivo* experiments comparing viraemia levels between chickens infected with rMd5 MDV and rMd5ΔLORF11 demonstrated a 10 to 100 fold reduction ($p<0.01$) in viraemia levels in MDV-susceptible chickens (Lee *et al.*, 2007). These findings suggest that LORF11 may be a virulence factor of MDV *in vivo*. Although no differences were found between rMd5ΔLORF11 and rMd5 infection *in vitro* there have been no studies examining the effect of post-transcriptional targeting of RLORF11 in a non-producer MDV infected cell line that would clarify whether the LORF11 gene product had a role in latency *in vitro*. LORF11 was selected for this study because it was unique to MDV-like viruses and appeared to be associated with MDV virulence factors.

MNFH (MDV ORF 49.1) was first described as a predicted protein in the CVI988 strain of MDV (Spatz *et al*., 2007b). It encodes a 93-94 aa gene product in the virulent MDV strains Md5, Md11 and GA but due to a mutation causing a stop codon it only encodes a 34 aa gene product in the attenuated CVI988 strain of MDV (Spatz *et al*., 2007b). The mutation, causing a stop codon in MNFH, was silent in the UL36 gene and apparently only affected the MNFH gene (Spatz *et al*., 2007b). MNFH was deemed a good candidate for study because the truncated gene product was found only in attenuated MDV strains and because relatively little was known about its function and expression.

The ANTISENSE gene (ORF 83.0 & 101.0) encodes two RNA transcripts (15 kb and 1.32 kb) which are strongly expressed *in vitro* in latent MDV transformed chicken lymphoblasts (Li *et al*., 1994). The α4 gene encoding ICP4 overlaps some of the ANTISENSE gene and is transcribed on opposite strands (table 1.9). As ICP4 is expressed only during lytic infection these findings imply that ANTISENSE may be expressed during latency when ICP4 would not be expressed and may be spliced as two RNA transcripts were identified. ANTISENSE was selected for study because of its association with selective expression during latency.

The US2 gene (MDV ORF 91.0) located in the unique short (Us) region of the genome is conserved in all α-herpesvirus genomes and a homologue has been described in the HSV-2 genome (Jiang *et al*., 1998). US2 is a tegument protein with an unknown

function (Kelly *et al.*, 2009; Pellett *et al.*, 2006; Spatz *et al.*, 2007b). The U$_S$ region in α-herpesviruses has been shown to encode a number of glycoprotein gene products that appear to be linked with determination of pathogenesis and virus-cell interaction (Brunovskis and Velicer, 1995). Excision of US2 in a deletion mutant virus did not appear to affect oncogenicity or the establishment and maintenance of latency (Parcells *et al.*, 1995) but deletion of US2 in conjunction with UL13 protein kinase and gC was linked to failure of horizontal transmission in chickens infected with strain Rb1b MDV (Jarosinski *et al.*, 2007). These findings led to the conclusion that US2 was conserved and present in latent and lytic infections, but non-essential for infection and latency (Parcells *et al.*, 1995). There have been no studies, however, analyzing at the effect of targeting US2 using siRNA in transformed lymphoblasts to assess whether it plays a role in maintenance of latency.

LORF6 (MDV ORF 49.5), LORF8 (MDV ORF 57.8) and LORF12 (MDV ORF 72.8) are all unique genes to MDV and are located in the repeat long region of the genome. The function of all three of these genes is unknown. Screening of LORF12 for MDV-chicken protein interactions as previously described revealed no matches (Niikura *et al.*, 2004).

### 1.4 <u>Project Aims</u>

This project intended to explore the molecular control mechanisms operating in latent MDV infections by studying selected uncharacterized MDV genes in order to identify

those affecting phenotype in MDV-transformed latent cell lines using siRNA techniques. The intention was to further characterise any genes that affected phenotype and determine whether they play a role in the maintenance of latency or transformation in MDV infected cells. Genes were selected for investigation based on a number of criteria that will be discussed further in chapter 3.

# Chapter 2 – Materials and Methods

## 2.1 Molecular Techniques

### 2.1.1 Restriction Endonuclease Digestion

Restriction digests were performed on plasmids and purified deoxyribonucleic acid

(DNA) using restriction enzymes according to manufacturer's instructions. All digests

were carried out in a 37ºC water bath for 1 to 6 hours.

### 2.1.2 DNA Isolation & Purification

Preparation of DNA from animal cell samples was, unless otherwise indicated,

performed using a commercially available kit (DNeasy Kit, Qiagen). Cells were lysed

with sodium dodecyl sulfate (SDS) followed by treatment with protease K which

degraded protein contaminants and nucleases. The DNA in the cell lysate was then

bound to a silica gel membrane, washed in two steps and eluted in water or low salt

buffer such as TE Buffer (section 2.1.10). After purification all DNA samples were

stored at either 4ºC for short-term or at -20ºC for long-term storage in TE Buffer or EB

Buffer (section 2.10.1). All DNA samples were checked for concentration and purity

after isolation (section 2.1.15). Samples that were deemed too dilute for their intended

purpose were concentrated using ethanol precipitation (section 2.1.6) before use.

### 2.1.3 RNA Isolation & Purification

Cells intended for ribonucleic acid (RNA) extraction were harvested and washed in

sterile phosphate buffered saline (SPBS) and frozen immediately at -80ºC in SPBS or

RNALater solution (Ambion, Invitrogen) until RNA was extracted.  Preparation of purified RNA from cell samples was, unless otherwise indicated, performed using a commercially available kit (RNeasy Kit, Qiagen) that utilized the selective binding of RNA to a silica gel membrane in the presence of a high salt buffer followed by washing and eluting the bound RNA.  All RNA samples were eluted in RNase-free water and stored at -80ºC and thawed slowly on ice before use.  All RNA samples were checked for concentration and purity with a low volume spectrophotometer (section 2.1.15). Samples were also analyzed using electrophoresis in a 2% agarose gel containing 0.8 µg/ml ethidium bromide (Thermo Fisher Scientific) (section 2.1.13) to assess purity before further treatment.  To decrease the likelihood of RNase contamination of samples, all RNA work was carried out using gloves, disposable RNase-free lab ware, RNase-free reagents and work surfaces were treated with RNase decontamination solution (RNaseZap, Ambion, Invitrogen) before use.


### 2.1.4  DNaseI Treatment of RNA Samples

Residual viral DNA contamination was removed from RNA samples by digestion with DNaseI.  Samples were treated with 2.4U DNaseI (Ambion, Invitrogen) or 3U Turbo DNaseI (Ambion, Invitrogen) per reaction (<10µg RNA/reaction) and incubated for 30 minutes at 37ºC.  This process was repeated 4 times (total of 12U/reaction for 120 minutes) to ensure complete removal of viral DNA after early experiments experienced problems with residual contaminating viral DNA in samples following shorter DNaseI treatment.  The DNaseI was inactivated, according to manufacturer instructions, either

with DNaseI inactivation reagent (Ambion, Invitrogen) or by phenol/chloroform

extraction (section 2.1.7). DNaseI treatment of RNA samples was always performed

before further treatment. To ensure that viral DNA had been completely removed, a

polymerase chain reaction (PCR) was performed as described in section 2.1.10 using

primers specific for Meq (Forward primer TGACCCTTGGACTGCTTACC and reverse

primer GAGCAATGTGGAGCGTTAGG) and the DNaseI treated RNA as a template.

No bands would be seen if the treatment had removed all viral DNA.


### 2.1.5  Synthesis of First Strand cDNA from RNA

Complementary DNA (cDNA) was prepared from DNaseI treated RNA using reverse

transcriptase according to standard manufacturer's instructions using either Stratascript

5.0 Multi-Temp Reverse Transcriptase (Stratagene) or AffinityScript Multiple

Temperature cDNA Synthesis Kit (Stratagene) using sequence specific primers. Once

synthesized the cDNA was used for either PCR or RTqPCR assays (sections 2.1.8 and

2.1.17). Samples were immediately stored at -80ºC after synthesis and kept on ice at all

times while setting up further experiments.


### 2.1.6  Concentration of DNA Samples

Dilute DNA samples were concentrated either by using the QIAEX II kit (Qiagen) using

standard manufacturer instructions or by ethanol acetate precipitation. Ethanol acetate

precipitation was performed by adding 1/10 volume 3M sodium acetate pH 5.2 and 3

volumes of 100% ethanol in a 1.5 ml microcentrifuge tube. Samples were then

centrifuged at 13,000 x g for 10 minutes and the supernate removed after pelleting the

DNA. The DNA pellet was washed with 70% ethanol, the supernate removed again and

the DNA pellet allowed to air dry for 10 to 15 minutes before resuspending in an

appropriate volume of either EB or TE buffer (section 2.10.1).

### 2.1.7    Purification of RNA by Phenol: Chloroform Extraction

Phenol chloroform extraction was performed by combining an equal volume of a

blended mix of phenol (49.5%), chloroform (49.5%) and isoamyl alcohol pH 8 (1%)

(Fluka Chemicals, Sigma-Aldrich) with a 1/10 volume of 3M pH 5.2 sodium acetate and

the RNA sample in the fume hood. The sample was mixed then centrifuged for 10

minutes at 13,000 x g at 4ºC. The top (aqueous) layer containing the RNA was removed

and placed into a fresh microfuge tube. An equal volume of chloroform and isoamyl

alcohol 24:1 mix was added in the fume hood. The sample was then thoroughly mixed

and centrifuged for 10 minutes at 13,000 x g at 4ºC. The top (aqueous) layer was

removed again, placed into a fresh microfuge tube, and mixed with 3 volumes of 100%

ethanol. After inverting the tube briefly to mix, the sample was then placed in a -80ºC

freezer. Once frozen, the sample was removed from the freezer and centrifuged for 20

minutes at 13,000 x g at 4ºC in a microfuge tube after which the supernate was decanted.

The RNA pellet was washed with an equal volume of 70% ethanol and centrifuged again

at 13,000 x g at 4ºC for 10 minutes. The supernate was carefully removed and the RNA

pellet was then air-dried on ice for a maximum of 5 minutes before resuspending in an

appropriate volume of RNase-free water.    This protocol was adapted from standard

laboratory protocol.  The purified RNA sample was assessed for concentration with a

low volume spectrophotometer and 2% agarose gel electrophoresis to assess for purity

(sections 2.1.13 and 2.1.15).


### 2.1.8  Polymerase Chain Reaction (PCR)

PCR was carried out using either recombinant *Taq* DNA polymerase (Invitrogen) for

non-proofreading reactions or Native *Pfu* DNA polymerase (Stratagene) for reactions to

be sequenced.  Forward and reverse primers were used at a concentration of 1-2 pmol

per reaction. The amount of template used for each reaction was 20-30 nmol per

reaction.  Both DNA polymerases were used according to standard manufacturer's

instructions.  DNA polymerase was added after the initial denaturing period of 5 minutes

at 95ºC ('hot start' program).  The program used for each cycle was as follows:  95ºC for

45 seconds, 55-62ºC for 45 seconds and 72ºC for 2 minutes.  The number of cycles per

reaction was 30-40 followed by a 5-10 minute cooling down period.  All reactions were

set up in a dedicated room using equipment and clothing used only for setting up PCR

reactions.  PCR reactions not used immediately were stored either at 4ºC or at -20 º C.

### 2.1.9  Reverse Transcriptase Quantitative PCR (RTqPCR)

cDNA (section 2.1.5) was diluted to 20-40 pMol/μl in RNase-free water and prepared in triplicate for RTqPCR using either Meq or Chicken β-actin primer sets as detailed in tables 2.1 and 2.2.  A standard curve of known concentrations of a Meq or Chicken β-actin plasmid was also prepared to quantify samples. The plasmids used for the standard curves were  made centrally in our laboratory by amplifying a target sequence approximately 50 base pairs upstream and downstream of the detection primers (see table 2.1 and 2.2) for either Meq (Meq forward = TACAGTC CCGCTGACGA TCCG and Meq reverse = GACCGTAGACTGAGTATCCG) or chicken β-actin (ChActin forward = GGGTGTGATGGTTGGTATGG and ChActin reverse = AAGAAAGATGGCTGGAAGAGG)  The PCR product from these primers was then cloned into TOPO pcDNA3.1 plasmid (Invitrogen) for Meq and TOPO PCR 4 plasmid (Invitrogen) for Chicken β-actin, sequenced for accuracy, linearised, purified and its concentration established so that copy number could be calculated for preparing the respective standard curves for each experiment.  The total reaction size used was 20 μl that consisted of 5 μl of diluted cDNA sample and 15 μl of a master mix containing all other components of the reaction which are detailed in tables 2.1 and 2.2.  Depending on the concentration of the original RNA, the cDNA used was between 0.5-1.5μg per reaction.  RTqPCR of all samples in each experiment were run at the same time and with the same batch of master mix to reduce variation resulting from pipetting variation and variable reverse transcriptase activity.  Equal amounts of RNA as determined by a low volume spectrophotometer were used to make cDNA, however the samples were not re-

normalized at the cDNA stage, leaving a small potential for error should the cDNA

reactions vary in their efficiency.  A 72-well thermocycler was used to process samples

(Rotor-Gene, Corbett Research, model 080316).   The software used for running the

thermocycler was Rotor-Gene 6 (Version 6.1, Build 71).  Cycle settings were as follows:

hold temperature 95ºC for 15 minutes followed by 45 cycles of 95ºC x 20 seconds, 60ºC

(Meq) or 62ºC (chicken β-actin) x 20 seconds and 72ºC x 20 seconds.  A second hold of

95ºC x 20 seconds was then performed followed by a melt curve from 60ºC to 95ºC

rising by 0.4ºC each step.  There was a wait of 20 seconds after the first step of the melt

curve and then a pause of 1 second between steps.

| (amounts in μl) | Per Reaction |
| --- | --- |
| 10x PCR Reaction Buffer with 2.0 mM $MgCl_2$ (Roche) | 2.0 |
| dNTPs 5 mM (Roche) | 0.4 |
| SYBR Green I Stock (Made by adding 5 μl SYBR Green reference solution (Bio Gene Ltd), 45 μl DMSO and 4950 μl DEPC-treated water) | 0.7 |
| *Taq* DNA Polymerase 5U/μl (Roche) | 0.15 |
| Forward Primer 3,200 nM 5' – TGA CCC TTG GAC TGC TTA CC – 3' | 2.5 |
| Reverse Primer 4,000 nM 5' – GAG CAATGT GGA GCG TTA GG – 3' | 2.5 |
| $MgCl_2$ 25 mM (Roche) | 1.6 |
| DEPC-treated Water | 5.1 |

**Table 2.1:** RTqPCR master mix for chicken β-actin (4.0mM Magnesium Chloride, 400nM forward primer, 500 nM reverse primer final concentrations and 62ºC anneal) for 15μl master mix and 5μl sample per reaction.

| (amounts in μl) | Per Reaction |
| --- | --- |
| 10x PCR Reaction Buffer with 2.0 mM $MgCl_2$ (Roche) | 2.0 |
| dNTPs 5 mM (Roche) | 0.4 |
| SYBR Green I Stock | 0.7 |
| *Taq* DNA Polymerase 5U/μl (Invitrogen) | 0.15 |
| Forward Primer 2,400 nM 5' – GAA TCC CAA AGC CAA TCG – 3' | 2.5 |
| Reverse Primer 1,600 nM 5' – CCC AGA GTC AAG CAC AAT CC – 3' | 2.5 |
| $MgCl_2$ 25 mM (Roche) | 0.4 |
| DEPC-treated Water | 6.3 |

**Table 2.2:** RTqPCR master mix for Meq (2.5 mM $MgCl_2$, 300nM forward primer, 200 nM reverse primer final concentrations and 60ºC anneal) for 15μl master mix and 5μl sample per reaction.

### 2.1.10  Normalization of cDNA Samples

A single control normalization error using the 75$^{th}$ percentile of housekeeping gene chicken β-actin was calculated for each sample using a formula described in (Vandesompele *et al.*, 2002).  This was done to allow more effective comparison of levels of gene expression between samples correcting for multiple factors such as transfection variation, differential RNA degradation and variable efficiency of the reverse transcription reaction.  Normalization of RTqPCR data consisted of comparing two runs, one with the primers specific for the gene of interest and another with primers specific for the housekeeping gene chicken β-actin.  Meq was expected to vary according to experimental treatment. Chicken β-actin was not expected to vary with the experimental groups as it should be expressed at consistent levels for all cells, thus making it a suitable housekeeping gene (Suzuki *et al.*, 2000; Yin *et al.*, 2011).

### 2.1.11  Purification of PCR Products

Contaminant primer, nucleotides, polymerase or salts were removed from PCR reactions using the QIAquick PCR Purification Kit (Qiagen) according to standard manufacturer instructions.

### 2.1.12  Agarose Gel Electrophoresis

1 to 2% (w/v) Agarose was prepared by mixing either 1 or 2g of LE agarose (SeaKem) per 100ml of TAE buffer (section 2.10.3) in a heat-resistant glass jar and heating in a microwave until completely dissolved.  The mixture was then allowed to cool slightly

and 3 μl of Ethidium Bromide stock solution 10 mg/ml (Thermo Fisher Scientific) was added and mixed thoroughly. The mixture was then poured into a block mould and combs for placement of the nucleic acid samples were positioned. Once cooled, the combs were removed and the gel block was placed into an electrophoresis tank filled with 1X TAE buffer with the sample wells situated at the negative pole of the tank. Nucleic acid samples were loaded into the wells combined with loading buffer (section 2.10.3) at a ratio of approximately 1:5. Current was then applied at the rate of 40-70mA (55-100V) for 30-90 minutes until the visible Orange G dye in the loading buffer had migrated to the bottom of the gel. After running, gels were visualized using long wavelength UV light and photographed. 2-log DNA ladder (New England Biolabs) or 1kb DNA ladder (New England Biolabs) was used to determine nucleic acid size on all agarose gels according to manufacturer instructions.

### 2.1.13 DNA Extraction from Agarose Gels

Purification of DNA fragments from agarose gels was performed by identifying the band of interest from the gel using long wavelength UV light. The band was then carefully cut from the agarose gel with a surgical scalpel and DNA purified using the QIAquick Gel Extraction Kit or QIAEX II Agarose Gel Extraction Kit (Qiagen) according to standard manufacturer instructions.

### 2.1.14  Quantification of Nucleic Acid by Spectrophotometry

Nucleic acid samples were checked for concentration and purity after isolation with a Nanodrop ND-1000 low volume spectrophotometer measuring 220nm to 750nm wavelength and version 3.7.1 software (Thermo Fisher Scientific).  The key ratios used to assess purity of nucleic acids was the 260/280 absorbance as an indicator of contaminants and the 260/230 absorbance as an indicator of both contaminants and co-products.

### 2.1.15  Sequencing of DNA

Sequencing of plasmids was performed by Mr. Ian Bennett in the University of Edinburgh Molecular Pathology Unit using a Licor 4000L Sequencer and an Epicenter Sequitherm Excel $^{tm}$ II Licor DNA Sequencing Kit (Li-Cor Biosciences).  Sequencing directly from PCR products was performed using a Big Dye Terminator Cycle v 3.1 (Applied Biosystems, Invitrogen) PCR reaction in our lab using the following protocol adapted from The Gene Pool website (www.wiki.ed.ac.uk/display/GenePool/Sequencing+Reactions, accessed 15/4/2008) which is detailed in tables 2.3 and 2.4.  The subsequent PCR product was then sequenced using an ABI3730 sequencer (Applied Biosystems, Invitrogen) by The Gene Pool, University of Edinburgh School for Biological Sciences Sequencing Service, UK.  The resulting sequence was analyzed using Vector NTI version 10 & 11 software (Invitrogen) to combine, align and overlay individual sequences to form longer sequences (2000-3000 bp).

| Big Dye Terminator Cycle PCR Reaction | μl |
|---|---|
| 5x Sequencing Buffer (Applied Biosystems, Invitrogen) | 2.0 |
| Double distilled H$_2$O | 4.7 |
| Primer (10μM) | 0.3 |
| Big Dye Terminator Cycle (Applied Biosystems, Invitrogen) | 1.0 |
| DNA Template | 2.0 |

**Table 2.3:** PCR Reaction for Big Dye Terminator Cycle (Applied Biosystems, Invitrogen).

| PCR Program | |
|---|---|
| 95ºC | 30 seconds |
| 50ºC | 20 seconds |
| 60ºC | 4 minutes |

**Table 2.4:** PCR program for Big Dye Terminator Cycle (Applied Biosystems, Invitrogen) Reactions were carried out for 25 cycles.

## 2.2  Bacterial Techniques

### 2.2.1  Bacterial Culture

Bacterial growth was carried out using either LB broth or LB agar plates (Merck) (section 2.10.4) as the culture medium in either a 37ºC still air incubator or a 37ºC shaking incubator rotating at 250 rpm.  Commercially available chemically competent *E.coli* cells were stored at -80ºC until use (Invitrogen or Stratagene).  Tubes of cells were thawed slowly on ice before cloning.  Transformed *E.coli* cells intended for short-term storage (less than 5 days) were stored at 4ºC.

### 2.2.2  Transformation of Chemically Competent *E. coli*

Plasmids were transformed into commercially prepared chemically competent *E.coli* (One Shot TOP10 & Stbl3 chemically competent *E.coli*, Invitrogen and Sure & Sure2 Supercompetent cells, Stratagene) according to standard manufacturer's instructions.

### 2.2.3  Plasmid DNA Isolation from Bacteria (Small-Scale)

Small-scale preparation of DNA from bacterial cells was performed using the commercially available QIAquick Miniprep Kit (Qiagen) according to standard manufacturer instructions.  All DNA samples were stored at either 4ºC for short-term or -20ºC for long-term storage in EB buffer (section 2.10.1).

### 2.2.4  Plasmid DNA Isolation from Bacteria (Large-Scale)

Large-scale preparation of DNA from bacterial cells was performed using the commercially available Endo-Free Maxiprep kit (Qiagen) or PureLink HiPure Plasmid Maxiprep Kit (Invitrogen) according to standard manufacturer instructions.  All DNA samples were stored at either 4ºC for short-term or -20ºC for long-term storage in TE Buffer (section 2.10.1).

### 2.2.5  Marek's Disease Virus Bacterial Artificial Chromosome Preparation

Marek's Disease Virus Bacterial Artificial Chromosomes (MDV BAC) were originally obtained from Dr. Venugopal Nair's Laboratory at the Institute for Animal Health in Compton, UK.  The MDV BAC was prepared in Dr. Nair's lab for use in transfection experiments by inserting the MDV genome into *E.coli* (Petherbridge *et al*., 2003).  A glycerol aliquot (section 2.2.6) of starter colony was continuously maintained at -80ºC for use in our experiments.  The MDV BAC transformed *E.coli* were streaked on a LB agar plate containing Chloramphenicol (15μg/ml) and grown at 37ºC overnight.  One colony was picked and placed in 10 ml of LB broth containing Chloramphenicol

(15μg/ml) and placed in a shaking incubator at 37ºC and 250 rpm until an optical density

of 0.6 was reached (usually 6-8 hours).  This starter colony was then placed on ice

overnight.  The following day 2 x 250 ml LB broth containing Chloramphenicol

(15μg/ml) were placed in 2 x 1 litre Erlenmeyer flasks and seeded with 5 ml starter

culture each.  The flasks were then placed in a shaking incubator at 37ºC and 250 rpm

until an optical density of 0.6 was reached (4-6 hours).  Cultures were transferred to 2 x

250 ml sterile centrifuge bottles and centrifuged at 4000-5000 x g for 10 minutes.

Supernate was then decanted and discarded leaving the bacterial cell pellets, which were

stored at -20ºC overnight if required.  A standard commercial kit was used to recover the

MDV BAC from the bacterial cells according to manufacturer's instructions (Phase Prep

BAC DNA Kit Midi Scale Preparation, Sigma-Aldrich or Large-Scale DNA Construct

Kit, Qiagen).  The bacteria cell pellets were resuspended and subjected to a modified

alkaline-SDS lysis procedure with RNase contained in the initial resuspension solution.

Afterwards the lysate was centrifuged at 15,000 x g for 30 minutes to remove cellular

debris from the sample.  The cleared lysate was removed and nucleic acids precipitated

by the addition of isopropanol.  Another centrifugation at >15,000g was performed to

pellet the DNA after which the supernate was discarded and the pellet was washed with

ethanol and air dried before eluting in TE buffer (section 2.10.1).  Residual RNA was

then removed from the DNA by a short digestion at 60ºC with an RNase cocktail

(Sigma-Aldrich or Qiagen).  A sodium acetate buffer solution (Sigma-Aldrich or

Qiagen) was added before endotoxins and impurities were removed with a single

temperature-mediated extraction and phase separation.  Finally, the BAC DNA was

precipitated from solution with isopropanol and washed with ethanol before air-drying

the pelleted product. The pelleted product was dissolved in 200 to 300 µl elution buffer

(Sigma-Aldrich or Qiagen) and stored at 4ºC or -20ºC for later use.

### 2.2.6 Preparation of Bacterial Stocks for Long Term Storage

Bacterial stocks were prepared for long-term storage at -80ºC by picking a fresh colony

from an overnight LB agar plate and placing it in 2-5 ml of sterile LB broth with

antibiotics and incubating it at 37ºC and 250 rpm in a shaking incubator for 6-10 hours.

0.85 ml of the subsequent culture was combined with 0.15 ml of sterile glycerol and

mixed thoroughly by pipetting up and down in a screw top microcentrifuge tube. The

microcentrifuge tube was then labelled and stored long term at -80ºC. Once frozen,

samples could be obtained from the master stock by scraping a small amount of frozen

sample with a sterile wire loop, spread on a fresh agar plate, and incubated overnight at

37 º C.

## 2.3  Protein Blots

The following protein blotting protocol was adapted from standard lab protocol used in

our lab and Molecular Cloning 3rd edition (Sambrook and Russell, 2001).

### 2.3.1 Protein Electrophoresis

CEF or RPL-1 cell pellets were harvested following experimental treatment by

immediately washing twice with SPBS and centrifuging at 480 x g at room temperature

for 5 minutes to pellet cells and then pouring off the supernate.  Following the washes all

SPBS was removed and 200-500 μl of Western Blot Lysis Buffer (section 2.10.2) was

added.  The sample was then placed in a screw-top microcentrifuge tube, mixed using a

vortex mixer and stored at -80ºC until use.   Just prior to electrophoresis the samples

were placed in a boiling water bath for 3-5 minutes.  8.5 cm x 7 cm plates with 7.5mm

spacers (Bio-rad Laboratories) were cleaned and assembled in a Mini Protean-3 Cell and

Electrophoresis Module Assembly (Bio-rad Laboratories).  A 12% Acrylamide resolving

gel (section 2.10.2) was poured to the level of the base of the combs immediately after

the addition of 10% ammonium persulphate and N, N, N', N'

Tetramethylethylenediamine (TEMED).  After pouring the gel was overlaid by 200 μl of

$H_2O$ saturated methyl butanol.  Once the gel had polymerized (approximately 5-15

minutes) the saturated butanol was removed and the top of the gel was rinsed with either

$H_2O$ or Tris-Glycine SDS Buffer (Severn Biotech).  After rinsing, a stacking gel (section

2.10.2) was prepared and poured on top of the resolving gel and the sample combs were

set in place.  The stacking gel was allowed to polymerise for an additional 5-15 minutes.

The gels were then assembled in the Bio-rad vertical gel tank and the tank was filled

with Tris-Glycine SDS Buffer (Severn Biotech).  The combs were removed and the

wells rinsed with Tris-Glycine SDS Buffer (Severn Biotech) before placing 10-20μl of

each sample per well using a 1 ml glass syringe (Hamilton).  10 μl of Broad Protein

Marker (Bio-rad Laboratories) or Pre-stained Protein Marker, Broad Range (7-175 kDa)

(New England Biolabs) was also placed in the far left lane as an aid to identifying target

protein bands.  Current was applied to the gel at approximately 70 mAmps (150 V) until

the blue dye front had reached the bottom of the gel (approximately 1 hour).  Gels were

then either stained with Coomassie stain for 30 minutes followed by de-stain overnight

(section 2.10.2) or the proteins were transferred to a nitrocellulose membrane for further

analysis (section 2.3.2).


### 2.3.2  Transfer of Protein to Nitrocellulose Membranes

Following protein electrophoresis the separated proteins were transferred to a

nitrocellulose membrane.  A piece of Hybond C nitrocellulose membrane (Amersham

Biosciences) was cut to the size of the resolving gel (approximately 9 x 6 cm) along with

6 pieces of slightly larger chromatography paper and these were all soaked briefly in

transfer buffer (section 2.10.2) prior to transfer.  The semidry electroblotter was then

prepared by placing 3 sheets of moistened chromatography paper in the centre of the bed

followed by the nitrocellulose membrane and the polyacrylamide gel.  The remaining 3

sheets of chromatography paper were placed on top and lightly rolled with the edge of a

plastic pipette to remove any residual air bubbles that might interfere with contact

between the sheets.  The cathode assembly was then placed on top and secured before

current was applied at 110 mA for 60 minutes.  Following transfer the nitrocellulose

membrane was carefully removed and the chromatography paper and polyacrylamide

gel discarded.    The nitrocellulose membrane was first rinsed with PBS + 0.1% (v/v)

Polyoxyethylenesorbitan Monolaurate (TWEEN 20, Sigma-Aldrich) and then washed

gently on a 20-50 rpm rocking table for 30 minutes in Blocking Buffer (section 2.10.2).

The nitrocellulose membrane was later sealed into a plastic envelope with 5-10ml of

fresh blocking buffer and stored flat at -20ºC until immunological detection of protein blots the following day.

### 2.3.3  Immunological Detection of Protein Blots

For detection of the Meq protein, nitrocellulose membranes were thawed from frozen to room temperature slowly and incubated in fresh blocking buffer for 20-30 minutes on a rocking table at 20-50 rpm.  The primary antibody Meq Monoclonal Mouse Anti-Meq FD7 (Kindly provided by Dr. Venugopal Nair's group at the Institute for Animal Health, Compton, UK) was diluted 1:150 in blocking buffer and incubated with the nitrocellulose membrane fully covered for 60 minutes on a rocking table at 20-50 rpm. After incubation the membrane was washed in PBS + 0.1 % TWEEN 20 for 3 washes of 5 minutes each on a rocking table at 20-50 rpm.  After the washes were complete the secondary antibody, Polyclonal Rabbit Anti-mouse Immunoglobulins HRP (Dako Cytomation) was made up at a 1:1000 dilution in Blocking Buffer (section 2.10.2).  5-10 ml of secondary antibody was incubated with the nitrocellulose membrane at room temperature on a rocking table at 20-50 rpm for 60 minutes.  After incubation the membrane was again washed in PBS + 0.1 % TWEEN 20 for 3 washes of 5 minutes each on a rocking table at 20-50 rpm.  After the final wash the proteins were visualized using Chemiglow West Substrate (Alpha Innotech) according to standard manufacturer instructions.  After incubating the nitrocellulose membrane for 1-2 minutes with a 1:1 mixture of the luminol/enhancer solution and stable peroxide solution the membrane was photographed using the Fluorchem HD2 Imaging System (Alpha Innotech).  A light

photograph was also taken to visualize the protein marker.  After photographing several different exposures over a period of up to 30 minutes the nitrocellulose membrane was discarded once the chemiluminescence reaction had faded.


## 2.4  Tissue Culture and Virus Growth Techniques

### 2.4.1  Growth of Established Cell Lines

Unless otherwise indicated all cell culture was performed under aseptic conditions in a Class II containment cabinet.  All cell lines unless otherwise noted were stored in liquid nitrogen for long-term storage and thawed for use.  Unless otherwise noted all cells were grown in either 6-well dishes (9.6 cm$^2$/well, Nunclon, Thermo Fisher Scientific) or tissue culture flasks (25 cm$^2$, 75 cm$^2$, 150 cm$^2$ Nunclon, Thermo Fisher Scientific or BD Falcon, BD Biosciences).  Unless otherwise noted all tissue culture protocols were adapted from standard laboratory protocols.


### 2.4.2  Preparation of Cell Lines for Liquid Nitrogen Storage

Healthy cells in log phase growth were suspended in a known volume of media (usually 10 ml) and counted using a haemocytometer.  Once counted the cells were centrifuged at 480 x g at room temperature for 5 minutes to pellet cells and the media poured off.  The amount of freezing media added to give a concentration of 2-5 x $10^6$ cells/ml was calculated and freezing media (90% Foetal Calf Serum (FCS) (Sera Laboratories International) and 10% DMSO mix) was combined with the cells and gently inverted to mix.  1 ml of the cell mixture was then added to each cryotube (Nunclon, Thermo Fisher

Scientific or BD Falcon, BD Biosciences) which was then placed in a -80ºC freezer for 24-72 hours before moving on dry ice to the appropriate liquid nitrogen freezer for long-term storage.

### 2.4.3  Growing Cell Lines from Liquid Nitrogen Frozen Stocks

Once removed from liquid nitrogen cells were thawed immediately and quickly in a 37ºC water bath.  When thawed the osmotic pressure was equilibrated by adding a few drops of pre-warmed media before transferring to a universal tube containing 20ml of pre-warmed cell culture media.  The cells were then centrifuged at 480 x g at room temperature for 5 minutes to pellet cells and then the media was poured off.  The cell pellet was subsequently resuspended in 5-10 ml of fresh media and moved to a T25 flask using a pipette.  Cells were then incubated overnight either at 38.5ºC for avian lines or 37ºC for mammalian lines in a 5% $CO_2$ humidified incubator.  The next day cells were checked for growth and contamination.  Once cells were growing well they were split and moved up to the next sized flask.

### 2.4.4  Counting Cells Using Haemocytometer

Cells to be counted were diluted 1:1 with 0.4% Trypan Blue stain and mixed thoroughly with a pipette before loading 50-100 µl into the haemocytometer by allowing the chamber to fill by capillary action, taking care not to flood the chamber.  A haemocytometer chamber divided into 25 smaller squares having an area of 1mm² total was used.  When the cover slip was loaded to form newton rings the depth of the

chamber was 0.1mm making the volume of each large square $0.1mm^3$ or 0.0001ml.

Total live cells in the 25 squares were counted and the number of cells/ml calculated

using the following formula:  Counted cells x dilution factor of $2 \times 10^4$.  For each cell

count determination at least 2 chambers were counted and the average was taken of the

chamber readings.


### 2.4.5  Transformed Chicken Lymphocyte Cells

Three different suspension transformed chicken lymphocyte cell lines were used:  RPL-

1, Rb1b T Cells and MSB-1.  The original cell stocks were all obtained from Dr.

Venugopal Nair's group at the Institute for Animal Health, Compton, UK and

maintained in liquid nitrogen until use.  RPL-1 cells are a non-producing chicken T cell

lymphoblastoid cell line transformed by MDV strain JM (Nazerian *et al*., 1976).  MSB-1

cells were developed from a chicken T cell line transformed by MDV (Akiyama and

Kato, 1974).  The Rb1b T cells had been isolated from an MDV infected chicken T cell

line by Dr. Nair's group.  Growing conditions for all 3 cell lines were the same.  The

media used was RPMI (Gibco, Invitrogen) supplemented with 10% (v/v) FCS (Sera

Laboratories International), 10% (v/v) tryptose phosphate broth (Gibco, Invitrogen), 100

mM sodium pyruvate (Gibco, Invitrogen) and 10 U/ml penicillin/streptomycin (Gibco,

Invitrogen) (section 2.10.5).  The incubation conditions were 38.5ºC and 5% $CO_2$.

Seeding density was around $1 \times 10^4/cm^2$ in a tissue culture flask.

### 2.4.6  Chicken Embryo Fibroblasts

CEFs were prepared by incubating eggs obtained from The Roslin Institute's laying flock for ten days under standard commercial conditions.  At day ten the eggs were transferred to the laboratory and prepared for harvesting aseptically in a tissue culture cabinet.  The eggs were cracked at the air space and embryos were immediately and humanely euthanized by severing the head.  Limb buds and internal organs were then removed and the embryo was washed twice in SPBS (section 2.10.5).  The embryo was then placed in an Erlenmeyer flask containing 20ml of 0.05% trypsin ethylenediaminetetraacetic acid (EDTA) solution (Gibco, Invitrogen) and incubated at 38ºC for 10 minutes with occasional mixing.  At the end of incubation the trypsin solution was removed using a 10 ml pipette and placed in a universal vial with 1 ml FCS (Sera Laboratories International) to neutralize the trypsin.  This process was repeated five times.  The first wash was discarded as it contains a high proportion of blood and damaged cells.  The tissue remaining after five washes was also discarded.  The cells were then centrifuged at 480 x g for five minutes and the trypsin solution was discarded. The cell pellets were then combined and resuspended in 10 ml of CEF standard Media 199 (Gibco, Invitrogen) with 5% added FCS (Sera Laboratories International) (section 2.10.5).  The cell suspension was then diluted 1:10 and counted with a haemocytometer. Once the cell density was known then six-well dishes were seeded at a density to give $3.5 \times 10^5$ cells/9.6 cm$^2$ well in 2 ml of CEF growth Media 199 (Gibco, Invitrogen) with 5% added FCS.  This was the density that was found to reliably grow to 80-85% confluence when incubated at 38.5ºC and 5% $CO_2$ in humidified air for 24-36 hours.

CEF were grown in M199 medium (Gibco, Invitrogen) supplemented with either 5%

(v/v) for growth or 2% (v/v) for maintenance FCS (section 2.10.5).  This protocol was

adapted from standard lab procedures developed by Dr. Venugopal Nair's group at the

Institute for Animal Health in Compton, UK.


## 2.5 *In vitro* Experiments

### 2.5.1 Transfection of CEFs using Lipofectamine

One day prior to transfection 3.5 x $10^5$ aseptically prepared CEF cells were seeded into

one well of a six-well dish and grown overnight at 38.5ºC and 5% $CO_2$ in humidified air.

The following day the cells were approximately 80% confluent and ready for

transfection.  For each well 1-2 μg of DNA to be transfected was brought to a total

volume of 100 μl with serum-free media (Optimem, Gibco, Invitrogen).  In a separate

tube 5 μl of Lipofectamine (Invitrogen) was combined with 100 μl serum-free media

and incubated at room temperature for 5 minutes to allow complexes to form.  The

contents of the tubes were then combined and incubated for 20 minutes at room

temperature.  During incubation the media in the CEF wells was changed to 0.8 ml

serum-free media.  After incubation the DNA/Lipofectamine mixtures were added to the

appropriate wells and incubated for three to four hours at 38ºC and 5% $CO_2$ in

humidified air.  The media was then changed to 2 ml CEF media M199 with 5% FCS

and incubated at 38ºC and 5% $CO_2$ in humidified air overnight.  The following morning

the media was changed to CEF media M199 with 2% FCS and incubated for an

additional two to three days. At three to four days post-transfection cells were harvested for analysis at 90-100% confluence.

### 2.5.2 Transfection of CEFs using Electroporation

CEF cells which had been either freshly prepared or thawed from liquid nitrogen were grown for 24-48 hours in standard tissue culture conditions (section 2.4.10). Immediately prior to electroporation cells were treated with 0.05% trypsin EDTA solution (Gibco, Invitrogen) to detach cells from the growing surface and counted using a haemocytometer (section 2.2.4). An average of two counts was taken for calculating cell numbers. Once counted the CEF cells were aliquoted to give $3.5 \times 10^6$ cells per single transfection or $1.05 \times 10^7$ cells per triplicate experiment and centrifuged at 100 x g for 10 minutes at room temperature. After centrifugation media was carefully and completely drained. 27mer siRNA oligonucleotides (Stealth siRNA, Invitrogen) were diluted with 500ul RNase-Free water (Invitrogen) to give a 40nM working stock which was flash frozen on dry ice and stored at -80ºC for later use. Before transfection 1.4 ml of fresh, antibiotic-free CEF growth media was placed in each well of a 6-well plate (Nunclon, Thermo Fisher Scientific) and stored in a 38.5ºC/5% $CO_2$ incubator until required. 500µl of fresh antibiotic-free CEF growth media was also placed in a 1.2ml microcentrifuge tube and placed in a 38.5ºC/5% $CO_2$ incubator until needed. After centrifugation 300µl Nucleofector Fibroblast Solution (Amaxa, Lonza) was added to each triplicate cell pellet along with 300pMol Stealth siRNA (100pMol/Reaction). Cells were then mixed briefly and gently to resuspend cell pellets and then 100µl per

transfection was pipetted into a cuvette. The cuvette was then placed into a

Nucleofector II electroporation unit (Amaxa, Lonza) and treated using program T-016.

Immediately after transfection a disposable plastic pipette was used to gently suck up

500μl media of pre-warmed media which was then added into the cuvette. The media

and cell mixture was then gently removed and added to 1.4 ml pre-warmed media in the

6-well dish. Total volume at this point was 2 ml containing approximately $3.5 \times 10^6$

cells. Cells were then incubated for 24 hours at 38.5ºC and 5% $CO_2$. This protocol was

adapted from standard manufacturer's protocol from Amaxa (Lonza) and Invitrogen.


