Norwegian University
of Life Sciences

Master's Thesis 2022 60 ECTS
Department of Animal and Aquacultural Sciences

Development of lentiviral technology for use in Atlantic salmon

Sara Stenmark Andreassen
Genome Science

Acknowledgements

The works presented in this thesis was performed at the Centre for Integrative Genetics (CIGENE) at Norwegian University of Life Sciences.

I would like to thank my supervisors Thomas Nelson Harvey, Guro Katrine Sandvik and Jacob Seilø Torgersen for the support and guidance. With a special thanks to Tom for all the help in the lab, and with writing of the thesis. Thank you for providing me with the tools to work independently and for being a great source of knowledge. To Guro and the researchers and students in her group, thank you for creating an including and safe community. Thank you to Prabin Sharma Humagain for providing us with Salmon fibroblasts for us to experiment on.

To my fellow student Thea Marie Låstad, thank you for the good company and for collaboration on several experiments.

Lastly, a great thanks to my partner for keeping my spirits high and encouraging me through the whole process.

Sara Stenmark Andreassen
Kråkstad, August 2022

Abstract

Lentiviral vectors are used to deliver and stably integrate genetic material into the genome of cells. When combined with CRISPR based gene editing technology, targeted editing of one or many host genes in a pooled format is possible, enabling massively parallel genetic screens. While this technology is commonplace in mammalian systems, non-mammalian non-model systems such as Atlantic salmon, lag behind. Atlantic salmon (*Salmo salar*) is one the most economically important farmed fish species, with one of the main constraints for continuous economic growth being infectious diseases. It is desirable to develop an effective method of gene editing and screening for candidate disease resistance genes in Atlantic salmon, such as lentiviral mediated genome wide CRISPR-Cas9 knockout screens. So far there has been limited success in lentiviral transduction of salmon. In this thesis we aim to enable lentiviral transduction of Atlantic salmon cells by identifying which steps in the process are preventing transduction. Confocal microscopy of Atlantic salmon kidney cells transduced with labeled virus revealed that the virus appears to be able to bind and form endosomes in salmon cells. It is unclear if the virus is able to fuse, but no reverse transcriptase activity was detectable by qPCR. Altering temperature and pH had no effect on that transduction of salmon cells, although lowering pH before transduction did increase transduction efficiency in human cells. We also created pseudotyped lentiviral vectors using glycoproteins derived from infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) and fusions of IHNV and VHSV glycoproteins with commonly used vesicular stomatitis virus glycoprotein. We found that two of the viruses pseudotyped with glycoprotein fusions were capable of transducing human cells, however we were unable to produce successful transduction of Atlantic salmon using any of the pseudotyped vectors.

Table of Contents

1. Intro

1.1. Lentiviral vectors

Lentiviral vectors are used to transfer genetic material into mammalian cells, using the retroviral ability to deliver and integrate its own genome into both dividing and non-dividing host cells (Lewis et al., 1992). In order to successfully integrate genetic material into the host cell genome, the virus must be able to detect surface receptors on target cells and bind in order to initiate cell entry. Lentiviral vectors have a broad spectrum of applications, both in research and gene therapy in clinical settings.

1.1.1. Retroviruses

Human immunodeficiency virus (HIV-1) derived lentiviral vectors belong to the Retroviridae family, a genus of viruses with a unique ability to integrate its genome into the genome of host cells using retroviral reverse transcriptase and integrase enzymes (Tang et al., 1999). Viral RNA is reverse transcribed into DNA, and integrated permanently into host cell genome, where the cellular transcription and translation machinery is utilized to produce viral particles (Tang et al., 1999).

Retroviruses are enveloped viruses, and their genome consists of a 7-12 kb positive sense single stranded RNA molecule (Cockrell & Kafri, 2007). The viral genome is contained within the capsid core, along with associated proteins including nucleocapsid protein, reverse transcriptase, and integrase (Figure 1). The capsid of HIV-1 is cone-shaped (Wills & Craven, 1991). The membrane associated matrix protein structure is a part of the multi domain structural polyprotein Gag and is located directly within the phospholipid envelope (Hamard-Peron & Muriaux, 2011). The matrix has interactions with the lipid bilayer and surface glycoproteins during assembly and cellular entry (Cosson, 1996).

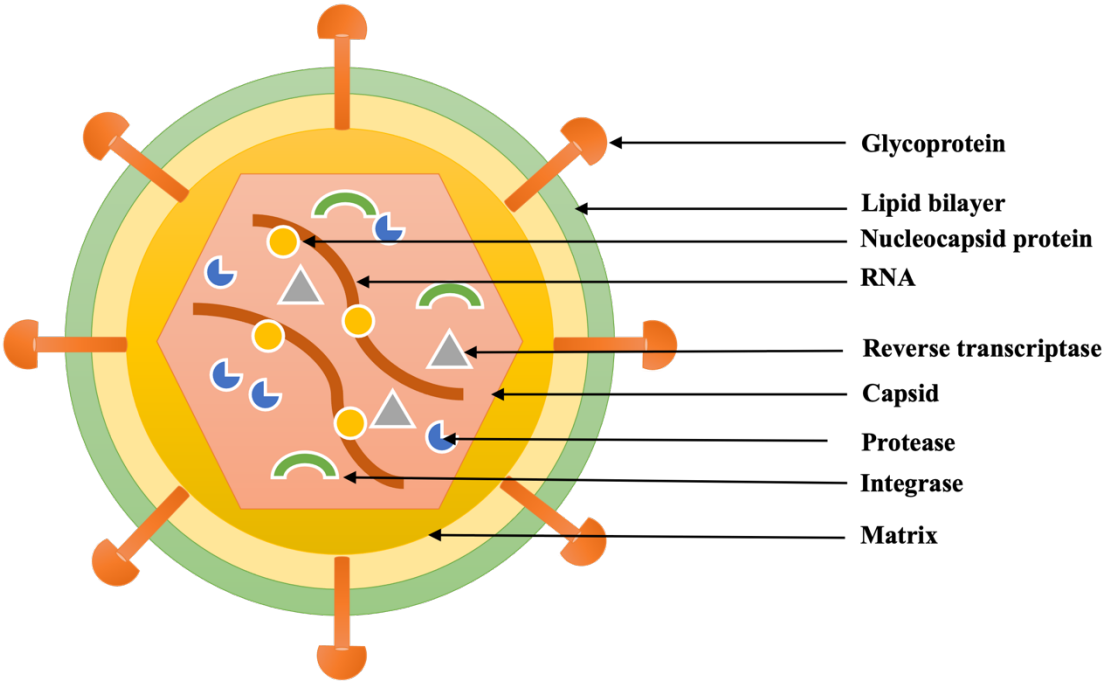


Figure 1: The retroviral virion.

The retroviral vector mode of cell entry begins when retroviral envelope proteins recognize and bind cellular receptors. Interactions between retroviral glycoprotein and receptor molecules are believed to trigger conformal changes in the protein, which for lentivirus leads to pH independent fusion of membranes (Stein et al., 1987). From there, the capsid is released and uncoated, revealing viral RNA and associated proteins (Ambrose & Aiken, 2014). Retroviral reverse transcriptase has two enzymatic functions, DNA polymerase which can use both DNA and RNA as a template, and ribonuclease H which cleaves the RNA strand of RNA-DNA duplex (Herschhorn & Hizi, 2010).

The viral RNA enters the cytoplasm, is reverse transcribed to double stranded DNA, and integrated permanently into host cell genome by viral integrase (Figure 2). Promoters within the integrated DNA initiate transcription of integrated viral DNA, synthesizing viral genomic RNA and mRNA for viral proteins (Herschhorn & Hizi, 2010). New virions are assembled and released by budding from host cells, meaning the phospholipid membrane of the viral envelope is derived from host cell membranes (Quigley et al., 1971).

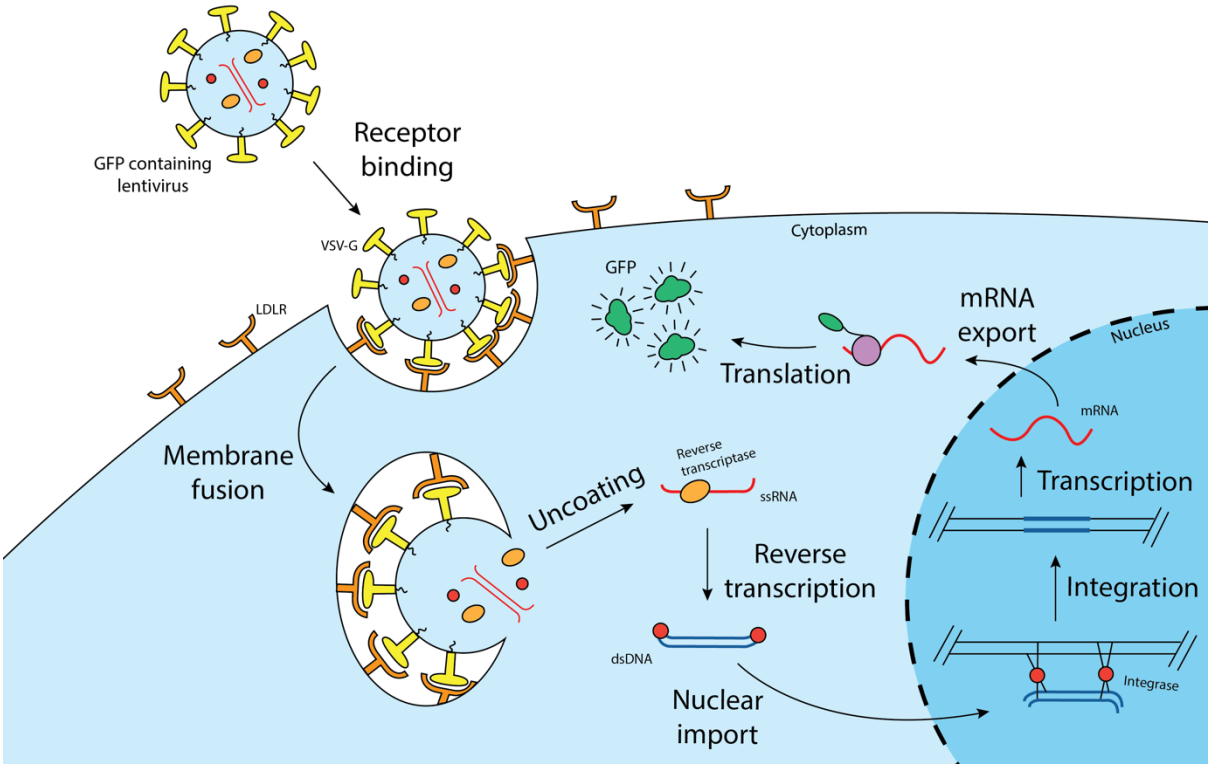


Figure 2: Lentiviral transduction. Figure was prepared by Thomas Nelson Harvey.

1.1.2. Production of lentiviral vectors

The safety of initial lentiviral vectors was ensured by separating the cis-acting elements in the genome from the trans-acting elements (Naldini et al., 1996b). This means separating the non-coding sequences needed for vector RNA synthesis, packaging, reverse transcription, and integration, from the coding sequences for enzymatic, structural, and accessory proteins (Naldini et al., 1996b). The result of this is a replication-incompetent lentiviral vector.

Lentiviral vectors are produced by transient transfection of packaging cell lines like HEK-293T with lentiviral plasmids (Naldini et al., 1996b). The second generation of lentiviral vectors consist of three plasmids, the envelope plasmid, the packaging plasmid, and the transfer plasmid. The envelope plasmid encodes the surface protein of the virus. The broad tropism of vesicular stomatitis glycoprotein and the stability of the protein has made this the standard surface protein for pseudotyped lentiviral vectors (Akkina et al., 1996). The packaging plasmid encodes the structural and enzymatic proteins of the virus. This includes the Gag and Pol genes (Naldini et al., 1996a; Naldini et al., 1996b). The transfer plasmid contains the transgene of

interest which will be integrated into host cell genome. For research of transduction efficiency, the gene sequence of the marker protein green fluorescent protein (GFP) is often used. The protein fluoresces green when exited with blue light and does not interfere with cell growth and function (Chalfie et al., 1994).

1.2. Use of lentiviral vectors in gene technology

Aquaculture is an important source of food and economic growth, with Atlantic salmon (*Salmo salar*) being one of the most economically important farmed fish species. Fresh fish is Norway's second largest export, earning 63.6 billion NOK in revenue in 2020 ((OEC), 2022). One of the main constraints of further expansion in aquaculture production is infectious diseases (Jennings et al., 2016). Selective breeding for increased disease resistance using quantitative trait loci (QTL), genetic markers tied to specific traits in the animal, or genomic selection using genome wide markers like SNPs to predict breeding values is used to increase the pace at which desired traits are enhanced (Zenger et al., 2018). In order to increase the rate in which these advances happen, implementing newer gene editing technology could have great impact.

One of these newer advances is the use of modified CRIPSR/Cas9 systems for targeted genome editing. The reprogrammed bacterial defense system can be used in genome engineering to create specific deletions or insertions in, as well as transcriptional regulation (Doudna & Charpentier, 2014). Using CRISPR in genomic selection means being able to test candidate genes and variants within QTL regions. It also means the possibility of editing unfavorable alleles to correspond to favorable alleles, and to introduce transgenes from closely related species or strains (Gratacap et al., 2019). CRISPR constructs are commonly delivered by lentiviral vectors.

Lentiviral technology would also enable CRISPR based genome-wide screening approaches in salmon. CRISPR screens are used to identify a small number of genes or genetic sequences in a large number of genetic sequences which gives a specific function or phenotype. (Doench, 2018). These screens are done for cell lines, and the process includes creating a library of single guide RNAs (gRNAs) to target all genes in the target organism and packaging these gRNA sequences into a lentiviral vector (Shalem et al., 2014). Further, a cell line expressing Cas9 is transduced with these lentiviral vectors, aiming at one gRNA per cell. The cells are screened

using a marker of selection, and the selected cells are sequences. Sequencing results will show either depletion or enrichment of gRNAs, which will give some insight if the target gene in the phenotype which is studied (Gratacap et al., 2019).

In order to efficiently utilize CRISPR/Cas9 screens in research to improve breeding programs and disease resistance in aquaculture, the lentiviral vectors need to efficiently transduce the cell lines of interest, in order to deliver the gRNAs. Some attempts have been made at optimizing transduction of salmonid cell lines, but with limited results.

In the study by Gratacap et al. (2020), the researchers had some positive results transducing Chinook salmon cells (CHSE-214) with GFP as a reporter gene by adjusting some of the incubation conditions. By increasing the incubation temperature from 17℃ to 22℃ they were able to increase transduction efficiency from 1% to 63%. By including a heat shock of 28℃ for 4 hours, the efficiency was further increased to 73%. Using a spinfection protocol also showed some improvement in transduction efficiency. The modified transduction protocol they developed for CHSE cells included spinfecting at 1000 xg for 2 hours for suspended cells and incubation at 24 hours at 22℃. The researchers used this protocol for transduction of Cas9 and GFP expressing CHSE cells. The lentivirus introduces gRNA with GFP gene as target, and transduction success was measured in loss of fluorescence. They recorded at 89.9% decrease in fluorescence. This study describes the first demonstration of lentivirus mediated editing in salmon cells (Gratacap et al., 2020), and these results can be used as a pointer of some adjustments which we can make to optimize transduction of salmon cells.

1.3. Pseudotyping

1.3.1. Concept

Viral tropism is the ability of a virus to detect and bind cellular receptors in order to produce a successful infection. The tropism of a virus determines which cell types in which host organism it can infect and reproduce in. Lentiviral tropism can be altered by pseudotyping with heterologous envelope proteins from viruses which have evolved the desired cell tropism(Cronin et al., 2005). Lentiviral vectors are most commonly pseudotyped with vesicular stomatitis glycoprotein (VSV-G) (Akkina et al., 1996). The resulting lentivirus has a broad tropism, infecting a wide range of host cells (Akkina et al., 1996).

The process of pseudotyping was first explored by infecting cells with two or more viruses to create phenotypic mixing, reviewed in Závada (1982). Human immunodeficiency virus was discovered to form hybrid progeny with broadened tropism by superinfecting cells infected with HIV-1 with vesicular stomatitis virus or herpes simplex virus (Závada, 1982). The phenotypic mixtures were able to infect both human and hamster cells (Zhu et al., 1990). During coinfection of cells with either RNA or DNA virus, HIV forms hybrid virions composed of the genetic information of HIV and envelope glycoproteins of the coinfecting viruses (Zhu et al., 1990).

When designing new pseudotyped lentiviral vectors, the pseudotyping is done by changing the gene sequence in the lentiviral envelope plasmid. This results in changes in the amino acid sequence and conformation of the surface protein located on the virion.

1.3.2. Chimeric proteins

VSV-G pseudotyped lentiviral vectors are the standard due to the broad tropism, and transduction efficiency. But this pseudotype can also be further adapted for new settings and contexts. In an experiment described in Carpentier et al. (2012) where the goal was to infect neurons in rats in order to treat neurodegenerative diseases, lentivirus was pseudotyped using rabies virus glycoprotein (RV-G). VSV-G pseudotyped vectors can transduce rodent neurons, but only locally around the injection site (Carpentier et al., 2012). RV-G pseudotyped vectors were proven to spread further in the nervous system, allowing for less invasive administration of gene therapy delivered to the central nervous system (Carpentier et al., 2012). Different combinations of domains from VSV-G and rabies virus glycoprotein were tested for their impact on transduction efficiency. The combination of domains proven to be most efficient was made by replacing the cytoplasmic region of RV-G with cytoplasmic region of VSV-G (Carpentier et al., 2012). The resulting surface glycoprotein was a chimeric protein containing extracellular and transmembrane domain from rabies virus glycoprotein and cytoplasmic domain of VSV-G.

It is unclear why this pseudotype with the cytoplasmic tail of VSV-G was the most efficient, but it was speculated that it may be due to interactions between the glycoprotein tail and the matrix protein (Carpentier et al., 2012). It was also theorized that the increased biological activity was due to a greater number of glycoproteins incorporated per viral particle due to the

ability of the VSV-G cytoplasmic tail to localize cholesterol rich microdomains, which is believed to be the main site for HIV-1 particle budding (Carpentier et al., 2012).

In this project, we will use Gibson cloning to create chimeric proteins of VSV-G and glycoproteins of the piscine rhabdoviruses infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHVS).

1.4. Rhabdoviruses

1.4.1. Classification and structure

The viruses used to pseudotype the lentiviral vectors (VSV, IHNV and VHSV) are members of the family Rhabdoviridae in order Mononegavirales. This family of viruses is comprised of many pathogens with great significance to both public health, agriculture and aquaculture (Kuzmin et al., 2009). Most rhabdoviruses are transmitted through arthropods to the vertebrate or plant hosts, but some are transmissible without a biological vector (Dietzgen et al., 2017). This includes lyssaviruses, for example rabies, which transmits through bites (Rupprecht et al., 2017). The aquatic viruses in the novirhabdovirus group, for example IHNV and VHSV are waterborne, or egg associated (Dietzgen et al., 2017).

The family of rhabdoviruses include thirteen genera, and their genomes contain non-segmented, negative sense, single stranded RNA of 11-16 kb length (Dietzgen et al., 2017). All rhabdoviruses have the same basic genome organization with some variations. The genome includes five different genes, which encode (from 3' to 5') the nucleoprotein (nucleocapsid protein, N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L, RNA-dependent RNA polymerase) (Dietzgen et al., 2017). Rhabdoviral genomes are dynamic, and there can be much variation between the different groups. Between the five basic genes, novel genes can be scattered, so that the number of genes can vary from 5-10 or more (Walker et al., 2011).

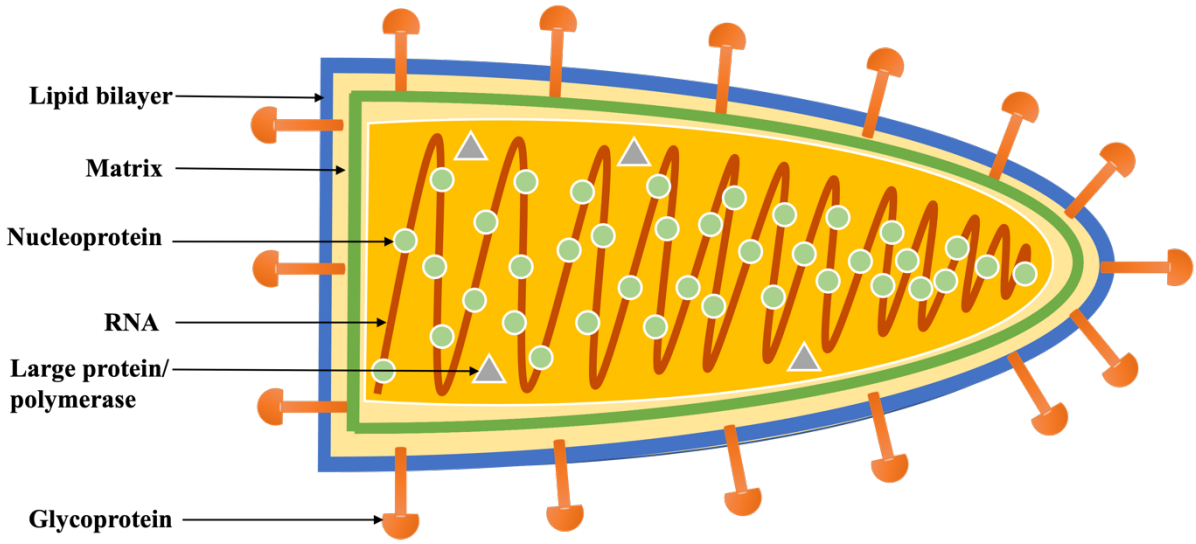


Figure 3: Rhabdoviral virion.

Rhabdoviruses with animal hosts have characteristic bullet or cone shaped enveloped virions (Figure 3) (Ge et al., 2010). Except for some plant infecting genera which replicate in the nucleus (e.g. nucleorhabdoviruses), rhabdovirus replication mechanisms are almost universal across the family (Dietzgen et al., 2017). The cytoplasmic replication pathway includes: (i.) cell entry, (ii.) uncoating, (iii.) transcription and translation, (iv.) genome replication and encapsidation, (v.) assembly and budding (Dietzgen et al., 2017). Cell entry in animal hosts is either clathrin-mediated or through receptor-binding endocytosis (Dietzgen et al., 2017).

1.4.2. Glycoprotein

The glycoprotein (G) is the only surface protein on the rhabdoviral virion. It can recognize receptors on host cells, bind, and induce the endocytosis pathway. The glycoprotein is organized in trimers (Coll, 1995). The glycoprotein is around 500 amino acids (511 aa for VSV-G and 505 aa for RABV-G), and forms around 400 homotrimers on the surface of the virion (Coll, 1995). The trimers are non-covalently bound, and consist of a globular head supported by a stalk region (Gaudin et al., 1992).

The first step in viral entry is attachment to host cell. The cellular receptor varies for different members of the rhabdoviral family. Clathrin mediated endocytosis with a pH dependent fusion of membranes is the most prominent route of entry for rhabdoviruses (Sun et al., 2005). The low pH inside the endosome leads to a series of structural rearrangements which results in fusion of virion and cellular membrane (Rucker et al., 2012).

1.4.3. Vesicular stomatitis virus

Vesiculoviruses comprise a group of rhabdoviruses with closely related morphology and genomes. The Vesicular stomatitis virus (VSV) is a term for a number of viruses within the same serogroup, with subgroups being New Jersey and Indiana serotypes (Liu et al., 2021). These viruses cause vesicular stomatitis disease in livestock, and the disease can transmit to human (Letchworth et al., 1999). The clinical signs of vesicular stomatitis are indistinguishable from foot-and-mouth disease (Letchworth et al., 1999). The glycoprotein derived form VSV has become the most commonly used glycoprotein in pseudotyped lentiviral vectors, due to its broad range of host cells and high infectivity (Aiken, 1997; Akkina et al., 1996; Cockrell & Kafri, 2007).

The cell surface low-density lipoprotein (LDL) receptor is the main receptor for vesicular stomatitis glycoprotein (Finkelshtein et al., 2013). The broad tropism of the virus is due to the LDL receptor being widely expressed in a broad number of cell types (Finkelshtein et al., 2013). The receptor is responsible for cellular uptake of cholesterol molecules bound to lipoproteins (Goldstein & Brown, 2009). Initiation of fusion of membranes by VSV glycoprotein is pH dependent, and the optimal pH for fusion is between 5 and 6 (Rucker et al., 2012).

1.4.4. Novirhabdovirus

IHNV and VHSV are both examples of novirhabdovirus, one of three genera of rhabdoviruses known to infect aquatic hosts, with the other two being perhabdoviruses and spriviviruses (Dietzgen et al., 2017). Both viruses have salmonid hosts, and they replicate at low temperatures. Phylogenetic studies of the L polymerase gene sequence of novirhabdoviruses indicates to the group having an ancestral role in the origin of rhabdoviruses (Dietzgen et al., 2017).

Novirhabdovirus genomes contain the same five genes as other genera of rhabdoviruses, 3'-N-P-M-G-L -5'. In addition to this, novirhabdoviruses have a unique gene encoding the non-virion protein (NV) (Kurath & Leong, 1985). This NV gene is located between the G and L genes and is expressed at low levels in infected cells (Kurath & Leong, 1985). The gene appears to have a role in blocking host innate immune response to viral infection (Thoulouze et al.,

2004). The NV protein has been shown to suppress apoptosis in an early stage of infection (Ammayappan & Vakharia, 2011). Suppression of apoptosis allows the virus more time to use the host cells to release more progeny virus.

1.4.4.1. Infectious Hematopoietic Necrosis Virus

Infectious hematopoietic necrosis virus (IHNV) causes the clinical disease infectious hematopoietic necrosis in salmonid species, which causes great economic losses (Bootland & Leong, 2011). The disease is monitored closely and is notifiable to the world organization for animal health (OIE) and the EU (European Commission, 2020). IHNV was initially identified in western North America where the disease now is endemic (Cieslak et al., 2017). The pathogen has since spread to Europe and Asia (Kurath et al., 2003). The disease has been reported in a variety of countries where salmonid species are farmed. The disease commonly produces significant mortality of up to 90% in hatcheries and is especially devastating for smolt (Kurath et al., 2016).

The disease typically presents by darkening of the skin because of hemorrhaging of the capillaries, pale gills, and lethargic behavior (Cieslak et al., 2017). The gills are considered to be the primary port of entry for the virus, but tissues in the digestive system may become infected by consumption of fish which have died from the disease (Drolet, 1994). In later stages of systemic viremia, IHNV infects epithelial cells of numerous organs, and especially connective tissue (Drolet, 1994). Infections usually happen at 8-14℃, and have not been reported above 15 ℃ (Lapatra, 1998).

1.4.4.2. Viral Hemorrhagic Septicemia Virus

Viral hemorrhagic septicemia virus (VHSV) is one of the most important pathogens for salmonid species, but also infects a broad range of hosts from different taxonomic families, with over 90 host species reported in both north Atlantic and north Pacific Ocean (Einer-Jensen et al., 2004). This disease is also notifiable to the world organization for animal health (OIE) and the EU (European Commission, 2020). Fry and juvenile fish are most sensitive to the disease, with mortalities of up to 100% (Skall et al., 2005). The virus is transmitted through contaminated water, or though contact with infected fish with optimal temperatures being 1-12℃ (Meyers & Winton, 1995). Symptoms of the disease includes hemorrhaging by the base of the fins, pale gills, bulging if the eyes, and darkening of the skin because of hemorrhaging of the capillaries (Kim & Faisal, 2011).