### 2.5.3   Transfection of RPL-1 and Rb1b T cells using Electroporation

Original stocks of RPL-1 and Rb1b T cells (section 2.4.5) obtained from Dr. Venugopal

Nair's lab at the Institute of Animal Health, Compton, UK were frozen in aliquots

(section 2.4.2 and 2.4.3) when growing well in log phase growth. For each experiment a

fresh aliquot of cells was thawed and passaged 7-10 times. The seeding density for each

passage was approximately 3 to $3.5 \times 10^5$/ml of media in a T75 flask with 20 ml of

media supplemented with penicillin/streptomycin. Cells were then grown in a T150

flask for the final passage (in 40ml media supplemented with penicillin/ streptomycin)

and harvested between $5 \times 10^5$ to $1 \times 10^6$ cell density per ml with >90% live viable cells

of cell total. Stock Stealth siRNA oligonucleotides (27mers) (Invitrogen) were prepared

as previously described (section 2.5.2). For the RPL-1 and Rb1b T cells a transfection

indicator SiGLO Red (Dharmacon, Thermo Fisher Scientific) was also used in the

experiments. SiGLO Red 50μl stock solutions were prepared by diluting with SPBS and

frozen at -20ºC in aliquots until use. RPL-1 and Rb1b T cells were counted and prepared for electroporation as described for CEF cells in section 2.5.2. After centrifugation 300μl Nucleofector Solution V (Amaxa, Lonza) was added to each triplicate cell pellet along with 300pmol Stealth siRNA (100pmol/Reaction) and SiGLO Red at 500nM overall concentration (3-6 μl of 50μM stock per triplicate). Electroporation of cells was done as described for CEF in section 2.5.2 except that cells were treated with program A-033. After transfection cells were incubated as described for CEF in section 2.5.2. Total volume per well after transfection was 2 ml containing approximately $3.5 \times 10^6$ cells. Cells were then incubated for 24 hours at 38.5ºC and 5% $CO_2$. 50μl aliquots of cells were removed for counting as described in section 2.4.4 at 24, 48, 72, 96 and 120 hours post-transfection. At 24 hours a 100μl aliquot of cells from each transfection was removed for determination of transfection efficiency. This was done by making a wet mount slide with the cell suspension on a glass slide and placing a cover slip over the sample. Four 20x fields were photographed and counted both under bright field and ultraviolet light with a red filter. A percentage determination of transfection efficiency was then made on the basis of a cumulative average of the 4 random fields. Depending on growth cells were split every 24 hours during the experiment into an appropriate volume to ensure an average density of $3.5 \times 10^5$ cells/ml to $1 \times 10^6$ cells per ml after the aliquots were removed. An additional aliquot of cells from each transfection of no more than 20% of total starting cell volume was removed at 48, 72 and 96 hours for RNA extraction as described in section 2.1.3. All cell samples taken for RNA were placed immediately on wet ice and processed as quickly as

possible.  This protocol was adapted from manufacturer's protocol from Amaxa (Lonza), Invitrogen and Dharmacon (Thermo Fisher Scientific).

2.6  **Dual Reporter Luciferase Assays**

Dual-Luciferase® Reporter Assay System (DLR) (Promega) was used to measure the response of an N-terminal fusion reporter plasmid containing a gene of interest transfected into CEF cells.  The use of two luciferase plasmids, one based on the firefly reporter (*Photinus pyralis*) and a renilla plasmid (*Renilla reniformis*) allow a correction factor for variable transfection efficiency and transcription activity.   The DLR assays were performed as follows:

CEF cells were prepared using standard methods (section 2.4.10) and passaged for 24-36 hours in 5% growth media (section 2.10.5) before being frozen and stored in liquid nitrogen in approximately $5 \times 10^6$ cell aliquots.  24 hours before starting the experiment CEF cells were thawed and counted as detailed in section 2.4.3.  Cells were then aliquoted into a 6-well plate (35mm diameter) with $3.5 \times 10^5$ cells per dish.  Cells were passaged in 5% growth media without antibiotics in a $38.5/5\%$ $CO_2$ incubator until reaching 50-60% confluence around 24 hours post-thawing.  Reporter plasmid transfection was carried out as follows:  100µl Optimem serum-free media (Gibco, Invitrogen) and 5µl Lipofectamine (Invitrogen) per well were gently mixed in 1.5 ml microcentrifuge tube and incubated for 5 minutes at room temperature (~25ºC) to form complexes.  In a separate microcentrifuge tube DNA and siRNA to be transfected were

mixed gently with Optimem (Gibco, Invitrogen) to form a total volume of 100μl

containing 100pmol stealth siRNA (Invitrogen) and 0.8 to 1.6 μg of N-terminal fusion

reporter plasmid DNA.  While the Optimem solutions were incubating, the cell culture

media from each experimental well was removed and replaced with 0.8 ml

Optimem/well and placed back in the 37ºC/5% $CO_2$ incubator.  After incubation the

dilute DNA/siRNA complex was gently mixed with the dilute Lipofectamine and

incubated a further 20 minutes at room temperature.  After incubation 0.2ml of the

Lipofectamine complex was added to each corresponding well and incubated at 38.5ºC /

5% $CO_2$ for 5-8 hours.  At end of the incubation media was changed to 2ml/well 5%

CEF growth media and incubated at 38.5ºC / 5% $CO_2$ to 24 hours post-transfection.  At

24 hours media was changed to 2% CEF maintenance media with antibiotics and

incubated at 38.5ºC / 5% $CO_2$ to 48 hours at 38.5C / 5% $CO_2$.  Cells were harvested at

80-90% confluence at approximately 48 hours.   At 48 hours cell lysates were prepared

using passive lysis buffer and scraping using a plastic pipette according to standard

manufacturer instructions.  If required, cell lysates were occasionally stored for up to 1

month at -80°C in a microcentrifuge tube before carrying out the DLR assay.

Luminescence readings were performed according to manufacturer instructions using a

Glomax 20/20 Luminometer (Promega).

**2.7** <u>**Confocal Laser Microscopy Techniques**</u>

### 2.7.1 Suspension Cell Lines

Suspension Rb1b T cells were transfected with plasmids as described in section 2.5.3 and grown at 38ºC and 5% $CO_2$ for 24 or 48h. Cells were harvested by placing an aliquot of approximately $3.5 \times 10^6$ cells in a microcentrifuge tube and pelleted using a centrifuge at 3000 x g for 3 minutes. The supernate was removed and 1 ml of SPBS was added. The cells were mixed gently then pelleted again using a microcentrifuge (3000 x g for 3 min). Supernate was removed and 500μl of 4% (w/v) paraformaldehyde solution was added. Samples were placed on a rotating wheel at 10 rpm to gently mix for 10 minutes at room temperature. After fixing cells were pelleted and washed in SPBS as described above. Cells were resuspended in 100μl of SPBS and dropped on to a lysine-coated microscope slide (Polysine, VWR International) and air-dried. Once dry the cells were fixed a second time on the microscope slide by dropping 200μl of 4% (w/v) paraformaldehyde on to the slide and incubating them at room temperature for 10 minutes. Slides were washed in SPBS for 3 minutes after incubation. Before staining, cells were permeabilised by incubation with 300μl SPBS containing 0.25% (v/v) Triton X-100 (t-Octylphenoxypolyethoxyethanol, Sigma-Aldrich) for 5 minutes at room temperature followed by a wash with SPBS as described above. Cells were counter-stained for nuclear DNA by incubating with 200μl of SPBS with 1% (w/v) bovine serum albumen, 10μg/ml RNaseA (Invitrogen) and TO-PRO-3 iodide (Molecular Probes®, Invitrogen diluted 1:1,000 to 1μM) at room temperature in the dark for 15 minutes. Cells were then gently rinsed with SPBS as described above. Cells were then air-dried and mounted with cover

slips using VectaShield Hard Mount Mounting Medium (Vector Labs) or Mowiol

Mounting Medium with DABCO (Sigma-Aldrich) (section 2.10.2) and sealed with nail

varnish.  Slides were stored in the dark at room temperature for the first 24 hours to allow

the mounting medium to harden and then at 4ºC.  Slides were examined using a Leica

Confocal Laser Scanning Microscope at either 63x or 100x power at the Impact Imaging

Facility, University of Edinburgh with the assistance of Mrs. Trudi Gillespie.  Images were

analyzed using both the Zeiss LSM Image Browser and the Carl Zeiss AxioVision Version

4.6 software.  Protocol was adapted from standard protocols used in our lab.


### 2.7.2  Adherent Cell Lines

CEFs (either freshly prepared or frozen) were transfected  with 0.5 to 2.0 μg plasmids

either using Lipofectamine (Invitrogen) (section 2.5.1)  or electroporation (section 2.5.2)

and grown at 38ºC and 5% $CO_2$ for 24 to 48h in 2 ml CEF 2% maintenance media on

sterile plain glass cover slips placed in a 6-well dish.  Cells were harvested by removing

the media and rinsing twice with 2 ml of SPBS.   After rinsing all excess SPBS was

drained and discarded.  After the supernate was removed 1.5 ml of 4%

paraformaldehyde solution was added.  Slides were incubated for 10 minutes at room

temperature.  After fixing cells were washed in SPBS as described above.  Excess SPBS

was removed and cover slips were air-dried.  Before staining cells were permeabilised

by incubation with 500ul SPBS containing 0.25% (v/v) Triton X-100 for 5 minutes at

room temperature followed by a wash with SPBS as described above.  Cells were

counter-stained for nuclear DNA, mounted and examined as previously described for

suspension cells (section 2.7.1).

## 2.8  Statistical Analysis

Data was subjected to statistical analysis using the MiniTab software program version

15.1.1.0 . Dr. Darren Shaw (University of Edinburgh, Royal (Dick) School for

Veterinary Studies) kindly assisted me in analyzing the data.

## 2.9  Recipes

### 2.9.1  Commonly Used Solutions

| Phosphate Buffered Saline Solution (Prepared centrally in our laboratory) | Amount |
|---|---|
| NaCl | 80.0 g |
| KCl | 2.0 g |
| $Na_2HPO_4$ | 11.5 g |
| $KH_2PO_4$ | 2.0 g |
| pH adjusted to 7.2 with HCl or NaOH | |
| $H_2O$ to 10 litres | |

| Mowiol Mount | Amount |
|---|---|
| Mowiol 4-88 | 2.4 g |
| Glycerol | 6.0 g |
| Distilled $H_2O$ | 6.0 ml |
| 0.2M Tris, pH 8.5 | 12.0 ml |
| 1,4-diazabicyclo-[2.2.2]octane (DABCO, Sigma-Aldrich)* | 2.5% (w/v) |

*1, 4-diazabicyclo-[2.2.2] octane (DABCO, Sigma) added as an anti-fade reagent prior to use. Aliquots of 1 ml were stored at -20ºC in screw top tubes.  Protocol adapted from our laboratory protocol and (Sambrook and Russell, 2001).

| 4% Paraformaldehyde Solution | Amount |
|---|---|
| Paraformaldehyde | 4.0 g |
| Distilled $H_2O$ | 50.0 ml |
| 1M NaOH | 1.0 ml |
| 10X PBS | 10.0 ml |
| pH adjusted to 7.4 using HCl and final volume brought to 100 ml with $H_2O$ | |

Aliquots of 10ml were stored at-20ºC before use.  Protocol adapted from (Sambrook and Russell, 2001).

| TE Buffer (Supplied in Qiagen Kits) | Amount |
|---|---|
| Tris, pH 8.0 | 10mM |
| EDTA | 1 mM |

| EB Buffer (Supplied in Qiagen Kits) | Amount |
|---|---|
| Tris-Cl, pH 8.5 | 10mM |

### 2.9.2  Protein Electrophoresis

| Western Blot Lysis Buffer | Amount |
|---|---|
| SDS 10% Solution | 4.0 ml |
| Glycerol | 4.0 ml |
| 0.125M Tris HCl pH 6.8 | 2.5 ml |
| Bromophenol Blue | 1-2 grains |
| $H_2O$ | 8.5 ml |
| 2-Mercaptoethanol | 1.0 ml |

Aliquot into 1ml screw top tubes and store at -20ºC for up to 6 months

| Ammonium Persulfate (10% w/v) | Amount |
|---|---|
| Ammonium Persulfate (Sigma) | 1g |
| $H_2O$ | To 10 ml |

Store at 4ºC for up to 7 days

| 12% Polyacrylamide Resolving Gel | Amount |
|---|---|
| $H_2O$ | 3.3 ml |
| 30% Acrylamide/Bis-Acrylamide (Sigma) | 4.0 ml |
| 1.5M Tris-HCl pH8.8 | 2.5 ml |
| 10% SDS | 100 $\mu$l |
| 10% Ammonium Persulphate | 100 $\mu$l |
| TEMED | 4 $\mu$l |

| Stacking Gel | Amount |
|---|---|
| $H_2O$ | 3.4 ml |
| 30% Polyacrylamide Mix | 830 $\mu$l |
| 1.0M Tris-HCl pH6.8 | 630 $\mu$l |
| 10% SDS | 50 $\mu$l |
| 10% Ammonium Persulphate | 50 $\mu$l |
| TEMED | 5 $\mu$l |

| Coomassie Stain | Amount |
|---|---|
| Coomassie Blue R250 | 2.5g |
| Methanol | 500 ml |
| Acetic Acid | 50 ml |
| $H_2O$ | To 1 litre |

Stain dissolved in methanol and added to acetic acid and water and passed through filter paper before use to remove any debris.

| Destaining Solution (for 1 litre) | Amount |
|---|---|
| Acetic Acid (10% v/v) | 100ml |
| Methanol (20% v/v) | 200ml |
| $H_2O$ | 700ml |

| SDS Running Buffer 5X | Amount |
|---|---|
| Tris | 60g |
| SDS | 10g |
| Glycine | 280g |
| $H_2O$ | to 2 litres |

| Semi-Dry Transfer Buffer 1X | Amount |
|---|---|
| 25mM Tris Base (w/v) | 1.5g |
| 150mM Glycine (w/v) | 5.6g |
| Methanol 10% (v/v) | 50 ml |
| $H_2O$ | To 500 ml |

| Blocking Buffer | Amount |
|---|---|
| Skimmed Milk Powder (5% w/v) | 5g |
| PBS + 0.1% v/v TWEEN 20 | 100 ml |

### 2.9.3  Nucleic Acid Electrophoresis

| Tris-Acetate EDTA (TAE) Running Buffer | Amount |
|---|---|
| Tris, pH 8.0 | 10mM |
| EDTA | 1 mM |

| 5 x Loading Buffer (Prepared centrally in our laboratory) | Amount |
|---|---|
| Ficoll dissolved in TAE buffer | 15 ml |
| Saturated solution of Orange G | 0.25 ml |
| $H_2O$ | To 100 ml |

### 2.9.4 Bacterial Media

| Luria-Bertani (LB) Broth (Marek) (Prepared centrally in our laboratory) | Amount |
|---|---|
| Tryptone | 1.0% |
| Yeast Extract | 0.5% |
| Sodium Chloride | 1.0% |
| pH 7.0 | |

| Luria-Bertani (LB) Agar Plates | Amount |
|---|---|
| LB Broth (as above) | 500 ml |
| Agar | 7.5 g |

Mixture was autoclaved and cooled to approximately 60ºC before adding antibiotic and pouring aseptically into plates. Plates were stored at 4ºC in the dark for up to 14 days.

### 2.9.5 Tissue Culture Media

| Transformed Chicken Lymphoblastic Cell Media | Amount |
|---|---|
| RPMI 1640 Media (Gibco, Invitrogen) | 500 ml |
| Tryptose Phosphate Broth (Gibco, Invitrogen) | 50 ml |
| Sodium Pyruvate 100 mM (Gibco, Invitrogen) | 5 ml |
| FCS | 50 ml |
| Penicillin/Streptomycin 10U/ml (Gibco, Invitrogen) | 5 ml |

Seeded at $1 \times 10^4$ cells/flask cm$^2$

| CEF Media (5% Growth/ 2% Maintenance) | Amount |
|---|---|
| M199 Media (Gibco, Invitrogen) | 500 ml |
| Tryptose Phosphate Broth (Gibco, Invitrogen) | 50 ml |
| 7% Sodium Bicarbonate Solution (Sigma-Aldrich) | 13.5 ml |
| FCS | 25 ml (growth)/ 10 ml maintenance) |
| Penicillin/Streptomycin 10U/ml (Gibco, Invitrogen) | 5 ml |

Seeded 6-Well Dish at $3.5 \times 10^5$ cells/dish

# Chapter 3 – siRNA Studies of Selected MDV Genes

**Results**

## 3.1  Project Objectives

The objective for this portion of the study was to identify uncharacterised MDV genes expressed during latent infection.  Following identification of such genes siRNA techniques were used to investigate their function.  Post-transcriptional targeting using a 25-mer siRNA oligonucleotide was used to remove the gene product from persistently MDV-infected chicken lymphoblastic cells *in vitro*.  The cells were then screened for any phenotypic changes when the gene product was removed.  Cell growth rate was used as the primary phenotypic indicator.


## 3.2  Selection of Genes for Study

The sequencing of the CVI988 (Rispens) vaccine strain of MDV (Spatz *et al*., 2007b) made it possible to compare the sequence of an attenuated strain with that of pathogenic strains such as Md5 (Tulman *et al*., 2000), Rb1b (Spatz *et al*., 2007a) and GA (Lee *et al*., 2000).  Multiple differences between the MDV strains have been identified, and some of these differences, particularly in Meq, have been extensively studied (Kingham *et al*., 2001; Spatz and Silva, 2007b).  However, there were many other genes that had not been fully characterised which became the selection pool for this study.  All candidate genes were subjected to a literature search to assess prior knowledge (chapter 1) and a protein motif scan to identify any recognized similarities between the protein sequence and known protein motifs using the CVI988 Rispens strain MDV genome (GenBank DQ530348.1)

and the "Prosite MyHits" online database (Pagni *et al*., 2007) and a prediction of

transmembrane protein regions generated by the ExPASy TMPred software based on the

TMbase online database (Hofmann and Stoffel, 1993). Proteins with a predicted motif or

potential transmembrane region were preferred over proteins without a recognizable motif.

Fifteen MDV ORFs were selected for characterization based on the criteria of predicted

protein motifs/ transmembrane regions and not being extensively characterised in the

literature at the time of selection and are listed in table 3.1. Some of the candidate genes

(RLORF5a, 23kDa, RLORF6, MLTI, RLORF12, MNFH, LORF12 and ANTISENSE)

contained strain differences in the number of codons present which are illustrated in table

3.1 (Spatz *et al*., 2007a; Spatz *et al*., 2007b; Spatz and Silva, 2007b). In the case of

23kDa, RLORF6, MLTI, RLORF12 and MNFH these differences in codon length were

observed between the attenuated CVI988 strain and one or more of the pathogenic strains

of MDV which could potentially play a role in determining virulence. Table 3.2 contains a

summary of the results of the protein motif scanning and transmembrane region prediction

of the 15 genes selected for study which will be discussed further in section 3.8.

| Reference | GenBank Reference ORF | Position CVI988 Start | End | CVI988 (Codons) | Md5 (Codons) | Md11 (Codons) | GA (Codons) | Rb1b (Codons) | JM/102W (Codons) |
|---|---|---|---|---|---|---|---|---|---|
| **L1** | 3.8 | 4,043 | 4,366 | 107 | 107 | 107 | 82 | 107 | NI |
| **(repeat)** | 78.1 | 138,072 | 138,395 | 107 | 107 | 107 | 82 | 107 | 107 |
| **23kDa** | 4.0 | 5,355 | 5,942 | 195 | 136 | 136 | 136 | 136 | NI |
| **(repeat)** | 77.0 | 136,496 | 137,083 | 195 | 136 | 136 | 136 | 136 | 196 |
| **RLORF6** | 5.1 | 5,141 | 5,935 | 264 | 205 | 205 | 205 | 205 | NI |
| **(repeat)** | 77.5 | 136,503 | 137,297 | 264 | 205 | 205 | 205 | 205 | 265 |
| **RLORF11** | 6.4 | 11,257 | 11,568 | 103 | 103 | 103 | 103 | 103 | NI |
| **(repeat)** | 75.4 | 130,870 | 131,181 | 103 | 103 | 103 | 103 | 103 | 103 |
| **MLTI** | 6.5 | 11,921 | 12,907 | 328 | 64 | 64 | 64 | 592 | NI |
| **(repeat)** | 75.2 | 129,531 | 130,517 | 328 | 64 | 64 | 64 | 372 | 64 |
| **RLORF12** | 7.0 | 13,493 | 13,840 | 115 | 115 | 115 | 115 | 115 | NI |
| **(repeat)** | 74.0 | 128,603 | 128,806 | 67 | 115 | 115 | 115 | 115 | 76 |
| **LORF1** | 9.0 | 13,785 | 14,786 | 333 | 333 | 333 | 333 | 333 | NI |
| **LORF3** | 12.0 | 18,276 | 19,430 | 384 | 384 | 384 | 398 | 384 | NI |
| **MNFH** | 49.1 | 80,737 | 80,872 | 34 | 93 | 93 | 94 | 94 | NI |
| **LORF6** | 49.5 | 88,510 | 88,977 | 155 | 155 | 155 | 155 | 155 | NI |
| **LORF8** | 57.8 | 104,760 | 105,386 | 208 | 208 | 208 | 208 | 208 | NI |
| **LORF11** | 72.0 | 123,905 | 126,616 | 903 | 903 | 903 | 903 | 903 | NI |
| **LORF12** | 72.8 | 126,818 | 127,198 | 126 | 126 | 126 | 148 | 126 | NI |
| **ANTISENSE** | 83.0 | 151,837 | 152,175 | 112 | 112 | 112 | 112 | 112 | NI |
| **(repeat)** | 101.0 | 168,574 | 168,912 | 112 | 112 | NP | 112 | 112 | NI |
| **US2** | 91.0 | 158,593 | 159,405 | 270 | 270 | 270 | 270 | 270 | NI |

**Table 3.1:** Comparison between the 15 selected ORFs in the CVI988 (Rispens) vaccine strain and pathogenic strains as adapted from (Spatz *et al.*, 2007a; Spatz *et al.*, 2007b; Spatz and Silva, 2007b) (GenBank DQ530348.1, EF523390.1 and DQ534539.1). NP denotes not present and NI denotes not included. Strains with a difference in codon length from consensus are highlighted in red.

| Gene | Protein Motif Matches Phosphorylation (P), N-Glycosylation (G), N-Myristoylation (M) & Amidation (A) Sites | Nuclear localization signals | Predicted Trans-membrane Helices | Other Motifs |
|---|---|---|---|---|
| **L1 (repeat)** | 4 P (weak match), 1 G (weak match), 0 M 0 A | None | None | 1 Ankyrin repeat (weak match) & 1 Microbodies C-terminal targeting signal (weak match) |
| **23kDa (repeat)** | 5 P (weak match) 2 G (weak match) 7 M (weak match) 4 A (weak match) | None | None | 3 FMRFamide related peptide family (weak match), 1 NUMOD3 motif (weak match),Arginine & Glycine-rich Regions |
| **RLORF6 (repeat)** | 10 P (weak match) 1 G (weak match) 2 M (weak match) 0 A | None | 1 | Proline-Rich Region (strong match) & RNA polymerase Rpb4 (weak match) |
| **RLORF11 (repeat)** | 2 P (weak match) 2 G (weak match) 1 M (weak match) 0 A | 2 (strong match) | None | 1 Microbodies C-terminal targeting signal (weak match) & 1 Cell Attachment Sequence Motif (weak match) |
| **MLTI (repeat)** | 0 P 7 G (weak match) 7 M (weak match) 0 A | None | 7 | None identified |
| **RLORF12 (repeat)** | 5 P (weak match) 2 G (weak match) 1 M (weak match) 0 A | None | 1 | None identified |
| **LORF1** | 8 P (weak match) 2 G (weak match) 2 M (weak match) 0 A | 2 (weak match) | 2 | Serine-rich region (weak match) |
| **LORF3** | 17 P (weak match) 3 G (weak match) 2 M (weak match) 1 A (weak match) | 1 (weak match) | 2 | 2 DUF1509 protein of unknown function (strong match), 1 Protein prenyltransferases $\alpha$ subunit repeat profile (weak match), Arginine and Proline-rich regions. |
| **MNFH** | Not Available | Not Available | Not Available | Truncated protein in CVI988 strain, but not pathogenic strains. |
| **LORF6** | 2 P (weak match) 0 G 2 M (weak match) 0 A | None | 2 | None identified |

| Gene | Protein Motif Matches<br>Phosphorylation (P), N-Glycosylation (G), N-Myristoylation (M) & Amidation (A) Sites | Nuclear localization signals | Predicted Trans-membrane Helices | Other Motifs |
|---|---|---|---|---|
| **LORF8** | 1 P (weak match)<br>2 G (weak match)<br>2 M (weak match)<br>0 A | 2 (weak match) | 3 | None identified |
| **LORF11** | 31 P (weak match)<br>2 G (weak match)<br>4 M (weak match)<br>0 A | None | 1 | 2 Major Vault Protein repeat profile (weak match) |
| **LORF12** | 4 P (weak match)<br>2 G (weak match)<br>1 M (weak match)<br>0 A | None | 1 | None identified |
| **ANTISENSE (repeat)** | 5 P (weak match)<br>0 G<br>3 M (weak match)<br>0 A | None | None | None identified |
| **US2** | 4 P (weak match)<br>0 G<br>3 M (weak match)<br>0 A | None | 1 | Strong match for US2 family proteins found in other herpesviruses |

**Table 3.2:** Summary of the protein motif scanning results of the 15 selected MDV genes using the CVI988 Rispens strain genome (GenBank DQ530348.1) and the online databases TMPred and Prosite Motif Scan (Hofmann and Stoffel, 1993; Pagni *et al.*, 2007).

### 3.3   PCR Screening for Expression in Latency

PCR primers specific for each of the 15 candidate genes were designed using WebPrimer software (GenScript) and sourced from a commercial supplier (Eurofins/MWG).  Primers for each ORF were between 18-22 nucleotides and are outlined in table 3.3.  A BLAST search against the chicken genome (Wallis *et al.*, 2004) for each primer was undertaken to make sure that there was no homology with the chicken genome that might cross-react in the PCR reactions.  Conventional PCR (section 2.1.8) was then used to determine expression of the selected ORFs in several chicken cell culture models as shown in table 3.3.  DNA from the CVI988 strain MDV BAC (Petherbridge *et al.*, 2003) was used as a template to optimize the PCR reactions, ascertain functionality of the primer sets and act as a positive control.  Primer sets were designed using the CVI988 strain of MDV (GenBank DQ530348.1) and checked for predicted function with the Primer3 (http://frodo.wi.mit.edu/primer3, accessed 2/7/2007) (Rozen and Skaletsky, 2000) and WebPrimer (http://www.yeastgenome.org/cgi-bin/web-primer, accessed 27/6/2007) software packages as detailed in table 3.4. Homologous sequences to all the PCR primers were present in the Rb1b strain MDV genome (GenBank EF523390.1), so the primer sets were expected to work in DNA or cDNA isolated from Rb1b MDV Infected CEFs and Rb1b T cells.  The $IR_L$ region of JM/102W strain MDV (GenBank DQ534539.1) (Spatz and Silva, 2007b), which was the partial sequence of a clone of the prototype strain JM MDV found in RPL-1 cells, also confirmed homologous regions corresponding to the primer sets of the Meq, RLORF6, 23kDa, MLTI, LORF1, RLORF11, RLORF12 and RLORF5a genes.  The context of the

selected ORFs within the genome is also illustrated in figures 3.5a-e.  Of the 15 genes

examined it is important to note that RLORF6, 23kDa, MLTI, RLORF12, LORF1 and

LORF3 had some degree of overlap with 1-2 other ORFs on the same strand which have

been detailed previously in table 1.9.

| DNA Sequence | Strain MDV | Model |
|---|---|---|
| MDV BAC DNA | Rb1b (US2 inactive) | Positive Control |
| Rb1b MDV Infected CEF cDNA | Rb1b | Lytic Infection Model |
| Rb1b T cell Line cDNA | Rb1b | Reactivating Latent Infection Model (Latent Model 1) |
| RPL-1 Cell Line cDNA | JM | Non-Reactivating Latent Infection Model (Latent Model 2) |

**Table 3.3:** Experimental groups for determination of expression in a latent and lytic MDV infection utilizing PCR

| MDV ORF | Forward Primer | Position in MDV CVI988 Genome | Reverse Primer | Position in MDV CVI988 Genome | Predicted PCR Product Size (bp) |
|---|---|---|---|---|---|
| DNA Polymerase* | CAGAATGTGGCAAGCGAGTA | 71094-71113 | CAAAGCAGTCTGCCGATACA | (71287-71306) | 270 |
| Meq** | TGACCCTTGGACTGCTTACC | 136425-136444 (5994-6013) | GAGCAATGTGGAGCGTTAGG | 5911-5930 (136508-136527) | 100 |
| RLORF6 | GACGAGCATAAAGCCTCTCC | 5321-5340 (137098-137117) | ATCTTCCCTGCATTGTGTCC | 136967-136986 (5452-5471) | 151 |
| 23kDa | GGGCAGAAGAGGGAATGG | 5497-5514 (136924-136941) | CATATTATCTACGCTCCGG | 136622-136640 136802-136820 (5798-5816) (5618-5636) | 240 |
| LORF1 | CGGTGGTGTAACCGTGTAGG | 14037-14056 (128387-128406) | AAAGACAAGGATGGCTGTGG | 128216-128235 (14208-14227) | 191 |
| LORF3 | ATTTCACCCACCTGATGACC | 18518-18537 | GGGAATGTTACGATGAGACG | (18742-18761) | 244 |
| LORF11 | GGCATCGTGTCGTTAAATGG | 124288-124307 | ACGTAGGCTCTCAACCATCG | (124498-124517) | 230 |
| LORF12 | GGGATAGGGAATCGTATGAAGG | 123818-123839 126967-126988 | AATCGAGGAAGAACCTGTGC | (127099-127118) | 152 |
| ANTISENSE | AAATCAGGCGGGTTGTCC | 151977-151993 (168756-168772) | GCACTTGAACCGTAGCTTCC | 168593-168612 (152137-152156) | 180 |
| US2 | GTCCCAGACACTTTGATTGC | 158918-158937 | CAGGATGTTCCACAGAATGG | (159073-159092) | 175 |

| MDV ORF | Forward Primer | Position in MDV CVI988 Genome | Reverse Primer | Position in MDV CVI988 Genome | Predicted PCR Product Size (bp) |
|---|---|---|---|---|---|
| **MLTI** | GCAGATCGTAAGCATTGCC | 11969-11987<br>12101-12119<br>12233-12251<br>12365-12383<br>12497-12515<br>12629-12647<br>12761-12779<br>12893-12911<br>(130451-130469)<br>(130319-130337)<br>(130187-130205)<br>(130055-130073)<br>(129923-129941)<br>(129791-129809)<br>(129659-129677)<br>(129527-129545) | CTTATTAAATGTGAGTTCGGC | 129509-129529<br>129641-129661<br>129773-129793<br>129905-129925<br>130037-130057<br>130169-130189<br>130301-130321<br>130433-130453<br>(12909-12929)<br>(12777-12797)<br>(12645-12665)<br>(12513-12533)<br>(12381-12401)<br>(12249-12269)<br>(12117-12137)<br>(11985-12005) | 110 |
| **RLORF11** | CAATACACACTCGGTCATTCC | 11363-11383<br>(131055-131075) | GCAACTCCTCCATCAACG | 130919-130936<br>(11502-11519) | 157 |
| **RLORF12** | AGAGAAGGAACCTCGCAACC | 13603-13622<br>(128816-128835) | CAAATGAGCAGTGCGAACG | 128612-128630<br>(13813-13831) | 229 |
| **MNFH** | ATGAACTTTCACTGGTCGG | 80768-80786 | GTGATCCGATTGATGAAACC | (80844-80863) | 57 |
| **LORF6** | AAGCGAACAGAGTCCAGACG | 88734-88753 | CAGCAGTGGAGCTTATGTCG | (88902-88921) | 188 |
| **LORF8** | CGATCATACACCGCATGG | 105022-105039 | ACCCTAATCCAATCCATTCC | (105165-105184) | 163 |
| **L1** | TGCCACATCGTAGAGAAAGC | 4070-4089<br>(138349-138368) | AAATACCTCATCGCAGAGACG | 138121-138141<br>(4297-4317) | 248 |

**Table 3.4:** Primers used for PCR to amplify the corresponding MDV gene in screening for expression during latency. Nomenclature of the ORFs based on MDV strain CVI988 (Spatz *et al.*, 2007b) and stated 5' to 3' unless in parentheses which denotes 3' to 5'. (*pol-1_for & pol-1_rev designed by Dr. Jeanette Webb; **MeqForQPCR, MeqRevQPCR, designed by Dr. Bernadette Dutia)
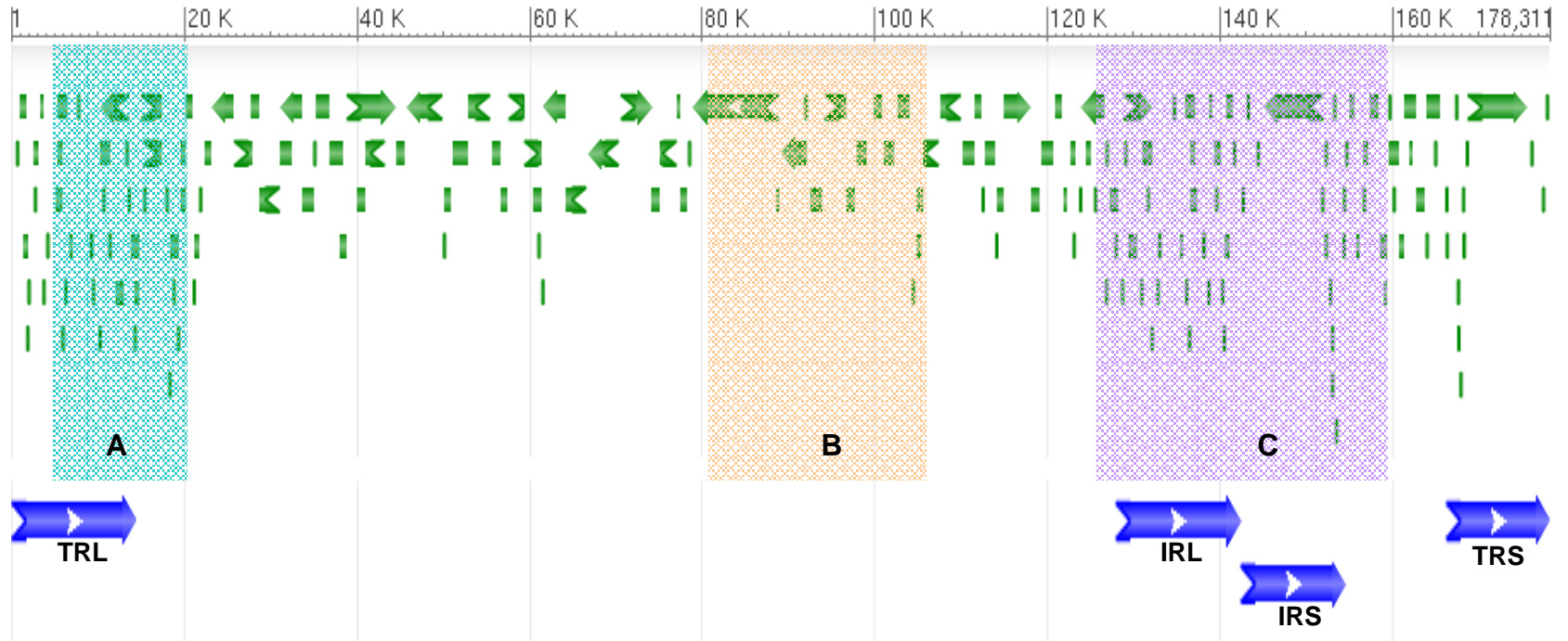
**Figure 3.5a:** Overview of CVI988 Rispens genome (GenBank DQ530348.1) and 3 regions of interest containing the ORFs investigated in this study as outlined in table 3.1. TRL = Terminal Repeat Long region, IRL = Internal Repeat Long region, IRS = Internal Repeat Short region, TRS = Terminal Repeat Short region. Diagram adapted from graphics interface of the NCBI GenBank database.
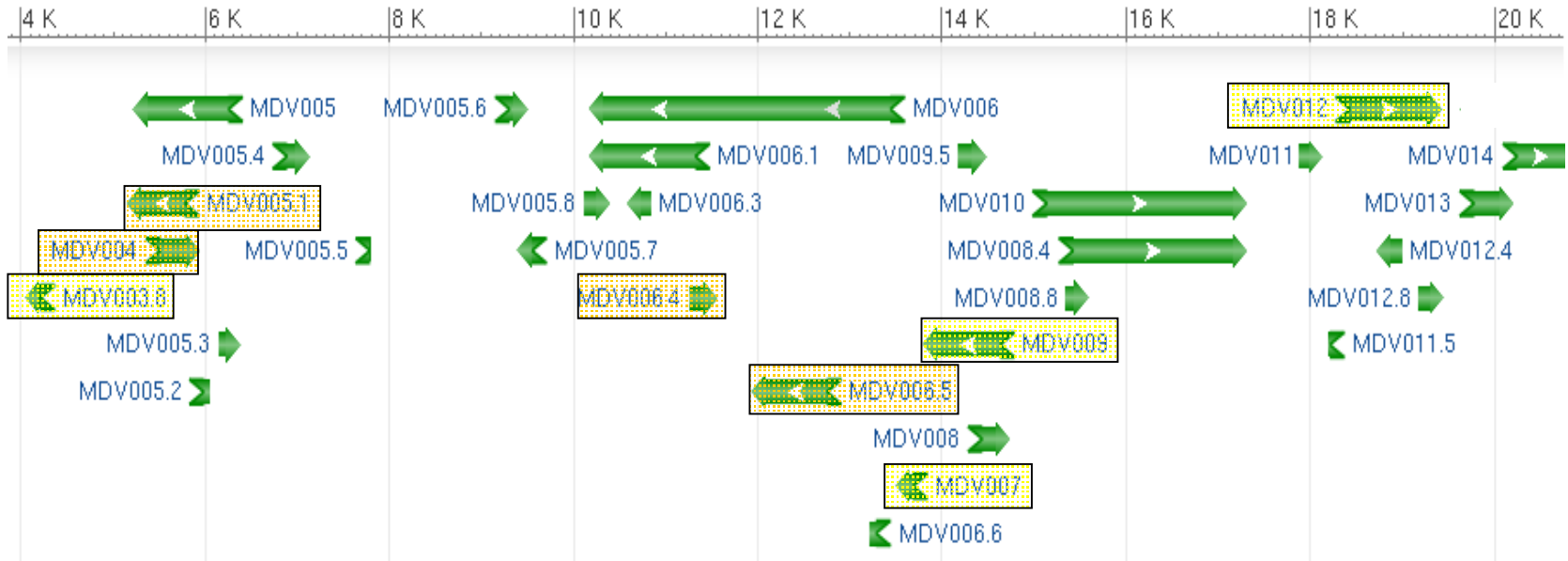
**Figure 3.5b:** Expanded Region A from figure 3.5a of MDV CVI988 genome showing genes of interest in the terminal repeat long and unique long region of the genome. Genes of interest in this region are shaded in yellow. MDV003.8 = L1, MDV004 = 23 kDa, MDV005 = Meq, MDV005.1 = RLORF6, MDV006.4 = RLORF11, MDV006.5 = MLTI, MDV007 = RLORF12, MDV009 = LORF1 and MDV0012 = LORF3. Diagram adapted from graphics interface of the NCBI GenBank database.
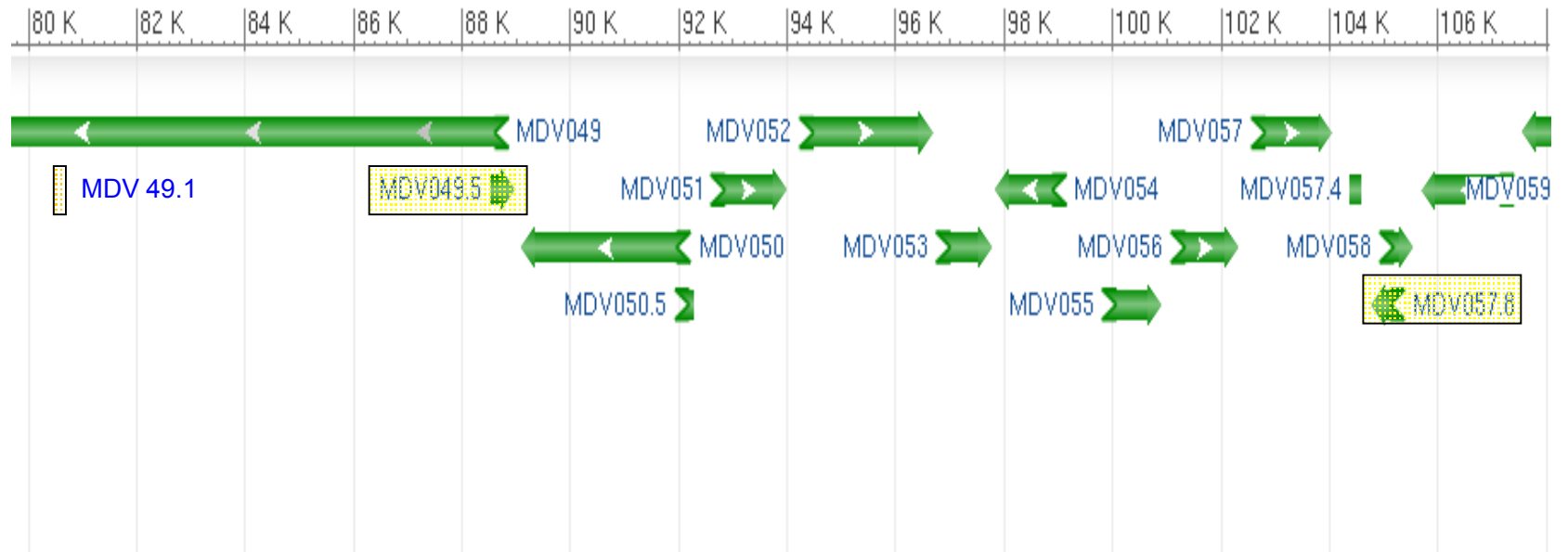
**Figure 3.5c:** Expanded Region B from figure 3.5a of MDV CVI988 genome showing genes of interest in the unique long region of the genome. Genes of interest in this region are shaded in yellow. MDV049.1 = MNFH, MDV049.5 = LORF6, MDV057.8 = LORF8. Diagram adapted from graphics interface of the NCBI GenBank database.
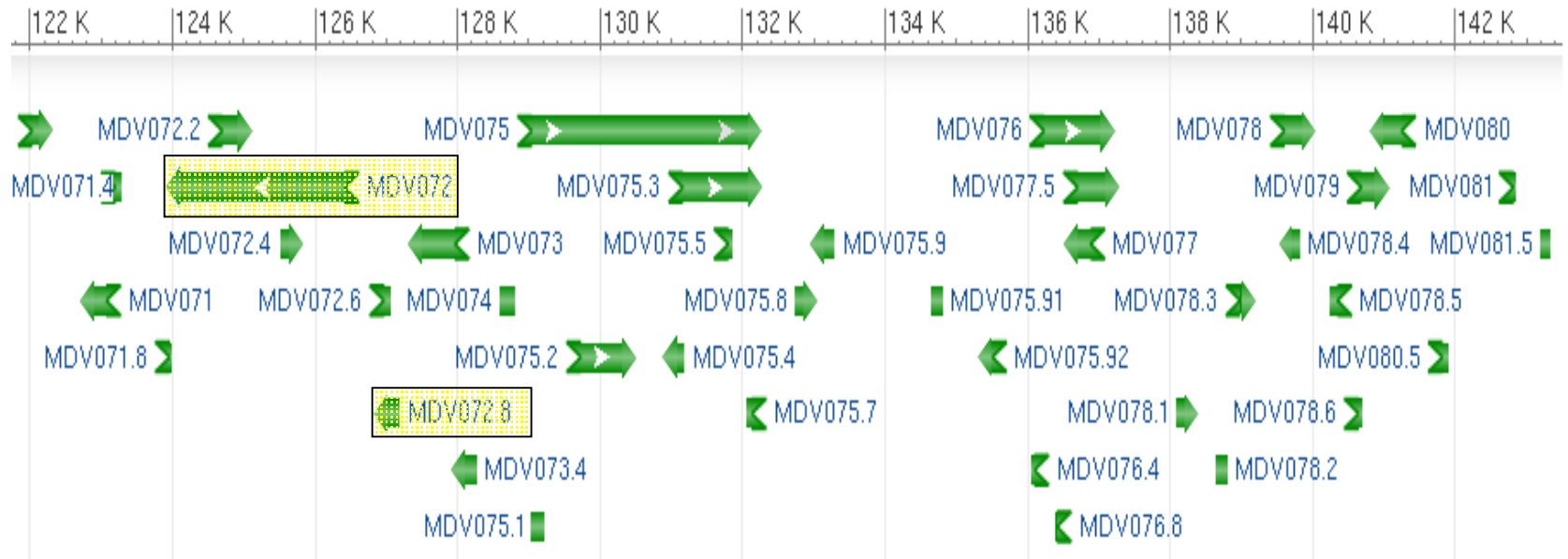
**Figure 3.5d:** Expanded Region C from figure 3.5a of MDV CVI988 genome showing genes of interest in the internal repeat long and internal repeat short region of the genome. Genes of interest in this region are shaded in yellow. MDV072 = LORF11, MDV072.8 = LORF12. Diagram adapted from graphics interface of the NCBI GenBank database.
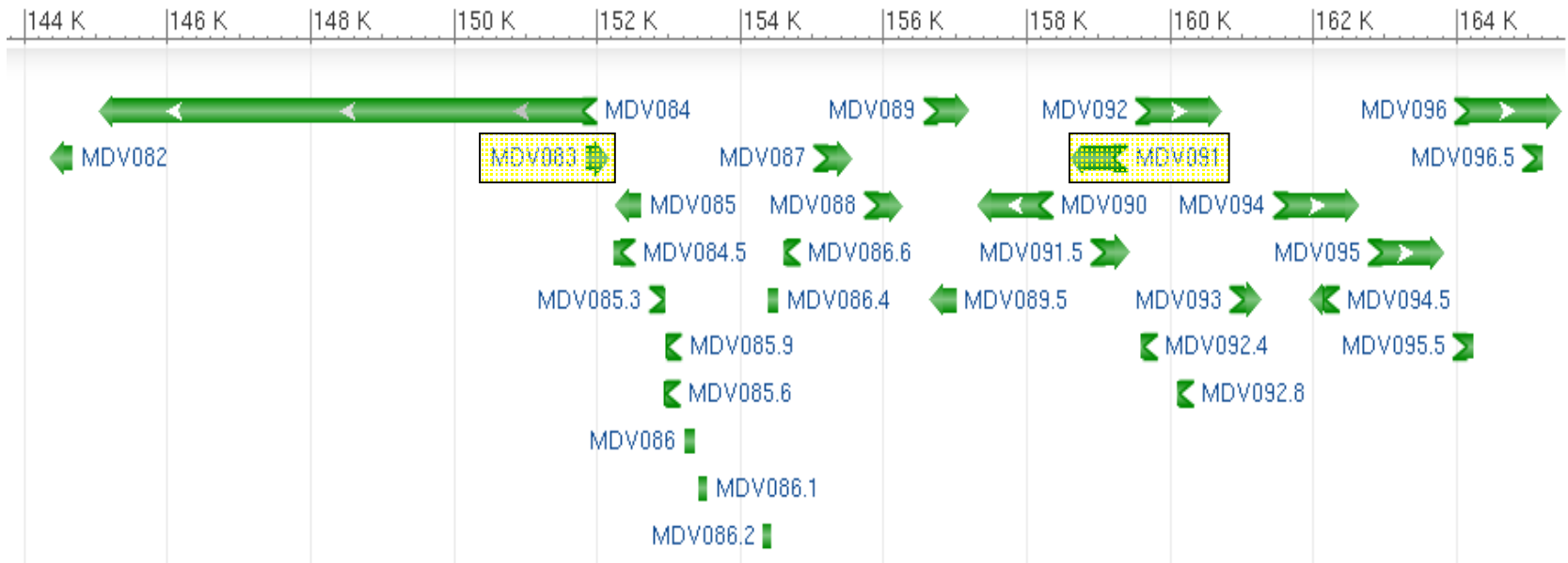
**Figure 3.5e:** Expanded Region C from figure 3.5a of MDV CVI988 genome showing genes of interest in the internal repeat short and unique short region of the genome. Genes of interest in this region are shaded in yellow. MDV083 = ANTISENSE and MDV091 = US2. Diagram adapted from graphics interface of the NCBI GenBank database.

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In all PCRs 60-180 ng of template DNA or a 1:10 dilution of cDNA made from equal amounts of RNA was used and the conditions were: 50-57ºC annealing temperature run for 30 cycles as detailed in section 2.1.8. The MDV BAC DNA contained the entire genome of the CVI988 strain except for US2 which had been deleted during the process of BAC construction and therefore the PCR would not be expected to work when using the MDV CVI988 BAC control DNA as a template (Petherbridge *et al.*, 2003). The CVI988 strain MDV was selected for a control as the complete genome had been published and a BAC was available from Dr. Venugopal Nair's Laboratory at the Institute for Animal Health, Compton, UK. To mimic a productive infection cDNA was prepared as detailed in section 2.1.5 from RNA isolated from Rb1b MDV infected CEFs. This model for lytic infection was predicted to express all genes in a productive MDV infection.

The first latent MDV model used (latent model 1) was Rb1b T cells which were derived from MDV strain Rb1b-infected lymphoblastic chicken tumours and kindly provided by Dr. Venugopal Nair at the Institute for Animal Health, Compton, UK. To test that these cells were truly latent, cDNA was prepared from purified Rb1b T cell mRNA (section 2.1.5) and used as a template in a PCR using primers specific for DNA polymerase. Dr. Jeanette Webb, Centre for Infectious Disease, University of Edinburgh kindly provided a proven primer set for DNA polymerase (table 3.4). DNA polymerase would not be expressed in latent MDV infections (Pellett *et al.*, 2006) and therefore should not be expressed in a true latent cell line model. The Rb1b T cells had been predicted to

express only genes that were expressed during latency, however some background expression of DNA polymerase was observed in PCRs which suggested a low level of productive infection in the cell line (table 3.6). The latent model 1 was subsequently replaced with a non-productive MDV cell line RPL-1 (designated latent model 2) which was also obtained from Dr. Venugopal Nair at the Institute for Animal Health, Compton, UK.

RPL-1 cells were derived from immortalized JMV MDV-infected chicken lymphoblastic tumours and while MDV viral sequences could be isolated from these cells they were a non-producer of virus (Nazerian *et al*., 1976; Stephens *et al*., 1976). Previous studies had also used RPL-1 cells as a latent infection model (Koptidesova *et al*., 1995). The virulent prototype JM strain in RPL-1 cells was originally isolated from a lymphomatous ovarian suspension of chicken cells isolated from a flock in Massachusetts, USA suffering from multiple cases of neurolymphomatosis and was the first MDV strain successfully used for experimental reproduction of the neural and visceral forms of MD (Sevoian *et al.*, 1962). When the PCR analysis was repeated for DNA polymerase using cDNA prepared from purified RPL-1 mRNA as a template there was no apparent expression of DNA polymerase (figure 3.7). Based on this evidence the decision was made to use the RPL-1 cells as the true latent infection model in the siRNA studies described in later sections rather than Rb1b T cells.

| ORF | Expected Size PCR Product (bp) | DNA CVI988 MDV BAC (Positive Control) | cDNA Rb1b Infected CEF (Productive Infection) | cDNA Rb1b T Cells (Reactivating Latent Infection) | cDNA RPL-1 Cells (Non-Reactivating Latent Infection) |
|---|---|---|---|---|---|
| DNA Polymerase | 270 | + | ND | Inconclusive | - |
| Meq | 100 | + | + | + | + |
| 23 kDa | 240 | Inconclusive | Inconclusive | Inconclusive | Inconclusive |
| RLORF6 | 151 | + | + | + | + |
| LORF1 | 191 | + | + | + | + |
| LORF3 | 244 | + | + | + | + |
| LORF11 | 230 | + | + | + | + |
| LORF12 | 152 | + | + | + | + |
| ANTISENSE | 180 | + | + | + | + |
| US2 | 175 | - | + | - | + |
| MLTI | 110 | Inconclusive | Inconclusive | Inconclusive | Inconclusive |
| RLORF11 | 157 | + | + | + | + |
| RLORF12 | 229 | + | - | Inconclusive | Inconclusive |
| MNFH | 57 | + | + | + | - |
| LORF6 | 188 | + | + | + | - |
| LORF8 | 163 | + | + | + | - |
| RLORF5a or L1 | 248 | + | + | + | - |

**Table 3.6:** Summary table showing MDV genes selected for study showing expression in a latent (RPL-1) cell line, a reactivating cell line (Rb1b T cells), a lytic infection (Rb1b Infected CEF) and a control (MDV BAC CVI988). ND = Not Done. Genes shaded in blue were located in the 'Meq Loci'. Genes shaded in pink were expressed in latency. Genes shaded in yellow were not expressed in latency.
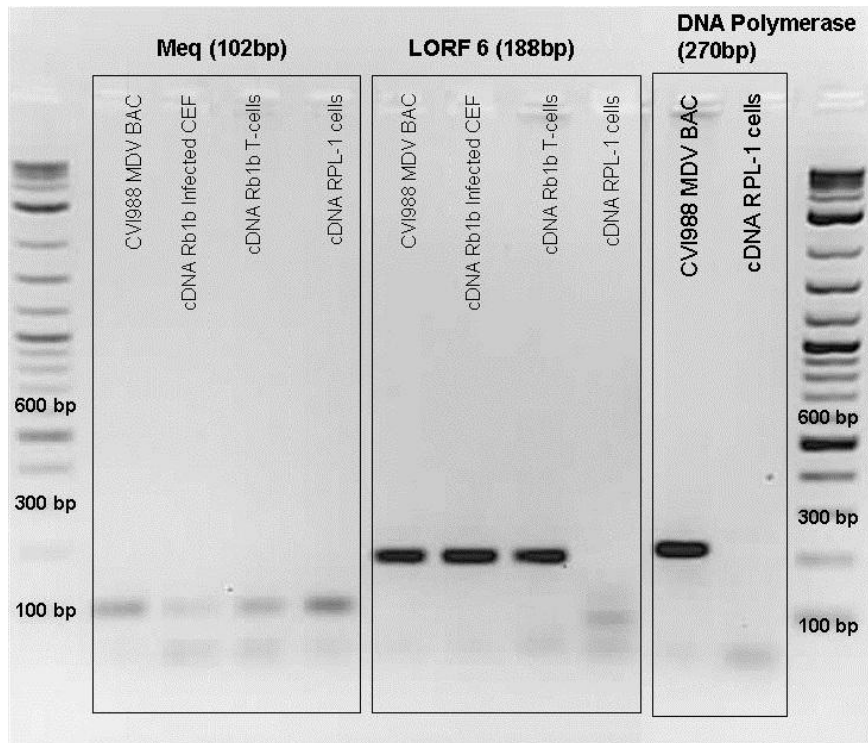
**Figure 3.7:** 2% agarose gel showing comparison between latent and lytic expression of Meq, LORF6 and DNA polymerase. Figure Key: Lane 1 shows the Positive control (strain CVI988 MDV BAC DNA), Lane 2 shows lytic infection (cDNA Rb1b Infected CEF), Lane 3 shows latent infection model 1 (cDNA Histopaque-treated Rb1b T cells) and Lane 4 shows latent infection model 2 (cDNA RPL-1). DNA polymerase shows only 2 lanes: Positive control (strain CVI988 MDV BAC) and latent infection model 2 (cDNA RPL-1). Ladder used was 2-log DNA Ladder (0.1-10 kb) (New England Biolabs).

Meq expression was used as a positive control in the selection of cell models and optimizing the PCR reactions as it would be expressed in both latent and productive MDV infections (Brown *et al.*, 2006). Dr. Bernadette Dutia from The Roslin Institute, University of Edinburgh, UK kindly provided a proven primer set for Meq which was described in table 3.4. PCRs using the Meq primer set and templates of MDV BAC DNA, Rb1b infected CEF cDNA, Rb1b T cell cDNA and RPL-1 cDNA all yielded a band signifying expression as expected on a 2% agarose gel (figure 3.7). Table 3.6 contains a summary of all the findings of the PCR studies and the individual comparative 2% agarose gels of the PCR products (section 2.1.13) are detailed in figures 3.7 to 3.12. Analysis of the agarose gels demonstrated the presence of clear bands of the expected size in PCRs performed with RPL-1 cDNA (latent model 2) as a template for the following genes: 23kDa, RLORF6, LORF1, LORF3, LORF11, LORF12, ANTISENSE, US2 and RLORF11. Based on these findings it was concluded that these genes were all expressed in latency. The ORF for both the 23kDa and RLORF6 significantly overlap Meq, however, so it was possible that the PCR primers may not have been able to accurately predict expression during latency due to the presence of Meq which is also expressed in latency (Brown *et al.*, 2006). MLTI did not yield any distinct bands (figure 3.10), only a faint multiple banding pattern on all templates except the CVI988 MDV BAC which could be from an ineffective primer set or possibly attributed to the repeating nature and small size of the target protein which made selection of primers expected to yield a single distinct band nearly impossible. The presence of a multiple banding pattern for MLTI was interpreted as inconclusive. The

results for expression of RLORF12 (figure 3.8) were not clear despite several rounds of optimizing the PCR. It was concluded that RLORF12 was probably expressed at a low level in latency based on the faint agarose gel band present in the latent model 2 lane compared to the strong band in the CVI988 MDV BAC positive control lane (indicating that the primers were effective). It was not possible to determine why no agarose gel bands for RLORF12 were seen in the lytic or reactivating latent model (latent model 1) as the RLORF12 primer sets were homologous to the Rb1b strain MDV found in these cell lines. The decision was made that despite the indeterminate results RLORF12 and MLTI would be included in the next phase of the project. No evidence of expression in latent model 2 was found for LORF6, LORF8, L1 or the hypothetical protein MNFH so no further studies were carried out on these genes.
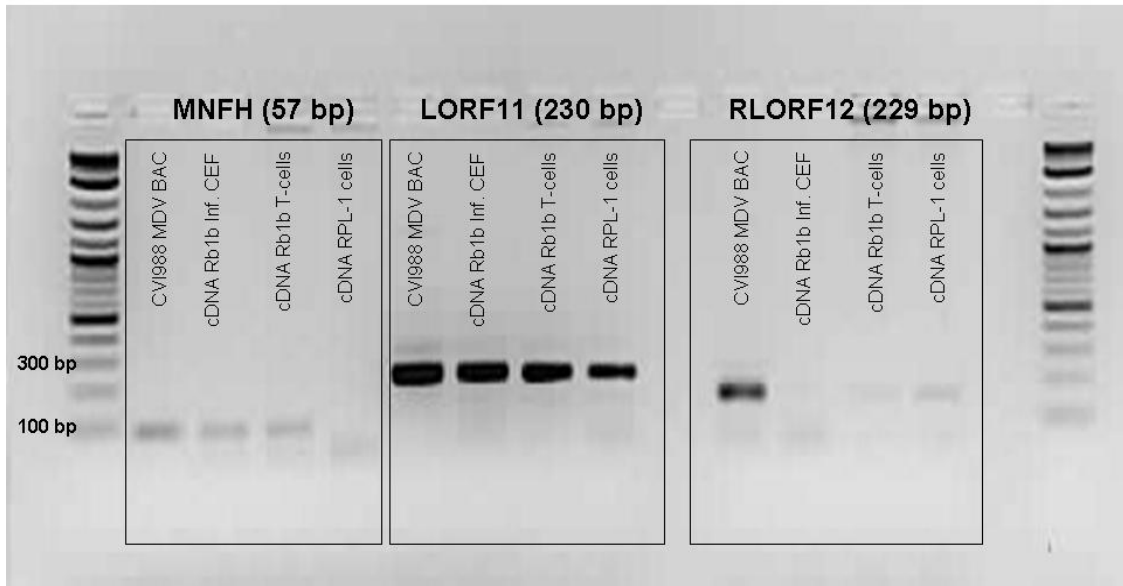
**Figure 3.8:** 2% agarose gel showing comparison between latent and lytic expression of MNFH (Primer MNFH), LORF 11 (Primer LORF11), RLORF12 (Primer RLORF12). Figure Key: Lane 1 shows the Positive control (MDV BAC DNA), Lane 2 shows lytic infection (cDNA Rb1b Infected CEF), Lane 3 shows latent infection model 1 (cDNA Histopaque-treated Rb1b T cells) & Lane 4 shows latent infection model 2 (cDNA RPL-1). Ladder used was 2-log DNA Ladder (0.1-10 kb).
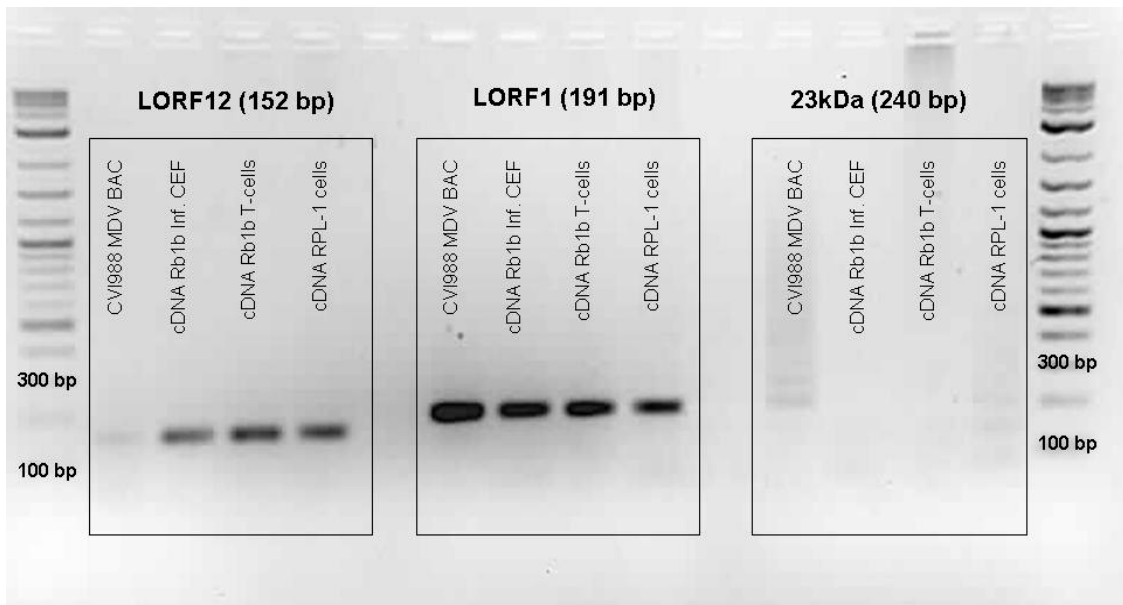


**Figure 3.9:** 2% agarose gel showing comparison between latent and lytic expression of LORF12 (Primer LORF12), LORF1 (Primer LORF1), 23kDa (Primer 23kDa). Figure Key: Lane 1 shows the Positive control (MDV BAC DNA), Lane 2 shows lytic infection (cDNA Rb1b Infected CEF), Lane 3 shows latent infection model 1 (cDNA Histopaque-treated Rb1b T cells) and Lane 4 shows latent infection model 2 (cDNA RPL-1). Ladder used was 2-log DNA Ladder (0.1-10 kb).

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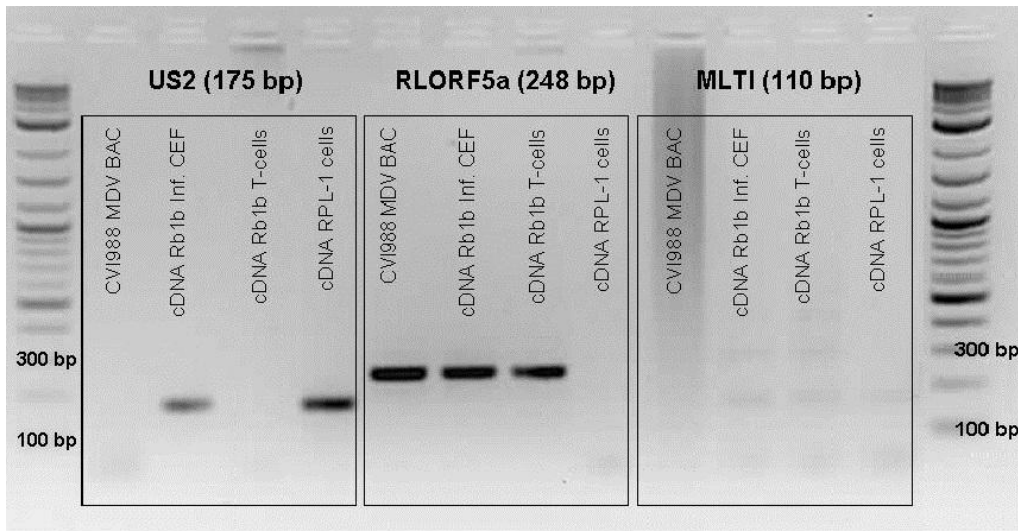
**Figure 3.10:** 2% agarose gel showing comparison between latent and lytic expression of US2 (Primer US2), RLORF5a or L1 (Primer L1), and MLTI (Primer MLTI). Figure Key: Lane 1 shows the Positive control (MDV BAC DNA), Lane 2 shows lytic infection (cDNA Rb1b Infected CEF), Lane 3 shows latent infection model 1 (cDNA Histopaque-treated Rb1b T cells) and Lane 4 shows latent infection model 2 (cDNA RPL-1). DNA polymerase shows only 2 lanes: Positive control (MDV BAC) and latent infection model 2 (cDNA RPL-1). Ladder used was 2-log DNA Ladder (0.1-10 kb).
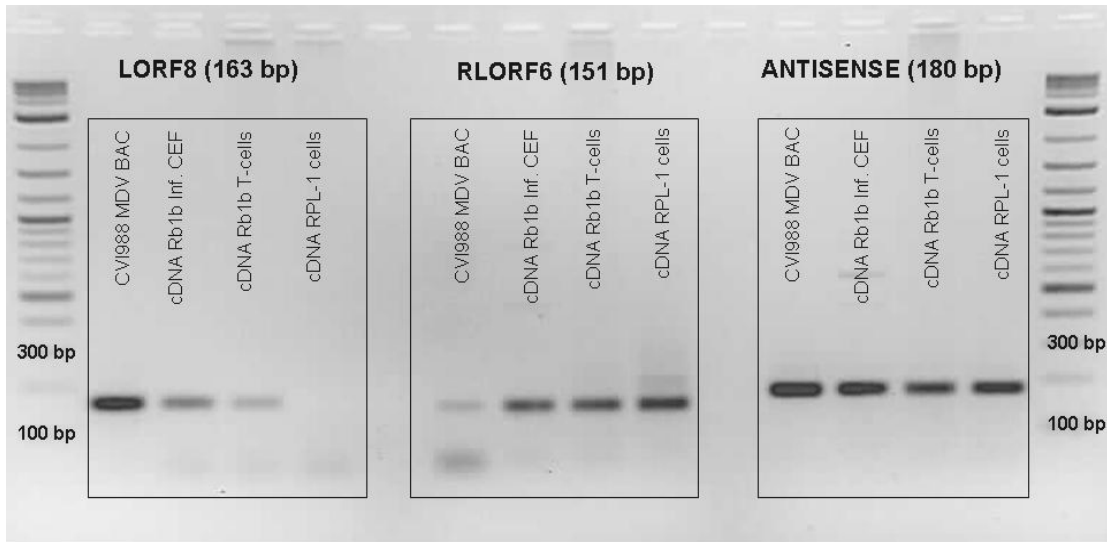


**Figure 3.11:** 2% agarose gel showing comparison between latent and lytic expression of LORF8 (Primer LORF8), RLORF6 (Primer RLORF6), ANTISENSE (Primer ANTISENSE). Figure Key: Lane 1 shows the Positive control (MDV BAC DNA), Lane 2 shows lytic infection (cDNA Rb1b Infected CEF), Lane 3 shows latent infection model 1 (cDNA Histopaque-treated Rb1b T cells) and Lane 4 shows latent infection model 2 (cDNA RPL-1). Ladder used was 2-log DNA Ladder (0.1-10 kb).

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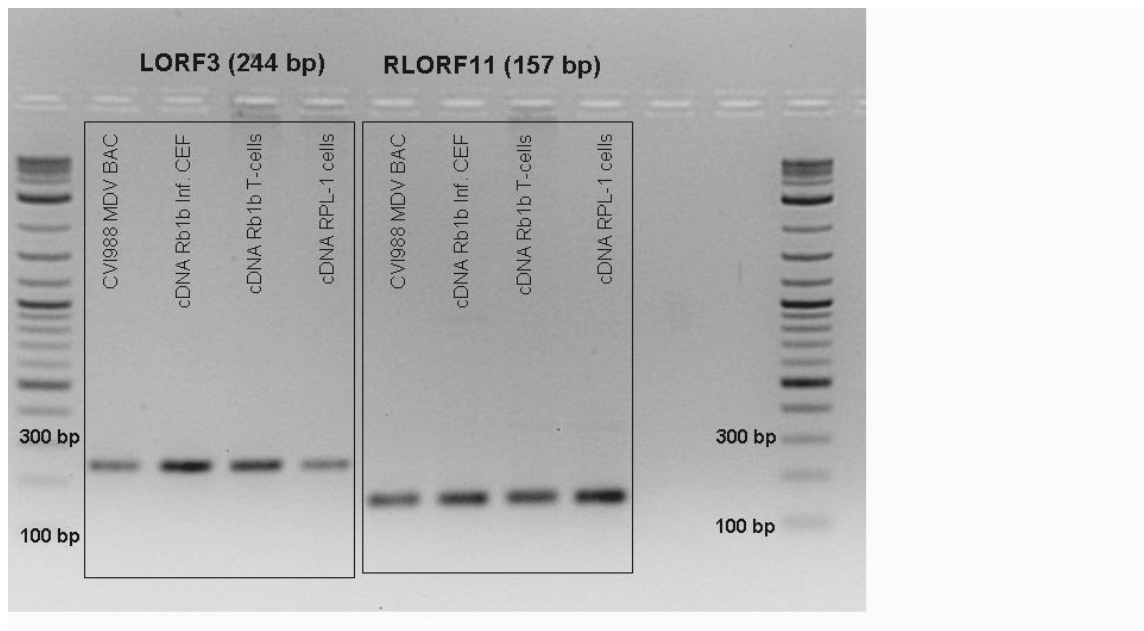
**Figure 3.12:** 2% agarose gel showing comparison between latent and lytic expression of LORF3 (Primer LORF3) and RLORF11 (Primer RLORF11). Figure Key: Lane 1 shows the Positive control (MDV BAC DNA), Lane 2 shows lytic infection (cDNA Rb1b Infected CEF), Lane 3 shows latent infection model 1 (cDNA Histopaque-treated Rb1b T cells) and Lane 4 shows latent infection model 2 (cDNA RPL-1). Ladder used was 2-log DNA Ladder (0.1-10 kb).

### 3.4  Sequencing of Genes of Interest in MDV JM Strain

Once the initial screening of the 15 ORFs had been completed (section 3.3) the potential

targets were narrowed down from 15 to the 11 expressed in latent model 2:  LORF1,

LORF3, LORF11, LORF12, ANTISENSE, US2, MLTI, RLORF11, RLORF12, 23kDa

and RLORF6.  These genes were selected for siRNA knockdown studies using RPL-1

cells which were persistently infected with the JM strain of MDV (Nazerian *et al*.,

1976).  The complete sequence of  strain JM MDV was not available at the time of this

study, but a partial sequence of strain JM/102W IR Long region (Spatz and Silva,

2007b) was available that encompassed the RLORF6, RLORF11, RLORF12, 23kDa and

MLTI ORFs.


The JM strain of MDV was used extensively in early studies of MDV (Calnek *et al.*,

1984; Churchill and Biggs, 1967; Churchill *et al.*, 1969; Dukes and Pettit, 1983;

Nazerian *et al.*, 1968; Witter and Burmester, 1967; Witter *et al.*, 1980) and many clones

of the prototype strain were used in the different labs working on MDV in the 1960's

and 1970's.  The MDV strain JM/102-W was a clone of the prototype strain JM virus

and was used to develop the JMV non-productively infected transplantable tumour

(Stephens *et al*., 1976) which was subsequently used to develop the RPL-1 cell line

(Nazerian *et al*., 1976).  Based on these early studies it was determined that the JM strain

described in (Sevoian *et al.*, 1962) and the JM/102W strain of MDV (Stephens *et al*.,

1976) were most likely homologous as they were clones of the same virus.  The decision

was made to use the published sequence of MDV strain JM/102W (Spatz and Silva,

2007b) for designing the siRNA oligonucleotides used for the *in vitro* studies in section 4.5 in RPL-1 cells.

The LORF 1, LORF 3, LORF 11, LORF 12, ANTISENSE and US2 ORFs were not covered in the published JM/102W sequence. In order to correctly design the siRNA oligonucleotides specific for RPL-1 cells the sequence of LORF1, LORF3, LORF11, LORF12, ANTISENSE and US2 was determined as illustrated in figure 3.13. Sequencing primers were selected using the Rispens (CVI988) MDV genome (Spatz *et al*., 2007b) (GenBank DQ530348.1) and were located outside the published beginning and end of each ORF. The online databases WebPrimer (http://www.yeastgenome.org/cgi-bin/web-primer, accessed 27/8/07) and Primer3 (http://frodo.wi.mit.edu/primer3, accessed 27/8/07) were used to assist the primer selection process. A forward and reverse sequencing reaction was done (section 2.1.16) using walking primers to ensure complete coverage of the gene to be sequenced. The sequences were then aligned using the align function in the Vector NTI v.10-11 software package (Invitrogen) to give a minimum of three overlapping sequences for each ORF. Each PCR sequenced between 200 and 600 bp so multiple primers were required in the longer ORFs to ensure complete and overlapping coverage. Sequencing primers used and their position in the CVI988 genome are detailed in table 3.14.

**Experimental flow chart of siRNA transfection studies**
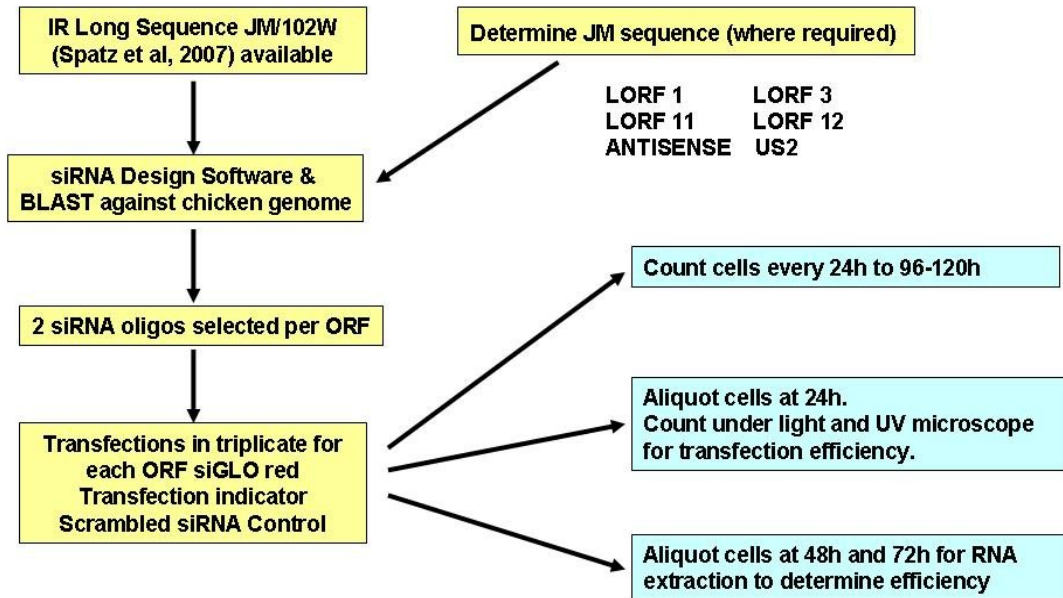


**Figure 3.13:** Experimental flowchart for siRNA transfection studies.

| MDV ORF | Primer Designation | Primer Sequence | Position in MDV CVI988 Strain |
|---|---|---|---|
| **LORF1** | LORF1 5' to 3' | CGTTCGCACCAGAGTCC | 13744-13760 (128683-128699) |
| | LORF1 3' to 5' | TGGCAAACCACGACTACC | (14794-14811) |
| | LORF1 REV#2 | TAGGAGTAGACTGACACG | (14938-14955) |
| **LORF3** | LORF3 5' to 3' | GGTCTTGCTGCTGAATGC | 18258-18275 |
| | LORF3 3' to 5' | CCCATAACAATACGTGAAGG | (19511-19530) |
| **LORF11** | LORF11 5' to 3' | ATTGTATCATCGTATGTGGG | 123850-123869 |
| | LORF11 3'to 5' | CCTTGATGTGGTTTGACG | (126678-126695) |
| | LORF11 FOR#2 | CTGCGATACTGTATATTATG | 125079-125098 |
| | LORF11 REV#2 | ATTTGGCATATCTTCGCTC | (126036-126054) |
| **LORF12** | LORF12 5' to 3' | CACAAGCGAGAAAGGAGC | 126748-126765 |
| | LORF12 3' to 5' | TGGGGAGGTGGAATCTG | (127316-127332) |
| **ANTISENSE** | ANTISENSE 5'to3' | CATGGTAACTGGAGTGG | 151813-151829 (168920-168936) |
| | ANTISENSE 3'to5' | CTAAATACAAAACCAGATGC | 168502-168521 (152228-152247) |
| **US2** | US2 5' to 3' | TAGCAAGTAGGTCTGTCG | 158567-158584 |
| | US2 3' to 5' | AAAGATTATTGGTGGAGGTG | (159409-159428) |

**Table 3.14:** Primers used for sequencing reactions of LORF1, LORF3, LORF11, LORF12, ANTISENSE and US2. Sequences are shown 5' to 3' direction.

Completed nucleotide and protein sequences for LORF3, LORF11, LORF12, ANTISENSE and US2 of the MDV JM strain aligned with the attenuated MDV CVI988 (Spatz *et al.*, 2007b) and highly pathogenic MDV Rb1b strain (Spatz *et al.*, 2007a) are detailed in Appendix 1. It was not possible to sequence the JM strain LORF1 gene due to difficulties in selecting specific primers for the sequencing reactions. This could have been due to multiple regions of partial homology in the LORF1 gene with other regions of the CVI988 genome which would lead to primers binding non-specifically in the sequencing reactions. After multiple unsuccessful attempts to sequence the LORF1 gene the decision was made to abandon sequencing it and use the Rb1b genome to design the siRNA oligonucleotides.

The sequencing of strain JM MDV genes LORF3, LORF12 and ANTISENSE showed 100% homology for MDV strains CVI988 and Rb1b. Analysis of the JM strain MDV LORF11 sequence revealed 4 different one bp substitutions between the CVI988 strain resulting in three aa substitutions in the final gene product. The aa substitutions between MDV LORF11 strain JM and CVI988 were: A to V at position 57, T to A at position 302 and P to L at position 780 which were detailed in Appendix 1. Comparison of strains JM and Rb1b MDV LORF11 showed 100% homology. Sequenced JM strain MDV US2 compared to strain CVI988 revealed 100% homology, but a G to A one bp substitution at position 159,005 was identified in the Rb1b strain resulting in an A to V aa substitution at position 51.

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The MDV JM strain LORF1 was not sequenced so the siRNA oligonucleotides were designed based on the MDV strain Rb1b (GenBank EF523390.1) and the gene knockdown studies were performed with Rb1b T Cells rather than RPL-1 cells which ensured that the siRNA targets selected would be present in the genome utilized. The completed strain JM MDV sequences were used to design siRNA 25-mer duplex oligo-ribonucleotides for gene knockdown studies which will be discussed in the next section.

### 3.5 Knockdown of Viral Gene Expression using siRNA

Two 25-mer siRNA oligonucleotides were designed for each target gene using BLOCK-IT ™ RNAi Designer software (Invitrogen) and either the JM/102 genome (Spatz and Silva, 2007b) or the JM strain sequence described previously for each of the selected ORFs expressed in latency (table 3.15). The only exception to this was the LORF1 gene which used the Rb1b genome (Spatz *et al*., 2007a) (GenBank EF523390) as there were technical problems in sequencing the JM strain as described in the previous section. The 25-mer siRNA oligonucleotides were sourced from a commercial supplier (Stealth™ siRNA, Invitrogen). The experimental groups for all the i*n vitro* studies were the two 25-mer siRNA oligonucleotide for each target gene which were both compared to a control siRNA sequence (Silencer™ Negative Control siRNA #2, Ambion, Invitrogen) which was a non-targeting siRNA oligonucleotide that had been validated to have minimal effects on gene expression profiles by the manufacturer. Each experimental group was transfected in triplicate for each experimental run to give three repetitions for each group. The siRNA oligonucleotides and a transfection indicator (siGloRed,

Dharmacon, Thermo Fisher Scientific) were transfected into RPL-1 cells as described in section 2.5.3. The only exception to this design was the LORF1 siRNA oligonucleotides which were transfected into Rb1b T cells instead of RPL-1 cells in order to match the MDV strain used to design the siRNA. Transfection efficiency was determined at 24 hours (section 2.5.3), cell counts were taken at 24, 48, 72, 96 and 120 hours post-transfection (section 2.4.4) and cell samples were taken for RNA isolation at 48, 72, 96 and 120 hours post-transfection (section 2.1.3). Transfection efficiencies calculated at 24 hours varied from 8.5 to 65.1% with an overall mean of 36.7%. All cell samples for RNA extraction were archived in RNALater solution and stored at -80ºC for possible analysis with RTqPCR at a later date. Due to the timescale and budget constraints siRNA studies for RLORF11, US2, 23kDa and LORF12 were not performed.

| Gene | Designation siRNA Oligonucleotide | MDV Strain | CVI988 Nucleotide Position | Sequence (RNA) Sense Strand |
|---|---|---|---|---|
| LORF1 | siRNA Rb1b LORF1 101 | Rb1b | (14662-14686)C | CAGGAACUUG GAUCUUUGUA CCUUU |
|  | siRNA Rb1b LORF1 644 | Rb1b | (14119-14143)C | CCGAGAGGG UCAAACUAUG UUAUAA |
| LORF3 | siRNA JM LORF3 9 | JM | 18284-18308 | CGGAGGAGG AACUAUUGCA UUAAUA |
|  | siRNA JM LORF3 217 | JM | 18492-18516 | CGGAAAUGUU UAGAUUGGU GCUGCG |
| LORF11 | siRNA JM LORF11 388 | JM | (126205-126229)C | CCAUGGUCAU UAUUGGGAAG GAUAA |
|  | siRNA JM LORF11 1087 | JM | (125506-125530)C | UCCGUUUCUC CAGAAUGCAU GUUAU |
| ANTI-SENSE | siRNA JM ANTISENSE 44 | JM | (168845-168869)C | UGUUUGCAG CGAGACGCCU UGAUAA |
|  | siRNA JM ANTISENSE 268 | JM | (168621-168645)C | CCUCCCAUGC UAGACCACAA GAUGU |
| MLTI | siRNA JM MLTI 7 | JM/102W | 16 Repeats, (8 Direct and 8 Complimentary) | UGGUGCUCG GCGAGCAUG UUCUGUA |
|  | siRNA JM MLTI 86 | JM/102W | 16 Repeats (8 Direct and 8 Complimentary) | GCAAUGCUUA CGAUCUGCC GAAACA |
| RLORF12 | siRNA JM RLORF12 69 | JM/102W | (13631-13655)C | GGGCGGCUC GGCUCUUGU GUAUAAA |
|  | siRNA JM RLORF12 186 | JM/102W | 128900-128924 & (13514-13538)C | GCUAGGCGA CGAACGAGCU GAAUUU |
| RLORF6 | siRNA RLORF6 JM 524 | JM/102W | 137023-137047 & (5391-5415)C | GGUUUACGC UCAGCUUUGU CCUGUU |
|  | siRNA RLORF6 JM 626 | JM/102W | 137125-137149 & (5289-5313)C | GGAUCCCGAA CAGGAUUCCU UGUAU |
| CONTROL | Silencer Negative Control siRNA #2 (Ambion, Invitrogen) | None | n/a | Not Available |

**Table 3.15:** siRNA oligonucleotides used for gene knockdown studies in RPL-1 cells. MDV strain CVI988 nucleotide position adapted from (Spatz *et al.*, 2007b).