1.5. Aim

While there have been some attempts at creating protocols for lentiviral transduction of salmon cells, success has been limited. There are no conclusive answers for what is hindering the transduction process. We will investigate this by varying the transduction conditions, with the hope of identifying which step in the transduction process is the main barrier. We will also produce pseudotyped lentiviral vectors using piscine rhabdovirus glycoprotein in an attempt to create a lentiviral vector with the desired tropism. The aim of this thesis is to enable lentiviral transduction in Atlantic salmon cells in order to make use of gene editing technology in Atlantic salmon, with the end goal of improved disease resistance and animal welfare.

2. Materials and method

2.1. Construct design

2.1.1. IHNV-G and VHSV-G

The gene sequences of Viral Hemorrhagic Septicemia Virus glycoprotein (VHSV-G) (Accession number: KM926343.1) and Infectious Hematopoietic Necrosis Virus glycoprotein (IHNV-G) (Accession number: L40883.1) were acquired from GenBank and imported into Benchling (Benchling, 2022).The IHNV-G and VHSV-G sequences were designed to be cloned seamlessly into the Vesicular Stomatitis Virus glycoprotein (VSV-G) containing lentiviral envelope plasmid pMD2.G (Addgene plasmid #12259). For this reason, we included non-coding sequence flanking glycoprotein coding sequence until the EcoRI sites in the plasmid (Figure 4) when designing DNA sequences to be synthesized. The sequences were human codon optimized using Genscript optimization software (Stothard, (2000)), the sequences were cloned in silico into the lentiviral envelope plasmid pMD2.G at the EcoRI restriction sites and ordered from Genscript. Both constructs were delivered cloned into pUC57 (Genscript #SD1176).

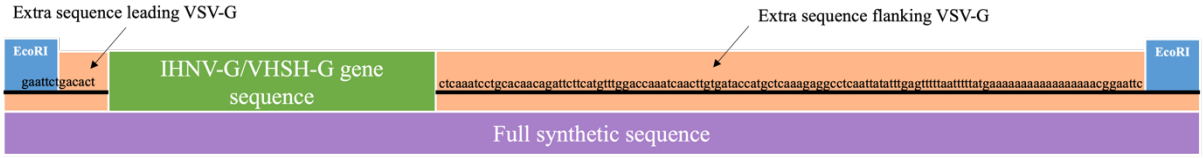


Figure 4: Design of synthetic sequences ordered in plasmid pUC57. Figure shows EcoRI restrictions sites, as well as the non-coding sequences leading and flanking the glycoprotein sequences.

2.1.2. VSV-G fusion proteins

We designed three different variants of the glycoprotein from both viruses: The native glycoprotein, the glycoprotein fused with the cytoplasmic tail from VSV-G, and the glycoprotein fused with the transmembrane domain and cytoplasmic tail from VSV-G (Figure 5).

Primers for Gibson cloning were designed for these four fusion proteins by using Geneious transmembrane domain predictions software (Geneious, 2022), and annotating gene sequences in Benchling. These annotations were used when cloning the sequences in silico to create the plasmids containing the fusion proteins. Primers were generated in Benchling to have 20-30 bp

binding regions and 20-30 bp of homology with the adjacent segment. The finished primers were ordered from Thermo Fisher Scientific (Table 1, Table 2).

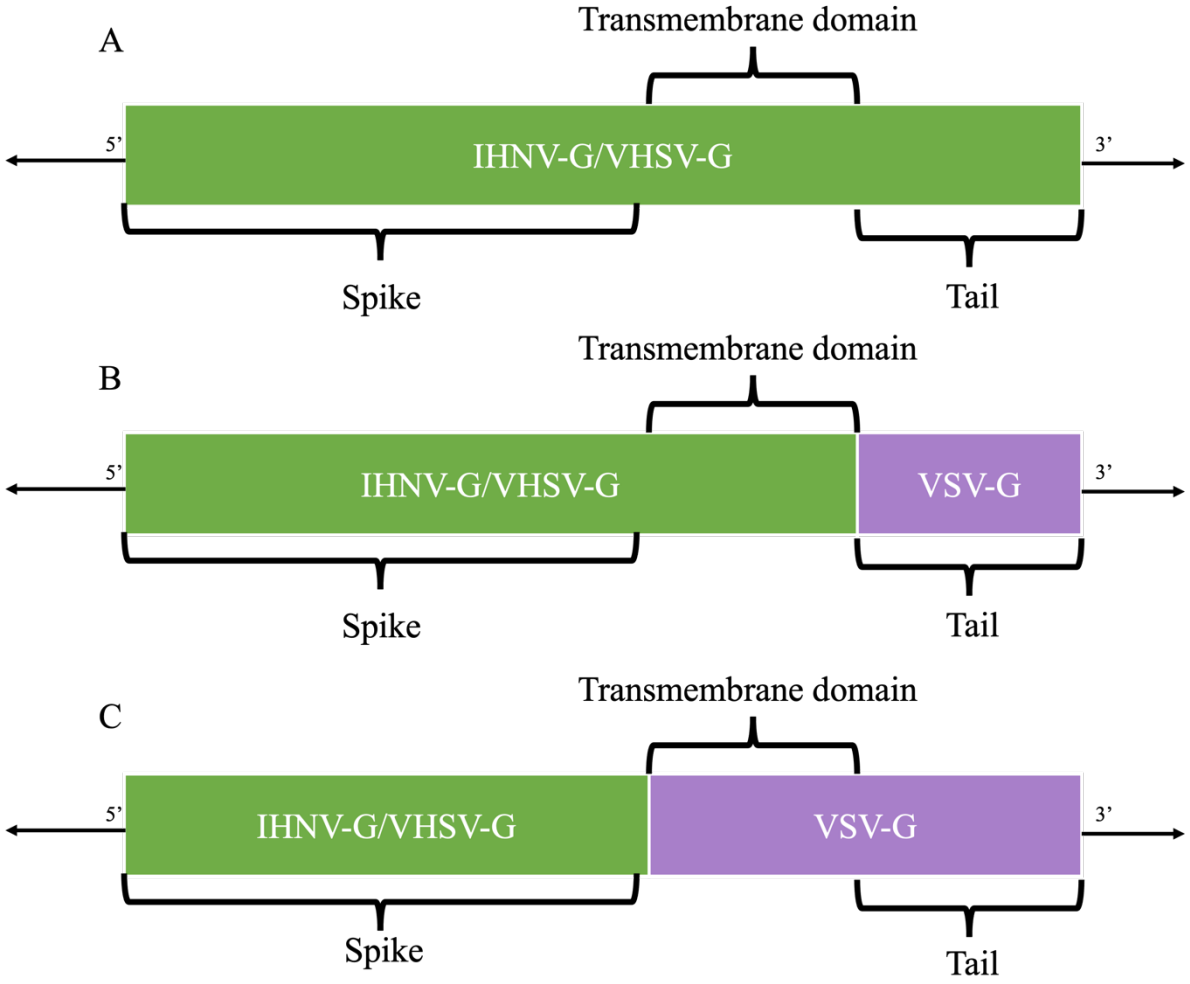


Figure 5: Design of glycoprotein sequences. A) native glycoprotein sequence, with spike, transmembrane domain, and cytoplasmic tail. B) fusion protein sequence with spike and transmembrane domain of IHNV-G or VHSV-G, and tail of VSV-G. C) fusion protein sequence with spike of IHNV-G or VHSV-G, and transmembrane domain and tail of VSV-G.

Table 1: Primers for Gibson cloning IHNV+VSV fusion glycoprotein. Binding regions are underlined.

Primer	Sequence	Tm ˚C
IHNV-G fwd	aagcacgtgagatctgaattctgacactatggacaccatgatcacca	68.59
IHNV-G rev	ataaagaaaaaagaggcaatggtgggccagaagctccaat	66.64
IHNV-G rev (including transmembrane domain)	ttggtgtgctttaatttaatggaccaacagcagcaagcgg	67.72
VSV-G fwd	ccgcttgctgctgttggtccattaaattaaagcacaccaagaaaa	68.09
VSV-G fwd (Including transmembrane domain)	attggagcttctggcccaccattgcctctttttctttatcatag	66.95
VSV-G rev	ctgcactggtggggtgaattccgttttttttttttttttcataaaaat	64.63

Table 2: Primers for Gibson cloning VHSV+VSV fusion glycoprotein. Binding regions are underlined.

Primer	Sequence	Tm ˚C
VHSV-G fwd	aagcacgtgagatctgaattctgacactatggaatggaaca	65.56
VHSV-G rev	ataaagaaaaaagaggcaatgatcagattggtgtggatgt	62.92
VHVS-G rev (Including transmembrane domain)	ttggtgtgctttaatttaattctacaacagcagcacagca	64.81
VSV-G fwd	tgctgtgctgctgttgtagaattaaattaaagcacaccaagaaaa	65.50
VSV-G fwd (Including transmembrane domain)	acatccacaccaatctgatcattgcctctttttctttatcatag	63.66
VSV-G rev	ctgcactggtggggtgaattccgttttttttttttttttcataaaaat	64.63

2.2. Cloning

2.2.1. Restriction cloning of synthetic IHNV-G and VHSV-G

One Shot Stbl3 Chemically Competent E. coli (Thermo Fisher) was transformed with the synthetic gene sequences. The constructs pUC57_IHNV-G and pUC57_VHSV-G arrived, and the 4 µg plasmid where reconstituted in 40 µL of elution buffer to make a 100 µg/mL concentration. The plasmid DNA was further diluted to 1ng/µL. One Shot Stbl3 (Invitrogen) chemically competent Escherichia coli was transformed with the two constructs using 1 ng of plasmid DNA according to the manufacturers protocol. The transformed cells were spread on agar plates prepared with 100 µg/mL ampicillin in volumes of 1 µL and 10 µL. The plates were incubated overnight at 37 ℃. Single colonies were picked the following day and inoculated in 5 ml LB broth. The tubes were incubated at 37 ˚C and shaken at 250 rpm overnight. The next day plasmid DNA was purified using the ZymoPURE Plasmid Miniprep Kit (ZYMO Research) following the manufacturer's instructions. The concentration of the samples was checked using Nanodrop, and samples were frozen at -20℃ for further use.

Restriction digests using EcoRI-HF (NEB) restriction enzyme was performed on the three plasmids pUC57_IHNV-G, pUC57_VHSV-G and pMD2.G in order to cut out the glycoprotein sequences from the backbones. For the pUC57 plasmids, the digested gene sequences were used further. The backbone in pMD2.G was kept and used further. EcoRI-HF produces sticky ends, which was used later when performing ligations. For 50 µL reactions, 50 units of EcoRI-HF and 5 µL 10X rCutSmart (NEB) buffer was used. The recommended amount of DNA for the reactions was 5 µg. Unfortunately, the concentration of pUC57_VHSV-G was too low. For this reaction 2 µg plasmid DNA was used, while 5 µg DNA was used for the two other

reactions. The digestions were performed at 37˚C for one hour followed by heat inactivation at 65 ˚C for 20 minutes.

A gel electrophoresis was performed on the restriction digested plasmids to separate the fragments by size and confirm the reactions were successful. A negative control in the form of undigested plasmid was added for each restriction digested plasmid. The ladder used was GeneRuler 1kb DNA Ladder (Thermo Fisher). The 1% agarose was run at 90 V for 30 minutes. The nucleic acid stain RedSafe (iNtRON Biotechnology) was added to the agarose gel to visualize DNA under UV light. Three other 1% agarose gels were prepared to separate gene sequences from backbone. These gels ran for 45 minutes at 70 V. The gene sequences were cut out from the gels of pUC57_IHNV-G and pUC57_VHSV-G, while the backbone was cut out from pMD2.G gel. The DNA fragments IHNV-G, VHSV-G and pMD2.G backbone were extracted from the agarose gel using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturers protocol, and Nanodrop was used to check concentration.

The backbone of pMD2.G was dephosphorylated to make sure the vector does not circularize during the ligation reaction. The enzyme used was 10X Antarctic phosphatase (NEB). The 20 µL reaction used 366 ng plasmid DNA, 5 units or 1 µL enzyme, and 2 µL buffer. The 20 µL reaction was incubated at 37 ˚C overnight on a heat block. The enzyme was heat inactivated at 80℃ for 2 minutes.

The gene sequences of VHSV-G and IHNV-G were ligated to pMD2.G backbone using T4 Ligase (NEB). The enzyme used was 10X T4 Ligase Reaction Buffer (NEB). The ligation of IHNV-G was done in a 1:3 molar ratio. For the ligation of VHSV-G the concentration of DNA was not high enough, so the reaction had to be done in a 1:2 molar ratio. A ligation reaction of only pMD2.G empty backbone was also done, so we could check whether the dephosphorylation was successful. For all 20 µL reactions, 50 ng of vector DNA was used along with 2 µL buffer and 1 µL enzyme. The reactions were incubated on the lab bench overnight, then heat inactivated at 65 ˚C for 10 minutes.

Transformation of E. coli with ligated vector containing synthetic gene sequences

Chemically competent E. coli was transformed using the ligated vector containing our gene sequences IHNV-G and VHVS-G. A transformation was also done using the ligated empty backbone. For each transformation of OneShot Stbl3, 5 µL of the ligation mix was used. The

transformation was carried out according to the manufacturer's instructions. For each of the three transformations, three agar plates containing 100 µg/mL ampicillin were streaked with 25 µL, 50 µL and 100 µL of transformation mix. The agar plates were incubated at 37 ˚C overnight, then placed in the fridge wrapped in parafilm.