Cell growth data was averaged for the 3 transfection repetitions and plotted for each 24 hour time point in the study with error bars equivalent to 1 standard error for LORF1, LORF3, LORF11, ANTISENSE, MLTI, RLORF12 and RLORF6 (figures 3.16-3.22). Analysis of the cell growth data showed a non-parametric distribution for LORF1, LORF3, LORF11, ANTISENSE, MLTI and RLORF12 and a normal distribution for RLORF6 (p=<0.005 at 72 and 96 hours). Statistical analysis of the cell growth data was performed using a Mann-Whitney test for all non-parametric data sets (LORF1, LORF3, LORF11, ANTISENSE, MLTI and RLORF12) or a general linear model ANOVA for normally distributed data (RLORF6) using MiniTab15 software as described in section 2.8. The criterion for statistical significance was a 95% confidence interval (p-value <0.05). Statistical findings and 24 hour transfection efficiencies were summarized at the bottom of figures 3.16 - 3.22 for each gene screened. The p-values for LORF1, LORF3, LORF11, ANTISENSE, MLTI and RLORF12 were all greater than 0.05 which did not meet the criteria for significance. Based on this analysis it was concluded that no significant differences existed *in vitro* between LORF1, LORF3, LORF11, ANTISENSE, MLTI or RLORF12 siRNA transfected RPL-1 cells and the siRNA negative control transfected RPL-1 cells. Due to timescale and budget constraints no follow-up studies for these genes were planned.
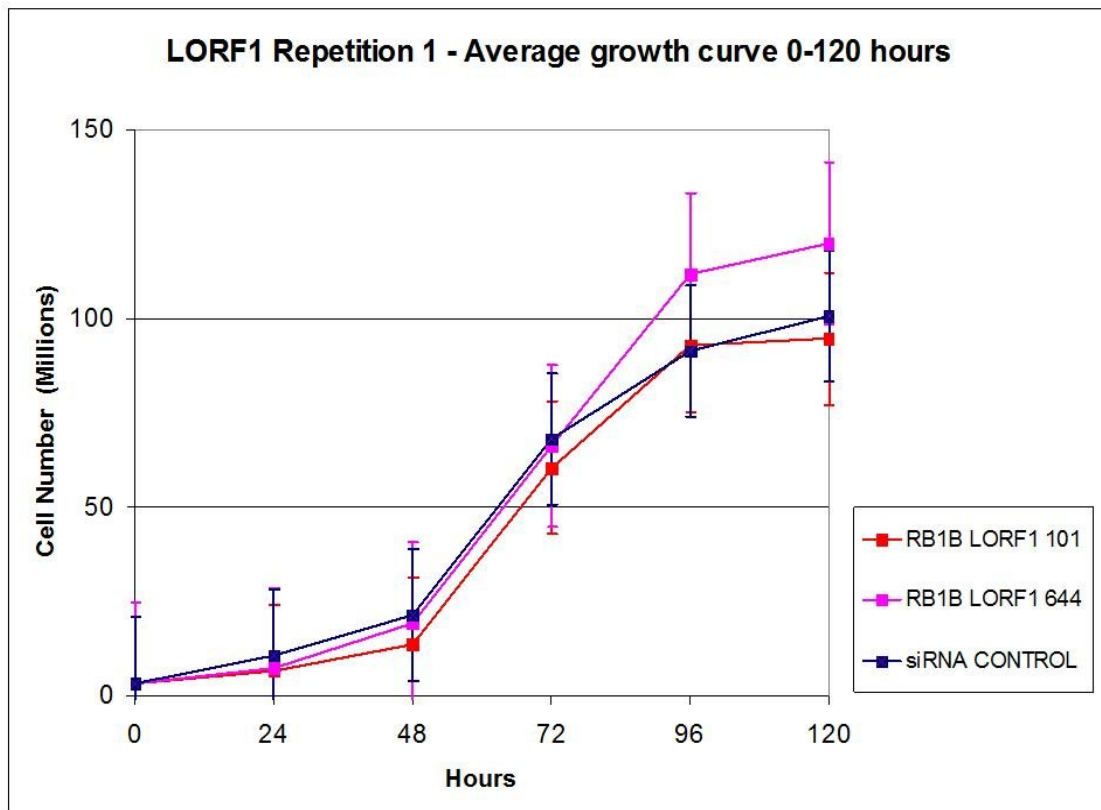
**LORF1 Repetition 1 - Average growth curve 0-120 hours**

| siRNA Oligonucleotide | Statistical Test | 72 hr p-value | 96 hr p-value | 24h Transfection Efficiency |
|---|---|---|---|---|
| siRNA Rb1b LORF1 101 | Mann-Whitney | 0.9362 | 1.0000 | 22.55% |
| siRNA Rb1b LORF1 644 | Mann-Whitney | 0.8102 | 0.4712 | 19.78% |
| siRNA Control | N/A | N/A | N/A | 20.88% |

**Figure 3.16a:** LORF1 cell growth data for first repetition of experiment in Rb1b T cells showing error bars equivalent to 1 standard error. Note the higher rate of cell growth compared to other siRNA experiments in this section. The Mann-Whitney test for significance incorporated all data points in the first and second repetitions and each experimental siRNA oligonucleotide was compared to the siRNA Control oligonucleotide. Transfection rates were determined as discussed in section 2.5.3 and calculated as an average value based on the first repetition only. N/A denotes not applicable.
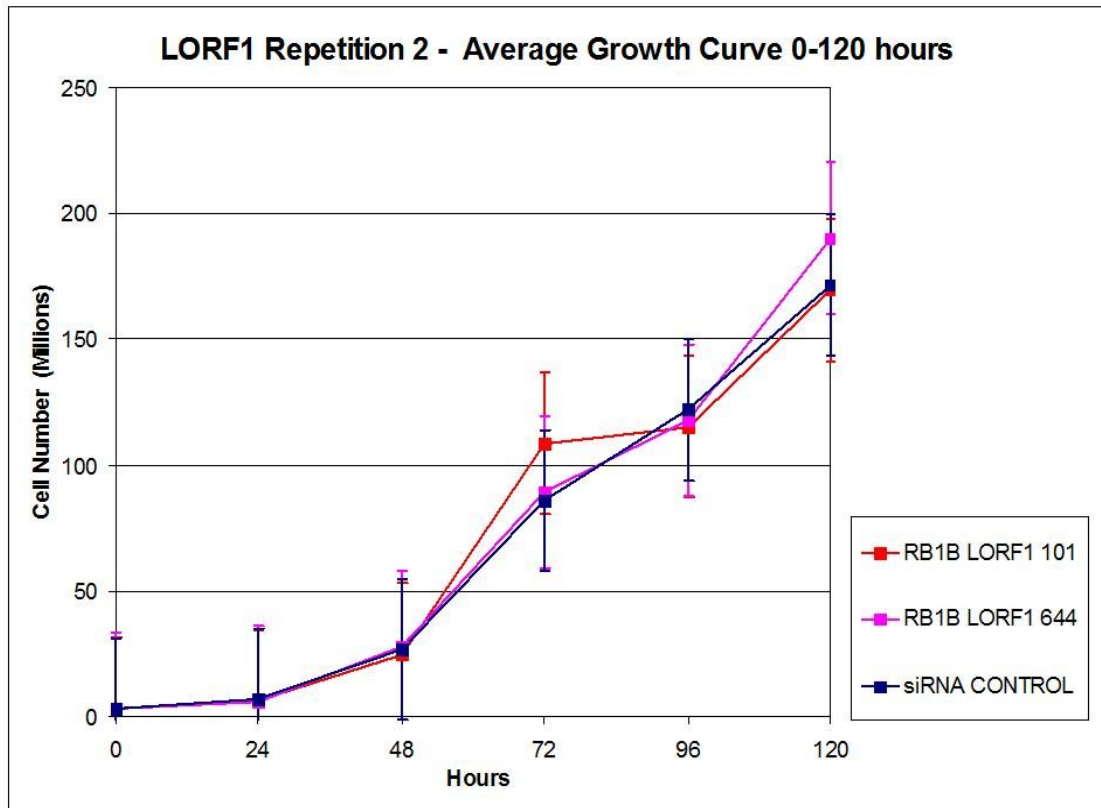
**Figure 3.16b:** LORF1 cell growth data for second repetition of experiment in Rb1b T cells showing error bars equivalent to 1 standard error. Note the difference in y-axis scale compared with figure 4.16a indicating an increased overall cell growth rate.
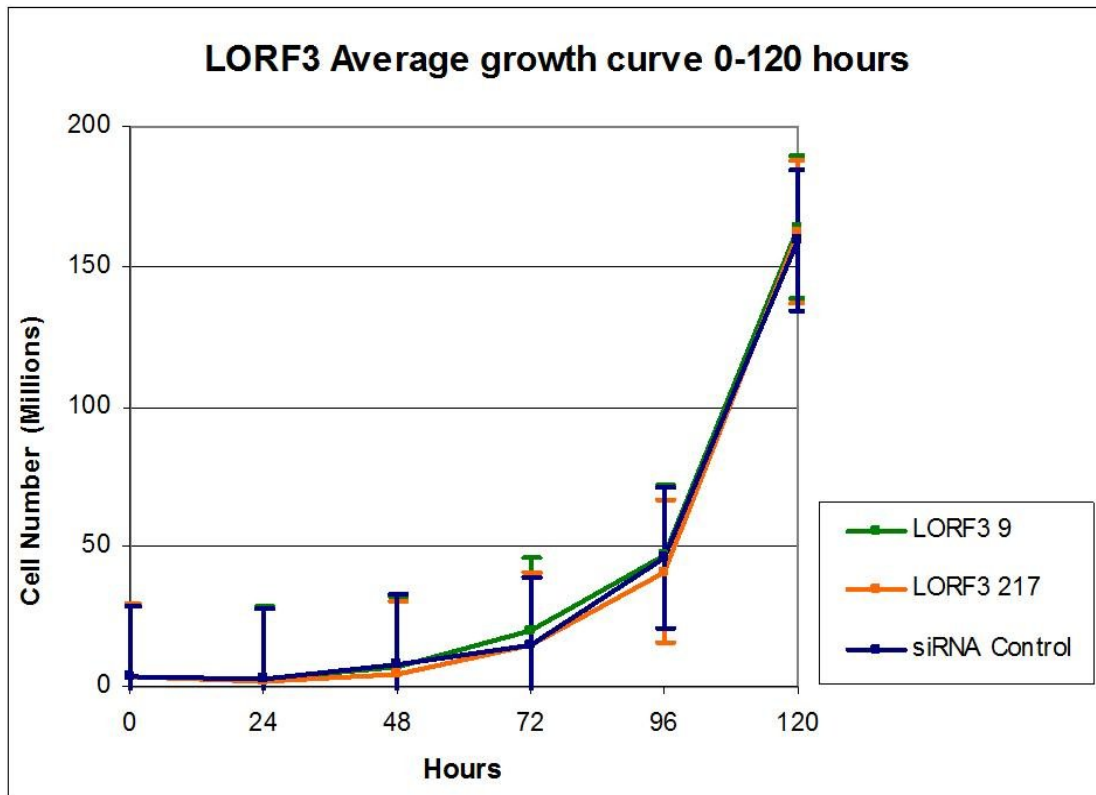
**LORF3 Average growth curve 0-120 hours**

| siRNA Oligonucleotide | Statistical Test | 72 hr p-value | 96 hr p-value | 24h Transfection Efficiency |
|---|---|---|---|---|
| siRNA JM LORF3 9 | Mann-Whitney | 0.0809 | 1.0000 | 32.55% |
| siRNA JM LORF3 217 | Mann-Whitney | 0.3827 | 0.1904 | 47.00% |
| siRNA Control | N/A | N/A | N/A | 40.52% |

**Figure 3.17:** LORF3 cell growth data for first repetition of experiment in RPL-1 cells showing error bars equivalent to 1 standard error. Note the higher rate of cell growth reflected by the y-axis scale compared to other siRNA experiments in this section. The Mann-Whitney test for significance compared each experimental siRNA oligonucleotide to the siRNA Control oligonucleotide. Transfection rates were determined as discussed in section 2.5.3 and calculated as an average value. N/A denotes not applicable.

**Figure 3.18a:** LORF11 cell growth data for first repetition of experiment in RPL-1 cells showing error bars equivalent to 1 standard error. The Mann-Whitney test for significance compared each experimental siRNA oligonucleotide to the siRNA Control oligonucleotide for all data points. Transfection rates were determined as discussed in section 2.5.3 and calculated as an average value based on the first repetition only. N/A denotes not applicable.
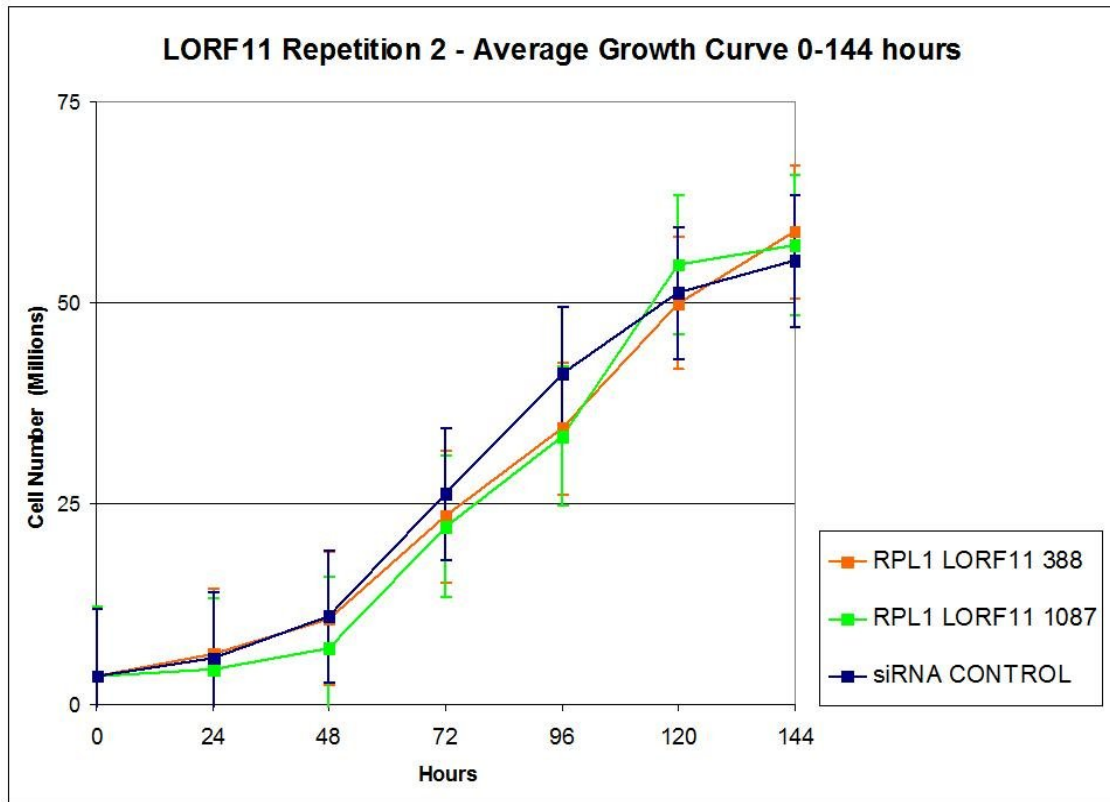
**Figure 3.18b:** LORF11 cell growth data for second repetition of experiment in RPL-1 cells showing error bars equivalent to 1 standard error.

**ANTISENSE Average growth curve 0-120 hours**
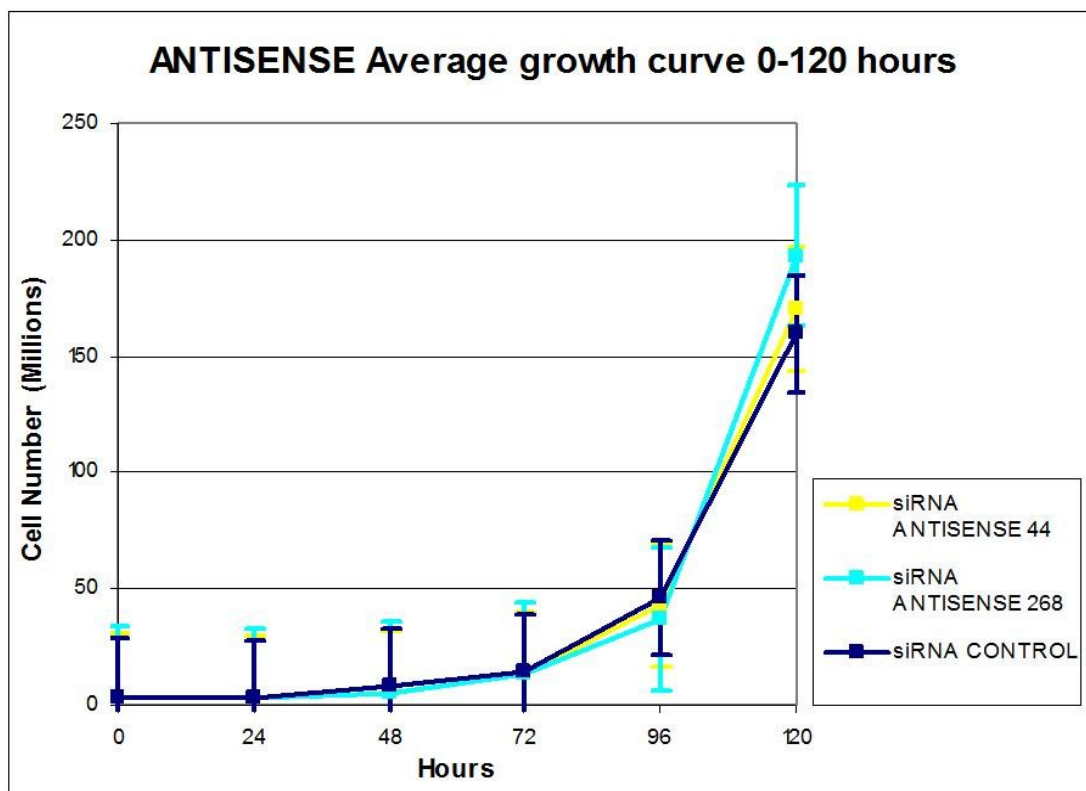
| siRNA Oligonucleotide | Statistical Test | 72 hr p-value | 96 hr p-value | 24h Transfection Efficiency |
|---|---|---|---|---|
| siRNA JM ANTISENSE 44 | Mann-Whitney | 0.1904 | 0.1904 | 33.81% |
| siRNA JM ANTISENSE 268 | Mann-Whitney | 0.6625 | 0.0809 | 34.71% |
| siRNA Control | N/A | N/A | N/A | 40.52% |

**Figure 3.19:** ANTISENSE cell growth data for first repetition of experiment in RPL-1 cells showing error bars equivalent to 1 standard error. Note the higher rate of cell growth reflected by the y-axis scale compared to other siRNA experiments in this section. The Mann-Whitney test for significance compared each experimental siRNA oligonucleotide to the siRNA Control oligonucleotide. Transfection rates were determined as discussed in section 2.5.3 and calculated as an average value. N/A denotes not applicable.

**MLTI Average Growth Curve 0-96 Hours**

| siRNA Oligonucleotide | Statistical Test | 72 hr p-value | 96 hr p-value | 24h Transfection Efficiency |
|---|---|---|---|---|
| siRNA JM MLTI 7 | Mann-Whitney | 0.1489 | 0.3865 | 46.27% |
| siRNA JM MLTI 86 | Mann-Whitney | 1.0000 | 0.3865 | 40.80% |
| siRNA Control | N/A | N/A | N/A | 40.27% |

**Figure 3.20:** MLTI cell growth data for first repetition of experiment in RPL-1 cells showing error bars equivalent to 1 standard error.  The Mann-Whitney test for significance compared each experimental siRNA oligonucleotide to the siRNA Control oligonucleotide. Transfection rates were determined as discussed in section 2.5.3 and calculated as an average value.  N/A denotes not applicable.

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**RLORF12 Repetition 1 - Average Growth Curve 0-96 Hours**
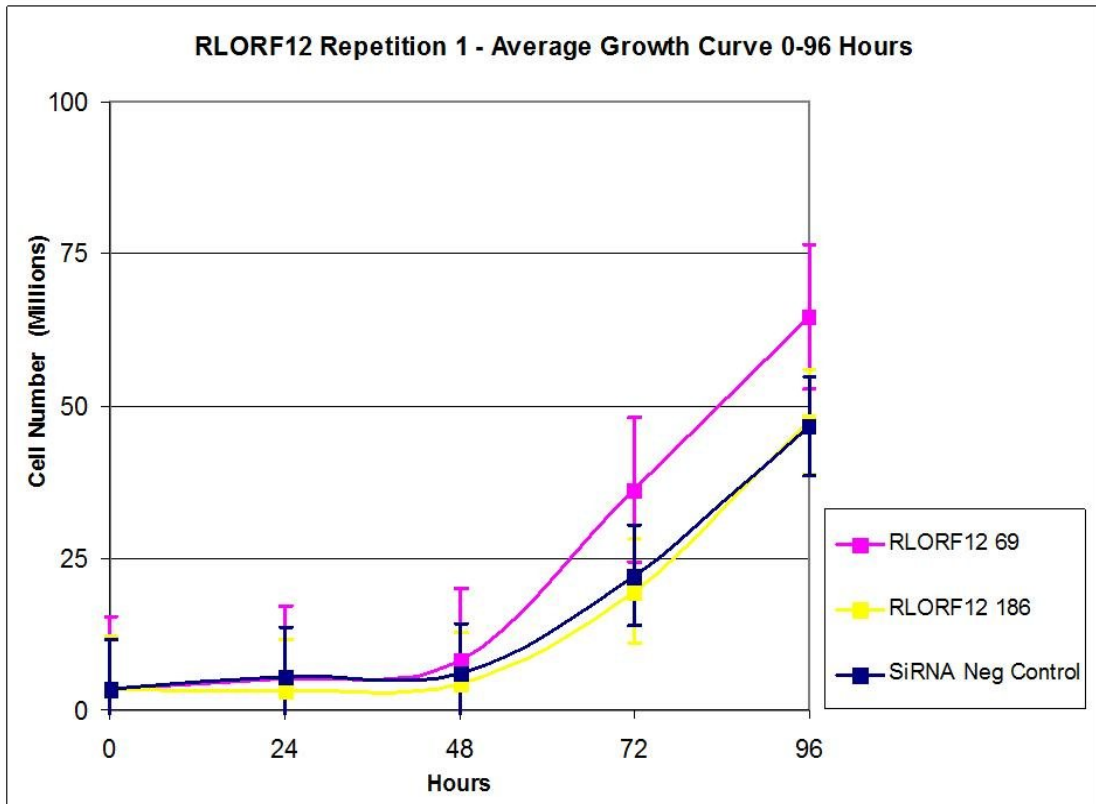
| siRNA Oligonucleotide | Statistical Test | 72 hr p-value | 96 hr p-value | 24h Transfection Efficiency |
|---|---|---|---|---|
| siRNA JM RLORF12 69 | Mann-Whitney | 0.4113 | 0.5228 | 55.42% |
| siRNA JM RLORF12 186 | Mann-Whitney | 1.0000 | 1.0000 | 49.88% |
| siRNA Control | N/A | N/A | N/A | 40.27% |

**Figure 3.21a:** RLORF12 cell growth data for first repetition of experiment in RPL-1 cells showing error bars equivalent to 1 standard error. The Mann-Whitney test for significance compared each experimental siRNA oligonucleotide to the siRNA Control oligonucleotide for all data points in both replications. Transfection rates were determined as discussed in section 2.5.3 and calculated as an average value for both repetitions. N/A denotes not applicable.
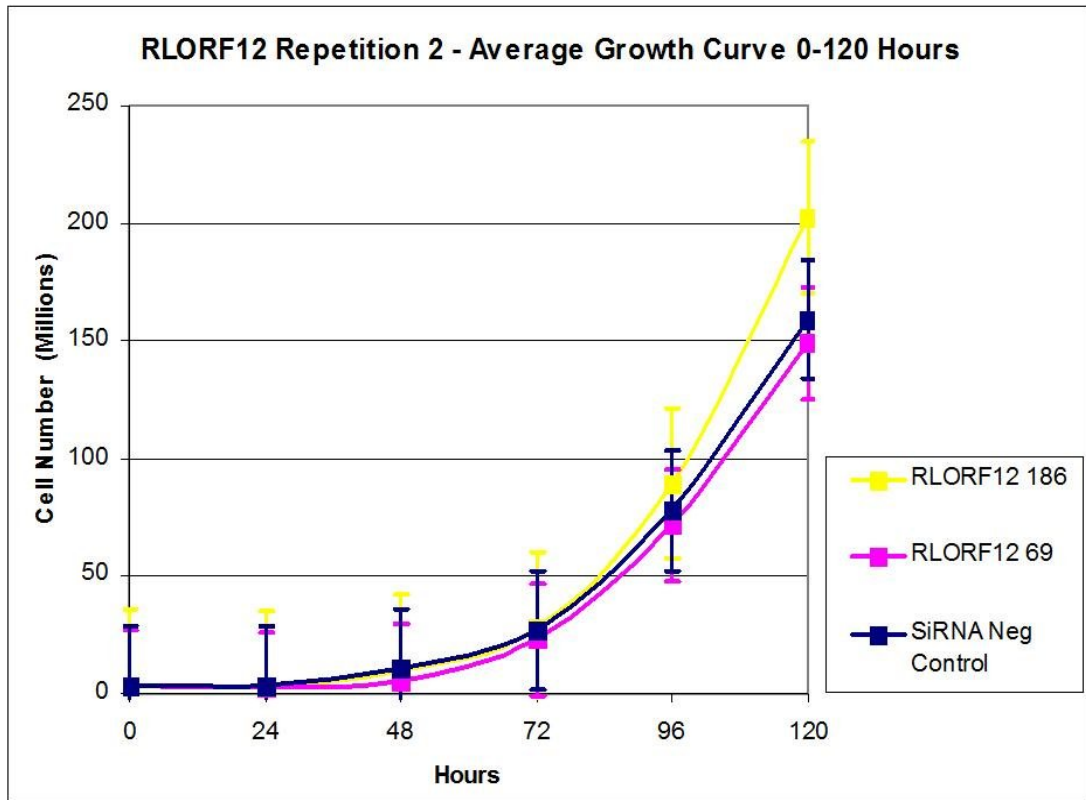
**Figure 3.21b:** RLORF12 cell growth data for second repetition of experiment in RPL-1 cells showing error bars equivalent to 1 standard error. Note the relatively higher rate of cell growth reflected by the higher y-axis scale.
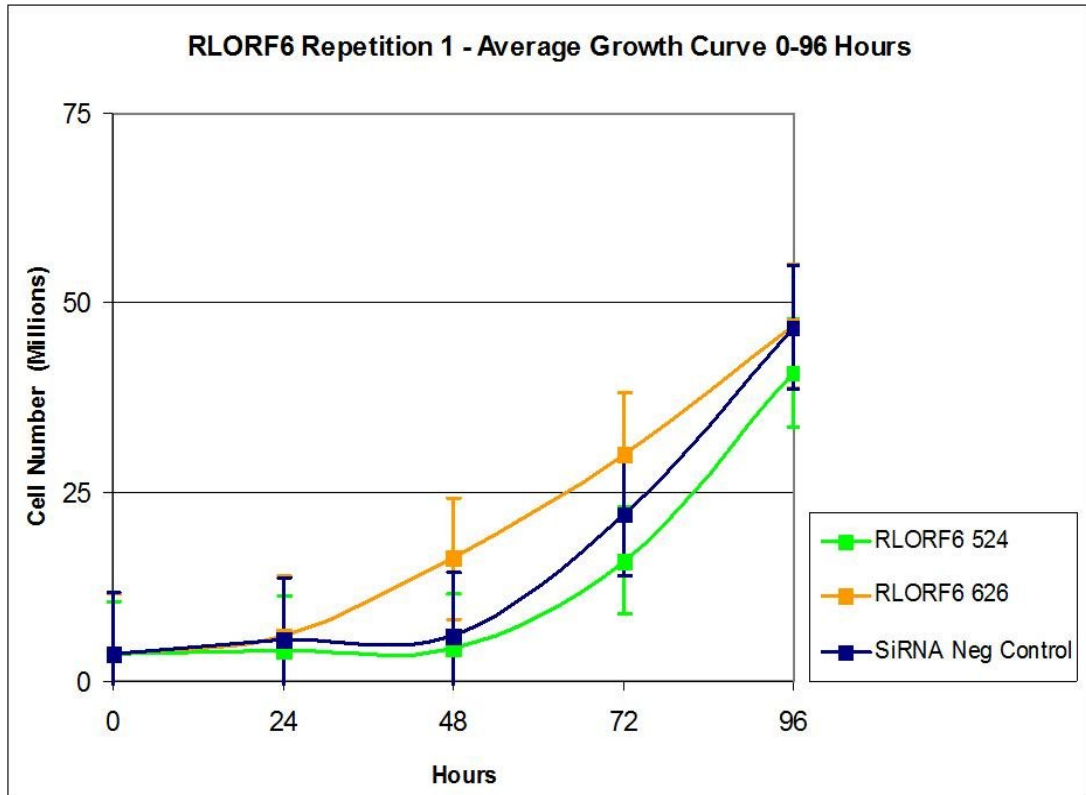
**RLORF6 Repetition 1 - Average Growth Curve 0-96 Hours**

| siRNA Oligonucleotide | Statistical Test | 72 hr p-value | 96 hr p-value | 24h Transfection Efficiency |
|---|---|---|---|---|
| siRNA RLORF6 JM 524 | ANOVA | 0.7265 | 0.4777 | 33.92% |
| siRNA RLORF6 JM 626 | ANOVA | 0.0094 | 0.4134 | 21.35% |
| siRNA Control | N/A | N/A | N/A | 31.29% |

**Figure 3.22a:** RLORF6 cell growth data for first repetition of experiment in RPL-1 cells showing error bars equivalent to 1 standard error. The general linear model ANOVA test for significance compared the experimental siRNA oligonucleotides to the siRNA Control oligonucleotide for all three repetitions and adjusted the p-value to take into account the replicate effect observed. Transfection rates were determined as discussed in section 2.5.3 and calculated as an average value. N/A denotes not applicable.
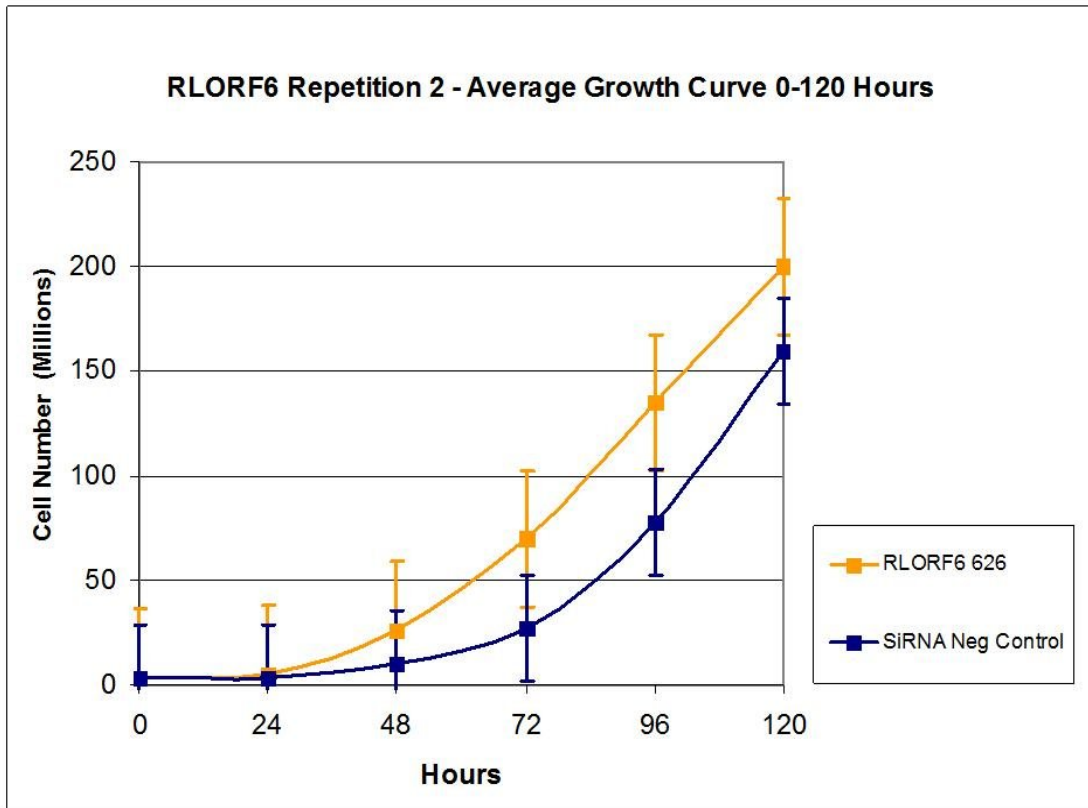
**Figure 3.22b:** RLORF6 cell growth data for second repetition of experiment showing error bars equivalent to 1 standard error. Note the relatively higher rate of cell growth reflected by the y-axis scale.
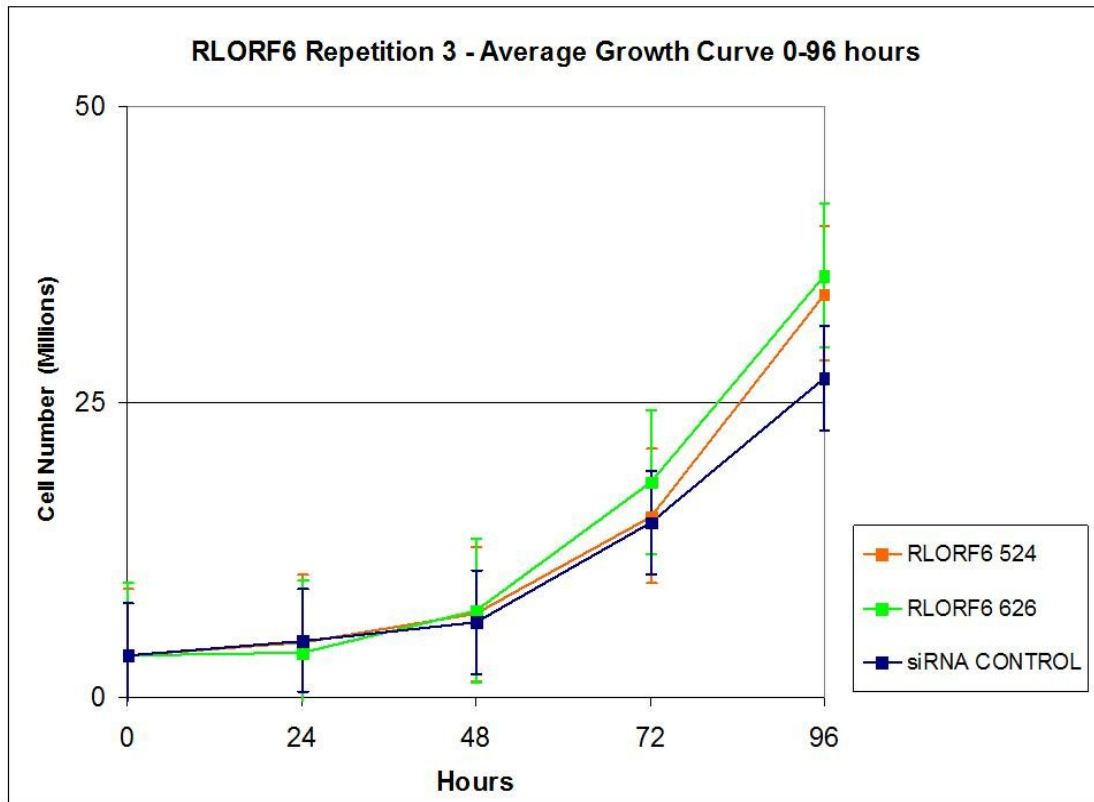
**Figure 3.22c:** RLORF6 cell growth data for third repetition of experiment showing error bars equivalent to 1 standard error.

**Individual Value Plot of data72**
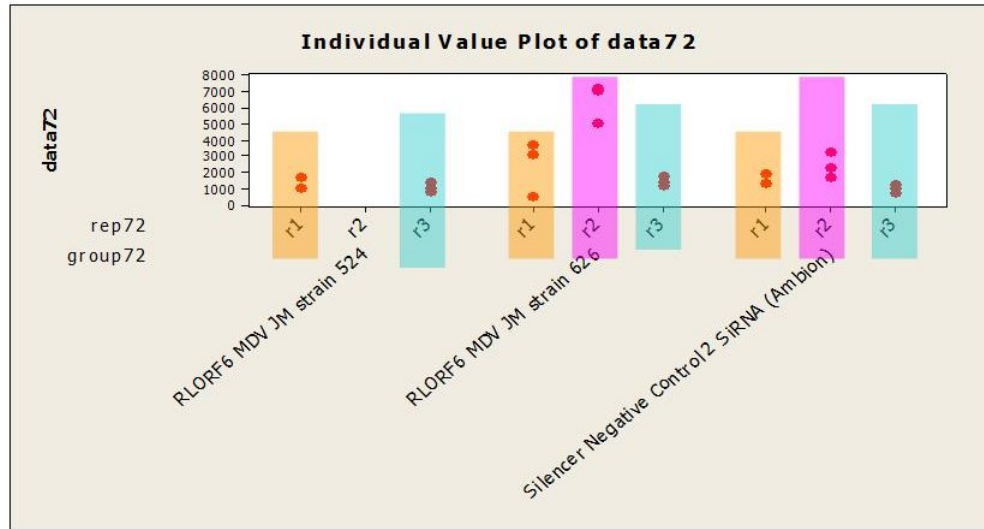
72 h Adjusted p-value RLORF6 524 (siRNA#1) = 0.7265
72 h Adjusted p-value RLORF6 626 (siRNA#2) = 0.0094 **SIGNIFICANT**

**Figure 3.22d:** RLORF6 cell growth data statistical analysis of 3 repetitions of experiment at 72 hours. Note the marked replicate effect existing between the three trial repetitions.

The residual graphs comparing the RLORF6 siRNA 524 and siRNA 626 to the negative control siRNA (figure 3.22d) showed a relatively normal distribution despite there being a marked replicate effect. To correct for the observed replicate effect and because the data was normally distributed statistical analysis was carried out using a general linear model ANOVA to analyze the raw cell growth data from the repetitions of the comparisons between the RLORF6 siRNA 524 and siRNA 626 with the negative control siRNA (figure 3.22d). When the ANOVA was performed on the 72 hour post-transfection data and adjusted for the replicate effect with pair wise comparisons the adjusted p-value for siRNA JM RLORF6 524 was 0.7265 and siRNA JM RLORF6 626 was 0.0094. The same calculations were also performed with the 96 hour post-transfection data and no significant differences were identified ($p >> 0.05$, figure 3.22a) Based on these calculations it was concluded that RPL-1 cells transfected with the oligonucleotide siRNA JM RLORF6 626 led to a significantly higher cell growth rate than the siRNA control oligonucleotide at 72 hours post-transfection, but not at 96 hours post-transfection. It was also concluded that a significant increase in RPL-1 cell growth rate was not seen for siRNA JM RLORF6 524 transfected cells over siRNA control at 72 or 96 hours post-transfection.

Some of the reasons for these observations could be due to the low number of replicates as the experiment was only repeated twice for the RLORF6 siRNA524 rather than the three times for the RLORF6 siRNA626. Other reasons for the lack of significant differences could have been related to variable efficiencies of the siRNA

oligonucleotides selected or variable cell transfection rates which will be discussed later in the chapter. Based on the significantly higher cell growth rate observed in siRNA JM RLORF6 626 transfected RPL-1 cells, follow-up studies utilizing RTqPCR, confocal microscopy and protein blotting were carried out for RLORF6 which will be detailed in the following chapter.