The transformation using empty pMD2.G backbone resulted in zero colonies, meaning the dephosphorylation reaction was successful. The number of colonies in the other two transformations which have an empty backbone should be minimal. Colonies were picked after two days and inoculated in 5 mL selective LB broth. From VHSV-G transformation, 6 colonies were picked. From IHNV-G transformation, both colonies were inoculated. The tubes were incubated at 37 ˚C and shaken at 250 rpm overnight. The remaining 100 µL of bacteria transformed with pMD2_IHNV-G was divided between two selective agar plates and incubated at 37 ˚C overnight. These plates were also placed in fridge wrapped in parafilm to have as backup in case the first two colonies had gene insert in the wrong orientation.

A plasmid prep was performed to harvest the ligated plasmids from transformed E. coli. The QIAprep Spin Miniprep Kit (Qiagen) was used, and the protocol for the kit was followed. Nanodrop was performed to determine concentration of plasmid DNA.

In order to determine the orientation of gene inserts in pMD2.G backbone, a restriction digest was performed using the restriction enzymes HindIII-HF (NEB), StuI (NEB) and SalI-HF (NEB). If the VHSV glycoprotein gene is inserted in the correct orientation, HindIII-HF and StuI should produce fragments of 2184 bp and 3626 bp by digesting pMD2.G_VHSH-G (Figure 6). If inverted, the fragments will be 888 bp and 4922 bp. If the IHNV glycoprotein gene is inserted the correct way, HindIII-HF and SalI will produce fragments of 1676 bp and 4137 bp when digesting pMD2.G_IHNV-G. If inverted, the fragments will be 1143 bp and 4670 bp.

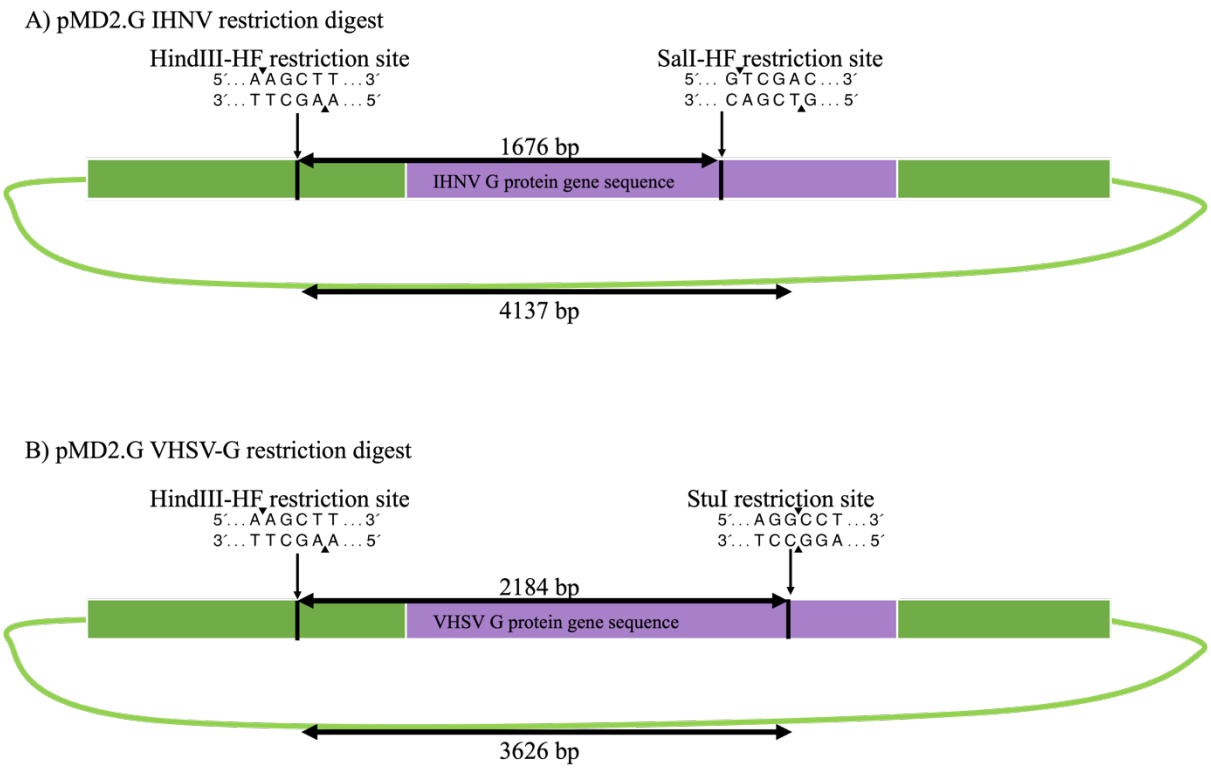


Figure 6: Analytical restriction digestions of pMD2.G_IHNV-G and pMD2.G_VHSV-G. A) pMD2.G_IHNV-G and B) pMD2.G_VHSV-G. Figures describe restriction sites for both plasmids and expected size of fragments produced by restriction digest.

A 1% agarose gel was run to determine the length of DNA fragments produced in the restriction digest on pMD2.G_VHSV-G and pMD2.G_IHNV-G. RedSafe was used to visualize DNA, and the ladder used was 1 kb GeneRuler (Thermo fisher). The gel ran for 1 hour on 80 V.

Sequencing primers were designed using Benchling. The primers were designed to cover the inserted gene sequences, plus 100-50 bp on each side (leading/flanking). For both genes, three primers were made for each strand (Table 3). Each 800 bp segment overlapping by at least 150 bp. Primer number 1 forward and reverse are located outside the gene insert, and therefore the same primers are used for sequencing both plasmids.

Mix2seq Sanger Sequencing kit from Eurofins Genomics was used, and twelve tubes were prepared according to the instructions provided by their website. For both DNA sequences, six samples, were needed in order to cover forward and reverse strand completely. Each tube contained 5 µL of 5 µm sequencing primer, and 5 µL of 50 ng/µL plasmid DNA. Results were aligned using Benchling. Plasmids could therefore be used further to produce lentivirus.

Table 3: Sequencing primers for cloned plasmids.

Primer		Sequence	CG%	Tm ˚C	Length
VHSV/IHNV fwd	1	tcttatcttcctcccacagc	50.00	52.70	20
IHNV 2 fwd		agatgccaatggtacaccg	52.63	54.08	19
IHNV 3 fwd		accaacagcgtgacaccatacc	54.55	59.22	22
VHSV/IHNV rev	1	tagtgatacttgtgggccaggg	54.55	57.55	22
IHNV 2 rev		gtcatgcacattccgtgg	55.53	53.31	18
IHNV 3 rev		atccacaccacgttggacc	58.89	56.46	19
VHSV 2 fwd		cggcaaagaccacgaatacc	55.00	55.77	20
VHSV 3 fwd		tggatggccagattatgcggg	57.14	58.82	21
VHSV 2 rev		tctgggatgatcagcttgtcgc	54.55	58.84	22
VHSV 3 rev		cgatagacacggtcttaggg	55.00	53.42	20

2.2.2. Gibson cloning of VSV-G fusion proteins

Gibson cloning was performed to produce the chimeric glycoproteins. The plasmids pMD2.G_VHSV-G and pMD2.G_IHNV-G produced by restriction cloning and pMD2.G were used in the Gibson cloning to produce the fours plasmids IHNVG(tm)-VSVG, IHNVG-VSVG(tm), VHSVG(tm)-VSVG, and VHSVG-VSVG(tm).

Using the Gibson primers (Table 1), a PCR reaction was performed using Phusion High Fidelity DNA Polymerase (Thermo Fisher). Using the 3-step protocol provided with the polymerase, eight reactions were set up for a temperature gradient for each of the fragments needed for cloning. For each plasmid to be produced, two fragments were needed. The first one containing IHNV-G or VHSV-G, with or without transmembrane domain. The second fragment containing VSV-G with or without transmembrane domain. The VSV-G fragments require overlapping segments with the IHNV-G/VHSV-G fragments. Negative control reaction was also set up without template DNA for each combination of primers. PCR reactions contained the following: 0.5 mM forward and reverse primers, 1 ng template DNA, 0.5 µL Phusion polymerase, 10 µL reaction buffer, and water to a final volume of 50 µl. The PCR reactions were run on the following cycle:

Table 4: PCR conditions for Gibson cloning

Step	Temp °C	Time	Cycles
Initial denaturation	98	30 seconds	1
Denaturation	98	8 seconds	25
Annealing	54-72	20 seconds	
Extension	72	45 seconds	
Final extension	72	5 minutes	1
Hold	4	∞	-

All samples were checked by agarose gel electrophoresis. The lengths of the fragments were checked, along with the occurrence of multiple bands. The sample with the most pronounced single band was chosen for gel extraction. The 8 chosen samples were separated on another agarose gel and excised. The samples were purified using QIAquick Gel Extraction Kit, following the protocol provided with the kit. The concentrations of all samples were checked by Nanodrop.

The fragments containing either IHNV-G or VHSV-G sequences with or without transmembrane domains were combined with the correct VSV-G fragments and pMD2.G backbone in Gibson cloning reactions. The backbone had been digested using EcoRI. The assembly was done according to the NEB Gibson Assembly Protocol for 2-3 fragment assembly. In the 20 µL reactions, 50 ng of backbone was used, and a 3:1 ratio of inserts. The samples were incubated at 50°C for 60 minutes. Following, the samples were placed on ice for use in transformation of E. coli.

One Shot Stbl3 Chemically Competent E. coli were transformed with the plasmids produced in the Gibson cloning reactions. The transformations were done according to the manufacturer's instructions. For each reaction, the whole volume of transformation mix was transferred to a single selective agar plate. The plate was stored at room temp for at least 48 hours before colonies were observed. Individual colonies were picked and inoculated in 5 mL selective LB broth at 22°C, 225 rmp, for 48 hours in preparation for plasmid prep. Glycerol stock was made for later use by adding 0.5 mL bacterial culture to 0.5 mL 50% glycerol solution in a cryovial and freezing at -80°C.

Each plasmid was prepped by using QIAprep Spin Miniprep Kit, following the manufacturer's instructions. Concentration of plasmid DNA was checked by Nanodrop, and samples were frozen at -20°C for further use.

Using the primers described in Table 3, samples were sent for sequencing using Mix2Seq Kit. The samples were prepared according to the instructions provided with the kit. Each tube contained 5 µL of 5 µm sequencing primer, and 5 µL of 50 ng/µL plasmid DNA. For each sequenced plasmid, six sequencing samples were prepared to cover the sequences completely. Three samples for sequencing forward strand, and three for the reverse strand. The results were aligned with the sequences in Benchling.

2.3. Tissue culture

2.3.1. ASK cells (Atlantic Salmon Kidney cells)

The Atlantic Salmon Kidney cells were (ATCC CRL-2747) grown in 8 ml ASK media containing Leibovitz's L-15 (Gibco), 20% fetal bovine serum (FBS) and 1 % penicillin streptomycin in ventilated T75 cell culture flasks. The cultures were incubated at 22 ˚C at atmospheric conditions and split at 90% confluency in a 1:2 or 1:3 ratio.

Prior to splitting, the cells were washed twice with 5 ml phosphate buffered saline (PBS) (Sigma) to remove all media. For cell dissociation 0,25 % Trypsin-EDTA (Gibco) was used. The solution was diluted to 0,05 % trypsin with PBS. To each flask, 5 ml was added, and let sit for 5 minutes. Following cell dissociation, 3 ml of ASK media was added to inactivate the trypsin. The cells were centrifuged at 150 xg for 5 minutes to remove trypsin and resuspended in 5 ml media. Cells were seeded in new flasks and incubated at 20 ˚C.

Excessive cells were frozen at -150 ˚C for later use. The protocol includes trypsinization and centrifugation at 150 xg. The freezing media was made by adding 10% dimethyl sulfoxide (DMSO) to the ASK cell media. In each tube, we froze 2 x 10^6 cells. The cryovials were placed in "Mr. Frosty" (Nalgene) in the -80 ˚C freezer overnight. The vials were then taken out of Mr. Frosty and moved to the -150 ˚C freezer the following day.

2.3.2. Salmon fibroblasts

Salmon fibroblasts were cultured according to protocols developed by the team at CIGENE. The salmon fibroblasts were grown in T75 ventilated culture flasks containing 8 ml media. The fibroblast media was prepared the by using 50% ASK cell media and 50% conditioned media. Conditioned media was prepared by collecting spent media from the culture flasks and through

a 0,2 µm syringe filter to sterilize. The cell cultures were incubated at 20 ˚C at atmospheric conditions.

The media was changed twice a week, and the cells where split in a 1:2 ratio at 80% confluency. Prior to dissociating the cells from the flasks, they were washed twice with 5 ml PBS. The cell cultures were split using 2 ml 0,05% trypsin. After 5 minutes of trypsinization, 3 ml fibroblast media was added to inactivate the trypsin. The cells were centrifuged at 150 xg for 5 minutes to remove trypsin and resuspended in 5 ml fibroblast media. The volume was distributed between the flasks, and filled with conditioned media to a total volume of 8 ml.

2.3.3. Human Embryonic Kidney cells, HEK-293T

Human embryonic kidney cells (HEK-293T) (ATCC CRL-3216) were cultured according to guidelines from European Collection of Authenticated Cell Cultures (ECACC) and protocols developed by the team at CIGENE. The HEK cells were grown in T75 ventilated cell culture flasks containing 8 ml media. The HEK cell media was made with Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) with 10 % FBS and 1 % penicillin streptomycin. Media was changes once per week, and cells were split 1:5 at 80 % confluency. The cells were kept at 37 ˚C at 5% CO_2.

When splitting the cells, 2 ml of 0,05 % Trypsin-EDTA was used. The cells were washed twice with 5 ml PBS, then dissociated by trypsinization for a maximum of 3 minutes. Following trypsinization, the cells were centrifuged at 150 xg to remove trypsin and resuspended in 5 ml HEK cell media. The cells where divided between flasks, and filled with media to a total volume of 8 ml.

2.4. Electroporation of ASK and HEK with pMD2.G

Transfection of Atlantic Salmon Kidney (ASK) and Human Embryonic Kidney (HEK-293T) cells with pLenti CMV GFP Puro using Neon Transfection System (Invitrogen). For each 10 µL transfection of both ASK and HEK-293T cells, 2 µg plasmid DNA and 2 $x10^5$ cells were used. The experiment was performed according to the Neon Transfection System user guide. The electroporation conditions were 1400 V for 20 ms, in two pulses. For both cell types, two electroporation transfections were performed. One negative control where plasmid DNA was added to the suspended cells was also included. ASK cells were plated in a 6 well plate with 2

ml ASK media. The plate was incubated at 20 ˚C at atmospheric conditions. HEK-293T cells were plated in a 6-well plate with 2 ml HEK media. The plate was incubated at 37 ˚C at 5% CO_2. Cells were observed the following days for GFP production.

2.5. Lentivirus packaging

2.5.1. Preparation of lentiviral packaging plasmids

E. coli from frozen glycerol stock was streaked on selective agar plates. The E. coli had been transformed with lentiviral plasmids psPAX2 (Addgene #12260), pLenti CMV GFP Puro (Addgene #17448), and pMD2.G (Addgene #12259). Single colonies were picked from the plates and inoculated in 5 mL selective LB broth. The tubes were incubated at 37 ˚C and shaken at 250 rpm for 7 hours. Following, 500 mL culture flasks were prepared with 100 mL selective LB broth. From the tubes, 100 µL bacterial culture was taken and transferred to culture flasks. These flasks were incubated at 37 ˚C and shaken at 250 rpm overnight.

To harvest the lentiviral plasmids produced in the previous step, a plasmid prep was performed using Plasmid Midiprep Kit (ZYMO Research). For each of the three bacterial cultures, 2 x 50 mL culture was centrifuged at 3220 xg for 10 minutes to remove media. The Midiprep was performed according to the manufacturers vacuum protocol. In order to get a higher concentration of plasmid, step 13 concerning elution in the protocol was altered slightly. The first sample containing each plasmid was eluted, then the eluate from this tube was used to elute the second sample of the same plasmid. Nanodrop was used to measure the concentration of plasmid DNA.

2.5.2. Lentivirus packaging at 37°C

Pseudotyped lentivirus was packaged using HEK cells according to protocol developed by scientists at CIGENE, Lentiviral packaging, Version 2.1.

The protocol includes seeding HEK cells in a six well plate with a density pf $7x10^5$ cells per well 24 hours before transfection. The cells are incubated in 2 mL HEK cell media at 37°C at 5% CO_2. For each transfection, a plasmid mix and a lipofectamine mix is needed. The plasmid mix required for each reaction is composed of 250 µL OptiMEM (Gibco), 1000 ng pLenti CMV GFP puro, 750 ng psPAX2 and 500 ng envelope plasmid. Either pMD2.G was used, or the cloned plasmids described earlier.

The lipofectamine mix is composed of 10 µL Lipofectamine 2000 (Thermo Fisher) and 250 µL OptiMEM. The lipofectamine is mixed together gently and incubated at room temperature for 5 minutes before mixing with the plasmid mix. The mix of plasmids and lipofectamine is incubated for 20 minutes at room temperature. Meanwhile, the media is removed from the HEK cells and replaced with fresh pre-warmed media. Following, 500 µL of the plasmid and lipofectamine mix is added directly to the cells dropwise. The cells are incubated for 4 hours under the same conditions as before.

After 4 hours, the media containing lipofectamine is removed and replaced with fresh pre-warmed media to avoid toxicity. Cells are then incubated for two days at the same conditions as before. After 24 hours incubation, GFP production is checked to confirm cells are transfected with the plasmids. Virus is harvested by aspirating supernatant form cells and filter through at 0.45 µM syringe filter. Filtered supernatant was transferred to cryovials and frozen at -80°C or used immediately.

Packaging of lentivirus with complete IHNV and VHSV glycoprotein sequences was done using envelope plasmids pMD2.G_VHSV-G and pMD2.G_IHNV-G. VSV-G pseudotyped lentivirus was also packaged as positive control, using envelope plasmid pMD2.G. The above method was followed, and two samples were made for each of the virus variants.

2.5.3. Low temperature lentivirus packaging

Lentivirus was packaged at lower temperature to prevent denaturation of glycoproteins at high temperature. As the viruses IHNV and VHSV are marine fish viruses which infect hosts at temperatures below 20°C, the virus glycoprotein might be activated at 37°C. For the second round of packaging the above protocol was followed with some modifications. HEK cells were moved to 22°C incubator 24 hours after transfection. Media was removed, and cells were washed twice with PBS before moving. Lentivirus was harvested after 4 days in a 22°C incubator. The envelope plasmids used were pMD2.G_VHSV-G and pMD2.G_IHNV-G. VSV-G pseudotyped lentivirus was packaged as positive control.

2.6. Staining of cells and virus

GFP lentivirus was stained with SYTO 82 fluorescent nucleic acid stain (Invitrogen). All centrifugations were performed at 1500 xg. The virus was thawed in a 37 ˚C water bath and transferred to a 30 kD cutoff column. The buffer was removed by centrifugation for 15 minutes, and the flowthrough was removed. The virus was washed twice by adding 4 mL Hank's Balanced Salt Solution (HBSS) (Gibco) followed by centrifugation for 15 minutes.
After resuspending the virus in 1 mL HBSS, 2 µL SYTO 82 was added. The column was mixed well by inversion and incubated for 15 minutes protected by light. The column was centrifuged for 30 minutes to remove excess stain, then resuspended in 1 mL HBSS and transferred to a microcentrifuge tube.

ASK and HEK cells were stained with CellMask plasma membrane stain (Invitrogen). Both cell types were seeded the day before the experiment in 30 mm dishes, with 7 x 10^5 cells per dish. ASK cells were incubated with 2 mL ASK media at 20 ˚C in atmospheric conditions. HEK cells were incubated with 2 mL HEK media at 37 ˚C with 5 % CO_2. The staining protocol was the same for both ASK and HEK cells. The staining solution was prepared by diluting CellMask stock 2000x. This was done by mixing 2,5 mL PBS with 1,25 µL CellMask. The media was removed from the cells, and 500 µL staining solution was added. The dishes were incubated for 10 minutes protected by light, then removed. The cells were washed 3 timed with media, and 2 mL media was added lastly. The dishes were kept protected from light in order to preserve the stain.

Both ASK and HEK cells were transduced using 50 µL stained virus (concentration of virus unknown). The confocal microscope was used to observe the cells after 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours and the following days.

2.7. Lentiviral transduction experiments

2.7.1. Transduction of ASK and HEK

Transduction of HEK cells at 20 ˚C and 37 ˚C, as well as ASK cells at 20 ˚C was performed to assess whether lentivirus can infect cells HEK cells at a lower temperature. We also wanted to see if VSV-G pseudotyped lentivirus could infect ASK cells under normal conditions. Polybrene Infection Reagent (Sigma) was added to cell media increase the chances of seeing a successful transduction. The day before the experiment, two 6-well plates were seeded with 5

x 10^5 HEK cells per well. Cells were incubated with 2 mL HEK cell media overnight at 37℃ with 5 % CO_2. ASK cells were also seeded in a 6-well plate, with 1 x 10^5 cells per well and 2 mL ASK cell media. ASK cells were incubated at 20℃ under atmospheric conditions.

Media was removed from all cells and replaced with fresh. ASK cells and HEK cells which were going to be kept at 20˚C were incubated with 2 mL ASK cell media, and 8 µg/mL polybrene. HEK cells kept at 37 ˚C were incubated with 2 mL HEK media. To each well in the 6-well plates, 25, 50, 100, 200 or 400 µL virus was added. One well was kept as negative control. Cells were incubated and observed the following days for GFP expression.

2.7.2. Transduction of HEK, gradual increase in temperature

Lentiviral vectors transduce cells at 37 ℃, the temperature at which most mammalian host cells thrive at. To investigate whether lentivirus is able to infect cells at lower temperatures, HEK cells were transduced at 20℃. The temperature was increased in two degree increments every 24 hours, in order to find the lowest temperature cells could be transduced at. The incubation temperature was increased until it reached 37℃. Following, the cells were incubated at 22 degrees for three days to investigate any change in GFP expression.

HEK cells were seeded in 6-well plate at 5 x 10^5 cells per well. Cells were incubated with 2 mL HEK cell media overnight at 37℃ with 5 % CO_2. Media was removed and exchanged for fresh. To each well in the 6-well plates, 25, 50, 100, 200 or 400 µL virus was added. One well was kept as negative control without virus. Cells were incubated with 5% CO_2 and imaged every 24 hours with the temperature change.

2.7.3. pH experiment, ASK

The goal of this part of the experiment was to determine the lowest pH value ASK cell could be exposed to without inducing cell death. Atlantic Salmon Kidney cells were seeded the day before in a 6-well plate using 3 x 10^5 cells per cell. In each well, 2 ml of ASK media was added. Acidic ASK cell media at pH 4, 4.5, 5, 5.5, and 6 was prepared for the experiment. Using a magnetic stirrer and a pH-meter, 37 % hydrochloric acid (HCL) and 1 M sodium hydroxide (NaOH) was added dropwise until desired pH was achieved. The media was removed from the cells and each well was exposed to 2 ml of media at different pH values, including one control with normal media. The cells were observed immediately after change of media for cell death,

and then every ten minutes following. After one hour, the media was removed from all the wells. The cells were washed with 2 ml of normal ASK cell media and incubated with 2 ml of normal media. The cells were incubated at 20 degrees and observed the following day, and after 5 days. Cell death was minimal at pH 5 and higher. Therefore, pH 5 will be used during transfection in part two of the experiment.

In this second part of the experiment, we will transfect Atlantic Salmon Kidney cells using media holding a pH value of 5. This is done to investigate if the lower pH allows lentiviral entry into ASK cells by triggering fusion of viral and cellular membranes. ASK were seeded the day before in a 6-well plate using 3 x 10^5 cells per cell. In each well, 2 ml ASK cell media was added. The plate was later incubated at 20 ˚C at atmospheric conditions. Lentivirus at 40 x 10^6 virus particles/ml were kept in dry ice and thawed in a 37 ˚C water bath right before transfection. All transductions were done with 75 µL of virus, at 5 MOI. The low pH media used had a pH of 5. This pH value was determined in the first part of the experiment. Media was removed from all wells and changed accordingly. There were four different conditions in the experiment: A) negative control, no virus, B) control, normal pH, virus added and media changed after 1 hour, C) low pH media and virus added, media change to normal pH after 1 hour, D) normal pH, added virus media changed to low pH after 1 hour, then changed to normal pH after another hour. The plate was later incubated at the same conditions as before and observed the following days for GFP production.

Two methods were tested out, changing pH of media before adding virus, and changing media after adding virus. This was done to check whether adding virus to a low pH environment would lead to the glycoprotein having changes in the conformation before attaching to receptors, therefore hindering glycoprotein-receptor interactions.

2.7.4. pH experiment HEK

The effect of lowered pH value during lentiviral transduction was investigated in HEK cells. Two methods were tried out, lowering pH before virus is added, and lowering pH after virus is added. We wanted to check whether it was more effective to lower pH after cells were already attached to receptors, or if the pH had to be lowered before virus could reach cellular receptors and endosomes are formed.

HEK cells were seeded the day before in a 6-well plate, 5 x 10^5 cells per well with 2 mL HEK cell media. Cells were incubated at 37℃ at 5% CO_2. Lentivirus at 40 x 10^6 virus particles/ml were kept in dry ice and thawed in a 37 ˚C water bath right before transfection. Samples were transduced with 75 µL virus. For the first sample, normal pH media was used when virus was added. Cells were incubated for 30 minutes. Then media was replaced with ASK media holding pH 5. Cells were incubated for 30 minutes again, then replaced with normal media. For the other sample, 2 mL ASK cell media at pH 5 was added before virus. Cells were incubated for 30 minutes, before media was removed and replaced with no0rmal media.
Cells were incubated at the same conditions as before and observed the following 5 days for GFP expression.

2.7.5. Temperature and pH experiment

Lower pH combined with higher temperatures were combined in an attempt to transduce ASK cells and fibroblasts. The experiment was done to investigate whether the low temperature is hindering VSV-G pseudotyped lentivirus form infecting salmon cells. Fibroblasts and ASK cells were seeded the day before in a 6-well plate using 3 x 10^5 cells per cell. Cells were incubated in 2 mL media overnight at 20 ˚C in atmospheric conditions.

Media was removed before the transductions, and 2 mL of ASK cell media holding pH of 5 was added to the wells. Lentivirus at 40 x 10^6 virus particles/ml were kept in dry ice and thawed in a 37 ˚C water bath right before transfection. Fibroblasts and ASK cells were transduced with 75 µL of virus, at 5 MOI. One sample of each cell type was kept as negative control, and no virus was added here. Media was exchanged for normal ASK media or fibroblast media after 30 minutes, and cells were incubated at 24℃ for 24 hours at atmospheric conditions. Cells were observed for GFP expression the following days, and temperature was increased by 2℃ every 24 hours.

2.7.6. Transduction with pseudotyped virus

2.7.6.1. Transduction with pseudotyped virus packaged at 37℃

ASK cells and fibroblasts were seeded in a 6-well plate with 3 x 10^5 cells/well with 2 mL of ASK cell media and fibroblast media, respectively. Cells were incubated at 20℃ under atmospheric conditions overnight. HEK cells were seeded at the same density with 2 mL HEK cell media. HEK cells were incubated at 37℃ with 5% CO_2 overnight. Cell media was

exchanged for fresh media before 200 µL virus was added. The three different viruses pseudotyped with VSV-G, IHNV-G and VHSV-G were added to the cells dropwise. Cells were incubated at the same conditions as before, and GFP production was monitored the following days.