## 3.6  <u>Summary</u>

This study analyzed several uncharacterised genes in MDV for expression in latency. Genes found to be expressed in latency were targeted with post-transcriptional siRNA knock down in a latent model MDV transformed cell line using growth rate as an indicator of changed phenotype. Fifteen uncharacterised MDV ORFs were screened using PCR for expression in latency in the non-producer MDV-transformed cell line RPL-1:  RLORF5a (L1), 23kDa, RLORF6, RLORF11, MLTI, RLORF12, LORF1, LORF3, MNFH, LORF6, LORF8, LORF11, LORF12, ANTISENSE and US2.  Eleven of these ORFs were found to be expressed in latency:  23kDa, RLORF6, RLORF11, MLTI, RLORF12, LORF1, LORF3, LORF11, LORF12, ANTISENSE and US2.  25-mer siRNA oligonucleotides were designed to target seven of these ORFs for gene knockdown studies in RPL-1 and Rb1b T cells:  LORF1, LORF3, LORF11, RLORF6, RLORF12, ANTISENSE and MLTI.  The non-producer MDV transformed chicken lymphoblast cell line RPL-1 was used as the latent model for the siRNA transfection studies and was persistently infected with the JM strain of MDV (Nazerian *et al*., 1976). The complete sequence of the JM strain was not available, however the JM/102W strain

was a clone of the prototype JM strain, and the IRL region sequence was available

(Spatz and Silva, 2007b). This sequence was used to design the RLORF6, RLORF11,

MLTI and RLORF12 25-mer siRNA oligonucleotides to use in the transfection studies.

The sequences of JM strain MDV LORF3, LORF11, LORF12, ANTISENSE and US2

were sequenced from a template of DNA purified from RPL-1 cells (sections 2.1.2 &

2.1.16). Sequencing of strain JM MDV LORF1 was unsuccessful due to homologous

regions in the MDV genome causing non-specific binding of the primers, and the

decision was made to utilize the published Rb1b genome (Spatz *et al*., 2007a) to design

the siRNA and transfect it into Rb1b T cells for siRNA *in vitro* studies. Two siRNA

oligonucleotides targeting each individual gene product and a negative control siRNA

were transfected in triplicate into MDV-transformed chicken lymphoblasts along with a

transfection indicator (RPL-1 cells for all but LORF1 which used Rb1b T cells) which

allowed the study of what the effect of gene product removal had on phenotype *in vitro*.

There were no significant differences in cell growth with post-transcriptional siRNA

knockdown identified in any of the genes at the 95% confidence interval except for

siRNA JM RLORF6 626 where there was an increase in cell growth over the siRNA

control at 72 hours with an adjusted p-value of 0.0094 (Figure 3.22d). As this result was

significant and the same trends were seen during 3 repetitions of the experiment the

decision was made to validate these results and further characterize the RLORF6 gene

which will be discussed in chapter 4.

**Discussion**

## 3.7  <u>Overview & Introduction</u>

There are at least 113 unique ORFs in the MDV genome.  To date barely a third have

been well-characterised in the literature.  This project intended to examine some of the

less-studied ORFs in MDV and find out if they were expressed in latency and look at

what effect post-transcriptional siRNA knock down would have in a latent MDV

transformed cell line using growth rate as an indicator of changed phenotype.  It was

hypothesized that more regulatory functions were operating in latent MDV infection

than those controlled by the well-studied genes such as vIL-8, Meq, pp38 and pp24 as

the complexity of MDV infection was likely to be controlled by many gene interactions

rather than the relatively few that had been studied to date.  Proteomic comparisons

between MDV infected and mock-infected chicken cells revealed large numbers of

chicken proteins unique to the MDV infected group.  Following MDV infection an over

20-fold greater number of proteins were found to be expressed or changed in the host

chicken cell, particularly among phosphorylated proteins, than there were present in the

MDV genome (Ramaroson *et al*., 2008).  This would suggest that many of the genes in

MDV play multiple roles and interact with multiple target molecules.  Many of these

interactions may not be apparent *in vitro* however, as many target molecules may play a

role in complex processes such as immune regulation in the infected animal.  With such

a large number of potential gene targets the decision was reached that there was a

reasonable likelihood that knockdown of some genes could produce phenotypic

differences that would be apparent *in vitro*.  Screening for gene interactions *in vivo* is a

very difficult and expensive process which is why *in vitro* studies such as the ones used

in this project can be useful to obtain information about some phenotypic changes

produced by the absence of a gene product.  The hypothesis was that any genes

producing a measurable phenotypic change *in vitro* would have changed the protein

interactions within the cell and therefore may act as a regulatory gene that should be

further characterised.


**3.8   Selection of Genes for Study**

Because of the timescale and scope of the project only a limited number of genes could be

studied.  The decision was made to focus mainly on the $TR_L$ and $IR_L$ regions of the

genome as they contain many genes unique to MDV which would be most likely to be

associated with latency and transformation.  Of particular interest were the uncharacterised

genes in the region of the origin of replication as many genes surrounding this region are

unique to MDV and have been implicated in latency, transformation and early lytic

replication (Parcells *et al*., 2003).  An initial literature search was undertaken on selected

candidate genes as discussed in chapter 1.  These selected genes were then subjected to

further analysis to identify possible target molecules or functions.  The motif scans in table

3.2 outlined multiple matches with the potential to affect regulatory functions in each of

the selected genes.  The "Prosite MyHits" online database (Pagni *et al*., 2007) used for the

protein motif scans utilized a series of algorithms to determine the probability of

sequences corresponding with recognized protein motifs.  The matches produced could

either be true positives that were genuinely homologous to recognized patterns or false positives which attributed the observed similarity to chance. The algorithm categorized these matches as strong, which was defined as being very unlikely to be due to a false positive result, and weak which required further biological evidence for definitive proof of function. These definitions were used to describe the results shown in table 3.2. Most of the results noted in table 3.2 were weak matches, so it is important to note that before any final conclusions could be made further biological evidence would be needed. Of particular note was the prevalence of various phosphorylation site motifs found for many functions such as casein kinase II phosphorylation, cAMP- and cGMP-dependent protein kinase phosphorylation, protein kinase C phosphorylation and tyrosine kinase phosphorylation in every gene analyzed with the exception of MLTI. Protein kinases are a large class of enzymes that modify other proteins by phosphorylation which can change the function of the target protein (Lehninger *et al.*, 1993). Proteomic comparisons between MDV infected and uninfected chicken cells (Ramaroson *et al*., 2008) have shown that there was a marked increase in phosphorylated proteins in the MDV-infected group over the uninfected group. This led to the hypothesis that genes containing multiple predicted phosphorylation sites could potentially play a role in regulation of MDV infection and would be appropriate targets for the study. MLTI was interesting in that it contained a repeating motif encompassing seven predicted transmembrane regions as well as 7 corresponding mystroylation and N-glycosylation sites in strain CVI988 MDV (8 transmembrane regions and 13 mystroylation and N-glycosylation sites in strain Rb1b MDV), all of which could be consistent with a transmembrane receptor molecule. The

undescribed MNFH (ORF49.1) was also interesting as in virulent strains it encodes a 93-94 aa protein, but in the attenuated CVI988 strain it was truncated to a 34 aa protein due to a point mutation (Spatz *et al*., 2007b). The MNFH protein was, however, not included in the published CVI988 genome (GenBank DQ530348.1) or any of the other pathogenic strains discussed in the article (Spatz *et al*., 2007b) and reconstruction of the aa sequence for a protein motif scan based on the published reference point of 80737-80872 bp was unsuccessful as the start and stop codons were not able to be determined from the information given. Even though there was no protein motif information the decision was made to include MNFH in the study based on the apparent sequence differences between the virulent and attenuated MDV strains. Many of the genes that were analyzed for protein motifs showed an additional variety of weak matches which could be consistent with functional proteins such as: Microbodies C-terminal targeting signal (a motif associated with retention signals for protein sorting, often in the endoplasmic reticulum), DUF1509 (a protein of unknown function found in MDV-like viruses), Ankyrin repeats (a common protein-protein interaction motif), Major Vault Protein repeat profiles (a novel component of multi-subunit structures that may act as scaffolds for proteins involved in signal transduction) and FMRFamide related peptide family (which has been associated with neurosecretory cells). Although most of the protein motifs were weak matches the decision was made that it was possible that the proposed uncharacterised target genes could be functional proteins and therefore appropriate for inclusion in the study.

### 3.9   PCR Screening for Expression in Latency

LORF6 has been shown to down-regulate MHC I in host cells which is thought to be a key step in immune evasion during MDV infection (Jarosinski *et al.*, 2010).  This finding suggested that LORF6 would most likely be expressed with the early genes during MDV infection and the study hypothesized that it may also operate during latency and reactivation.  However, we could find no evidence of expression of RLORF6 in RPL-1 cells and therefore it seems that it may not function in this way in the latent model RPL-1 cells.  MNFH, RLORF5a/L1 and LORF8 were also not found to be expressed in latent RPL-1 cells, but as limited information on their function was available no hypothesis could be formed on the possible significance of these findings. It is important to note that there was some overlap on the same strand with other ORFs for MLTI, RLORF 12, LORF1 and LORF3 (table 1.9) which could have interfered with PCR based methods of quantification and affected PCR based screening assays.
 All of the remaining genes screened appeared to be expressed at some level in RPL-1 cell cDNA indicating that the gene products were expressed during latency, but again no hypothesis could be formed regarding the significance of these findings due to the limited information available for these genes.


### 3.10   Sequencing of Genes of Interest in JM Strain MDV

The attenuated CVI988 MDV genome was used for the sequencing reaction primer selection as the JM/102W strain MDV IRL contained many similarities.  Both strains contained a 177 bp insertion in the Meq gene region and a 10 bp deletion in the

RLORF12 gene which were not found in comparisons of JM/102W with more pathogenic strains such as Rb1b (Spatz *et al*., 2008; Spatz and Silva, 2007b). Based on these observations the decision was made that the likelihood of strains CVI988 and JM being homologous for the selected primer sequences was greater than the likelihood of homology with one of the more pathogenic strains (Spatz *et al*., 2007a).

A comparison of the sequenced strain JM MDV with attenuated CVI988 and pathogenic Rb1b strains revealed 100% homology for the LORF3, LORF12 and ANTISENSE genes. This indicated that these genes were probably conserved between strains of MDV. In any future follow-up studies it would be possible to use the siRNA oligonucleotides designed for JM strain MDV for post-transcriptional gene product targeting in other cell lines based on the Rb1b or CVI988 strains of MDV. Comparison of the strain JM MDV LORF11 and US2 sequences were found to be homologous with strain Rb1b MDV. Analysis of strain JM attenuated strain CVI988 MDV LORF11 revealed 4 one bp substitutions resulting in 3 aa substitutions. Comparisons of the strain JM and CVI988 MDV US2 sequences revealed a one bp substitution resulting in a one aa substitution.

In LORF11 the first aa substitution was alanine to valine at position 57 which both carry no charge and are hydrophobic (hydrophobicity index +1.0 and +2.3 respectively) (Lehninger *et al.*, 1993). This probably would not change the orientation of a membrane-bound protein, however, as both aa were hydrophobic. The second

substitution identified was threonine to alanine at position 302 which represents a change from a hydrogen-bonding hydrophilic molecule to a non- hydrogen bonding hydrophobic molecule (Lehninger *et al.*, 1993) which could potentially change the affinity of a molecule to water and interfere with the orientation of a membrane-bound protein. Comparison of protein motif scans of the sequenced strain JM and CVI988 MDV LORF11 revealed the loss of one casein kinase II phosphorylation site motif at aa position 302 due to this mutation which could impact protein function. The third substitution was proline to leucine at position 780 which denotes a change from a rigid hydrophilic aa to a strongly hydrophobic aa with less steric hindrance (hydrophobicity index -1.6 and +3.8 respectively) (Lehninger *et al.*, 1993). This substitution had the potential to change the shape and orientation of the LORF11 protein at this site. As the exact structure and function of LORF11 was not fully known at the time of this study it can only be speculated as to whether these aa substitutions and the loss of a casein kinase II phosphorylation site had any bearing on attenuation in the CVI988 strain.

Comparison of US2 sequence between MDV JM & Rb1b strains and CVI988 strain identified one aa substitution of alanine to valine at position 51. This substitution represents a substitution of a mildly hydrophobic aa for a strongly hydrophobic one (hydrophobicity index +1.8 and +4.2 respectively) (Lehninger *et al.*, 1993). As the fundamental properties of the CVI988 substituted aa were not substantially different from the original aa there were probably no changes between the gene product targets of

strain CVI988 US2 and strain JM or Rb1b MDV US2.  Comparison of protein motif

scans of the US2 gene of JM and CVI988 strains also revealed no differences.

### 3.11   Knockdown of Viral Gene Expression Using siRNA

The lack of significant changes in cell growth rate following post-transcriptional gene

targeting of LORF1, LORF3, LORF11, RLORF12, ANTISENSE and MLTI was

disappointing and could indicate that these genes did not play a role in determining cell

proliferation.  However, the lack of phenotypic changes seen for *in vitro* post-

transcriptional targeting could have been due to extraneous factors such as the effects

produced by gene product knockdown not effecting cell proliferation or that the siRNA

oligonucleotides used were not effective in knocking down their target.  Transfection

efficiency also could have played a role in the lack of observed changes in cell growth.

Transfection rates achieved for MDV transformed chicken lymphoblasts using the

Nucleofector II transfection system (Amaxa, Lonza) had been reported to be in the

region of 20-30% with 99% viability by the manufacturer's online database

(http://www.lonzabio.com/no_cache/meta/cell-database/cell-details/cell/1149, accessed

4/10/10) which was somewhat lower than the overall average of 36.71% +/- 14.04%

calculated in this study.  Variations in transfection rates observed during the study

appeared to be more dependent on the replicate rather than the transfected siRNA

oligonucleotide group which indicated that the source of variation was probably not due

to cytotoxic effects produced by particular siRNA oligonucleotides.  It is tempting to

speculate that this could have had an influence on the replicate effect observed in the

RLORF6 cell growth data, but further studies would be needed for confirmation.


Transfection rates as high as 75% with an 80% viability have been documented in EBV

transformed lymphocytes using the Nucleofector transfection system which is regarded

as one of the most reliable and reproducible methods available for transfecting

lymphoblastic cell lines (Maurisse *et al*., 2010).  Reported transfection efficiency and

viability can vary a great deal between apparently similar cell lines, however, so these

efficiencies would probably not be reproducible in RPL-1 cells.  Optimization of another

MDV transformed chicken lymphoblastic cell line, MSB-1, undertaken in our lab by Dr.

Jeanette Webb, showed a transfection rate of 67% efficiency with a 50% viability using

Nucleofector program X-001.  These rates could not be reproduced on this program with

RPL-1 cells transfected with siRNA however so a separate optimization was carried out

which is detailed in Appendix 3.  After optimizing the RPL-1 cells in our lab as detailed

in section 2.5.3 the most efficient program identified was A-033 using solution V which

produced an efficiency of 35.7% and 18.3% viability using the pmaxGFP reporter

plasmid (Amaxa, Lonza).  These results raised the possibility that the transfection

efficiency was too low for any effects of siRNA post-transcriptional gene knockdown in

the transfected cells to be detected in mixed populations with untransfected cells.  A

second attempt to optimize transfection of RPL-1 cells, undertaken after the siRNA

transfection studies had been completed, identified another program A-30 that produced

a 26.8% efficiency and a 96.6% viability which may be useful for increasing transfection

efficiency in future siRNA studies in RPL-1 cells. The poor transfection and viability rates of the RPL-1 cells coupled with the other factors identified above make the finding of an increased growth rate in siRNA JM RLORF6 626 transfected cells despite these conflicting factors remarkable.

The overlapping nature of RLORF6 and Meq on the L to R strand and 23kDa on the complimentary strand makes targeting and measuring expressed levels of one gene without measuring or affecting the others very difficult-hence the name 'Meq loci'. Because of the extensive gene overlap in this location conventional PCR and RTqPCR were of limited use in assessing gene knockdown as primers specific for one gene would also be specific for the overlapping genes and it would not be possible to determine relative levels of individual transcripts. An antibody specific to Meq was kindly made available by Dr. Venugopal Nair (Institute of Animal Health, Compton, UK), but no antibodies were available specific to 23KdA or RLORF6. The Meq antibody was used for protein immunoblots in some of the follow up studies which will be discussed in chapter 4, but relative levels of these 3 genes could not be accurately determined with the tools available in the study.

The results showing an increased RPL-1 cell growth rate following siRNA JM RLORF6 626 transfection were particularly interesting because post-transcriptional targeting of Meq in MDV transformed cell lines had been shown to result in cell death as Meq is necessary for the maintenance of latency (Brown *et al*., 2006). Therefore any post-

transcriptional targeting of Meq inadvertently produced by siRNA JM RLORF6 oligonucleotides would be expected to cause cell death rather than the observed increase in cell growth.  Clarification is needed as to why post-transcriptional targeting of RLORF6 in these experiments produced a significant increase in cell growth despite the substantial background factors of poor transfection rates, poor cell viability and potential siRNA cross targeting of Meq.  Future siRNA studies of RLORF6 need to address these issues of low cell transfection efficiency and viability in order to decrease the potential for error due to the transfection process which would depict a more accurate picture of the role that RLORF6 plays in latent MDV infection.


**3.12** **<u>Conclusions</u>**

Targeting of RLORF6 in the RPL-1 cell line appeared to have a measurable effect on RPL-1 cell growth in at least one of the siRNA oligonucleotides used in the transfection studies despite complicating factors such as mixed cell populations and poor transfection rates.  This unanticipated finding raised several questions such as what was the host cell target of RLORF6 in latent and lytic MDV infections and does RLORF6 interact directly or indirectly with Meq in latent MDV infections?  Before these questions could be addressed though studies should be done to confirm the observed RLORF6 siRNA cell growth results were genuine preferably in a 90-100% transfected cell population and to ascertain that the RLORF6 siRNA used was both effective and specific against its intended target.  Cell lines other than RPL-1, such as Rb1b T cells, could be trialled in future studies in an attempt to increase cell transfection rates as they appear to vary

substantially between different cell lines.  If higher transfection rates and increased viability could be achieved it may be possible to sort transfected and untransfected cell populations using methods such as fluorescence activated cell sorting. This approach was not tried in the current studies as the observation was made that RPL-1 cells did not grow well at low densities and it would not be possible to isolate sufficient numbers of cells without multiple replications which were not possible in the timescale of the study. Most of the uncharacterised gene product targets in the host cell were still unknown at the time of this study, but given the large number of protein changes in the chicken genome relative to the number of MDV genes following MDV infection (Ramaroson *et al.*, 2008) it is very likely that most MDV genes have multiple targets and functions.  It is possible that some of the genes studied that did not produce any measurable phenotypic changes when targeted *in vitro* would effect phenotypic changes when targeted *in vivo*.  Unfortunately *in vivo* experiments for the uncharacterised genes in this study and determination of the molecular targets of RLORF6 were beyond the timescale and budget of the current project.

# Chapter 4 – Characterization of MDV RLORF6

**Results**

## 4.1  Project Objectives

The objective of the work undertaken in this section was to confirm that the siRNA JM

RLORF6 oligonucleotides used in the experiments outlined in chapter 3 selectively

targeted RLORF6.  This was done in two phases:  Firstly performing a dual-reporter

luciferase assay using the RLORF6 Luc firefly luciferase fusion plasmid co-transfected

with the pRL-SV40 *Renilla* luciferase plasmid to confirm that the siRNA RLORF6

oligonucleotides effectively targeted RLORF6 and secondly using protein

immunoblotting and gene-specific RTqPCR to confirm that Meq was not co-targeted by

siRNA RLORF6 JM 524 and 626.  A secondary objective was to determine the

localization of RLORF6 in the MDV infected cell.  This was undertaken with

transfection experiments with the pEGFP RLORF6 and pDSRed Meq fusion plasmids

and analysis using confocal laser microscopy.


## 4.2  Determination of siRNA Knockdown of RLORF6

A key assumption in interpreting the cell growth data in chapter 3 was that the siRNA

oligonucleotides used were effectively knocking down expression of their target gene.  If

the oligonucleotides had not effectively targeted their intended gene product then any

observations regarding their effect on cell growth would be invalid.  In order to validate

this assumption a dual reporter luciferase assay was used to confirm the effectiveness of

siRNA JM RLORF6 524 and 626 in reducing post-transcriptional expression of

RLORF6.  An N-terminal firefly luciferase fusion plasmid based on the pGL3 plasmid vector (Promega) and encoding the MDV strain CVI988 RLORF6 gene (designated RLORF6 Luc) was kindly provided by Ola Ali Hassanin (Division of Pathway Medicine, University of Edinburgh, UK) for use in the assay.  The pRL-SV40 *Renilla* luciferase control reporter vector (Promega) encoded the *Renilla* luciferase gene and was used as the *Renilla* control plasmid in the assay.  The pGL3 plasmid (Promega) encoded the firefly luciferase gene and was used as the luciferase control plasmid.  The full protocol used for the dual reporter luciferase assay was detailed previously in section 2.6 and the experimental design is outlined in figure 4.1.
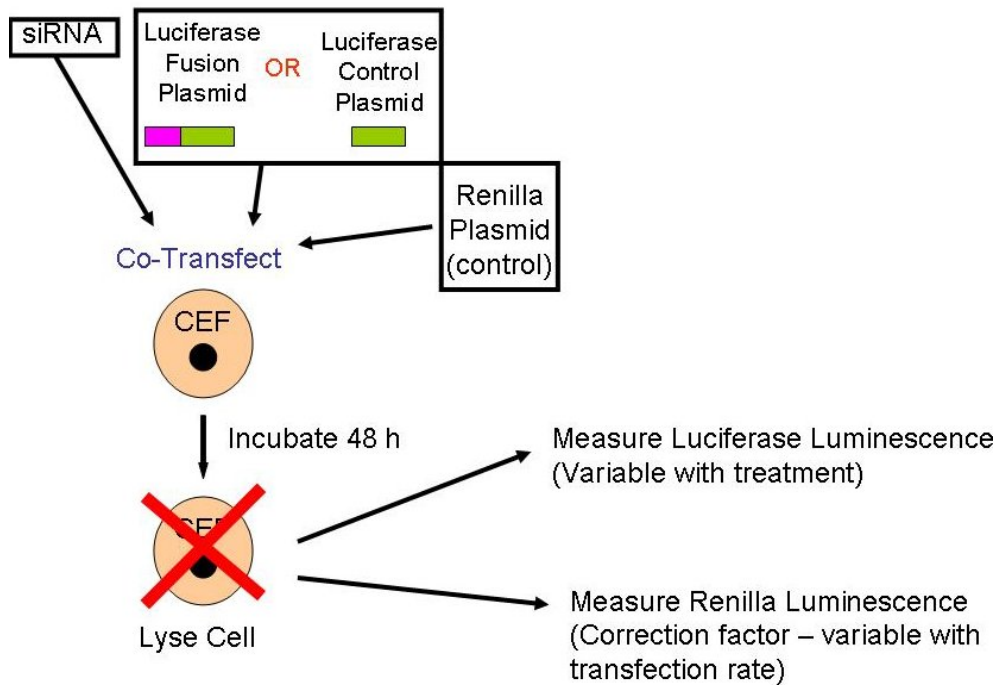
| Experimental Groups | Description |
|---|---|
| Non-Transfected Control (NTC) | Luciferase Background control |
| RLORF6 Luc + Renilla SV40 | RLORF6 Luc w/ no siRNA |
| RLORF6 Luc + Renilla SV40 + siRNA RLORF6 524 | RLORF6 Luc w/ siRNA RLORF6 524 |
| RLORF6 Luc + Renilla SV40 + siRNA RLORF6 626 | RLORF6 Luc w/ siRNA RLORF6 626 |
| RLORF6 Luc + Renilla SV40 + siRNA Control | RLORF6 Luc w/ siRNA Control |
| pGL3 + Renilla SV40 | Luciferase Control w/ no siRNA |
| pGL3 + Renilla SV40 + siRNA RLORF6 524 | Luciferase Control w/ siRNA RLORF6 524 |
| pGL3 + Renilla SV40 + siRNA RLORF6 626 | Luciferase Control w/ siRNA RLORF6 626 |
| pGL3 + Renilla SV40 + siRNA Control | Luciferase Control w/ siRNA Control |

**Figure 4.1:** Schematic diagram of dual reporter Luciferase assay experimental design.

Non-transfected control (NTC) samples were used to calculate a correction factor for background luminescence from instrumentation and sample tubes for each data point. Readings for both firefly luciferase and *Renilla* luminescence were taken for each sample. The siRNA JM RLORF6 oligonucleotides 524 and 626 were expected to target and degrade the RLORF6 Luc fusion protein which would result in reduced firefly luciferase luminescence in treated samples. Firefly luciferase readings were used as a measure of siRNA targeting of RLORF6. *Renilla* luciferase produced a luminescence distinct from firefly luciferase and was expected to be expressed at a constant rate in co-transfected cells as siRNA JM RLORF6 oligonucleotides were not designed to target the pRL-SV40 plasmid. Any observed variation in *Renilla* luciferase luminescence would therefore be due to variations in transfection rate. The *Renilla* luciferase luminescence was used to calculate a correction factor for the firefly luciferase reading to adjust for variations in transfection rate which was designated the adjusted luminescence. The primary aim of this experiment was to compare the adjusted luminescence produced in CEF cells by co-transfecting with one of the following: siRNA JM RLORF6 524, siRNA JM RLORF6 626, siRNA Control or no siRNA and both the RLORF6 Luc and pRL-SV40 plasmids. If the siRNA JM RLORF6 oligonucleotides effectively targeted RLORF6 then a reduction in luminescence would occur only in siRNA JM RLORF6 treated samples and not in siRNA Control or no siRNA samples. Two control experiments were incorporated into the experimental design to determine correction factors for the luminescence readings produced by extraneous factors not related to siRNA targeting of RLORF6. The first experimental control was a comparison of

samples transfected with RLORF6 Luc and pRL-SV40 (*Renilla*) with samples

transfected with siRNA Control, RLORF6 Luc and pRL-SV40 (*Renilla*) which

determined the change in measured luminescence produced by transfection with non-

targeting siRNA.  The second control was to duplicate the experiment substituting the

pGL3 plasmid which encoded only firefly luciferase for the RLORF6 Luc plasmid

which encoded both firefly luciferase and RLORF6.  This was undertaken to determine

whether there was any reduction of luminescence attributable to non-specific targeting

by siRNA JM RLORF6 oligonucleotides.  The effect of siRNA transfection on

luminescence was determined by comparing cells transfected with siRNA Control

oligonucleotide and cells transfected only with DNA plasmid.  Non-specific targeting of

firefly luciferase by siRNA JM RLORF6 would be expected to reduce luminescence in

the duplicate experiment transfected with pGL3 which did not encode any MDV viral

genes.  Three repetitions of each experimental group were prepared and the adjusted

luminescence readings were summarized in figure 4.2.

## Adjusted Luciferase Readings

Luciferase Units

89%

76%

32%

22%

- RLORF6 luc & pRL-SV40 Renilla
- RLORF6 luc & siRNA RLORF6 524 & pRL-SV40 Renilla
- RLORF6 luc & siRNA RLORF6 626 & pRL-SV40 Renilla
- RLORF6 luc & siRNA Control & pRL-SV40 Renilla
- pGL3 & pRL-SV40 Renilla
- pGL3 & siRNA RLORF6 524 & pRL-SV40 Renilla
- pGL3 & siRNA RLORF6 626 & pRL-SV40 Renilla
- pGL3 & siRNA Control & pRL-SV40 Renilla
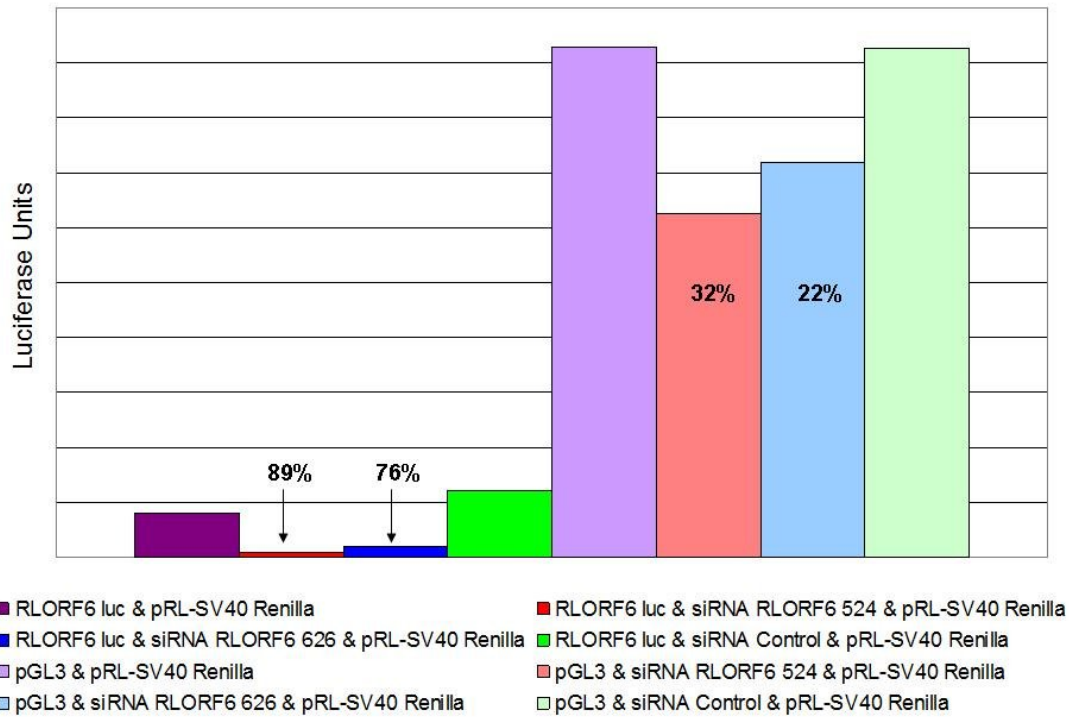
**Figure 4.2:** Averaged results of the dual-reporter luciferase assay for RLORF6. Percentages shown are the reduction of adjusted luminescence compared with samples treated with siRNA Control.

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The results of this experiment (figure 4.2) showed an 89% reduction of adjusted

luminescence in cells treated with siRNA JM RLORF6 524 over siRNA Control #2.  A

76% reduction was measured for siRNA JM RLORF6 626.  In CEFs transfected with

pGL3 instead of RLORF6 Luc there was a non-specific reduction in luminescence of

32% for siRNA JM RLORF6 524 and a 22% for siRNA JM RLORF6 626.  This was

compared to the higher percentage of luminescence reduction seen in the RLORF6 Luc

experiment and the overall targeting efficiency of siRNA JM RLORF6 524 was

calculated to be 57% and JM RLORF6 626 was 54%.  Percentages were used for this

comparison rather than absolute luminescence as the overall amount of luminescence

produced by the pGL3 plasmid was considerably higher than RLORF6 Luc so a direct

comparison would not accurately represent the differences between the 2 groups.


## 4.3   Effect of RLORF6 Knockdown on Meq Expression

Due to the extensive overlap on the same strand of the predicted Meq and RLORF6

mRNAs (figure 4.3) it was possible that siRNA designed to target RLORF6 could also

inadvertently target Meq.  If this was correct then the siRNA RLORF6 cell growth study

data described in chapter 3 would be difficult to interpret as there would be no way to

determine which gene had affected cell growth.  Two experiments were designed to

investigate this hypothesis by comparing the levels of Meq gene expression and protein

in RPL-1 cells transfected with siRNA oligonucleotides targeting either RLORF6 or a

non-targeting control.  If no significant variations in Meq gene expression or protein

synthesis were found then this would be supporting evidence that siRNA JM RLORF6 oligonucleotides 524 and 626 did not co-target Meq.
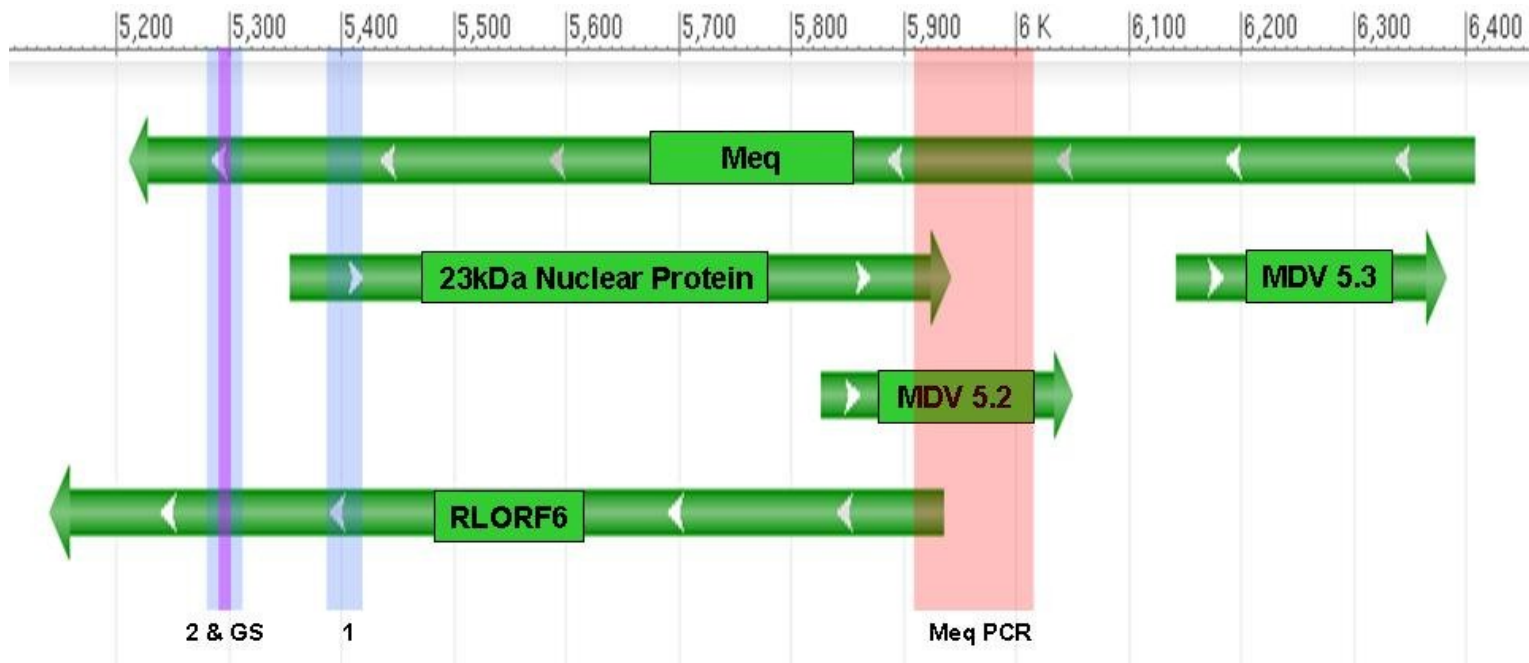
**Figure 4.3:** Depiction of the "Meq Loci" in the TRL region of MDV strain CVI988 (GenBank DQ534539.1) showing in the context in the genome of the location of the Meq gene specific primer (depicted by GS and shaded lavender, bp 5286-5301), siRNA RLORF6 JM oligonucleotides 524 (depicted as 1, bp 5391-5415) and 626 (depicted as 2, bp 5289-5313) which are both shaded in blue and the region amplified by the RTMeq primer set (shaded pink, bp 5911-6013). Arrows indicate the direction of transcription. Overlapping regions of the Meq gene and the RLORF6 gene are clearly visible. Homologous regions to those shown above were identified in the IRL region of MDV strain JM/102W (GenBank DQ534539.1). Diagram was adapted from GenBank online material accessed 11/9/10 (Benson *et al.*, 2008).

### 4.3.1 RTqPCR Studies Utilizing a Directional cDNA Template for Determination of Meq Expression Levels

PCR and RTqPCR assays were used to analyze whether there was any variation in expression of Meq using a cDNA template made with a directional primer specific to the RLORF6 predicted mRNA (designated directional primer). No suitable primers could be identified in the region that did not overlap the Meq predicted mRNA as well so the directional primer was selected in the extreme 3' region of the Meq/RLORF6 genes as close as possible to the non-overlapping RLORF6 region using the JM/102W strain MDV IRL sequence (Spatz and Silva, 2007b). The primer (5'-CGA ATA CAA GGA ATC C-3') which overlapped the Meq/RLORF6 coding regions by approximately 80 bp was chosen with the assistance of Dr. Robert Dalziel (The Roslin Institute, University of Edinburgh, UK). Figure 4.3 shows the relative location in the MDV genome of the directional primer (homologous region in MDV strain CVI988 shaded yellow) and the region of the genome amplified by the Meq primer set (described in table 3.4 and shaded red). The PCR product amplified by the Meq primer set was located approximately 700 bp upstream from the directional primer and would only produce a full length 102 bp PCR product for the Meq and MDV ORF 05.2 genes. Since the directional cDNA template contained only the Meq and RLORF6 genes then only Meq would be amplified in a PCR or RTqPCR assay. The RLORF6 gene would not be expected to produce a PCR product as there would only be one primer site present in the RLORF6 gene rather than the forward and reverse primer site required for a full-length PCR product. Therefore the Meq/RLORF6 specific directional cDNA (designated directional cDNA)

used as a template in a PCR or RTqPCR using the Meq primer set would be specific for Meq only.

To obtain the RNA samples used to synthesize the directional cDNA RPL-1 cells were transfected in triplicate (section 2.5.3) either with siRNA RLORF6 JM 524, siRNA RLORF6 JM 626 or siRNA Control #2 (Ambion, Invitrogen) as part of the third repetition of the cell growth studies in RPL-1 cells previously described in section 3.5. Cell counts were carried out (section 2.4.4) at 24, 48 and 72 hours post-transfection and showed an increase in cell growth of the siRNA JM RLORF6 transfected cells over the siRNA Control transfected cells which was illustrated previously in figure 3.21a. RNA was extracted (section 2.1.3) from cells harvested at 48 and 72 hours post-transfection and used as a template to prepare the directional cDNA as follows: 100 ng of directional primer was used for each AffinityScript Multiple Temperature cDNA Synthesis Kit reaction (Stratagene) in the place of oligo (dT) primers used in non-specific cDNA reactions. The full protocol for synthesis of first strand cDNA was described previously in section 2.1.5. The resulting directional cDNA was then used as a template in two PCR assays (section 2.1.8), using primer sets specific for either RLORF11 or Meq described previously in figure 3.4. The RLORF11 primer set was selected as it had been shown to be effective in previous PCR assays (figure 3.11) and the region of the MDV genome amplified was approximately 5.3 kb downstream from the Meq and RLORF6 genes and was not expected to be represented in the directional cDNA template. The conditions used for the PCR reactions were as follows: 2 ng of template, 30 pg of each

primer, 57ºC annealing temperature and 40 cycles as described in section 2.1.8.  A

higher number of PCR cycles were selected than the standard 25-30 as the aim of these

reactions were to demonstrate the presence of a PCR product and the high cycle number

would maximize amplification.  The resulting PCR products were analyzed using a 2%

agarose gel (section 2.1.13).  The results confirm that the cDNA was specific to

Meq/RLORF6 as an approximately 100 bp band was seen for all the Meq primer

samples (figure 4.4a) and no bands were present for the RLORF11 primer samples

(figure 4.4b).