2.7.6.2. Transduction with pseudotyped virus packaged at 22°C

ASK cells were seeded in a 12-well plate with 1,5 x 10^5 cells/well with 1 mL of ASK cell media and. Cells were incubated at 20 °C under atmospheric conditions overnight. HEK cells were seeded at the same density with 1 mL HEK cell media. HEK cells were incubated at 37°C with 5% CO_2 overnight. Cell media was exchanged for fresh media before 100 µL virus was added. The seven different viruses pseudotyped with VSV-G, IHNV-G, VHSV-G and chimeric glycoproteins of IHNV-G/VHSV-G and VSV-G were added to the cells dropwise. Cells were incubated at the same conditions as before, and GFP production was monitored the following days.

2.8. qPCR experiment

The qPCR experiment was set up to detect GFP expression in ASK and HEK cells, as well as plasmid contamination. Housekeeping genes for both salmon and human cells were included in the experiment to account for differences in expression levels. Primers for sequences GFP, ampicillin resistance, and housekeeping genes $EF1A_A$ and GAPDH were chosen and ordered from Thermo Fisher Scientific for qPCR (Table 5).

Table 5: Primer references, names, sequences and melting temperature for qPCR.

Reference	Primer name	Sequence	Tm ˚C
Ikeda et al. (2002)	GFP fwd	CAACAGCCACAACGTCTATATCAT	55.17
	GFP rev	ATGTTGTGGCGGATCTTGAAG	55.21
Olsvik et al. (2005)	$EFIA_A$ fwd	CCCCTCCAGGACGTTTACAAA	56.16
	$EFIA_A$ rev	CACACGGCCCACAGGTACA	58.79
Verschoor et al. (2014)	GAPDH fwd	GCACAGTCAAGGCCGAGAAT	57.10
	GAPDH rev	GCCTTCTCCATGGTGGTGAA	56.31
Sastry et al. (2002)	AMP fwd	ACTCGCCTTGATCGTTGGG	56.65
	AMP rev	GTTGCCATTGCTACAGGCATC	56.63

ASK cells were seeded in two T25 flasks with a density of 6 x 10^5 cells per flask and 5 mL ASK cell media. Cells were incubated at 20°C under atmospheric conditions for two days. HEK cells were seeded at the same density and with 5 mL HEK cell media. HEK cells were incubated

at 37℃ at 5% CO_2 overnight. After exchanging the media for fresh, 500 µL virus of unknown concentration was added to one HEK flask, and one ASK flask. Cells were incubated overnight at same conditions as before. Cells were washed three times with PBS and then trypsinized. Each flask was split into two samples, one for RNA extraction and one for DNA extraction.

DNA and RNA was extracted from HEK and ASK cells. DNA was extracted from cells using DNeasy Blood and Tissue Kit (Qiagen). The extraction was done using the spin protocol in the handbook provided with the kit. RNA was extracted using RNeasy Mini Kit (Qiagen), and DNA was digested using RNase-Free DNase (Qiagen). DNA and RNA concentrations were found using Nanodrop, and frozen at -80 ˚C to preserve unstable RNA.

iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize cDNA from RNA samples. Two cDNA samples were made from each of the treatments. The 20 µL reactions were set up with 50 ng RNA template and cycled according to the manufacturers protocol. Concentration of cDNA was measured by Nanodrop before freezing the samples at -20℃.

In order to optimize the conditions for qPCR, a temperature gradient was done to find melting temperature for the different primers. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used, and the 10 µL reactions were set up with 5 ng plasmid DNA and 1 µm final concentration of primers, with 5 replicates for each sample. The thermal cycler was set up according to manufacturer's instructions (Table 6). The gradient covered 50℃ to 65℃ with an interval of 1.5℃. The optimal temperatures were determined to be 55.9℃ for GFP and GAPDH, and 62.5℃ for $EFIA_a$

For the experiment, two plates were set up. For both samples treated with lentivirus and untreated, four replicates were done of each for DNA, cDNA and RNA. At 55.9℃ GFP and GAPDH samples were included, as well as standard curves. At 62.5℃ $EF1A_a$ and AMP saples were set up, as well as standard curves. Positive controls for AMP and GFP were also included, as well as no template control for all primers. The 10 µL reactions using SsoAdvanced Universal SYBR Green were set up with 5ng DNA and 1µm final concentration of primers, with 4 replicates for each sample. The same thermal cycler protocol was used (Table 6), with the optimal annealing temperatures determined by the gradient.

Table 6: Thermal cycling protocol for qPCR

Step	Temp ℃	Time	Cycles
Initial denaturation and polymerase activation	98	30 seconds	1
Denaturation	98	10 seconds	35 cycles
Annealing, extension, plate read	Gradient	15 seconds	
Melt curve analysis	65-95	-	1

3. Results

3.1. Cloning

3.1.1. Restriction cloning of synthetic IHNV-G and VHSV-G

IHNV-G and VHSV-G was synthesized and cloned into pUC57 by Genscript. Upon receipt, the constructs were immediately transformed into E. coli for propagation and storage. In order to confirm the identity of the synthesized constructs and the VSV-G containing plasmid pMD2.G, an analytical digestion was performed using EcoRI. Bands were obtained at the expected sizes for all plasmids (Figure 7A). The gel confirmed plasmids pMD2.G, pUC57_IHNV-G and pUC57_VHSV-G were cut in the restriction digest reaction. Due to human error, the gel ran for 2 minutes without ladder added. Therefore, DNA fragments appear to be of slightly greater size than they are.

The EcoRI digested glycoprotein sequences of IHNV and VHSV was cloned into EcoRI digested backbone. Transformation of E. coli with ligated vector containing synthetic gene sequences resulted in 17 colonies for pMD2.G_VHSV-G, and 2 colonies for pMD2.G_IHNV-G.

To verify the glycoproteins gene sequences were inserted with the correct orientation, plasmid pUC57_IHNV-G was digested using HindIII and SalI. Plasmid pUC57_VHSV-G was digested using HindIII. The length of the fragments Figure 7B indicates that IHNV glycoprotein gene sequence was inserted correctly in both samples. VHSV glycoprotein sequence was inserted correctly in four of the tested samples. These samples were sequenced, and alignments in Benchling showed that the genes were inserted correctly, with no mutations.

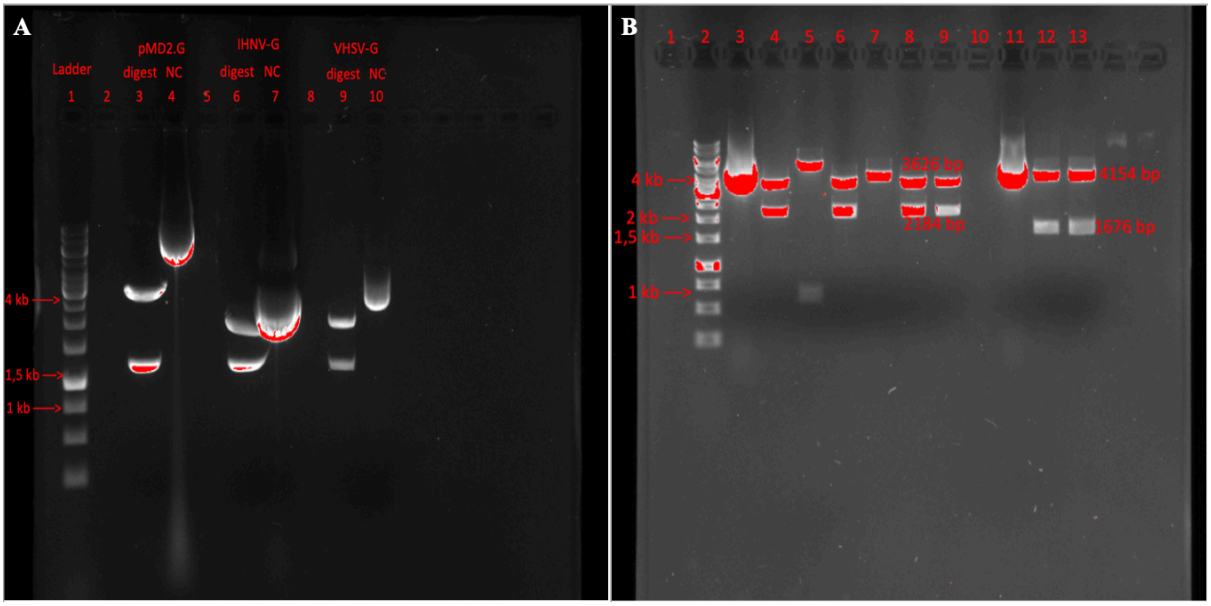


Figure 7: Analytical digestions of plasmid DNA. A) Analytical digestion of glycoprotein plasmids and pMD2.G. The expected band size for digested pMD2.G is 4.1 kB for backbone, and 1.7 kb for gene insert. The expected band sizes for pUC57_IHNV-G and pUC57_VHSV-G is 2.7 kB for backbone and 1.7 kB for gene insert. B) Analytical digestion to screen for orientation of glycoprotein sequences in pMD2.G backbone. 2: Ladder 1 kb GeneRuler, 3: uncut control pMD2.G_VHSV-G, 4-9: restriction digested pMD2.G_VHSV-G, 11: uncut control pMD2.G_IHNV-G, 12-13: restriction digested pMD2.G_IHNV-G. Expected size for digested pMD2.G_IHNV-G is 4.1 kB and 1.7 kB. Expected size for digested pMD2.G_VHSV-G is 3.6 kB and 2.2 kB. The length of products in well 4, 6, 8 and 9 are of the correct length for pMD2.G_VHSV-G. Product in well 5 had an inverted insert. Product in well 7 did not have any insert. Product in well 12 and 13 are correct length of pMD2.G_IHNV-G.

3.1.2. Gibson cloning of VSV-G fusion proteins

The PCR products produced for Gibson cloning were verified by gel electrophoresis (Figure 8). IHNV/VHSV glycoprotein spike fragments with transmembrane domains should be 1.5 kb. Without transmembrane domains, these fragments should be 1.4 kb. For the VSV glycoproteins tail fragments with transmembrane domains the length should be 144 bp. The VSV fragments without transmembrane domain should be 69 bp. The samples with the clearest single band were selected and purified for cloning. The PCR products were cloned into plasmids and the cloning was verified by sequencing. Sequencing results were aligned in Benchling and confirmed segments were cloned into plasmid with no mutations.

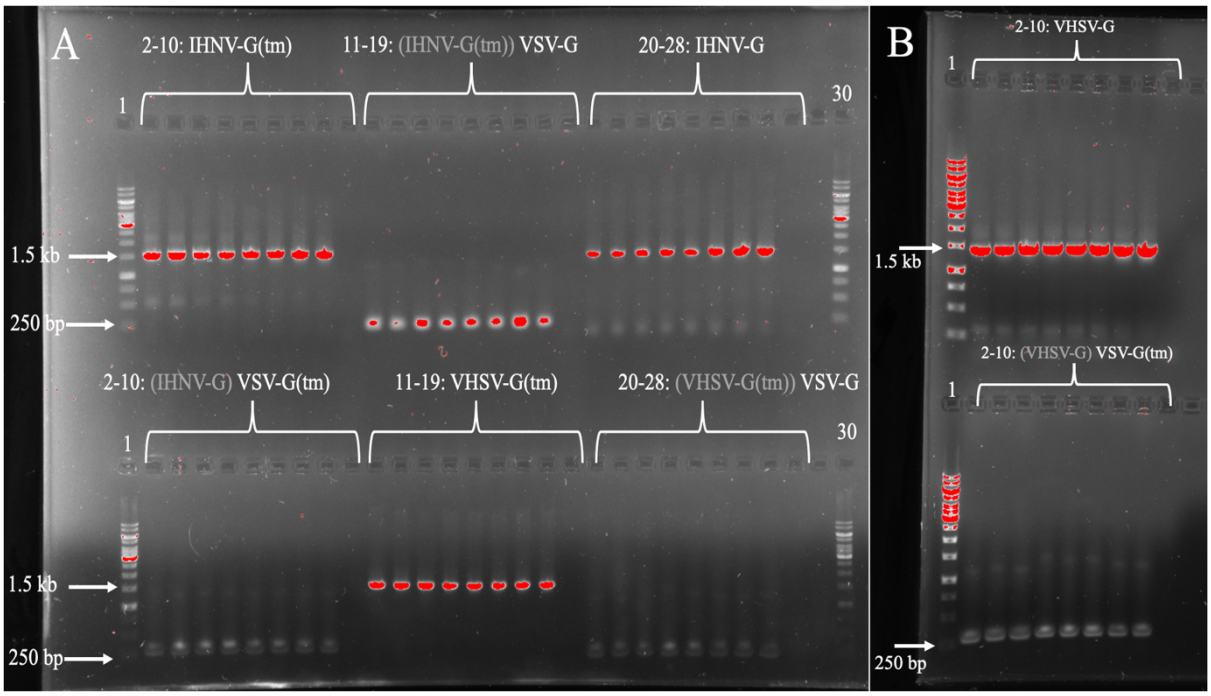


Figure 8: Gel electrophoresis of PCR products for Gibson cloning. A) Top row includes PCR products IHNV-G(tm) (wells 2-10), and the corresponding VSV-G fragment (wells 11-19). It also includes IHNV-G fragment (wells 20-28). Bottom row includes the VSV-G(tm) fragment for IHNV-G (wells 2-10). It also includes VHSV-G(tm) (wells 11-19) and the corresponding VSV-G fragment (wells 20-28). Wells 1 and 30 in both rows has GeneRuler 1 kb ladder. B) Top row has VHSV-G fragment (well 2-10) and bottom row had the corresponding VSV-G fragment (well 2-10). Row 1 is 1 KB ladder. The last well of each fragment is no template control.