To extend the PCR findings RTqPCR was undertaken as described in section 2.1.10

utilizing the directional cDNA samples as templates and the Meq primer set (table 2.2

and figure 4.4) to more accurately determine the variation in Meq expression.  A second

RTqPCR assay was also performed using primers specific for chicken β-actin (table 2.1)

and the results were normalized against the 75th percentile of the housekeeping gene as

described in section 2.1.10 to correct for RNA and cDNA variations.  The normalized

results were statistically analyzed using a Mann-Whitney test for non-parametric data as

described in section 2.8 and the p-values are shown in figures 4.5a and 4.5b.  None of

the results were deemed to be statistically significant as all of the p-values were greater

than 0.05 which represents the 95% confidence interval criteria for significance.  Meq

expression at 72 hours was notably lower than at 48 hours in all the experimental

groups, but as the variability between the replicates was high no conclusions could be

drawn as to the significance of these findings.
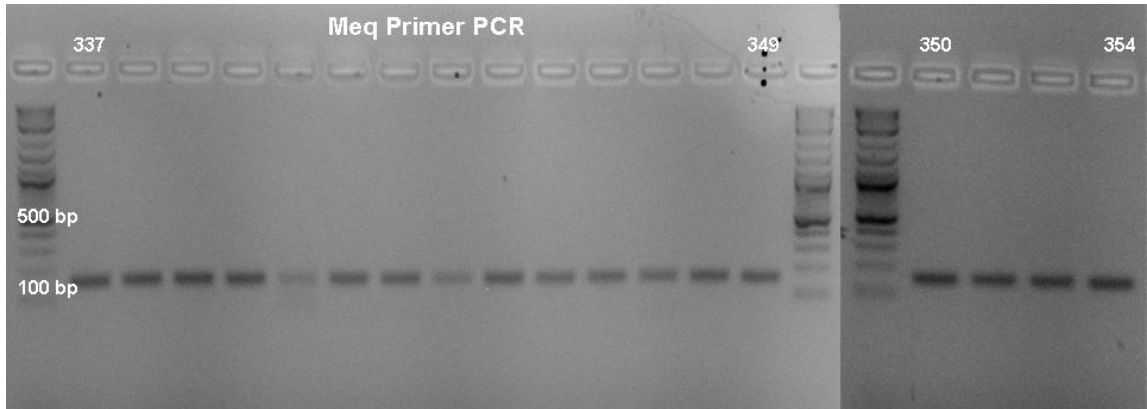
**Figure 4.4a:** PCR results for Meq/RLORF6 specific cDNA using Meq primer set and analyzed on a 2% agarose gel. Bands at approximately 100 bp were identified for every sample which indicated Meq amplification. Ladder used was 2-log DNA ladder (New England Biolabs).
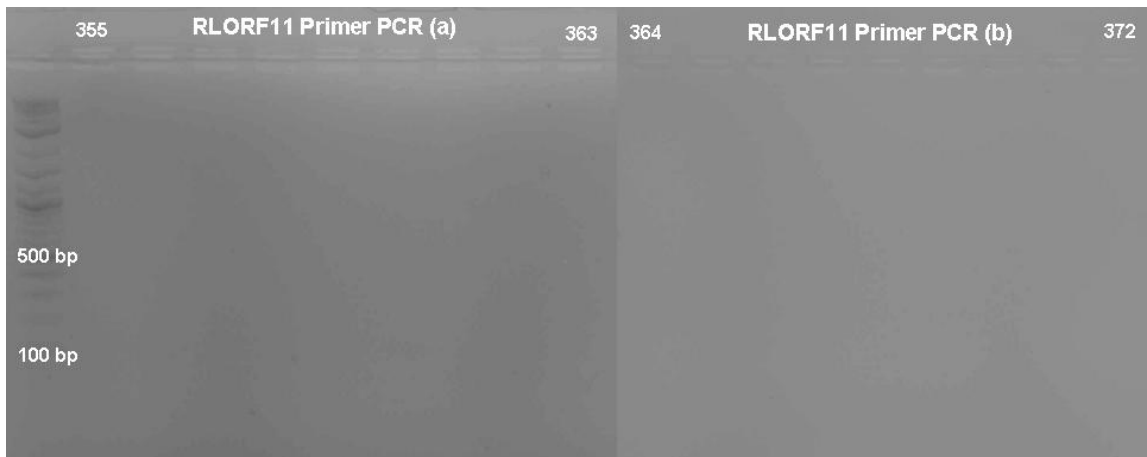


**Figure 4.4b:** PCR results for Meq/RLORF6 specific cDNA using RLORF11 primer set and analyzed on a 2% agarose gel. No bands were identified indicating no amplification of RLORF11. Ladder used was 2-log DNA ladder (New England Biolabs).
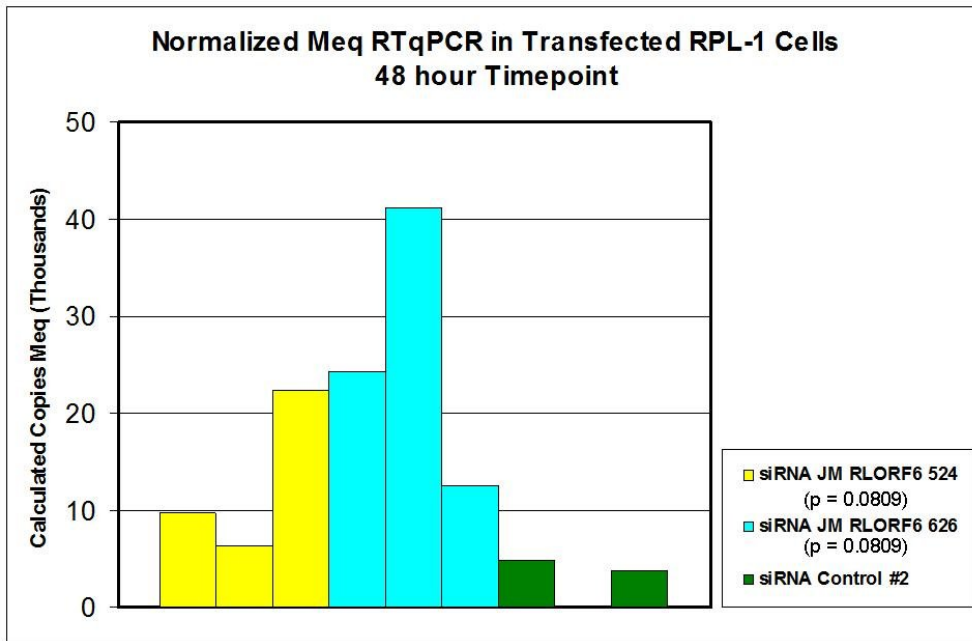
**Figure 4.5a:** RTqPCR data from gene-specific Meq/RLORF6 cDNA at 48 hours. Raw data was normalized against the 75th percentile of the housekeeping gene Chicken β-Actin. Statistical analysis was done using a Mann-Whitney test comparing siRNA RLORF6 JM 524 & 626 with siRNA Control #2.
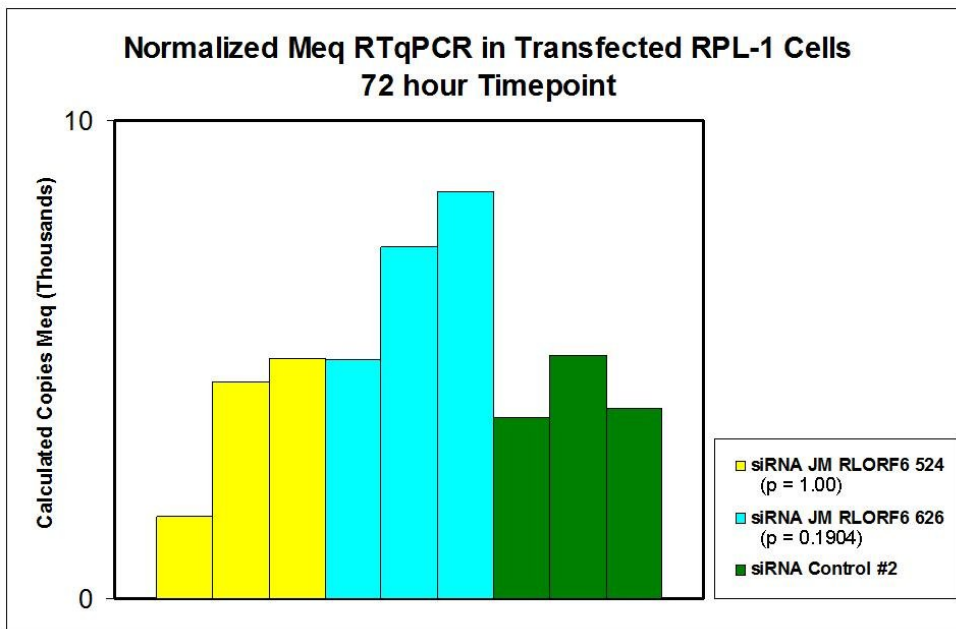


**Figure 4.5b:** RTqPCR data from gene-specific RLORF6 cDNA at 72 hours. Raw data was normalized against the 75th percentile of the housekeeping gene Chicken β−Actin. Statistical analysis was done using a Mann-Whitney test comparing siRNA RLORF6 JM 524 & 626 with siRNA Control #2. Note that y-axis scale is 5-fold less than figure 4.5a.

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### 4.3.2  Protein Immunoblotting Studies to Determine Meq Protein Levels

The results of the previous experiment did not show clear evidence of reduction in Meq expression between the experimental groups.  These findings were encouraging, but were not conclusive.  In an attempt to expand on these results an experiment was designed to assess whether Meq protein synthesis was affected by transfection of siRNA targeting RLORF6 in RPL-1 cells.  Dr. Venugopal Nair (Institute of Animal Health, Compton, UK) kindly provided an antibody to the Meq protein for these studies (Meq Monoclonal Mouse Anti-Meq FD7, described in section 2.3.3).  A protein immunoblot was performed using the antibody to Meq protein to measure Meq protein produced in RPL-1 cells transfected with either siRNA JM RLORF6 524, siRNA JM RLORF626 or siRNA Control oligonucleotides.

Three experimental groups of RPL-1 cells in log phase growth were prepared and transfected in triplicate as previously described (section 2.5.3) with SiGLO red (Dharmacon, Thermo Fisher Scientific) and 100 pmol of one of the following:  siRNA JM RLORF6 524, siRNA JM RLORF6 626 or siRNA Control #2.  Full descriptions of these siRNA oligonucleotides can be found in section 3.5.  Aliquots of cells were taken at 24 hours to determine transfection efficiency as described in section 2.5.3.  Figure 4.6 shows the transfection efficiency results which varied from 8.5 to 29.9%.  This was low but within the general range of transfection rates reported previously in chapter 3.  One replicate from each experimental group was harvested at 24, 48 and 72 hours, washed two times in SPBS and stored in Western Blot Sample Buffer at -80ºC (section 2.10.2)

in preparation for protein immunoblotting which was carried out as detailed in section 2.3. Figure 4.7 shows the resulting protein blot comparing Meq expression levels in RPL-1 cells transfected with either non-targeting siRNA Control, siRNA JM RLORF6 524 or siRNA JM RLORF6 626 at 24, 48 and 72 hours. No quantification of the results was possible but the intensity of the band was assumed to be indicative of the relative amount of Meq protein present. A common source of error in interpreting protein immunoblots is unequal loading of protein samples in the acrylamide gel which can result in variable intensity of immunoblot bands independent of antibody targeting. The amounts of protein in each lane of the gel appeared approximately equal (figure 4.8) when treated with coomassie stain as described in section 2.3.1 to visualize total protein. The assumption was made that equal amounts of sample protein were loaded into the immunoblot protein gel based on these results and therefore any differences in intensity of the immunoblot bands were due to variable amounts of Meq protein. Meq protein bands of samples treated with siRNA JM RLORF6 524 & 626 appeared less intense than cells transfected with siRNA Control at 24 hours indicating a degree of reduction of Meq protein in the siRNA JM RLORF6 treated samples compared to the siRNA Control samples. No obvious differences were noted between the immunoblot bands at 48 and 72 hours post-transfection.

**Figure 4.6:** Transfection efficiency calculated at 24 hours for RPL-1 cells transfected with either RLORF6 JM 524 siRNA, RLORF6 JM626 siRNA or Control #2 siRNA and analyzed by protein immunoblotting with a MDV Meq antibody as described in Section 4.2.2.
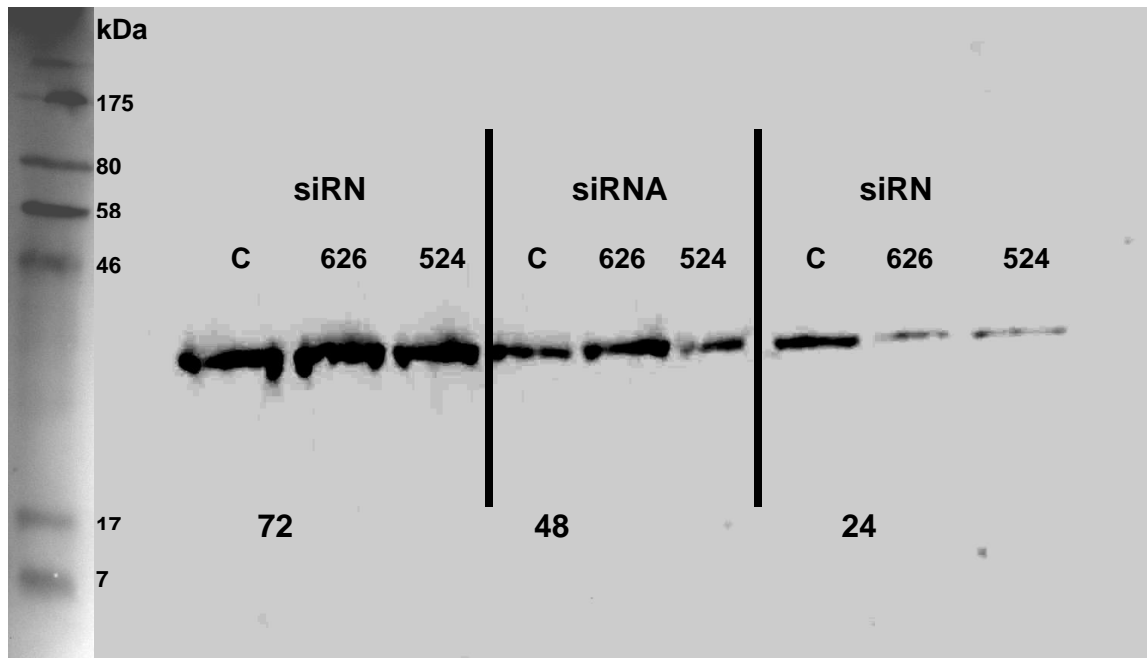
**Figure 4.7:** Protein immunoblot for Meq expression (39 kDa) in RPL-1 cells transfected with a negative control siRNA, RLORF6 siRNA 524 or RLORF6 siRNA 626 at 24, 48 and 72 hours post-transfection. Antibodies used for protein blotting were Meq Monoclonal Mouse Anti-Meq FD7 (Dr. Venugopal Nair) (primary) and Polyclonal Rabbit Anti-mouse Immunoglobulins HRP (Dako Cytomation) (secondary). The proteins were visualized using chemiluminescence (Chemiglow West, Alpha Innotech) and photographed 3 minutes post-reaction.
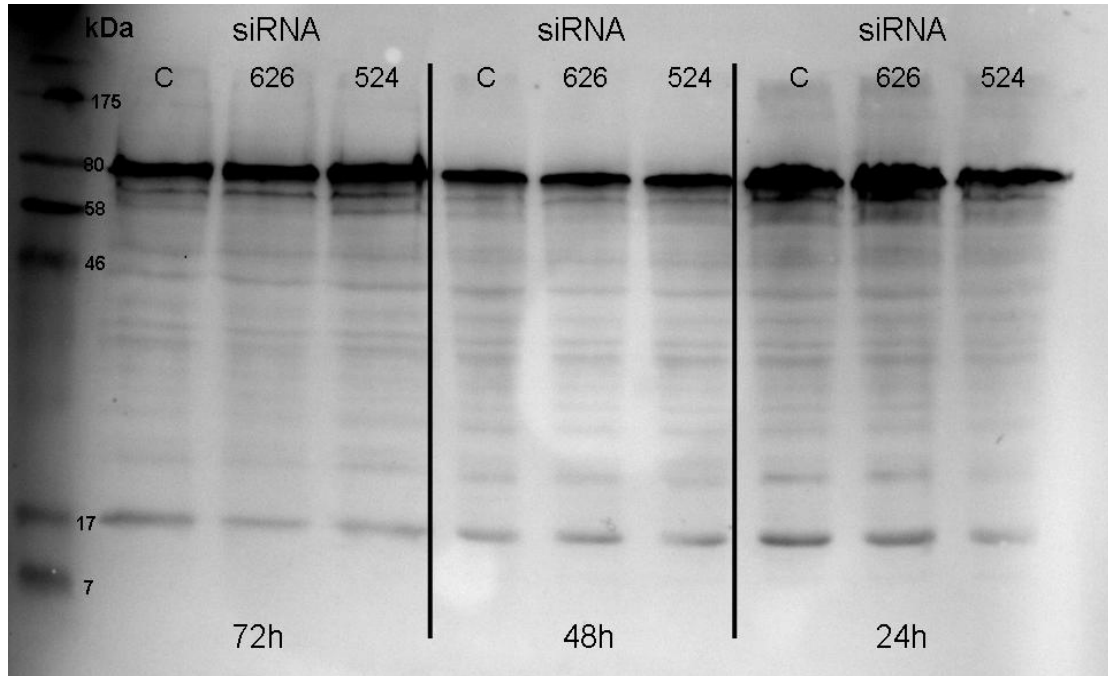
**Figure 4.8:** Coomassie stained protein gel with sample loading as in figure 5.5 which shows approximately equal loading of lanes with protein sample.   The Meq protein size is approximately 39-44 kDa as it exists in several different dimers.  The protein ladder used in far left lane was Pre-stained Protein Marker, Broad Range (7-175 kDa) (New England Biolabs).

The interpretation of this experiment was that transfection of siRNA JM RLORF6 oligonucleotides 524 and 626 appeared to reduce Meq protein expression at 24 hours post-transfection but not at 48 or 72 hours.  The protein immunoblot results were consistent with the results of the directional cDNA RTqPCR discussed previously (section 4.2.1) which found no significant differences in Meq gene expression between the siRNA RLORF6 and control siRNA groups at 48 or 72 hours post-transfection. Both of these findings appeared to disprove the hypothesis outlined previously in section 4.2 that siRNA designed to target RLORF6 could inadvertently target Meq; however a definite conclusion could not be drawn from these studies alone due to the high variability observed between replicates.

## 4.4   Determination of RLORF6 Cellular Localization using Confocal Laser Microscopy

As no data was available in the literature regarding the function of RLORF6 it was decided that determining its host cellular localization would be useful in defining its function.  An N-terminal EGFP fusion plasmid encoding the MDV strain Rb1b RLORF6 gene was kindly provided by Ola Ali Hassanin (Division of Pathway Medicine, University of Edinburgh, UK) for use in confocal laser microscopy studies to determine the cellular localization of RLORF6.  The fusion plasmid was designated pEGFP Rb1b RLORF6 and was based on a pEGFP-C1 (Clontech) plasmid backbone.  The pEGFP-C1 plasmid (GenBank U55763) encodes a red-shift variant of the wild-type green florescence (designated EGFP) optimized for brightness and high expression in mammalian cells (Cormack *et al.*, 1996).  To prepare the pEGFP Rb1b RLORF6

plasmid Ola Ali Hassanin had inserted a GATEway® cassette (Invitrogen) encoding the strain Rb1b MDV RLORF6 gene into pEGFP-C1 at the multiple cloning site (MCS) between the EGFP and SV40 Poly A coding sequences. Genes cloned into the pEGFP-C1 plasmid at the MCS would be over expressed as a fusion protein in transfected cells as long as they had been cloned in the same reading frame as EGFP (Clontech). The resulting expression of fusion protein would be visible under UV green light and this feature was exploited in order to determine the cellular localization of RLORF6.

CEF cells were prepared and grown to 70-80% confluence on glass coverslips as previously described (section 2.4.8) and transfected using electroporation (section 2.5.2) with either 1 or 2 μg of the pEGFP Rb1b RLORF6 plasmid or the pEGFP-C1 control plasmid. Three replicates were prepared for each of the four experimental groups. One replicate from each experimental group was harvested at 24, 36 and 48 hours post-transfection, stained using the nuclear stain TO-PRO-3 iodide (Suzuki *et al*., 1997) and fixed on lysine-coated glass microscope slides as previously described in section 2.7.1. The slides were photographed using a Leica Confocal Laser Scanning Microscope at either 63x or 100x power at the Impact Imaging Facility, University of Edinburgh with the assistance of Mrs. Trudi Gillespie.

The control pEGFP-C1 transfected cells showed a diffuse distribution of the EGFP protein in the cytoplasm and occasionally also in the nucleus (figure 4.9). The EGFP CVI988 RLORF6 fusion protein was clearly visible in the cell nucleoplasm, but not the

nucleolus at 24, 36 and 48 hours post-transfection (36 hour images shown in figure

4.10). The experiment was repeated using suspension Rb1b T Cells instead of adherent

CEFs according to the protocol described in section 2.7.2. Figure 4.11 shows the EGFP

RLORF6 fusion protein was also visible in the Rb1b T cell nucleoplasm but not the

nucleolus although the visualization was not as clear as in the CEF cells due to the large

irregular nucleus present in transformed lymphoblasts. The EGFP RLORF6 fusion

protein was visible from 24 hours post-transfection onwards in all cell types tested. No

differences in cell viability or signal intensity were observed between cells transfected

with either 1 μg or 2 μg of DNA. Some degree of cytotoxicity and nuclear degradation

was observed in pEGFP RLORF6 transfected cells from 48-72 hours post-transfection.
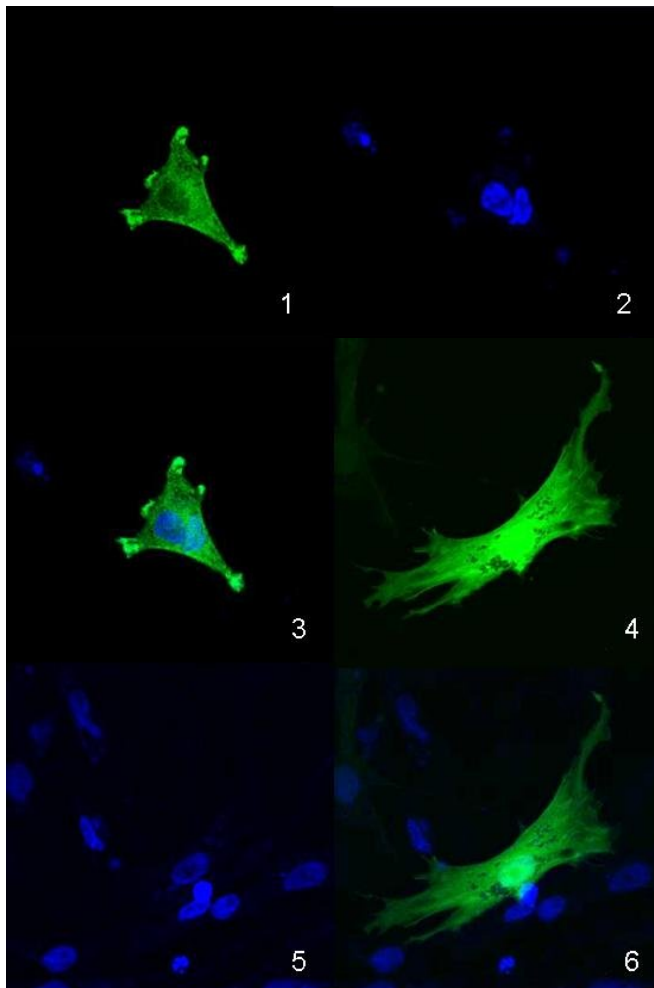
**Figure 4.9:** Series of confocal laser microscopy images of CEF cells 36 hours post-transfection with pEGFP-C1 control plasmid at 63x objective. Views depicted: 1) UV green filter showing EGFP control protein cytoplasmic localization 2) DAPI filter showing TO-PRO-3 iodide nuclear stain 3) Overlay image showing views 1 & 2 4) UV green filter showing EGFP control protein cytoplasmic and nuclear localization 5) DAPI filter showing TO-PRO-3 iodide nuclear stain. 6) Overlay image showing views 4 & 5. Photos were taken at the Confocal Imaging Facility, University of Edinburgh with the assistance of Trudi Gillespie.
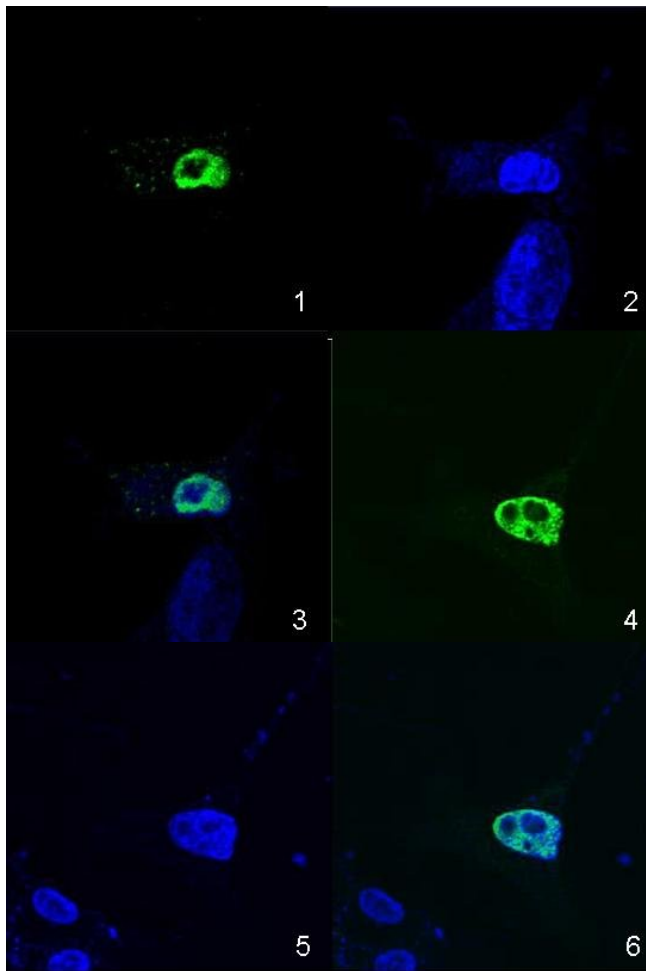
**Figure 4.10:** Series of confocal laser microscopy images of CEF cells 36 hours post-transfection with pEGFP Rb1b RLORF6 plasmid at 63x objective. Views depicted: 1) UV green filter showing pEGFP RLORF6 fusion protein nucleoplasm localization 2) DAPI filter showing TO-PRO-3 iodide nuclear stain 3) Overlay image showing views 1 & 2 4) UV green filter showing pEGFP Rb1b RLORF6 control protein nucleoplasm localization 5) DAPI filter showing TO-PRO-3 iodide nuclear stain 6) Overlay image showing views 4 & 5. Photos were taken at the Confocal Imaging Facility, University of Edinburgh with the assistance of Trudi Gillespie.
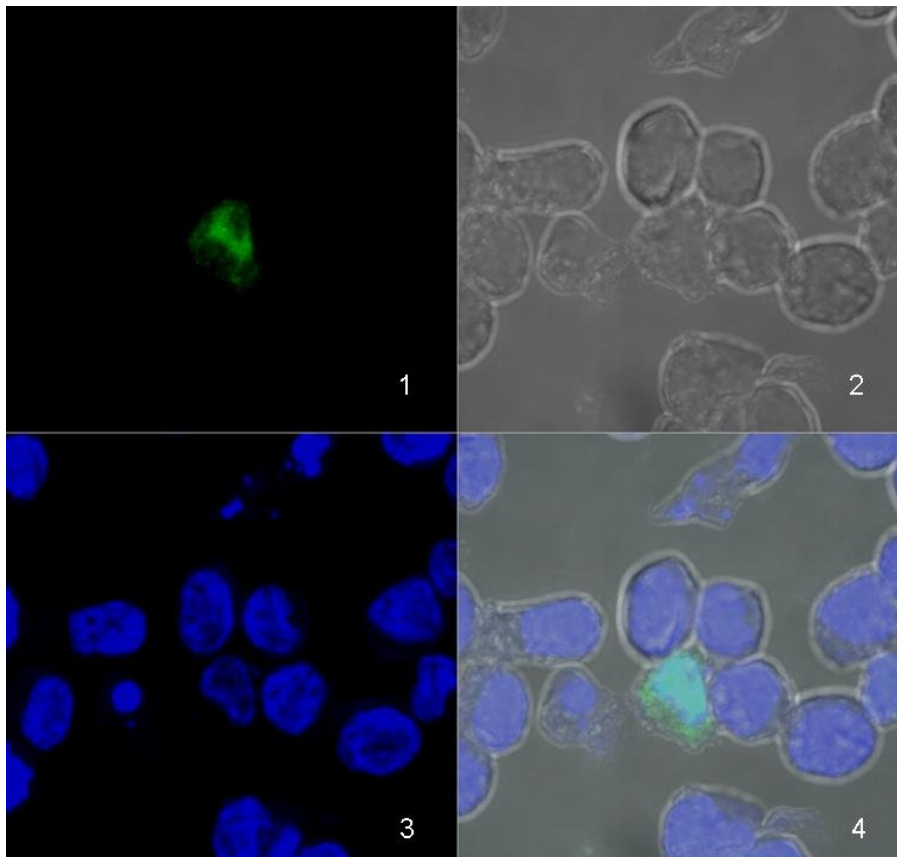
**Figure 4.11:** Series of confocal laser microscopy images of Rb1b T cells 48 hours post-transfection with pEGFP Rb1b RLORF6 plasmid at 100x objective. Views depicted: 1) UV green filter showing pEGFP RLORF6 fusion protein localization 2) White light showing cell outlines 3) DAPI filter showing TO-PRO-3 iodide nuclear stain 4) Overlay image showing views 1-3. Photos were taken at the Confocal Imaging Facility, University of Edinburgh with the assistance of Trudi Gillespie.

Due of the proximity of RLORF6 in the genome to Meq and since both genes appear to be expressed in latency it is possible that the two viral proteins may interact in the cell. To clarify whether this was the case a co-localization experiment was designed for Meq and RLORF6. An N-terminal fusion plasmid based on the pDSRed (Clontech) plasmid backbone encoding the strain Rb1b MDV Meq gene was kindly provided by Ola Ali Hassanin (Division of Pathway Medicine, University of Edinburgh, UK) for RLORF6/Meq co-localization studies. The pDSRed plasmid encodes a red fluorescent protein first isolated from *Discosoma sp*. marine corals and fluoresces red at wavelength 558-583 nm (Matz *et al.*, 1999) which is spectrally distinct from EGFP which fluoresces green at 488-507 nm (Cormack *et al.*, 1996). Therefore both pEGFP-C1 and pDSRed fusion plasmids can be co-transfected into cells and the encoded fusion proteins would be distinct using different filters in UV confocal laser microscopy.

CEF cells were transfected in triplicate with one of the following: 2 μg p-EGFP-C1 control, 2 μg pEGFP Rb1b RLORF6 or 1 μg pDSRed Meq and 1 μg pEGFP Rb1b RLORF6 as described in section 2.7.2. Each experimental group was harvested, fixed and photographed at 24, 36 and 48 hours post-transfection as previously described. Both pEGFP RLORF6 and pDSRed Meq appeared to localize to the nucleus from 24 hours in co-transfected cells, but the Meq fusion protein was apparent in both the nucleoplasm and nucleolus whereas the RLORF6 fusion protein was visible only in the nucleoplasm. In some of the co-transfected cells the pDSRed Meq fusion protein was also visible diffusely in the cytoplasm. When the two plasmids were co-transfected a very marked

cytotoxic effect was observed with significant nuclear degradation and cell death

occurring in some cells anywhere from 33-48 hours post-transfection (figure 4.12 and

4.13).  This effect was not observed in cells transfected with pEGFP-C1 or pEGFP Rb1b

RLORF6 even though they were transfected with an equivalent amount of plasmid DNA

(2μg).
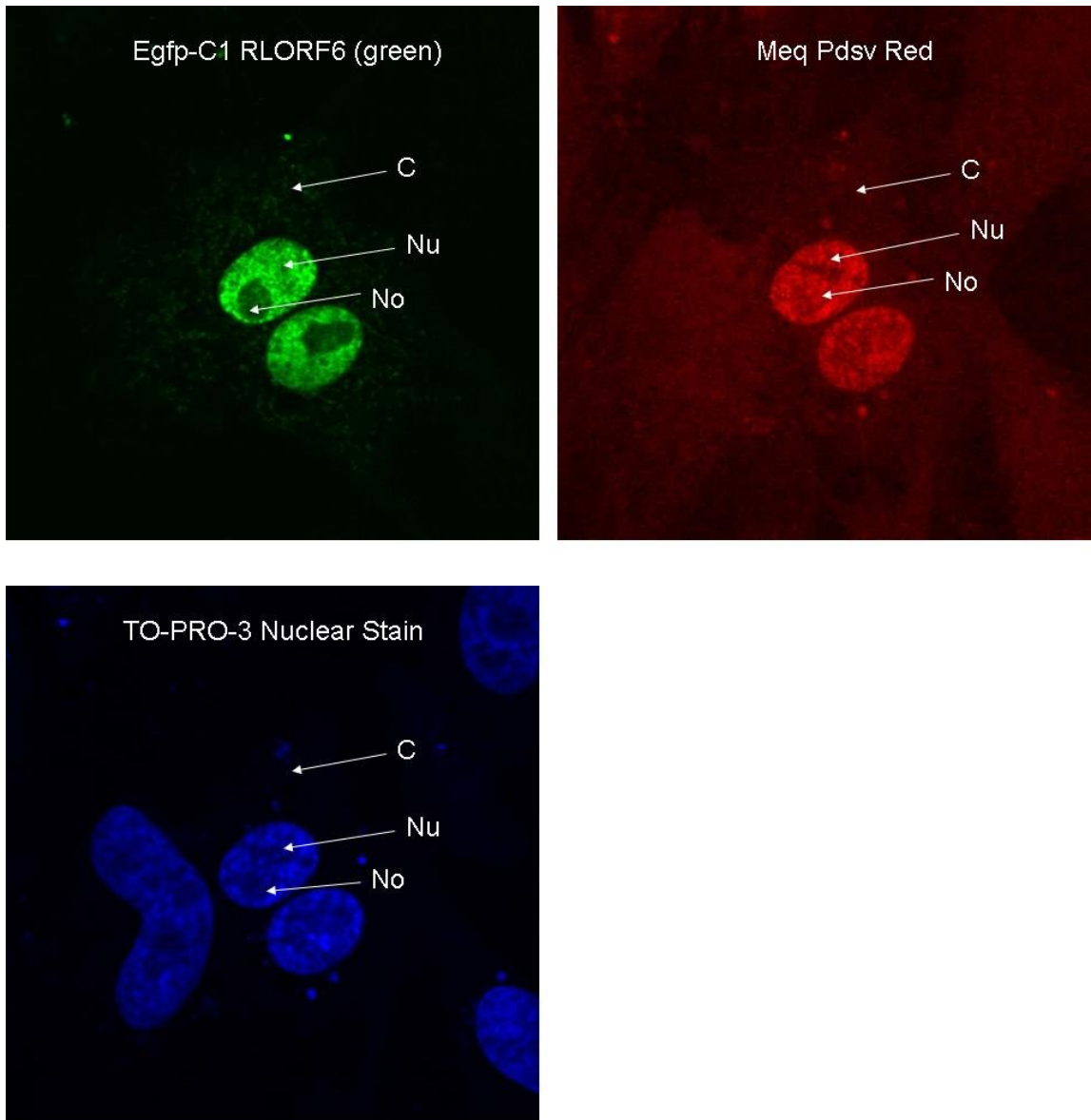
**Figure 4.12:** Series of confocal laser scanning microscopy images depicting two CEF cells co-transfected with pEGFP Rb1b RLORF6 plasmid (green fluorescent) and pDSRed Meq plasmid (red fluorescent) and stained with TO-PRO-3 iodide (DAPI, nuclear stain) at 100x objective. C = cytoplasm, Nu = Nucleoplasm and No = Nucleolus. Image series taken 33 hours post-transfection at the Confocal Imaging Facility, University of Edinburgh with the assistance of Trudi Gillespie.
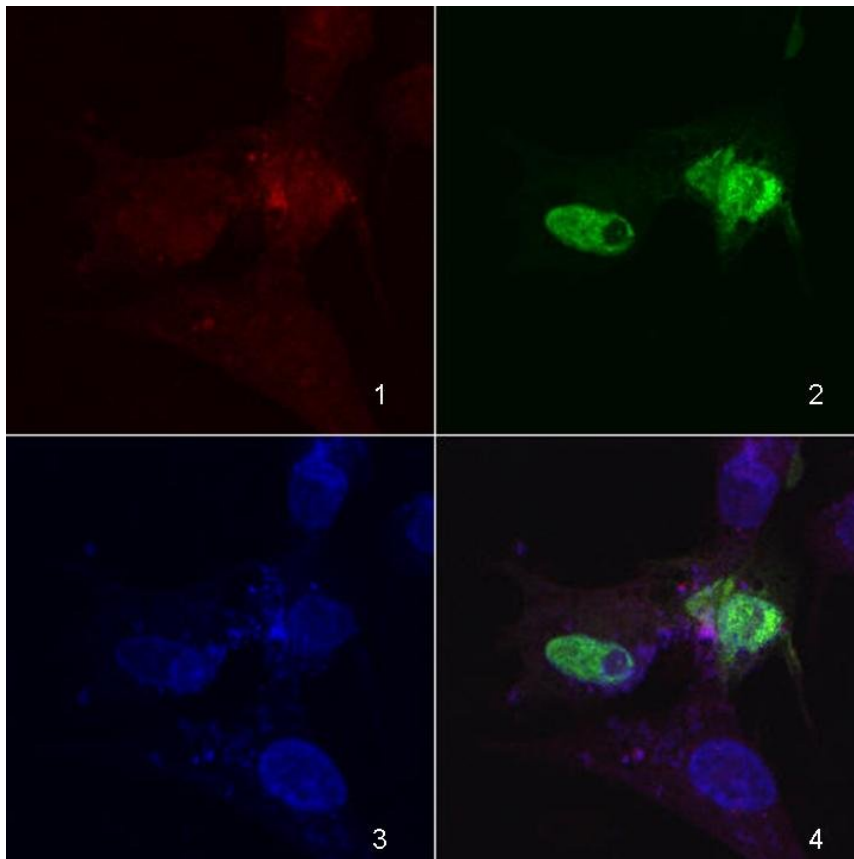
**Figure 4.13:** Series of confocal laser microscopy images of CEF cells co-transfected with 1 μg pEGFP Rb1b RLORF6 plasmid and 1 μg pDSRed Meq plasmid at 63x objective.  Note that some degree of nuclear degradation is apparent.  Views depicted:  1) UV red filter showing pDSRed Meq fusion protein diffuse localization  2) UV green filter showing pEGFP Rb1b RLORF6 control protein nucleoplasm localization  3)  DAPI filter showing TO-PRO-3 iodide nuclear stain  4)  Overlay image showing views 1-3  Photos were taken 33 hours post-transfection at the Confocal Imaging Facility, University of Edinburgh with the assistance of Trudi Gillespie.

**4.5** <u>**Summary**</u>

The efficacy of the siRNA JM RLORF6 oligonucleotides 524 and 626 were confirmed using a dual reporter luciferase assay which showed a reduction in luminescence of 57% for siRNA JM RLORF6 524 and 54% for siRNA JM RLORF6 626 when readings were adjusted to correct for variation in transfection rates, background luminescence and non-specific targeting of siRNA oligonucleotides. These results all supported the assumption that siRNA JM RLORF6 oligonucleotides were effective and specifically targeted RLORF6.

As the RLORF6 and Meq genes extensively overlap in the MDV genome it was necessary to confirm that the siRNA JM RLORF6 specific oligonucleotides 524 and 626 did not inadvertently cross-target the Meq gene in order to support the finding that post-transcriptional targeting of RLORF6 using siRNA resulted in an increased cell growth rate *in vitro* (section 3.5). cDNA specific to both RLORF6 and Meq was prepared using RNA isolated from RPL-1 cells 48 and 72 hours post-transfection as part of the third repetition of the RLORF6 cell growth studies which were presented previously (figure 3.22c). This was used in a RTqPCR assay with primers specific to Meq only. The results showed no significant differences in Meq expression between siRNA Control and siRNA JM RLORF6 oligonucleotides 524 or 626 at the 95% confidence interval (48 hour p=0.0809/0.0809 and 72 hours p=1.00/0.1904 respectively). Protein immunoblotting utilizing an antibody specific to Meq showed a small reduction in Meq protein expression in RPL-1 cells transfected with siRNA JM RLORF6 oligonucleotides

524 and 626 compared to siRNA Control at 24 hours but not at 48 or 72 hours post-transfection.

Confocal laser microscopy studies in CEF and Rb1b T cells utilizing a pEGFP RLORF6 fusion plasmid showed localization of RLORF6 in the nucleoplasm but not the nucleolus.  A second fusion plasmid pDSRed Meq was also used in co-localization studies and appeared to localize mostly to the nucleus, but the interpretation of these results were complicated by a marked cytotoxic effect in CEF cells.

**Discussion**

### **4.6 Introduction**

The significant difference in growth rate (p=0.0094) found in RPL-1 cells 72 hours post-transfection with oligonucleotide siRNA JM RLORF6 626 compared to a siRNA Control #2 reported in the previous chapter suggested that MDV RLORF6 may play a role in regulating cell growth rate *in vitro* but clarification was required before any final conclusions could be made as the RLORF6 ORF overlapped with other ORFs in the Meq loci and the siRNA oligonucleotides designed to target RLORF6 may have also inadvertently targeted one or more of the overlapping ORFs as they shared portions of the same sequences (figure 4.3). Additional experiments were also necessary to confirm that the cell growth results found were due to the actions of the siRNA oligonucleotides and not other extraneous factors.

**4.7** **Determination of siRNA Knockdown of RLORF6**

The dual reporter Luciferase assay demonstrated a >75% reduction of RLORF6

expression in cells treated with siRNA JM RLORF6 524 & 626 in two independent

repetitions of the experiment. These findings were interpreted as confirmation that the

siRNA JM RLORF6 oligonucleotides were effective in targeting RLORF6 post-

transcriptional expression. However, a small reduction in luminescence was observed

for the siRNA JM RLORF6 transfected groups in the control experiment utilizing pGL3

rather than the RLORF6 Luc plasmid. As the pGL3 plasmid and CEF cells should not

encode either MDV or the MDV RLORF6 gene it was determined that there was some

unintended cross-targeting of firefly luciferase by the siRNA JM RLORF6

oligonucleotides independent of post-transcriptional targeting of RLORF6. In an

attempt to correct the siRNA efficiency results for this non-specific cross-targeting the

percentage reduction in firefly luciferase luminescence observed in cells transfected with

pGL3 was subtracted from the luminescence results of cells transfected with the

RLORF6 Luc fusion plasmid. Even with this correction there was a >50% efficiency

calculated for both the siRNA JM RLORF6 oligonucleotides indicating that they were

effective against their intended target gene RLORF6.


**4.8** **Effect of RLORF6 Knockdown on Meq Expression**

The overlapping nature of the multiple genes in the Meq loci (figure 4.3) made it very

difficult and complex to confirm siRNA targeting of one gene without inadvertently

measuring the expression of other overlapping genes. The multi step approach utilizing

directional cDNA followed by RTqPCR utilizing a Meq primer set was laborious but appeared to be effective for detecting Meq gene expression. The normalized results for the directional cDNA RTqPCR experiments in figure 4.5a superficially appeared to show some increase of Meq expression in cells treated with siRNA JM RLORF6 524 & 626 over the siRNA Control at 48 hours (p = 0.0809) or 72 hours (p=1.0000 & 0.1904 respectively) post-transfection, but these differences did not meet the criteria for significance. This was probably due to a genuine lack of cross-targeting, however it can not be ruled out that the lack of significance was due to the low number of replicates (n=3) as well as a high degree of variability observed between the replicates. The observation of replicate variability could be random or it could be due to variations in transfection efficiency which were discussed previously in section 4.5. For definitive proof multiple replicates of the experiment would be necessary to give sufficient data for robust statistical analysis, but this was beyond the timescale and budget of the study. Because these results were not conclusive the decision was made to analyze the levels of Meq protein present to further assess siRNA targeting specificity.

The results for protein immunoblotting showed a small degree of Meq protein reduction in the siRNA JM RLORF6 524 and 626 samples compared to the scrambled control siRNA samples at 24 hours but not at 48 or 72 hours. These findings were interpreted as the presence of a limited amount of cross-targeting of Meq and the subsequent reduction of Meq protein by the siRNA RLORF6 oligoribonucleotides at 24 hours but not at 48 or 72 hours post-transfection. The reason why a reduction in Meq protein was observed

only at 24 hours but not at 48 or 72 hours could not be conclusively determined.

Further assessment of knockdown efficiency utilizing additional means such as northern

blots to assess relative amounts of Meq and RLORF6 mRNA was considered but not

undertaken due to the timescale of the project.


## 4.9   Determination of RLORF6 Cellular Localization using Confocal Laser Microscopy Studies

The confocal laser microscopy studies comparing the pEGFP-C1 control plasmid with

the pEGFP-C1 CVI988 RLORF6 fusion plasmid showed an apparent nuclear

localization of the RLORF6 fusion protein in both CEF and Rb1b T cells at 24, 36 and

48 hours post transfection.  Some of the transfected cells fixed at 24 hours additionally

showed a nodular cytoplasmic localization of RLORF6.  This was most likely due to

cytoplasmic synthesis of the RLORF6 protein with subsequent export and sequestration

to the nucleoplasm, but further studies would be required before any definitive

conclusions could be reached.  Localization of the pEGFP-C1 control EGFP was

observed in some cell nuclei as well as the expected cytoplasmic localization which

could complicate interpretation of these results.  Nuclear localization of EGFP had been

documented in the literature (Seibel *et al*., 2007) and the observation of some diffuse

nuclear localization of small EGFP fusion proteins such as RLORF6 in isolation would

not usually be sufficient for conclusive proof of nuclear localization.  It is important to

note that EGFP RLORF6 strongly localized only to the nucleoplasm which was very

different to the diffuse localization pattern seen in the pEGFP-C1 plasmid controls, and

therefore the observation of nuclear localization was most likely genuine.  After 48

hours it was noted that in cells transfected with pEGFP RLORF6 the nucleus became increasingly degraded in proportion to the amount of RLORF6 sequestration. Degraded cell nuclei did not have a clearly defined shape and in many cells the nucleus appeared to have ruptured and nuclear material was seen in the cytoplasm in images taken with the DAPI filter which illuminates the TO-PRO-3 iodide nuclear stain. Cells displaying these changes were obviously not healthy and were probably dead or dying when the slide was fixed. Interpretation of gene overexpression studies can be difficult as the gene product is often expressed at high levels and without normal degradation or in the context of any regulation that would be present in natural infections. It is possible that the cytotoxic effects observed in pEGFP RLORF6 and pDSRed Meq transfected cells were due to the lack of context and regulation provided in natural MDV infections, but this could not be confirmed without further experiments which were beyond the timescale of the project. It was noted that the cytotoxic effect was much more marked in cells transfected with the pDSRed Meq plasmid with nuclear degradation being noted from 24 hours post-transfection rather than 48-72 hours observed in pEGFP RLORF6. This could have been due to overexpression effects as outlined above but other factors such as backbone plasmid cytotoxicity could not be ruled out. The pDSRed plasmid vector was originally intended to be included in these transfection studies in order to investigate the possibility of backbone plasmid cytotoxicity but sufficient quantities of plasmid DNA for control studies could not be obtained in the timescale of the experiment. This was due to the transformed *E.coli* bacteria used to prepare the pDSRed plasmid clone yielding very low amounts of plasmid DNA that was of poor quality. In

any future repetitions of this experiment it would be worthwhile to include a control

group transfected only with the pDSRed backbone plasmid in order to rule out backbone

plasmid cytotoxicity.


**4.10**  **Conclusions**

The results of the directional cDNA RTqPCR and protein immunoblotting achieved the

objective of assessing whether there was any cross-targeting of Meq by siRNA JM

RLORF6 524 and 626 oligonucleotides.  Both experiments showed no significant

differences in Meq protein expression at 48 or 72 hours post-transfection.  An

unquantifiable reduction in Meq protein was seen at 24 hours only in the protein

immunoblotting experiments.  As the significant differences in the cell growth studies

outlined in chapter 4 were measured at 72 hours post-transfection the assumption was

made that there was no cross-targeting of Meq by the siRNA JM RLORF6

oligonucleotides at the time of the increased cell growth observation.


The confocal laser microscopy studies also achieved their objectives by demonstrating

that RLORF6 localizes in the cell nucleoplasm but not the nucleolus.  Meq also appeared

to localise to the nucleus in co-transfection studies which correlates with prior studies

demonstrating localization of Meq to the nucleus (Anobile *et al.*, 2006). These findings

indicate that the RLORF6 gene product and Meq localize to the cell nucleus making it

theoretically possible for them to interact although much more detailed biological

studies would have to be undertaken for confirmation which was beyond the timescale of the current study.

Ideally a dual-reporter luciferase assay would have been undertaken for every gene screened in chapter 4, but due to timescale and the availability of luciferase fusion plasmids the decision was made to screen only RLORF6 as it was the gene identified with significant differences in cell growth rates. The results of the RLORF6 dual reporter luciferase assays supported the hypothesis that the siRNA JM RLORF6 oligonucleotides effectively targeted RLORF6 and therefore could be expected to reduce gene expression. The findings in chapter 4 for RLORF6 were supported by these experiments and lent proof to the assumption that the siRNA JM RLORF6 oligonucleotides used in these experiments were both effective and target-specific.

# Chapter 5 – Final Summary and Future Work

The objective of these studies was to investigate the function of previously

uncharacterised genes during the MDV life cycle (chapters 3 and 4).

Work described in this thesis used siRNA techniques to investigate the function of

selected uncharacterised genes during the MDV life cycle.  It is established that certain

well-characterised genes such as Meq play a large role in determining the outcome and

establishment of latent infections in MDV, but relatively little is known about the role of

other genes expressed during latent infection, many of which are unique to MDV.  It

seems logical to assume that genes expressed during latency have a function.  This study

focused on screening an initial list of 15 genes that were deemed under-characterised

and interesting (section 1.3) for expression in latent and lytic MDV infections using

several different cell line models (section 3.3).  Out of the 15 genes screened for

expression LORF1, LORF3, LORF11, LORF12, ANTISENSE, US2, MLTI, RLORF11,

RLORF12, 23kDa and RLORF6 were all found to be expressed in MDV latently

infected RPL-1 cells.  siRNA oligonucleotides were designed and obtained for post-

transcriptional gene targeting for the LORF1, LORF3, LORF11, ANTISENSE, MLTI,

RLORF12 and RLORF6 genes as the timescale and budget of the project did not allow

for screening of all the latently expressed genes identified.  siRNA transfection studies

with RPL-1 cells were undertaken for each of these genes incorporating a transfection

marker and measuring cell growth rate as the phenotypic marker (chapter 3).  The

control group for each of these experiments was RPL-1 cells transfected with a non-

targeting siRNA oligonucleotide.  Only the RLORF6 gene was found to affect the cell

growth rate in one of the two oligonucleotides tested (adjusted p=0.0094, General Linear ANOVA). None of the other targeted genes analyzed appeared to change cell growth parameters compared with the control group in the MDV-transformed latent cell lines tested (Chapter 3). Confirmation of siRNA targeting efficacy and specificity was undertaken for RLORF6 (chapter 4). The timescale of the project did not allow for confirmation of siRNA efficacy for oligonucleotides that did not produce any effect on growth rate in transformed cells. Before any conclusions could be drawn regarding the effect of post-transcriptional targeting of these genes on growth rate in RPL-1 cells it would be necessary to confirm the efficacy of the siRNA oligonucleotides used for each gene with a dual-reporter luciferase assay (section 2.6). It is important to note that the reason no effect was observed in all but RLORF6 could be due to other factors such as inefficient gene targeting by the siRNA or low transfection rates instead of the function of the intended target gene not affecting cell growth rate *in vitro*. The finding that post-transcriptional targeting of RLORF6 positively affected cell growth (chapter 3 and 4) was unexpected as its location in the genome overlapping Meq, whose knock down had been shown to decrease cell growth rate in latent MDV infections (Brown *et al.*, 2006). Analysis using confocal laser microscopy confirmed that RLORF6 localizes to the nucleus similar to Meq (chapter 4), which could be consistent with a regulatory gene. To continue the work in this study characterizing RLORF6 it would be desirable to repeat the cell growth experiments outlined in chapter 4 with sufficient repetitions to give statistics that are more robust in a population of 90-100% transfected cells. This would confirm whether the observed increase in cell growth when RLORF6 was

targeted in a partially-transfected latent cell population was due only to the effects of

siRNA knockdown of the targeted gene. The transfection efficiencies reported in

chapters 4 and 5 were relatively low (<35%) and not consistent between groups or

repetitions compared to the high transfection efficiencies reported in some transformed

lymphocytic cell lines using similar methods (Maurisse *et al.*, 2010). One method of

obtaining a completely transfected cell population would be to either transfect cells with

a fluorescent transfection marker and isolate transfected cells 24-36 hours post-

transfection utilizing Fluorescence-activated cell sorting (FACS). It might also be

worthwhile to assess the effect of post-transcriptional targeting of RLORF6 on cell

growth rate in other MDV transformed cells to confirm that the growth rates observed

were not exclusive to RPL-1 cells. An assessment of RLORF6 activity during lytic

infection could be done by transfecting CEF or DF-1 cells with siRNA followed by

infection with MDV or co-transfection with MDV BAC and assess the effect on cell

growth rate and viral plaque numbers. This would help to clarify whether RLORF6 has

a similar function in latent and lytic infection. Construction of a double hairpin

miRNA30-like plasmid expressing siRNA sequences targeting RLORF6 in a lentiviral

plasmid similar to pRFPRNAiC Meq T1/T14 and making stable cell lines for testing *in

vitro* would be another method of generating a population of cells all carrying siRNA

targeting RLORF6. This could be used to generate transgenic chickens allowing further

studies on the function of RLORF6 *in vivo* if it were deemed worthwhile. All of these

methods would further characterize RLORF6 and clarify issues related to poor

transfection rates but they were beyond the timescale of this project. The definitive

function of MDV RLORF6 remains unknown at this time, but this study concluded that

the RLORF6 gene product in both attenuated and very virulent MDV strains localizes to

the nucleus but not the nucleolus.  Targeting of the RLORF6 gene product using siRNA

in RPL-1 cells appears to increase cell growth.  These findings and the location of

RLORF6 in the 'Meq loci' region of genome, associated with latent and transforming

infection (Parcells *et al*., 2003), could be consistent with a gene regulating

transformation between latent and lytic infection.  While this project could not prove

that RLORF6 was involved with the regulation of cell growth, it introduced the

possibility that it may have a regulatory effect on cell growth distinct from Meq and

further study would be warranted.

# Appendix 1 – Sequencing Results

**LORF3**

```
            18471                                                  18520
Rb1b        TTGTCGAGGT CTTGCTGCTG AATGCATGTT TACCGGAGGA GGAACTATTG
JM          ........GGT CTTGCTGCTG AATGCATGTT TACCGGAGGA GGAACTATTG
CVI 988     TTGTCGAGGT CTTGCTGCTG AATGCATGTT TACCGGAGGA GGAACTATTG
            18261                                                  18310

            18521                                                  18570
Rb1b        CATTAATAGA ACGTCTAGCG ACTTCCTGGC TAACGGCCAT AAGATTGATT
JM          CATTAATAGA ACGTCTAGCG ACTTCCTGGC TAACGGCCAT AAGATTGATT
CVI 988     CATTAATAGA ACGTCTAGCG ACTTCCTGGC TAACGGCCAT AAGATTGATT
            18311                                                  18360

            18571                                                  18620
Rb1b        TTATCCTGGC ATCCCATTCA CGCTCCCAAT CGTAATCAAG AGCCTCTCGA
JM          TTATCCTGGC ATCCCATTCA CGCTCCCAAT CGTAATCAAG AGCCTCTCGA
CVI 988     TTATCCTGGC ATCCCATTCA CGCTCCCAAT CGTAATCAAG AGCCTCTCGA
            18361                                                  18410

            18621                                                  18670
Rb1b        TAGCTTATGC CGCGAAGGTC GCGAATATAT TGTAATGATT TCTGGGACGG
JM          TAGCTTATGC CGCGAAGGTC GCGAATATAT TGTAATGATT TCTGGGACGG
CVI 988     TAGCTTATGC CGCGAAGGTC GCGAATATAT TGTAATGATT TCTGGGACGG
            18411                                                  18460

            18671                                                  18720
Rb1b        TTCACCCACG ACATGCTACA TGGCCATTTT GGCAAGTGAT GCGGAAATGT
JM          TTCACCCACG ACATGCTACA TGGCCATTTT GGCAAGTGAT GCGGAAATGT
CVI 988     TTCACCCACG ACATGCTACA TGGCCATTTT GGCAAGTGAT GCGGAAATGT
            18461                                                  18510

            18721                                                  18770
Rb1b        TTAGATTGGT GCTGCGCATT TCACCCACCT GATGACCACA GTTGTGAATT
JM          TTAGATTGGT GCTGCGCATT TCACCCACCT GATGACCACA GTTGTGAATT
CVI 988     TTAGATTGGT GCTGCGCATT TCACCCACCT GATGACCACA GTTGTGAATT
            18511                                                  18560

            18771                                                  18820
Rb1b        CGGAGCACCA CGCATTGGAA TCCGGCTAGA GGGCGAAAAT CATTTTTTCG
JM          CGGAGCACCA CGCATTGGAA TCCGGCTAGA GGGCGAAAAT CATTTTTTCG
CVI 988     CGGAGCACCA CGCATTGGAA TCCGGCTAGA GGGCGAAAAT CATTTTTTCG
            18561                                                  18610

            18821                                                  18870
Rb1b        CACCAATTTT GGGATTGTAT TCTGTAGTAA TGACATGGAG TCCAATTTCA
JM          CACCAATTTT GGGATTGTAT TCTGTAGTAA TGACATGGAG TCCAATTTCA
CVI 988     CACCAATTTT GGGATTGTAT TCTGTAGTAA TGACATGGAG TCCAATTTCA
            18611                                                  18660

            18871                                                  18920
Rb1b        TGTTACCGAG AATTCCCAAT ACGTCAAAAT TCTAAGGAGC CTGATCCCCA
JM          TGTTACCGAG AATTCCCAAT ACGTCAAAAT TCTAAGGAGC CTGATCCCCA
CVI 988     TGTTACCGAG AATTCCCAAT ACGTCAAAAT TCTAAGGAGC CTGATCCCCA
            18661                                                  18710
```

197

## LORF3

```
                18921                                                     18970
Rb1b            ACCATCAACA TCATCCGAAC CTGAGCCTCA GCCATCGACA TCGTCTCATC
JM              ACCATCAACA TCATCCGAAC CTGAGCCTCA GCCATCGACA TCGTCTCATC
CVI 988         ACCATCAACA TCATCCGAAC CTGAGCCTCA GCCATCGACA TCGTCTCATC
                18711                                                     18760

                18971                                                     19020
Rb1b            GTAACATTCC CGTAGCACGT GTAAGACCTC TTGTCGCTCA GCAAAAGGTA
JM              GTAACATTCC CGTAGCACGT GTAAGACCTC TTGTCGCTCA GCAAAAGGTA
CVI 988         GTAACATTCC CGTAGCACGT GTAAGACCTC TTGTCGCTCA GCAAAAGGTA
                18761                                                     18810

                19021                                                     19070
Rb1b            CCCAAAACTA GACCTTTGGA TACAGAAATA CACAGGCCCG GACCAATTGC
JM              CCCAAAACTA GACCTTTGGA TACAGAAATA CACAGGCCCG GACCAATTGC
CVI 988         CCCAAAACTA GACCTTTGGA TACAGAAATA CACAGGCCCG GACCAATTGC
                18811                                                     18860

                19071                                                     19120
Rb1b            AATCCAGAAC CCAACAGATA CGGATGAACC TGAACTTCGC TTGAACCCGA
JM              AATCCAGAAC CCAACAGATA CGGATGAACC TGAACTTCGC TTGAACCCGA
CVI 988         AATCCAGAAC CCAACAGATA CGGATGAACC TGAACTTCGC TTGAACCCGA
                18861                                                     18910

                19121                                                     19170
Rb1b            GACCACGCCC GGGTCCTTCG GGGCAAAATA CACGTCCAAG GACTCCCACT
JM              GACCACGCCC GGGTCCTTCG GGGCAAAATA CACGTCCAAG GACTCCCACT
CVI 988         GACCACGCCC GGGTCCTTCG GGGCAAAATA CACGTCCAAG GACTCCCACT
                18911                                                     18960

                19171                                                     19220
Rb1b            TTGGACTTGG ATACCGTTGT CGTTCGAGAT CACCCAGTAA CACATCGTCG
JM              TTGGACTTGG ATACCGTTGT CGTTCGAGAT CACCCAGTAA CACATCGTCG
CVI 988         TTGGACTTGG ATACCGTTGT CGTTCGAGAT CACCCAGTAA CACATCGTCG
                18961                                                     19010

                19221                                                     19270
Rb1b            TCCGCGTTCT CCTAGCCCAC CTGAGGAAGA TTATACTAAC CAAGATGAAA
JM              TCCGCGTTCT CCTAGCCCAC CTGAGGAAGA TTATACTAAC CAAGATGAAA
CVI 988         TCCGCGTTCT CCTAGCCCAC CTGAGGAAGA TTATACTAAC CAAGATGAAA
                19011                                                     19060

                19271                                                     19320
Rb1b            ATCTCTCATA TACTCCCCAA TTAATCCATT CTTCCCCAGA TAGTGAAGTT
JM              ATCTCTCATA TACTCCCCAA TTAATCCATT CTTCCCCAGA TAGTGAAGTT
CVI 988         ATCTCTCATA TACTCCCCAA TTAATCCATT CTTCCCCAGA TAGTGAAGTT
                19061                                                     19110

                19321                                                     19370
Rb1b            GCTGAGGAGA TTTATGCTCA GCCCGACCCT TGGGGTACAC AAGAACTGCT
JM              GCTGAGGAGA TTTATGCTCA GCCCGACCCT TGGGGTACAC AAGAACTGCT
CVI 988         GCTGAGGAGA TTTATGCTCA GCCCGACCCT TGGGGTACAC AAGAACTGCT
                19111                                                     19160

                19371                                                     19420
Rb1b            ATTAGCAAAT CGTGAACGCA CTCCAGATGA TCAAACAGAT ATTACGGATG
JM              ATTAGCAAAT CGTGAACGCA CTCCAGATGA TCAAACAGAT ATTACGGATG
CVI 988         ATTAGCAAAT CGTGAACGCA CTCCAGATGA TCAAACAGAT ATTACGGATG
                19161                                                     19210
```

## LORF3

```
           19421                                                    19470
Rb1b       ATAGCGCAGA CTGGTCTGAG GGCGAAACAC GTCGACCATC ACATAGTGAA
JM         ATAGCGCAGA CTGGTCTGAG GGCGAAACAC GTCGACCATC ACATAGTGAA
CVI 988    ATAGCGCAGA CTGGTCTGAG GGCGAAACAC GTCGACCATC ACATAGTGAA
           19211                                                    19260

           19471                                                    19520
Rb1b       GTTGGGGAAC GTAGATTGTC CAGAGAAAAT AACAGTGAAG ATCCAAACCG
JM         GTTGGGGAAC GTAGATTGTC CAGAGAAAAT AACAGTGAAG ATCCAAACCG
CVI 988    GTTGGGGAAC GTAGATTGTC CAGAGAAAAT AACAGTGAAG ATCCAAACCG
           19261                                                    19310

           19521                                                    19570
Rb1b       TAGTCGGAGC CGGAGTCGAT CTAGGGAGCG TAGGCGAAGA CGGCCACGAG
JM         TAGTCGGAGC CGGAGTCGAT CTAGGGAGCG TAGGCGAAGA CGGCCACGAG
CVI 988    TAGTCGGAGC CGGAGTCGAT CTAGGGAGCG TAGGCGAAGA CGGCCACGAG
           19311                                                    19360

           19571                                                    19620
Rb1b       TTAGGCCTGG GCGTAGGAGT ACGGCTACAA CTATACGAGA TCTTGTGGTT
JM         TTAGGCCTGG GCGTAGGAGT ACGGCTACAA CTATACGAGA TCTTGTGGTT
CVI 988    TTAGGCCTGG GCGTAGGAGT ACGGCTACAA CTATACGAGA TCTTGTGGTT
           19361                                                    19410

           19621                                                    19670
Rb1b       CTTGGGATGT CGAGTTCAGA TGATGAATAG CATTTGTGTA TATGTTGTGT
JM         CTTGGGATGT CGAGTTCAGA TGATGAATAG CATTTGTGTA TATGTTGTGT
CVI 988    CTTGGGATGT CGAGTTCAGA TGATGAATAG CATTTGTGTA TATGTTGTGT
           19411                                                    19460

           19671                                                    19720
Rb1b       GATTGCAAAT CCACTGTATG GTTATATAGT CAGAATTTAA TAAAATGTTC
JM         GATTGCAAAT CCACTGTATG GTTATATAGT CAGAATTTAA TAAAATGTTC
CVI 988    GATTGCAAAT CCACTGTATG GTTATATAGT CAGAATTTAA TAAAATGTTC
           19461                                                    19510

           19721                                                    19770
Rb1b       GAAGTTTACA CCTTCACGTA TTGTTATGGG TATTTATGGG TGGGCGAGGG
JM         GAAGTTTACA CCTTCACGTA TTGTTATGGG .......... ..........
CVI 988    GAAGTTTACA CCTTCACGTA TTGTTATGGG TATTTATGGG TGGGCGAGGG
           19511                                                    19560
```

**Appendix 1a:** LORF3 nucleotide sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain. 100% consensus was found with both strains of MDV. Start and stop codons are shown in **purple**

## LORF3 AMINO ACID

```
        1                                                           50
Rb1b    MFTGGGTIAL IERLATSWLT AIRLILSWHP IHAPNRNQEP LDSLCREGRE
JM      MFTGGGTIAL IERLATSWLT AIRLILSWHP IHAPNRNQEP LDSLCREGRE
CVI988  MFTGGGTIAL IERLATSWLT AIRLILSWHP IHAPNRNQEP LDSLCREGRE

        51                                                          100
Rb1b    YIVMISGTVH PRHATWPFWQ VMRKCLDWCC AFHPPDDHSC EFGAPRIGIR
JM      YIVMISGTVH PRHATWPFWQ VMRKCLDWCC AFHPPDDHSC EFGAPRIGIR
CVI988  YIVMISGTVH PRHATWPFWQ VMRKCLDWCC AFHPPDDHSC EFGAPRIGIR

        101                                                         150
Rb1b    LEGENHFFAP ILGLYSVVMT WSPISCYREF PIRQNSKEPD PQPSTSSEPE
JM      LEGENHFFAP ILGLYSVVMT WSPISCYREF PIRQNSKEPD PQPSTSSEPE
CVI988  LEGENHFFAP ILGLYSVVMT WSPISCYREF PIRQNSKEPD PQPSTSSEPE

        151                                                         200
Rb1b    PQPSTSSHRN IPVARVRPLV AQQKVPKTRP LDTEIHRPGP IAIQNPTDTD
JM      PQPSTSSHRN IPVARVRPLV AQQKVPKTRP LDTEIHRPGP IAIQNPTDTD
CVI988  PQPSTSSHRN IPVARVRPLV AQQKVPKTRP LDTEIHRPGP IAIQNPTDTD

        201                                                         250
Rb1b    EPELRLNPRP RPGPSGQNTR PRTPTLDLDT VVVRDHPVTH RRPRSPSPPE
JM      EPELRLNPRP RPGPSGQNTR PRTPTLDLDT VVVRDHPVTH RRPRSPSPPE
CVI988  EPELRLNPRP RPGPSGQNTR PRTPTLDLDT VVVRDHPVTH RRPRSPSPPE

        251                                                         300
Rb1b    EDYTNQDENL SYTPQLIHSS PDSEVAEEIY AQPDPWGTQE LLLANRERTP
JM      EDYTNQDENL SYTPQLIHSS PDSEVAEEIY AQPDPWGTQE LLLANRERTP
CVI988  EDYTNQDENL SYTPQLIHSS PDSEVAEEIY AQPDPWGTQE LLLANRERTP

        301                                                         350
Rb1b    DDQTDITDDS ADWSEGETRR PSHSEVGERR LSRENNSEDP NRSRSRSRSR
JM      DDQTDITDDS ADWSEGETRR PSHSEVGERR LSRENNSEDP NRSRSRSRSR
CVI988  DDQTDITDDS ADWSEGETRR PSHSEVGERR LSRENNSEDP NRSRSRSRSR

        351                                       385
Rb1b    ERRRRRPRVR PGRRSTATTI RDLVVLGMSS SDDE.
JM      ERRRRRPRVR PGRRSTATTI RDLVVLGMSS SDDE.
CVI988  ERRRRRPRVR PGRRSTATTI RDLVVLGMSS SDDE.
```

**Appendix 1b:** LORF3 aa sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain.

## LORF11

```
                124194                                                      124243
Rb1b    TTGTATCATC GTATGTGGGC AACAGTGCGG AATTATTGTA TCTGGTAACC
JM      TTGTATCATC GTATGTGGGC AACAGTGCGG AATTATTGTA TCTGGTAACC
CVI 988 TTGTATCATC GTATGTGGGC AACAGTGCGG AATTATTGTA TCTGGTAACC
        123861                                                      123910

                124244                                                      124293
Rb1b    GATTCTAATT GTAAATGGAT ATATTCTGTG TGTCTATGTC TACATGTAAA
JM      GATTCTAATT GTAAATGGAT ATATTCTGTG TGTCTATGTC TACATGTAAA
CVI 988 GATTCTAATT GTAAATGGAT ATATTCTGTG TGTCTATGTC TACATGTAAA
        123911                                                      123960

                124294                                                      124343
Rb1b    GAGCTTTCTG CATATTTTTG AGCTTCGGTC AATGCACGTG TTAGTATAGC
JM      GAGCTTTCTG CATATTTTTG AGCTTCGGTC AATGCACGTG TTAGTATAGC
CVI 988 GAGCTTTCTG CATATTTTTG AGCTTCGGTC AATGCACGTG TTAGTATAGC
        123961                                                      124010

                124344                                                      124393
Rb1b    CACGATCCGG CTCTCCCTCT CTGGGGTCGG AACACAATGC ATATGAAATA
JM      CACGATCCGG CTCTCCCTCT CTGGGGTCGG AACACAATGC ATATGAAATA
CVI 988 CACGATCCGG CTCTCCCTCT CTGGGGTCGG AACACAATGC ATATGAAATA
        124011                                                      124060

                124394                                                      124443
Rb1b    ATATGCCACA GCCAAAAAAG TTATTAGTCA TGCAAGCATC TGTCAAATAG
JM      ATATGCCACA GCCAAAAAAG TTATTAGTCA TGCAAGCATC TGTCAAATAG
CVI 988 ATATGCCACA GCCAAAAAAG TTATTAGTCA TGCAAGCATC TGTCAAATAG
        124061                                                      124110

                124444                                                      124493
Rb1b    CAATCACATA ATGGAATCAA ATGCCTCAAA GCTGCATAAT GAGGGGAACC
JM      CAATCACATA ATGGAATCAA ATGCCTCAAA GCTGCATAAT GAGGGGAACC
CVI 988 CAATCACATA ATGGAATCAA ATGCCTCAAA GCTGCATAAT GAGGGGAACC
        124111                                                      124160

                124494                                                      124543
Rb1b    TTCTCCCCAT TGAAACCATT TATGGAACCT ACGAGTATAT TCTGCCAACC
JM      TTCTCCCCAT TGAAACCATT TATGGAACCT ACGAGTATAT TCTGCCAACC
CVI 988 TTCTCCCCAT TGAAACCATT TATGGAACCT ACGAGTATAT TCTGCCAACC
        124161                                                      124210

                124544                                                      124593
Rb1b    ATCGTTTGGT TTCTGCAGTT ATCACCCGAA ATCCTTTAAA TGGAAAGAAT
JM      ATCGTTTGGT TTCTGCAGTT ATCACCCGAA ATCCTTTAAA TGGAAAGAAT
CVI 988 ATCGTTTGGT TTCTGCAGTT ATCACCCGAA ATCCTTTAAA TGGAAAGAAT
        124211                                                      124260

                124594                                                      124643
Rb1b    AAGCCCAAGT ACCAGTCATT ATTTTCAGGC TTTAGGGGGC ATCGTGTCGT
JM      AAGCCCAAGT ACCAGTCATT ATTTTCAGGC TTTAGGGGGC ATCGTGTCGT
CVI 988 AAGCCCAAGT ACCAGTCATT ATTTTCAAGC TTTAGGGGGC ATCGTGTCGT
        124261                                                      124310

                124644                                                      124693
Rb1b    TAAATGGGTA ACAAATTCGA TCTGAGAAGG TATGTATGTC AAATATTCAG
JM      TAAATGGGTA ACAAATTCGA TCTGAGAAGG TATGTATGTC AAATATTCAG
CVI 988 TAAATGGGTA ACAAATTCGA TCTGAGAAGG TATGTATGTC AAATATTCAG
        124311                                                      124360
```

## LORF11

```
          124694                                                      124743
Rb1b      AGCTTCTAAC AATACACTCT TCCCAGTGAG ATTGCAATAC ATTATAATTC
JM        AGCTTCTAAC AATACACTCT TCCCAGTGAG ATTGCAATAC ATTATAATTC
CVI 988   AGCTTCTAAC AATACACTCT TCCCAGTGAG ATTGCAATAC ATTATAATTC
          124361                                                      124410

          124744                                                      124793
Rb1b      TCACATCTAA CTACCTTGGG TGTCATACAA GCCGTAATAT GTGTTGTTCG
JM        TCACATCTAA CTACCTTGGG TGTCATACAA GCCGTAATAT GTGTTGTTCG
CVI 988   TCACATCTAA CTACCTTGGG TGTCATACAA GCCGTAATAT GTGTTGTTCG
          124411                                                      124460

          124794                                                      124843
Rb1b      TTGTAGAATA CACCTATACG GACTTGTTCC AATATCAGGA ATCGATTCGA
JM        TTGTAGAATA CACCTATACG GACTTGTTCC AATATCAGGA ATCGATTCGA
CVI 988   TTGTAGAATA CACCTATACG GACTTGTTCC AATATCAGGA ATCGATTCGA
          124461                                                      124510

          124844                                                      124893
Rb1b      TGGTTGAGAG CCTACGTATT CCTGATACTG CTAAGAGATC ACACAAAACA
JM        TGGTTGAGAG CCTACGTATT CCTGATACTG CTAAGAGATC ACACAAAACA
CVI 988   TGGTTGAGAG CCTACGTATT CCTGATACTG CTAAGAGATC ACACAAAACA
          124511                                                      124560

          124894                                                      124943
Rb1b      AGTAACTTAT TAATTCCATC AATCAAAGTT GTCATATCTA TTGCAGCACC
JM        AGTAACTTAT TAATTCCATC AATCAAAGTT GTCATATCTA TTGCAGCACC
CVI 988   AGTAACTTAT TAATTCCATC AATCAAAGTT GTCATATCTA TTGCAGCACC
          124561                                                      124610

          124944                                                      124993
Rb1b      CCCAAAAGAA AATAATGTTT CTGTACATGT TCTAGCACCG TTTTCAACAT
JM        CCCAAAAGAA AATAATGTTT CTGTACATGT TCTAGCACCG TTTTCAACAT
CVI 988   CCCAAAAGAA AATAATGTTT CTGTACATGT TCTAGCACCG TTTTCAACAT
          124611                                                      124660

          124994                                                      125043
Rb1b      TGCTCGTTAC GACCGAAAGA CTAAAAATAA AAGCCAATTC CGGTTTAGAC
JM        TGCTCGTTAC GACCGAAAGA CTAAAAATAA AAGCCAATTC CGGTTTAGAC
CVI 988   TGCTCGTTAC GACCGAAAGA CTAAAAATAA AAGCCAATTC CGGTTTAGAC
          124661                                                      124710

          125044                                                      125093
Rb1b      TTCTCAAGTG GGACGCTTAT GCGGCGCACT GTACTTTCCA GCTCTTTCCT
JM        TTCTCAAGTG GGACGCTTAT GCGGCGCACT GTACTTTCCA GCTCTTTCCT
CVI 988   TTCTCAAGTG GGACGCTTAT GCGGCGCACT GTACTTTCCA GCTCTTTCCT
          124711                                                      124760

          125094                                                      125143
Rb1b      GAAGTTAGAC CTCCCACATT TTCCAGCTCT GCTCCAATCT GCTGAAAAAA
JM        GAAGTTAGAC CTCCCACATT TTCCAGCTCT GCTCCAATCT GCTGAAAAAA
CVI 988   GAAGTTAGAC CTCCCACATT TTCCAGCTCT GCTCCAATCT GCTGAAAAAA
          124761                                                      124810

          125144                                                      125193
Rb1b      ATTTACGTAC AATTGGGGTT ATAGACGTAC CAATGAACAC CTTAAAACAT
JM        ATTTACGTAC AATTGGGGTT ATAGACGTAC CAATGAACAC CTTAAAACAT
CVI 988   ATTTACGTAC AATTGGGGTT ATAGACGTAC CAATGAACAC CTTAAAACAT
          124811                                                      124860
```

## LORF11

```
             125194                                                125243
Rb1b     AAATCCTGTG GAGGGTATAC TTCAGTATTA TCGATATCTA GATCTTCTAA
JM       AAATCCTGTG GAGGGTATAC TTCAGTATTA TCGATATCTA GATCTTCTAA
CVI 988  AAATCCTGTG GAGGGTATAC TTCAGTATTA TCGATATCTA GATCTTCTAA
         124861                                                124910

             125244                                                125293
Rb1b     CTTTTGTTTC ATCGATAGTA GAACACGTTC ACAATCTCTA TTCGCGGGCG
JM       CTTTTGTTTC ATCGATAGTA GAACACGTTC ACAATCTCTA TTCGCGGGCG
CVI 988  CTTTTGTTTC ATCGATAGTA GAACACGTTC ACAATCTCTA TTCGCGGGCG
         124911                                                124960

             125294                                                125343
Rb1b     GGTTGCACAA TCTTCTCAAA AAAGGTTTGA TCAATGCAAC TGCACCGCAT
JM       GGTTGCACAA TCTTCTCAAA AAAGGTTTGA TCAATGCAAC TGCACCGCAT
CVI 988  GGTTGCACAA TCTTCTCAAA AAAGGTTTGA TCAATGCAAC TGCACCGCAT
         124961                                                125010

             125344                                                125393
Rb1b     CTCGACACAT GTAACGGGGG CATAGTTGAA CGTGCAACAG GTCTTTCTGT
JM       CTCGACACAT GTAACGGGGG CATAGTTGAA CGTGCAACAG GTCTTTCTGT
CVI 988  CTCGACACAT GTAACGGGGG CATAGTTGAA CGTGCAACAG GTCTTTCTGT
         125011                                                125060

             125394                                                125443
Rb1b     AAACAAATCT AGACGTATAC GCCGGGAGCT GCGATACTGT ATATTATGTT
JM       AAACAAATCT AGACGTATAC GCCGGGAGCT GCGATACTGT ATATTATGTT
CVI 988  AAACAAATCT AGACGTATAC GCCGGGAGCT GCGATACTGT ATATTATGTT
         125061                                                125110

             125444                                                125493
Rb1b     CGGCTGTATA CGTAGAATGA CTACGATGGA GCCAATCATC CCATGTGCCA
JM       CGGCTGTATA CGTAGAATGA CTACGATGGA GCCAATCATC CCATGTGCCA
CVI 988  CGGCTGTATA CGTAGAATGA CTACGATGGA GCCAATCATC CCATGTGCCA
         125111                                                125160

             125494                                                125543
Rb1b     GTGAAGTACA TTATAGGTGG AACTTTTTTT CTTTGCCTCA CCTTAGAAGT
JM       GTGAAGTACA TTATAGGTGG AACTTTTTTT CTTTGCCTCA CCTTAGAAGT
CVI 988  GTAAAGTACA TTATAGGTGG AACTTTTTTT CTTTGCCTCA CCTTAGAAGT
         125161                                                125210

             125544                                                125593
Rb1b     GATGGTGGTA CTATCCCCAC TGATGTGTAA ATTGAGGGTT TCTTTCCAGG
JM       GATGGTGGTA CTATCCCCAC TGATGTGTAA ATTGAGGGTT TCTTTCCAGG
CVI 988  GATGGTGGTA CTATCCCCAC TGATGTGTAA ATTGAGGGTT TCTTTCCAGG
         125211                                                125260

             125594                                                125643
Rb1b     GTTTTAGTTT TTCGGATATT AACATATCGT TTGCCCGTCG ACAACACTCT
JM       GTTTTAGTTT TTCGGATATT AACATATCGT TTGCCCGTCG ACAACACTCT
CVI 988  GTTTTAGTTT TTCGGATATT AACATATCGT TTGCCCGTCG ACAACACTCT
         125261                                                125310

             125644                                                125693
Rb1b     TCAACTACCC ATTTTAAATC GTCTAAGTAC ACTTCAGATA ATTCCTTGAC
JM       TCAACTACCC ATTTTAAATC GTCTAAGTAC ACTTCAGATA ATTCCTTGAC
CVI 988  TCAACTACCC ATTTTAAATC GTCTAAGTAC ACTTCAGATA ATTCCTTGAC
         125311                                                125360
```

## LORF11

```
          125694                                                      125743
Rb1b      ATAGCTTTCA TCTGCATCGC AATCACAACC ATTCGGAAAA TAATTGCCCT
JM        ATAGCTTTCA TCTGCATCGC AATCACAACC ATTCGGAAAA TAATTGCCCT
CVI 988   ATAGCTTTCA TCTGCATCGC AATCACAACC ATTCGGAAAA TAATTGCCCT
          125361                                                      125410

          125744                                                      125793
Rb1b      GATTATTATG TGTAGCTGCG GAACCATCAA GCTCCATTTT ATGATATAAG
JM        GATTATTATG TGTAGCTGCG GAACCATCAA GCTCCATTTT ATGATATAAG
CVI 988   GATTATTATG TGTAGCTGCG GAACCATCAA GCTCCATTTT ATGATATAAG
          125411                                                      125460

          125794                                                      125843
Rb1b      CGAGATACAG TATCCCTGGT ATAACGATAA CAGGGGGCAA TATCCTTACT
JM        CGAGATACAG TATCCCTGGT ATAACGATAA CAGGGGGCAA TATCCTTACT
CVI 988   CGAGATACAG TATCCCTGGT ATAACGATAA CAGGGGGCAA TATCCTTACT
          125461                                                      125510

          125844                                                      125893
Rb1b      AGGGTATAAC ATGCATTCTG GAGAAACGGA CGTTATATAT GTGTTTGGAT
JM        AGGGTATAAC ATGCATTCTG GAGAAACGGA CGTTATATAT GTGTTTGGAT
CVI 988   AGGGTATAAC ATGCATTCTG GAGAAACGGA CGTTATATAT GTGTTTGGAT
          125511                                                      125560

          125894                                                      125943
Rb1b      CTCTGGGTTC TAAATTTTCA CTCGCTCGTA TCCAGCCATA TTTATCCTCT
JM        CTCTGGGTTC TAAATTTTCA CTCGCTCGTA TCCAGCCATA TTTATCCTCT
CVI 988   CTCTGGGTTC TAAATTTTCA CTCGCTCGTA TCCAGCCATA TTTATCCTCT
          125561                                                      125610

          125944                                                      125993
Rb1b      ACGGGTTGGA GTATATCGGA ATGTTTCGGT GGGGGAGGAT CCTCTATCAG
JM        ACGGGTTGGA GTATATCGGA ATGTTTCGGT GGGGGAGGAT CCTCTATCAG
CVI 988   ACGGGTTGGA GTATATCGGA ATGTTTCGGT GGGGGAGGAT CCTCTATCAG
          125611                                                      125660

          125994                                                      126043
Rb1b      AAAATCAATT TTCGAGATTT CTGATGTTCT AGTATTTGAG CTATGTGGTT
JM        AAAATCAATT TTCGAGATTT CTGATGTTCT AGTATTTGAG CTATGTGGTT
CVI 988   AAAATCAATT TTCGAGATTT CTGATGTTCT AGTATTTGAG CTATGTGGTT
          125661                                                      125710

          126044                                                      126093
Rb1b      TATCCACCGT AGCTGCCGAA TCATACATTA ACATCTCAGC TAGCATGACG
JM        TATCCACCGT AGCTGCCGAA TCATACATTA ACATCTCAGC TAGCATGACG
CVI 988   TATCCACCGT AGTTGCCGAA TCATACATTA ACATCTCAGC TAGCATGACG
          125711                                                      125760

          126094                                                      126143
Rb1b      TCAATATTTT CAAATGCCCC TGCATACACC GCACCCATTC CCATTTGTGT
JM        TCAATATTTT CAAATGCCCC TGCATACACC GCACCCATTC CCATTTGTGT
CVI 988   TCAATATTTT CAAATGCCCC TGCATACACC GCACCCATTC CCATTTGTGT
          125761                                                      125810

          126144                                                      126193
Rb1b      ACAAGTATTT GTGTCGACAT CGAGCTCCCA AAACGTTGTT AGGTAATCTC
JM        ACAAGTATTT GTGTCGACAT CGAGCTCCCA AAACGTTGTT AGGTAATCTC
CVI 988   ACAAGTATTT GTGTCGACAT CGAGCTCCCA AAACGTTGTT AGGTAATCTC
          125811                                                      125860
```