3.2. Electroporation of ASK and HEK

Both ASK and HEK cells were electroporated with the lentiviral transfer plasmid pLenti CMV GFP puro to investigate whether the GFP transgene could be expressed by both cell types driven by the CMV promoter. ASK cells showed GFP expression after 24 hours, confirming the GFP gene located on the transfer plasmid pLenti CMV GFP puro can be expressed by the cells (Figure 9). The expression was strongest after 24 hours and had decreased after 48 hours. HEK cell GFP expression was also highest after 24 hours. The negative controls where plasmid DNA had been added to cells but not electroporated did not show any GFP expression.

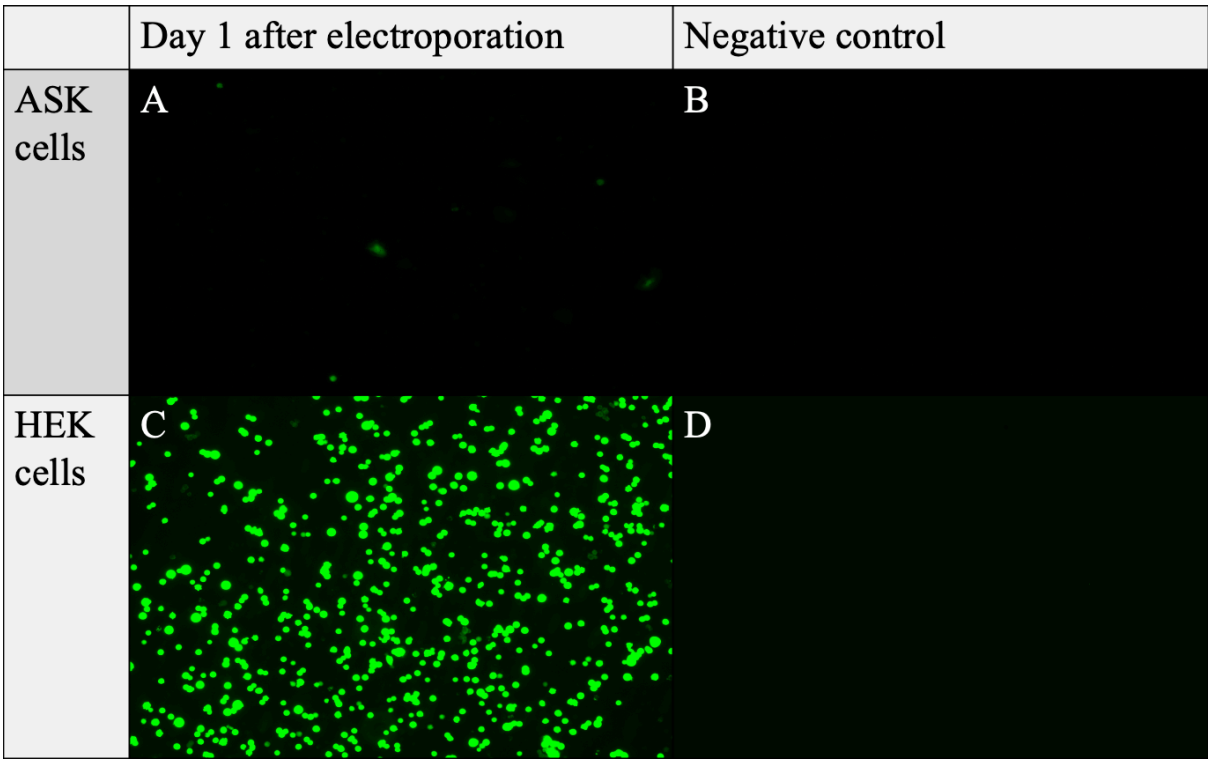


Figure 9: Electroporation results. A) ASK cells 24 hours after transfection with pLenti CMV GFP puro by electroporation. Some GFP expression is visible. B) ASK cells negative control day 1 no visible GFP. C) HEK cells 24 hours after electroporation. GFP expression is visible. D) HEK cells negative control day 1, no GFP is visible.

3.3. Lentivirus packaging

3.3.1. Lentivirus packaging at 37°C

Lentivirus pseudotyped with native glycoprotein sequences were packaged through co-transfection of HEK cells with lentiviral plasmids. Following 24-hour incubation, all samples of HEK cells were expressing GFP (Figure 10), indicating they were successfully transfected with plasmids for lentiviral production.

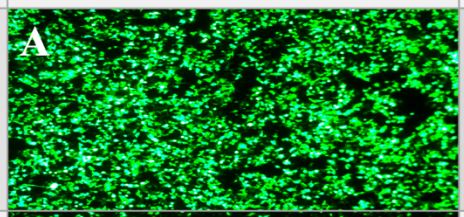
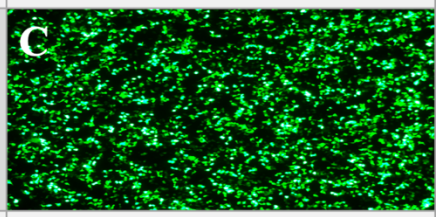
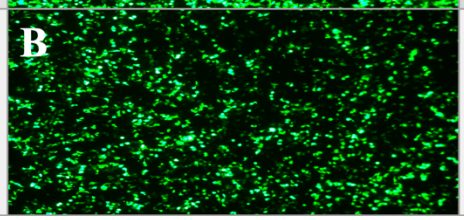
Glycoprotein	GFP	Glycoprotein	GFP
VSV-G (positive control)		VHSV-G	
IHNV-G			

Figure 10: GFP production in HEK cells transfected with lentiviral plasmids for production of virus at 37℃. A, B, and C) cells transfected with plasmids for production of VSV-G, IHNV-G and VHSV-G pseudotyped lentivirus respectively.

3.3.2. Virus packaging at 22℃

Lentivirus pseudotyped with native glycoprotein sequences as well as chimeric glycoprotein sequences were packaged through co-transfection of HEK cells with lentiviral plasmids. HEK cells were incubated at 22℃ to prevent denaturation of glycoprotein structure by high temperatures. All samples of HEK cells were green fluorescent following 24 hours incubation (Figure 11). Indicating they were successfully transfected with plasmids for lentiviral production.

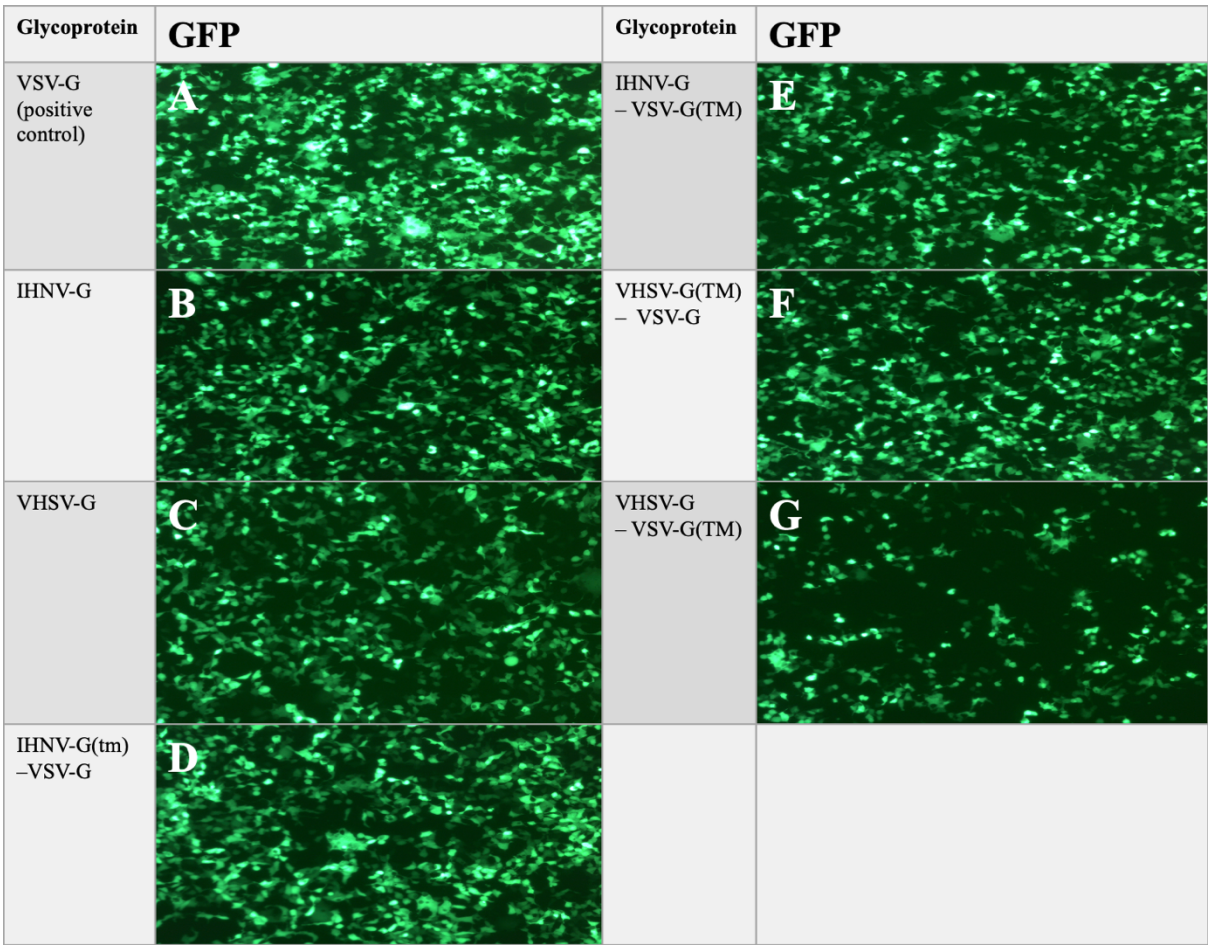


Figure 11: GFP production in HEK cells transfected with lentiviral plasmids for production of virus at 22°C. A, B and C) cells transfected with plasmids for production of VSV-G, IHNV-G and VHSV-G pseudotyped lentivirus respectively. D, E, F and G) cells transfected with lentiviral plasmids for packaging viruses with chimeric glycoproteins. These proteins are in order IHNV-G(tm)–VSV-G, IHNV-G–VSV-G(tm), VHSV-G(tm)–VSV-G and VHSV-G–VSV-G(tm).

3.4. Staining of cells and virus

ASK and HEK cells were stained with plasma membrane stain CellMask, and virus was stained with orange fluorescent nucleic acid stain SYTO 82. As seen in pictures A and B (

Figure ***12***), the stained HEK and ASK cells are red. The stained virus is visible as a yellow fog on and around cells in pictures C and D. Already right after virus is added, the virus appears to gather in round clusters. This could be endosomes. These round clusters are still visible in pictures E and F, taken three days after virus is added. HEK cells were expressing GFP day three.

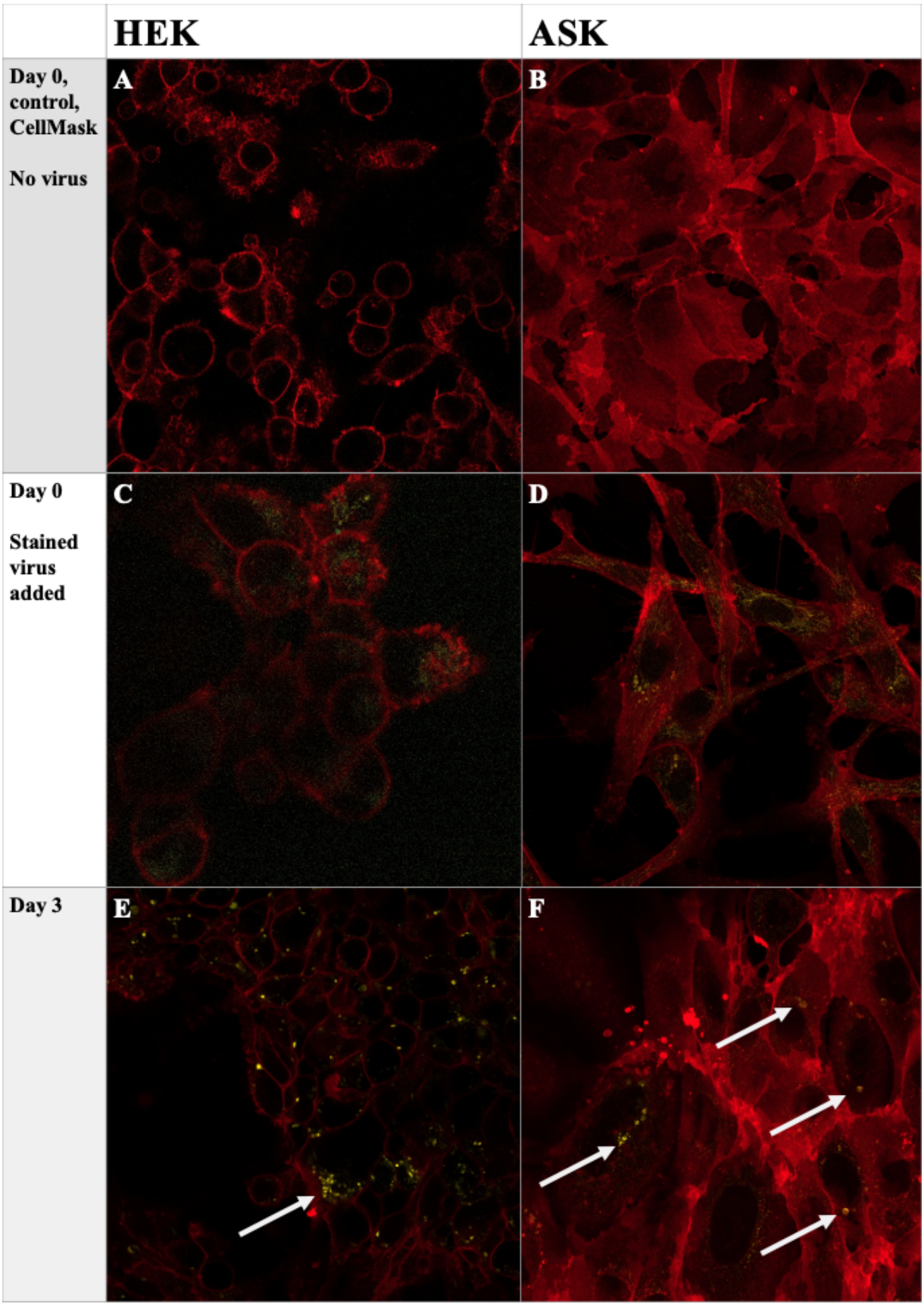

HEK
ASK
Day 0, control, CellMask
No virus
A
B
Day 0
Stained virus added
C
D
Day 3
E
F

Figure 12: HEK and ASK cells stained with CellMask, with SYTO 82 stained lentivirus. A and B) stained HEK and ASK cells respectively. No virus is added to these cells. Cells are red. C and D) HEK and ASK cells respectively right after stained lentivirus is added. E and F) HEK and ASK cells respectively day three after transduction. Arrows indicate clusters of virus in cells.