## LORF11

```
        126194                                                    126243
Rb1b    CATAGAGAGC AAAATCTGCA TCTGTGGTAT TTCGTACAAA GTTGACCACA
JM      CATAGAGAGC AAAATCTGCA TCTGTGGTAT TTCGTACAAA GTTGACCACA
CVI 988 CATAGAGAGC AAAATCTGCA TCTGTGGTAT TTCGTACAAA GTTGACCACA
        125861                                                    125910

        126244                                                    126293
Rb1b    TGACTCCACC GGGTAGATCC CAGGCGTATA TTGAGCCATG CTATTGAGTT
JM      TGACTCCACC GGGTAGATCC CAGGCGTATA TTGAGCCATG CTATTGAGTT
CVI 988 TGACTCCACC GGGTAGATCC CAGGCGTATA TTGAGCCATG CTATTGAGTT
        125911                                                    125960

        126294                                                    126343
Rb1b    TCTCAATTCA TCATAATCAT TTCCGTGAAA TGTTTTATGA TCCCATTCCA
JM      TCTCAATTCA TCATAATCAT TTCCGTGAAA TGTTTTATGA TCCCATTCCA
CVI 988 TCTCAATTCA TCATAATCAT TTCCGTGAAA TGTTTTATGA TCCCATTCCA
        125961                                                    126010

        126344                                                    126393
Rb1b    GTAAAATTAT TGTATACAAT TTAGACTTGT ATAAAGAGCG AAGATATGCC
JM      GTAAAATTAT TGTATACAAT TTAGACTTGT ATAAAGAGCG AAGATATGCC
CVI 988 GTAAAATTAT TGTATACAAT TTAGACTTGT ATAAAGAGCG AAGATATGCC
        126011                                                    126060

        126394                                                    126443
Rb1b    AAATCTGTGG AAGGGTTTGT AGACTCAGGC AGGGGTGTCA CTGTAACAGC
JM      AAATCTGTGG AAGGGTTTGT AGACTCAGGC AGGGGTGTCA CTGTAACAGC
CVI 988 AAATCTGTGG AAGGGTTTGT AGACTCAGGC AGGGGTGTCA CTGTAACAGC
        126061                                                    126110

        126444                                                    126493
Rb1b    CTCGAGATTT CTACCAAAGT GAGTCCTACG TTCGAATCGT ATCAAACCTG
JM      CTCGAGATTT CTACCAAAGT GAGTCCTACG TTCGAATCGT ATCAAACCTG
CVI 988 CTCGAGATTT CTACCAAAGT GAGTCCTACG TTCGAATCGT ATCAAACCTG
        126111                                                    126160

        126494                                                    126543
Rb1b    GCATACCCGC AGCATCGGTA ACCTGAACAC ATCGTAGGAC TGTGACTTCT
JM      GCATACCCGC AGCATCGGTA ACCTGAACAC ATCGTAGGAC TGTGACTTCT
CVI 988 GCATACCCGC AGCATCGGTA ACCTGAACAC ATCGTAGGAC TGTGACTTCT
        126161                                                    126210

        126544                                                    126593
Rb1b    GTTCTTATCC TTCCCAATAA TGACCATGGA GGCCCACAAT TTATGTTATA
JM      GTTCTTATCC TTCCCAATAA TGACCATGGA GGCCCACAAT TTATGTTATA
CVI 988 GTTCTTATCC TTCCCAATAA TGACCATGGA GGCCCACAAT TTATGTTATA
        126211                                                    126260

        126594                                                    126643
Rb1b    TAAAGCTTGG ATTATGGTAG ATCTGGTTGA CGTACATTGA AATTCTGGAA
JM      TAAAGCTTGG ATTATGGTAG ATCTGGTTGA CGTACATTGA AATTCTGGAA
CVI 988 TAAAGCTTGG ATTATGGTAG ATCTGGTTGA CGTACATTGA AATTCTGGAA
        126261                                                    126310

        126644                                                    126693
Rb1b    ACCATTCTGC AGACGGTTTA GGTGGGTTTA AATTCTGAAT TGGCCCGCTT
JM      ACCATTCTGC AGACGGTTTA GGTGGGTTTA AATTCTGAAT TGGCCCGCTT
CVI 988 ACCATTCTGC AGACGGTTTA GGTGGGTTTA AATTCTGAAT TGGCCCGCTT
        126311                                                    126360
```

## LORF11

```
            126694                                                    126743
Rb1b        ACTTGCATCC ATTCATACAT TGAGCAAATT AGAGTATGAG GATCGGGAAC
JM          ACTTGCATCC ATTCATACAT TGAGCAAATT AGAGTATGAG GATCGGGAAC
CVI 988     ACTTGCATCC ATTCATACAT TGAGCAAATT AGAGTATGAG GATCGGGAAC
            126361                                                    126410

            126744                                                    126793
Rb1b        ATCGTCAAAA TGGGCAAATA GAGCTATTGT ACTACGTTCC GCCGGTACGG
JM          ATCGTCAAAA TGGGCAAATA GAGCTATTGT ACTACGTTCC GCCGGTACGG
CVI 988     ATCGTCAAAA TGGGCAAATA GAGCTATTGT ACTACGTTCC GCCGGTGCGG
            126411                                                    126460

            126794                                                    126843
Rb1b        AGCCTATAGA CATAACATAT CTCGGATTTG ATGGCAATCT TTTGAGTTGG
JM          AGCCTATAGA CATAACATAT CTCGGATTTG ATGGCAATCT TTTGAGTTGG
CVI 988     AGCCTATAGA CATAACATAT CTCGGATTTG ATGGCAATCT TTTGAGTTGG
            126461                                                    126510

            126844                                                    126893
Rb1b        TCCAGGAACA ACACTGCACC CGATGAGAAT ACAGTCCTTT CAGGATCGTT
JM          TCCAGGAACA ACACTGCACC CGATGAGAAT ACAGTCCTTT CAGGATCGTT
CVI 988     TCCAGGAACA ACACTGCACC CGATGAGAAT ACAGTCCTTT CAGGATCGTT
            126511                                                    126560

            126894                                                    126943
Rb1b        GTCTTGGTTG GGGTGCCTAT TACAATCCAC CTTTTTTCTC CTTATAGAAG
JM          GTCTTGGTTG GGGTGCCTAT TACAATCCAC CTTTTTTCTC CTTATAGAAG
CVI 988     GTCTTGGTTG GGGTGCCTAT TACAATCCAC CTTTTTTCTC CTTATAGAAG
            126561                                                    126610

            126944                                                    126993
Rb1b        AAGCGAAGCA AGACATCCTG GCGCAGCTCT CGCCAACAGT TGTCTGAAAC
JM          AAGCGAAGCA AGACATCCTG GCGCAGCTCT CGCCAACAGT TGTCTGAAAC
CVI 988     AAGCGAAGCA AGACATCCTG GCGCAGCTCT CGCCAACAGT TGTCTGAAAC
            126611                                                    126660

            126994                                                    127043
Rb1b        AAGCAATATA TTCTTAAGTT ATAGGTACGT CAAACCACAT CAAGGTATAT
JM          AAGCAATATA TTCTTAAGTT ATAGGTACGT CAAACCACAT CAAGG. . . . .
CVI 988     AAGCAATATA TTCTTAAGTT ATAGGTACGT CAAACCACAT CAAGGTATAT
            126661                                                    126710
```

**Appendix 1c:** LORF11 nucleotide sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain. 100% consensus with strain Rb1b and 4 one bp substitutions with strain CVI988 were found. Note that the LORF11 sequence is complimentary. Start and stop codons are shown in **purple**

## LORF11 AMINO ACID

```
          1                                                              50
Rb1b      MSCFASSIRR KKVDCNRHPN QDNDPERTVF SSGAVLFLDQ LKRLPSNPRY
JM        MSCFASSIRR KKVDCNRHPN QDNDPERTVF SSGAVLFLDQ LKRLPSNPRY
CVI988    MSCFASSIRR KKVDCNRHPN QDNDPERTVF SSGAVLFLDQ LKRLPSNPRY

          51                                                             100
Rb1b      VMSIGSVPAE RSTIALFAHF DDVPDPHTLI CSMYEWMQVS GPIQNLNPPK
JM        VMSIGSVPAE RSTIALFAHF DDVPDPHTLI CSMYEWMQVS GPIQNLNPPK
CVI988    VMSIGSAPAE RSTIALFAHF DDVPDPHTLI CSMYEWMQVS GPIQNLNPPK

          101                                                            150
Rb1b      PSAEWFPEFQ CTSTRSTIIQ ALYNINCGPP WSLLGRIRTE VTVLRCVQVT
JM        PSAEWFPEFQ CTSTRSTIIQ ALYNINCGPP WSLLGRIRTE VTVLRCVQVT
CVI988    PSAEWFPEFQ CTSTRSTIIQ ALYNINCGPP WSLLGRIRTE VTVLRCVQVT

          151                                                            200
Rb1b      DAAGMPGLIR FERRTHFGRN LEAVTVTPLP ESTNPSTDLA YLRSLYKSKL
JM        DAAGMPGLIR FERRTHFGRN LEAVTVTPLP ESTNPSTDLA YLRSLYKSKL
CVI988    DAAGMPGLIR FERRTHFGRN LEAVTVTPLP ESTNPSTDLA YLRSLYKSKL

          201                                                            250
Rb1b      YTIILLEWDH KTFHGNDYDE LRNSIAWLNI RLGSTRWSHV VNFVRNTTDA
JM        YTIILLEWDH KTFHGNDYDE LRNSIAWLNI RLGSTRWSHV VNFVRNTTDA
CVI988    YTIILLEWDH KTFHGNDYDE LRNSIAWLNI RLGSTRWSHV VNFVRNTTDA

          251                                                            300
Rb1b      DFALYGDYLT TFWELDVDTN TCTQMGMGAV YAGAFENIDV MLAEMLMYDS
JM        DFALYGDYLT TFWELDVDTN TCTQMGMGAV YAGAFENIDV MLAEMLMYDS
CVI988    DFALYGDYLT TFWELDVDTN TCTQMGMGAV YAGAFENIDV MLAEMLMYDS

          301                                                            350
Rb1b      AATVDKPHSS NTRTSEISKI DFLIEDPPPP KHSDILQPVE DKYGWIRASE
JM        AATVDKPHSS NTRTSEISKI DFLIEDPPPP KHSDILQPVE DKYGWIRASE
CVI988    ATTVDKPHSS NTRTSEISKI DFLIEDPPPP KHSDILQPVE DKYGWIRASE

          351                                                            400
Rb1b      NLEPRDPNTY ITSVSPECML YPSKDIAPCY RYTRDTVSRL YHKMELDGSA
JM        NLEPRDPNTY ITSVSPECML YPSKDIAPCY RYTRDTVSRL YHKMELDGSA
CVI988    NLEPRDPNTY ITSVSPECML YPSKDIAPCY RYTRDTVSRL YHKMELDGSA

          401                                                            450
Rb1b      ATHNNQGNYF PNGCDCDADE SYVKELSEVY LDDLKWVVEE CCRRANDMLI
JM        ATHNNQGNYF PNGCDCDADE SYVKELSEVY LDDLKWVVEE CCRRANDMLI
CVI988    ATHNNQGNYF PNGCDCDADE SYVKELSEVY LDDLKWVVEE CCRRANDMLI

          451                                                            500
Rb1b      SEKLKPWKET LNLHISGDST TITSKVRQRK KVPPIMYFTG TWDDWLHRSH
JM        SEKLKPWKET LNLHISGDST TITSKVRQRK KVPPIMYFTG TWDDWLHRSH
CVI988    SEKLKPWKET LNLHISGDST TITSKVRQRK KVPPIMYFTG TWDDWLHRSH

          501                                                            550
Rb1b      STYTAEHNIQ YRSSRRIRLD LFTERPVARS TMPPLHVSRC GAVALIKPFL
JM        STYTAEHNIQ YRSSRRIRLD LFTERPVARS TMPPLHVSRC GAVALIKPFL
CVI988    STYTAEHNIQ YRSSRRIRLD LFTERPVARS TMPPLHVSRC GAVALIKPFL

          551                                                            600
Rb1b      RRLCNPPANR DCERVLLSMK QKLEDLDIDN TEVYPPQDLC FKVFIGTSIT
JM        RRLCNPPANR DCERVLLSMK QKLEDLDIDN TEVYPPQDLC FKVFIGTSIT
CVI988    RRLCNPPANR DCERVLLSMK QKLEDLDIDN TEVYPPQDLC FKVFIGTSIT
```

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## LORF11 AMINO ACID

```
              601                                                    650
Rb1b          PIVRKFFSAD WSRAGKCGRS NFRKELESTV RRISVPLEKS KPELAFIFSL
JM            PIVRKFFSAD WSRAGKCGRS NFRKELESTV RRISVPLEKS KPELAFIFSL
CVI988        PIVRKFFSAD WSRAGKCGRS NFRKELESTV RRISVPLEKS KPELAFIFSL

              651                                                    700
Rb1b          SVVTSNVENG ARTCTETLFS FGGAAIDMTT LIDGINKLLV LCDLLAVSGI
JM            SVVTSNVENG ARTCTETLFS FGGAAIDMTT LIDGINKLLV LCDLLAVSGI
CVI988        SVVTSNVENG ARTCTETLFS FGGAAIDMTT LIDGINKLLV LCDLLAVSGI

              701                                                    750
Rb1b          RRLSTIESIP DIGTSPYRCI LQRTTHITAC MTPKVVRCEN YNVLQSHWEE
JM            RRLSTIESIP DIGTSPYRCI LQRTTHITAC MTPKVVRCEN YNVLQSHWEE
CVI988        RRLSTIESIP DIGTSPYRCI LQRTTHITAC MTPKVVRCEN YNVLQSHWEE

              751                                                    800
Rb1b          CIVRSSEYLT YIPSQIEFVT HLTTRCPLKP ENNDWYLGLF FPFKGFRVIT
JM            CIVRSSEYLT YIPSQIEFVT HLTTRCPLKP ENNDWYLGLF FPFKGFRVIT
CVI988        CIVRSSEYLT YIPSQIEFVT HLTTRCPLKL ENNDWYLGLF FPFKGFRVIT

              801                                                    850
Rb1b          AETKRWLAEY TRRFHKWFQW GEGSPHYAAL RHLIPLCDCY LTDACMTNNF
JM            AETKRWLAEY TRRFHKWFQW GEGSPHYAAL RHLIPLCDCY LTDACMTNNF
CVI988        AETKRWLAEY TRRFHKWFQW GEGSPHYAAL RHLIPLCDCY LTDACMTNNF

              851                                                    900
Rb1b          FGCGILFHMH CVPTPERESR IVAILTRALT EAQKYAESSL HVDIDTQNIS
JM            FGCGILFHMH CVPTPERESR IVAILTRALT EAQKYAESSL HVDIDTQNIS
CVI988        FGCGILFHMH CVPTPERESR IVAILTRALT EAQKYAESSL HVDIDTQNIS

              901
Rb1b          IYN.
JM            IYN.
CVI988        IYN.
```

**Appendix 1d:** LORF11 aa sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain.

## LORF12

```
                    127040                                                    127089
Rb1b     TGTATCACAT ACGTCAATTA TGTTTCTGTG ATAATAGTTA CAAGGGTTGC
JM       TGTATCACAT ACGTCAATTA TGTTTCTGTG ATAATAGTTA CAAGGGTTGC
CVI 988  TGTATCACAT ACGTCAATTA TGTTTCTGTG ATAATAGTTA CAAGGGTTGC
         126801                                                    126850

                    127090                                                    127139
Rb1b     AGAAACTACC GTCAAAAAAA GTATTAATAA TGTAGCGATC GCACATCCAT
JM       AGAAACTACC GTCAAAAAAA GTATTAATAA TGTAGCGATC GCACATCCAT
CVI 988  AGAAACTACC GTCAAAAAAA GTATTAATAA TGTAGCGATC GCACATCCAT
         126851                                                    126900

                    127140                                                    127189
Rb1b     CATGTCGAAT GATAAACGC AAGTCAACTT CTGCGCAACT TTCAGAATTG
JM       CATGTCGAAT GATAAACGC AAGTCAACTT CTGCGCAACT TTCAGAATTG
CVI 988  CATGTCGAAT GATAAACGC AAGTCAACTT CTGCGCAACT TTCAGAATTG
         126901                                                    126950

                    127190                                                    127239
Rb1b     TTAACATTAT GTATCGGGGA TAGGGAATCG TATGAAGGGG GTGGGTCAAC
JM       TTAACATTAT GTATCGGGGA TAGGGAATCG TATGAAGGGG GTGGGTCAAC
CVI 988  TTAACATTAT GTATCGGGGA TAGGGAATCG TATGAAGGGG GTGGGTCAAC
         126951                                                    127000

                    127240                                                    127289
Rb1b     TGCATCATCG TATGTGGGCA ACAGTGCGGA ATTATTGTAT CTGGTAACCG
JM       TGCATCATCG TATGTGGGCA ACAGTGCGGA ATTATTGTAT CTGGTAACCG
CVI 988  TGCATCATCG TATGTGGGCA ACAGTGCGGA ATTATTGTAT CTGGTAACCG
         127001                                                    127050

                    127290                                                    127339
Rb1b     ATTCTAATTG TAAATGAATA TATTCTGATT GTTCATCAAT ATCTGAAAGC
JM       ATTCTAATTG TAAATGAATA TATTCTGATT GTTCATCAAT ATCTGAAAGC
CVI 988  ATTCTAATTG TAAATGAATA TATTCTGATT GTTCATCAAT ATCTGAAAGC
         127051                                                    127100

                    127340                                                    127389
Rb1b     ACAGGTTCTT CCTCGATTGG TGGGGAGATA GTCTCGGGTA TAGAAACGGC
JM       ACAGGTTCTT CCTCGATTGG TGGGGAGATA GTCTCGGGTA TAGAAACGGC
CVI 988  ACAGGTTCTT CCTCGATTGG TGGGGAGATA GTCTCGGGTA TAGAAACGGC
         127101                                                    127150

                    127390                                                    127439
Rb1b     ATCTGCATCC AAATCGAGAC TTTCGTGCTG CAGCTGTCCA TTTTCCATTT
JM       ATCTGCATCC AAATCGAGAC TTTCGTGCTG CAGCTGTCCA TTTTCCATTT
CVI 988  ATCTGCATCC AAATCGAGAC TTTCGTGCTG CAGCTGTCCA TTTTCCATTT
         127151                                                    127200

                    127440                                                    127489
Rb1b     TTTGGTCTTT GTCTGCACCA ATATTCGTAA AGGTGAGAAT TCGCTTAATC
JM       TTTGGTCTTT GTCTGCACCA ATATTCGTAA AGGTGAGAAT TCGCTTAATC
CVI 988  TTTGGTCTTT GTCTGCACCA ATATTCGTAA AGGTGAGAAT TCGCTTAATC
         1227201                                                   127250
```

**Appendix 1e:** LORF12 nucleotide sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain. Note that the LORF12 sequence is complimentary. Start and stop codons are shown in **purple**

## LORF12 AMINO ACID

```
          1                                                          50
Rb1b      MENGQLQHES LDLDADAVSI PETISPPIEE EPVLSDIDEQ SEYIHLQLES
JM        MENGQLQHES LDLDADAVSI PETISPPIEE EPVLSDIDEQ SEYIHLQLES
CVI988    MENGQLQHES LDLDADAVSI PETISPPIEE EPVLSDIDEQ SEYIHLQLES

          51                                                         100
Rb1b      VTRYNNSALL PTYDDAVDPP PSYDSLSPIH NVNNSESCAE VDLRFIIRHD
JM        VTRYNNSALL PTYDDAVDPP PSYDSLSPIH NVNNSESCAE VDLRFIIRHD
CVI988    VTRYNNSALL PTYDDAVDPP PSYDSLSPIH NVNNSESCAE VDLRFIIRHD

          101                     127
Rb1b      GCAIATLLIL FLTVVSATLV TIITET.
JM        GCAIATLLIL FLTVVSATLV TIITET.
CVI988    GCAIATLLIL FLTVVSATLV TIITET.
```

**Appendix 1f:** LORF12 aa sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain.

```
                 151770                                            151819
Rb1b     GCGAGCGTTG TCCATGGTAA CTGGAGTGGG GGCGGTATGT ACCCGATTGT
JM       .......... ..CATGGTAA CTGGAGTGGG GGCGGTATGT ACCCGATTGT
CVI 988  GCGAGCGCTG TCCATGGTAA CTGGAGTGGG GGCGGTATGT ACCCGATTGT
         151801                                            151850

                 151820                                            151869
Rb1b     CCGGCTAGCG ATGGTACGGG GACACAGGGT GTTTGCAGCG AGACGCCTTG
JM       CCGGCTAGCG ATGGTACGGG GACACAGGGT GTTTGCAGCG AGACGCCTTG
CVI 988  CCGGCTAGCG ATGGTACGGG GACACAGGGT GTTTGCAGCG AGACGCCTTG
         151851                                            151900

                 151870                                            151919
Rb1b     ATAAGGGTCC CCCGGAGTGG CTGGCGTATG TGGAAAAATC CCCACGTCTT
JM       ATAAGGGTCC CCCGGAGTGG CTGGCGTATG TGGAAAAATC CCCACGTCTT
CVI 988  ATAAGGGTCC CCCGGAGTGG CTGGCGTATG TGGAAAAATC CCCACGTCTT
         151901                                            151950

                 151920                                            151969
Rb1b     CGTCAAAAGC TGCCAACAAG CTGTCGAAAT CAGGCGGGTT GTCCATTTTT
JM       CGTCAAAAGC TGCCAACAAG CTGTCGAAAT CAGGCGGGTT GTCCATTTTT
CVI 988  CGTCAAAAGC TGCCAACAAG CTGTCGAAAT CAGGCGGGTT GTCCATTTTT
         151951                                            152000

                 151970                                            152019
Rb1b     AGGGTTTAGG AGGGGCGCAA ATAGATGGTG GGTGGGCCGA TGTGAGGGGA
JM       AGGGTTTAGG AGGGGCGCAA ATAGATGGTG GGTGGGCCGA TGTGAGGGGA
CVI 988  AGGGTTTAGG AGGGGCGCAA ATAGATGGTG GGTGGGCCGA TGTGAGGGGA
         152001                                            152050

                 152020                                            152069
Rb1b     ATACGGAAGG CGTAGCTGTC TATGACTCCC TTTGGGGGTC TCGTGGAAAT
JM       ATACGGAAGG CGTAGCTGTC TATGACTCCC TTTGGGGGTC TCGTGGAAAT
CVI 988  ATACGGAAGG CGTAGCTGTC TATGACTCCC TTTGGGGGTC TCGTGGAAAT
         152051                                            152100

                 152070                                            152119
Rb1b     GGCCCTCCCA TGCTAGACCA CAAGATGTGG GGGGAGGGAA GCTACGGTTC
JM       GGCCCTCCCA TGCTAGACCA CAAGATGTGG GGGGAGGGAA GCTACGGTTC
CVI 988  GGCCCTCCCA TGCTAGACCA CAAGATGTGG GGGGAGGGAA GCTACGGTTC
         152101                                            152150

                 152120                                            152169
Rb1b     AAGTGCGGAA ATTTCGATGT GCTGAAAGTC GAAACATAAA TGTAGGTGTC
JM       AAGTGCGGAA ATTTCGATGT GCTGAAAGTC GAAACATAAA TGTAGGTGTC
CVI 988  AAGTGCGGAA ATTTCGATGT GCTGAAAGTC GAAACATAAA TGTAGGTGTC
         152151                                            152200

                 152170                                            152219
Rb1b     AGCTGCGGAT ATAATCCCGA ACAGCTAGCA TCTGGTTTTG TATTTAGGCC
JM       AGCTGCGGAT ATAATCCCGA ACAGCTAGCA TCTGGTTTTG TATTTAG...
CVI 988  AGCTGCGGAT ATAATCCCGA ACAGCTAGCA TCTGGTTTTG TATTTAGGCC
         152201                                            152250
```

**Appendix 1g:** ANTISENSE nucleotide sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain. Start and stop codons are shown in **purple.**

## ANTISENSE AMINO ACID

```
           1                                                          50
Rb1b       MYPIVRLAMV RGHRVFAARR LDKGPPEWLA YVEKSPRLRQ KLPTSCRNQA
JM         MYPIVRLAMV RGHRVFAARR LDKGPPEWLA YVEKSPRLRQ KLPTSCRNQA
CVI988     MYPIVRLAMV RGHRVFAARR LDKGPPEWLA YVEKSPRLRQ KLPTSCRNQA

           51                                                        100
Rb1b       GCPFLGFRRG ANRWWVGRCE GNTEGVAVYD SLWGSRGNGP PMLDHKMWGE
JM         GCPFLGFRRG ANRWWVGRCE GNTEGVAVYD SLWGSRGNGP PMLDHKMWGE
CVI988     GCPFLGFRRG ANRWWVGRCE GNTEGVAVYD SLWGSRGNGP PMLDHKMWGE

           101        113
Rb1b       GSYGSSAEIS MC.
JM         GSYGSSAEIS MC.
CVI988     GSYGSSAEIS MC.
```

**Appendix 1h:**  ANTISENSE aa sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain.

## US2

```
                   158302                                              158351
Rb1b      AATTATATCT AATTGGTAGC AAGTAGGTCT GTCGAATAAC AGCTAATGAC
JM        .......... ......TAGC AAGTAGGTCT GTCGAATAAC AGCTAATGAC
CVI 988   AATTATATCT AATTGGTAGC AAGTAGGTCT GTCGAATAAC AGCTAATGAC
          158551                                              158600

                   158352                                              158401
Rb1b      TACCGGCTCT ACATTTTTTC TGTATTCGTG ACTTTCCTGT CGCAGTGTAA
JM        TACCGGCTCT ACATTTTTTC TGTATTCGTG ACTTTCCTGT CGCAGTGTAA
CVI 988   TACCGGCTCT ACATTTTTTC TGTATTCGTG ACTTTCCTGT CGCAGTGTAA
          158601                                              158650

                   158402                                              158451
Rb1b      CGAACCGGAA TTGCAATCGC ATCTCTATCT TCTTTCTTGC AACATTTTCC
JM        CGAACCGGAA TTGCAATCGC ATCTCTATCT TCTTTCTTGC AACATTTTCC
CVI 988   CGAACCGGAA TTGCAATCGC ATCTCTATCT TCTTTCTTGC AACATTTTCC
          158651                                              158700

                   158452                                              158501
Rb1b      ACAACAGAAT AATCTGCCGG GTGTACTACT CATTTGAGGT GGTTCGATTT
JM        ACAACAGAAT AATCTGCCGG GTGTACTACT CATTTGAGGT GGTTCGATTT
CVI 988   ACAACAGAAT AATCTGCCGG GTGTACTACT CATTTGAGGT GGTTCGATTT
          158701                                              158750

                   158502                                              158551
Rb1b      CCGGAGGTTT TAGAGGATTG GGTGGGGACC CGAGGATTTT GTATACACAT
JM        CCGGAGGTTT TAGAGGATTG GGTGGGGACC CGAGGATTTT GTATACACAT
CVI 988   CCGGAGGTTT TAGAGGATTG GGTGGGGACC CGAGGATTTT GTATACACAT
          158751                                              158800

                   158552                                              158601
Rb1b      ACCATATCAC TGTCGCAAAA ATGCGCTCTA TCTTCTGGGG TGTCGAACTT
JM        ACCATATCAC TGTCGCAAAA ATGCGCTCTA TCTTCTGGGG TGTCGAACTT
CVI 988   ACCATATCAC TGTCGCAAAA ATGCGCTCTA TCTTCTGGGG TGTCGAACTT
          158801                                              158850

                   158602                                              158651
Rb1b      CGGTTCCCAT GTAGATGTCA AGAGAGTTTG AATATTGTCG GGAATGGCCC
JM        CGGTTCCCAT GTAGATGTCA AGAGAGTTTG AATATTGTCG GGAATGGCCC
CVI 988   CGGTTCCCAT GTAGATGTCA AGAGAGTTTG AATATTGTCG GGAATGGCCC
          158851                                              158900

                   158652                                              158701
Rb1b      ACGGCATACC GGACCAGGTC CCAGACACTT TGATTGCAAG TAACCTTTTT
JM        ACGGCATACC GGACCAGGTC CCAGACACTT TGATTGCAAG TAACCTTTTT
CVI 988   ACGGCATACC GGACCAGGTC CCAGACACTT TGATTGCAAG TAACCTTTTT
          158901                                              158950

                   158702                                              158751
Rb1b      GGCAAAGGAA TACATTCGAG CGCAATGCGA CATATATCTG CCGCCCCAAC
JM        GGCAAAGGAA TACATTCGAG CGCAATGCGA CATATATCTG CCGCCCCAAC
CVI 988   GGCAAAGGAA TACATTCGAG CGCAATGCGA CATATATCTG CCGCCCCAAC
          158951                                              159000

                   158752                                              158801
Rb1b      TATCCACAAG CTATGTGGAG CATTACCAGA AACTTCAGAT TCCAACATCA
JM        TATCCACAAG CTATGTGGAG CATTACCAGA AACTTCAGAT TCCAACATCA
CVI 988   TATCCACAAG CTATGTGGAG CATTACCAGA AACTTCAGAT TCCAACATCA
          159001                                              159050
```

213

## US2

```
            158802                                                  158851
Rb1b        AATATCCAGA TAGAACATCC TGCCATTCTG TGGAACATCC TGCAACATCT
JM          AATATCCAGA TAGAACATCC TGCCATTCTG TGGAACATCC TGCAACATCT
CVI 988     AATATCCAGA TAGAACATCC TGCCATTCTG TGGAACATCC TGCAACATCT
            159051                                                  159100

            158852                                                  158901
Rb1b        TCAAATAGCC GCACTATAAA CGAATCCCTA GTTCCGGCCA ATCCGGTACC
JM          TCAAATAGCC GCACTATAAA CGAATCCCTA GTTCCGGCCA ATCCGGTACC
CVI 988     TCAAATAGCC GCACTATAAA CGAATCCCTA GTTCCGGCCA ATCCGGTACC
            159101                                                  159150

            158902                                                  158951
Rb1b        ACGAACTCCA GTTCCATCTG GTGGCTTTGT CCTTACTATC GGTCGATGTT
JM          ACGAACTCCA GTTCCATCTG GTGGCTTTGT CCTTACTATC GGTCGATGTT
CVI 988     ACGAACTCCA GTTCCATCTG GTGGCTTTGT CCTTACTATC GGTCGATGTT
            159151                                                  159200

            158952                                                  159001
Rb1b        GCCGAGGAAG AATTAACATG GGTTTGGCAA AACGGAATAG GTCTGCAGCT
JM          GCCGAGGAAG AATTAACATG GGTTTGGCAA AACGGAATAG GTCTGCAGCT
CVI 988     GCCGAGGAAG AATTAACATG GGTTTGGCAA AACGGAATAG GTCTGCAGCT
            159201                                                  159250

            159002                                                  159051
Rb1b        CTGACGATTA TGGGCACACC CACATCATCC TGTATTTGTT CCATACATTG
JM          CTGGCGATTA TGGGCACACC CACATCATCC TGTATTTGTT CCATACATTG
CVI 988     CTGGCGATTA TGGGCACACC CACATCATCC TGTATTTGTT CCATACATTG
            159251                                                  159300

            159052                                                  159101
Rb1b        CTTTATAAGG AATATCCATA AAGTAGATGC AGCATCTCTA GATCTTCCTG
JM          CTTTATAAGG AATATCCATA AAGTAGATGC AGCATCTCTA GATCTTCCTG
CVI 988     CTTTATAAGG AATATCCATA AAGTAGATGC AGCATCTCTA GATCTTCCTG
            159301                                                  159350

            159102                                                  159151
Rb1b        GCAATCGATC GCATTCATCT AGAAGTGTGA CTATAGTTAT CATGGACACA
JM          GCAATCGATC GCATTCATCT AGAAGTGTGA CTATAGTTAT CATGGACACA
CVI 988     GCAATCGATC GCATTCATCT AGAAGTGTGA CTATAGTTAT CATGGACACA
            159351                                                  159400

            159152                                                  159201
Rb1b        CCCATCTTCA CCTCCACCAA TAATCTTTTT TATTGTTAAT AACTGGGCCG
JM          CCCATCTTCA CCTCCACCAA TAATCTTT.. .......... ..........
CVI 988     CCCATCTTCA CCTCCACCAA TAATCTTTTT TATTGTTAAT AACTGGGCCG
            159401                                                  159450
```

**Appendix 1i:** US2 nucleotide sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain. Note that the US2 sequence is complimentary. Start and stop codons are shown in **purple** and mutations are denoted in **green**.

## US2 AMINO ACID

```
          1                                                          50
Rb1b    MGVSMITIVT LLDECDRLPG RSRDAASTLW IFLIKQCMEQ IQDDVGVPII
JM      MGVSMITIVT LLDECDRLPG RSRDAASTLW IFLIKQCMEQ IQDDVGVPII
CVI988  MGVSMITIVT LLDECDRLPG RSRDAASTLW IFLIKQCMEQ IQDDVGVPII

          51                                                         100
Rb1b    VRAADLFRFA KPMLILPRQH RPIVRTKPPD GTGVRGTGLA GTRDSFIVRL
JM      ARAADLFRFA KPMLILPRQH RPIVRTKPPD GTGVRGTGLA GTRDSFIVRL
CVI988  ARAADLFRFA KPMLILPRQH RPIVRTKPPD GTGVRGTGLA GTRDSFIVRL

          101                                                        150
Rb1b    FEDVAGCSTE WQDVLSGYLM LESEVSGNAP HSLWIVGAAD ICRIALECIP
JM      FEDVAGCSTE WQDVLSGYLM LESEVSGNAP HSLWIVGAAD ICRIALECIP
CVI988  FEDVAGCSTE WQDVLSGYLM LESEVSGNAP HSLWIVGAAD ICRIALECIP

          151                                                        200
Rb1b    LPKRLLAIKV SGTWSGMPWA IPDNIQTLLT STWEPKFDTP EDRAHFCDSD
JM      LPKRLLAIKV SGTWSGMPWA IPDNIQTLLT STWEPKFDTP EDRAHFCDSD
CVI988  LPKRLLAIKV SGTWSGMPWA IPDNIQTLLT STWEPKFDTP EDRAHFCDSD

          201                                                        250
Rb1b    MVCVYKILGS PPNPLKPPEI EPPQMSSTPG RLFCCGKCCK KEDRDAIAIP
JM      MVCVYKILGS PPNPLKPPEI EPPQMSSTPG RLFCCGKCCK KEDRDAIAIP
CVI988  MVCVYKILGS PPNPLKPPEI EPPQMSSTPG RLFCCGKCCK KEDRDAIAIP

          251              271
Rb1b    VRYTATGKSR IQKKCRAGSH .
JM      VRYTATGKSR IQKKCRAGSH .
CVI988  VRYTATGKSR IQKKCRAGSH .
```

**Appendix 1j:** US2 aa sequence of JM strain of MDV sequenced from DNA isolated from RPL-1 cells (Nazerian *et al.*, 1976) aligned and compared with the attenuated CVI988 Rispens MDV strain and the pathogenic Rb1b MDV strain.

# Appendix 2 – Supplier Information

**Alpha Innotech Inc.**

    2401 Merced Street,
    San Leandro, California  94577, USA

**Amersham Biosciences**

    GE Healthcare Life Sciences
    Amersham Place
    Little Chalfont, Buckinghamshire
    HP7 9NA, UK

**BD Biosciences**

    Edmund Halley Road
    Oxford Science Park
    Oxford OX4 4DQ, UK

**Bio-rad Laboratories**

    Bio-Rad House
    Maxted Road
    Hemel Hempstead
    Hertfordshire HP2 7DX, UK

**Bio Gene Ltd.**

    BioGene House
    6 The Business Centre
    Harvard Way
    Kimbolton, Cambridgeshire
    PE28 0NJ, UK

**Clontech Laboratories, Inc.**

    Takara Bio Company
    1290 Terra Bella Avenue
    Mountain View, California  94043, USA

**Daco Cytomation UK Ltd.**

    Cambridge House,
    St Thomas Place, Ely,
    Cambridgeshire CB7 4EX, UK

**Eurofins MWG Operon**
Anzingerstr. 7a
85560 Ebersberg
Germany

**Hamilton Bonaduz AG (designated as Hamilton)**
P.O. Box 26
CH-7402 Bonaduz GR
Switzerland

**Invitrogen Ltd. (incorporating Gibco, Ambion and Applied Biosystems)**
Life Technologies Corporation
European Headquarters
3 Fountain Drive,
Inchinnan Business Park,
Paisley PA4 9RF, UK

**Li-Cor Biosciences**
St. John's Innovation Centre
Cowley Road, Cambridge
CB4 0WS, UK

**Lonza (incorporating Amaxa)**
Lonza Cologne AG
Nattermannallee 1
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Germany

**Merck & Co., Inc.**
One Merck Drive
P.O. Box 100
Whitehouse Station, New Jersey
08889-0100, USA

**Minitab Ltd.**
Brandon Court, Unit E1-E2
Progress Way
Coventry CV3 2TE, UK

**New England Biolabs**
75-77 Knowl Piece
Wilbury Way
Hitchin,
Hertsfordshire SG4 0TY, UK

**Promega Corporation**
2800 Woods Hollow Road
Madison, Wisconsin
53711-5399  USA

**Qiagen (Incorporating Corbett Life Sciences)**
QIAGEN House
Fleming Way
Crawley
West Sussex
RH10 9NQ, UK

**F. Hoffmann-La Roche AG (designated as Roche)**
Konzern-Hauptsitz,
Grenzacherstrasse 124,
CH-4070 Basel, Switzerland

**SeaKem**
Cambrex Bio Science Rockland, Inc.,
191 Thomaston Street,
Rockland, Maine 04841 USA

**Sera Laboratoires International**
Unit 44, Bolney Grange Business Park
Haywards Heath, West Sussex
RH17 5PB  UK

**Severn Biotech, Ltd.**
Unit 2, Park Lane,
Kidderminster,
Worcestershire,
DY11 6TJ, UK

**Sigma-Aldrich (incorporating Fluka Chemicals)**
3050 Spruce Street,
St. Louis, MO 63103 USA

**Stratagene**
Aligent Technologies,
11011 N. Torrey Pines Road,
La Jolla, California 92037, USA

**Thermo Fisher Scientific (incorporating Nunclon and Dharmacon)**
    Fisher Scientific UK Ltd
    Bishop Meadow Road,
    Loughborough,
    Leicestershire, LE11 5RG UK


**VWR International Ltd**
    Hunter Boulevard, Magna Park,
    Lutterworth, Leicestershire
    LE17 4XN, UK

**Vector Laboratories**
    30 Ingold Road,
    Burlingame, California 94010, USA

Amaxa Biosystems worksheet                                          20.06.2007
Short Optimization with the Cell Line Optimization Protocol (VCO-1001):
Cell Line: RPL-1

Clone/origin:                                                       Researcher:  G. Hunter
Passage no.:  P8 after thawing
Used culture medium: RPMI, 10%FCS, Tryptose Phosphate Broth, Sodium Pyruvate,
        University of Edinburgh
Density/confluence before Nucleofection: Short Protocol
Cell no./sample: 3.5 million          Total cells needed: 57   million
Max. centrifugal force: 200g, 10min, Room Temperature

6-well plates for plating out cells after Nucleofection,
1 well per Nucleofection (2-4 plates needed);   2 ml media per well

DNA/RNA:
Used construct, supplier (e.g. pEGFP, Clontech): pmaxGFP      reporter gene (e.g. eGFP): maxGFP
Used promoter (e.g. CMV):   CMV   size: 3,5kb                special sequences (e.g. IRES, LTR): no
Used amount: 2µg                                            purification method: endotoxin-free
A260/A280:  >1.8

RESULTS post Nucleofection:

Time point of analysis:  16-24h        method: fluorescent. Microscope

| Sample | | Solution: | Cell Line Nucleofector Solution V | | |
|---|---|---|---|---|---|
| # | Program: | plasmid | Trans. Efficiency (%): V | Viability (%): V |
| 1 | A-033 | pmaxGFP | 35.7 | 18.3 |
| 2 | X-002 | pmaxGFP | 29.0 | 14.9 |
| 3 | T-001 | pmaxGFP | 35.0 | 16.6 |
| 4 | U-001 | pmaxGFP | 32.8 | 9.1 |
| 5 | X-001 | pmaxGFP | 13.4 | 11.4 |
| 6 | w/o Nucleofection | pmaxGFP | 0 | 100 |

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