3.5. Lentiviral transduction experiments

3.5.1. Transduction of ASK and HEK

HEK and ASK cells were initially transduced with VSV-G lentivirus to observe the reaction to the virus. HEK and ASK cells were transduced with increasing amounts of added virus. One sample of HEK cells were incubated at 37°C, while another was incubated with ASK cells at 20°C. For HEK cells transduced at 37°C, fluorescence increased with the increasing volume of virus added (Figure 13). Higher level of cell death was also seen in cells transduced with a higher volume of virus (Figure 14). Transduction of ASK cells and HEK cells at 20°C resulted in no detectible GFP production. HEK cell transduction at 20°C resulted in cell death for all samples.

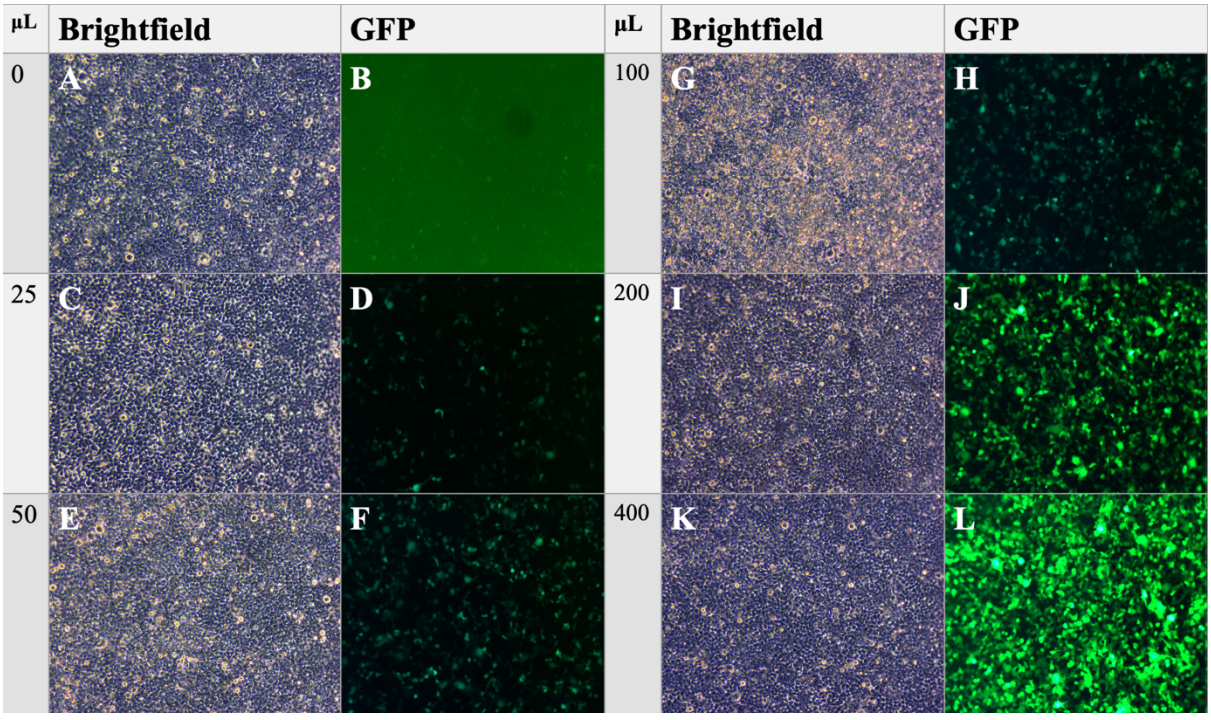


Figure 13: HEK cells day 1 after transduction by VSV-G lentivirus. B, D, F, H, J and L) florescent HEK cells transduced with 0, 25, 50, 100, 200 and 400 µL virus of unknown concentration. A, C, E, G, I and K) the same cells in brightfield image.

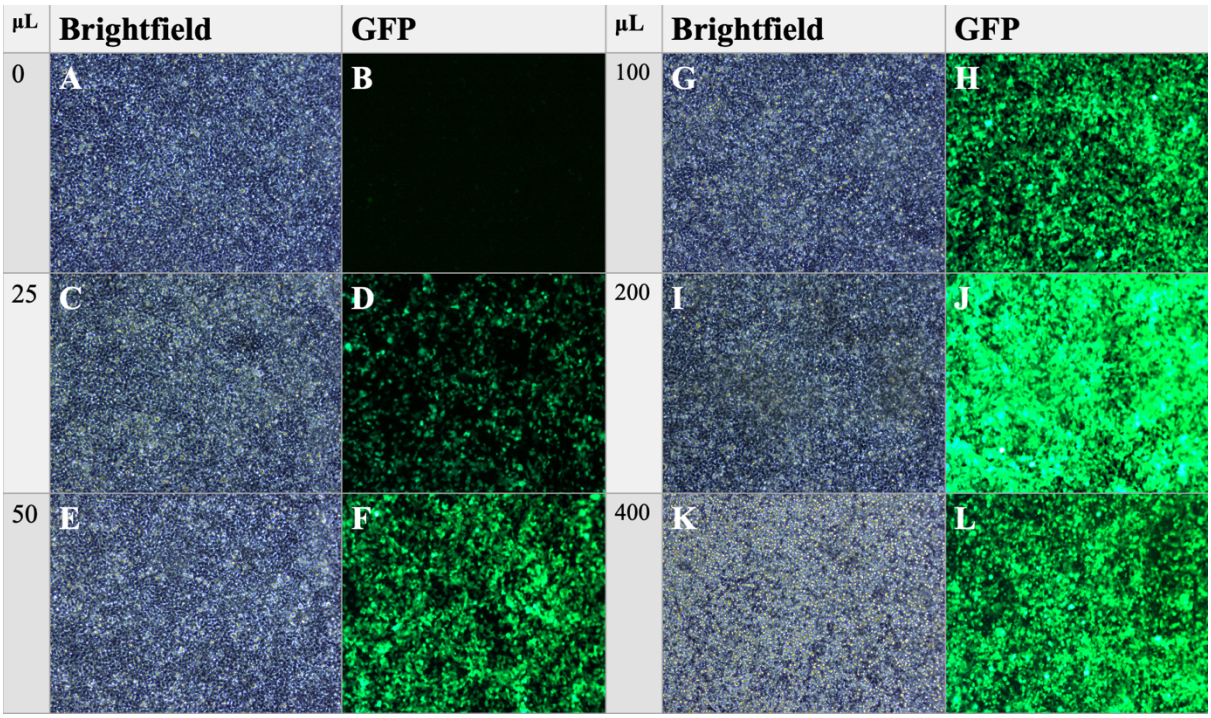


Figure 14: HEK cells day 8 after transduction by VSV-G lentivirus. B, D, F, H, J and L) florescent HEK cells transduced with 0, 25, 50, 100, 200 and 400 µL virus of unknown concentration. A, C, E, G, I and K) the same cells in brightfield image.

3.5.2. Transduction of HEK, gradual increase in temperature

HEK cells were transduced at 20℃, and temperature was increased in 2 degree increments every 24 hours until incubation temperature reached 37℃. GFP expression was monitored to find the lowest temperature HEK cells could be transduced by lentivirus. Following, temperature was reduced to 22℃ HEK cells start expressing GFP at 26 degrees, three days after transduction by VSV-G lentivirus. GFP expression increased until it reached 37 degrees (Figure 15). After turning temperature back down to 22 degrees expression decreased but persisted.

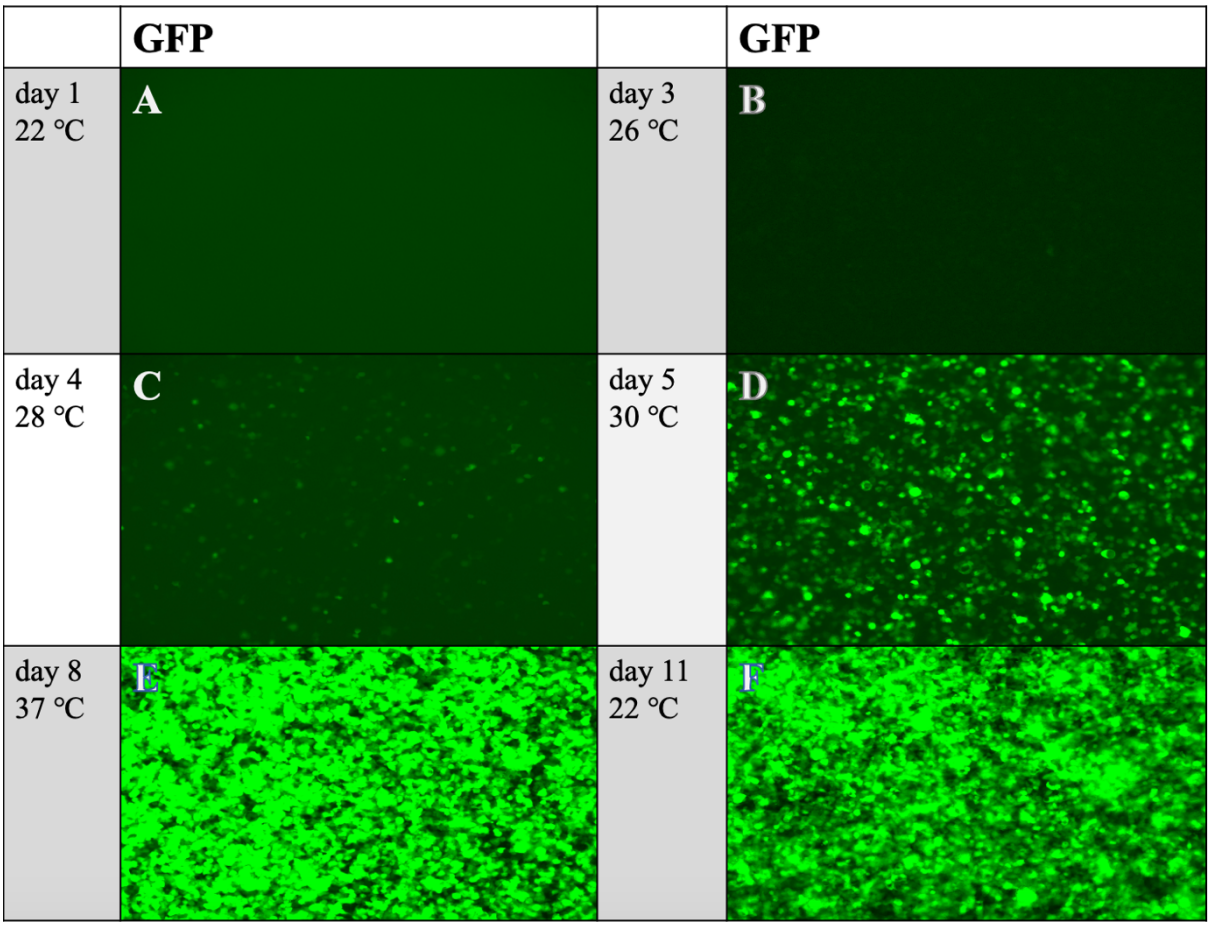


Figure 15: GFP expression in HEK cells transduced with 400 µL VSV-G lentivirus. A) cells day one after transduction, after incubation at 22°C. B) cells day three, after incubation at 26°C. Some weak GFP fluorescence can be seen. C) cells day four after transduction, after incubation at 28°C. D) cells day five after transduction, after incubation at 30°C. E) cells eight days after transduction, after incubation at 37°C. F) cells eleven days after transduction, after incubation at 22 degrees.

3.5.3. pH experiment

3.5.3.1. Determining pH tolerance of ASK cells

In this experiment, ASK cells were transduced at a lower pH environment in an attempt to trigger pH induced conformal changes in glycoprotein to initiate fusion of viral and cellular membranes. The first step in the process was to determine the lowest pH cells could survive in. ASK cells were exposed to pH levels ranging from pH 4 to pH 6. The lowest pH value ASK cells could tolerate was determined to be pH 5. ASK cells treated with media holding pH 4 and pH 4.5 resulted in a large number of cells dying (Figure 16). Cells were viewed in microscope 5 minutes after exchange of media, and cells were already detaching from the plate. Significant cell death could be seen after 60 minutes.

After 24 hours, even more cells treated with pH 4.5 had died. This can be seen as a large number of cells detaching from the plate. Cells treated with media holding pH 5, 5.5 and 6 did not show more cell death compared to cells treated with normal media. Cells exposed to pH 5 were indiscernible to control in number of dead cells after 24 hours.

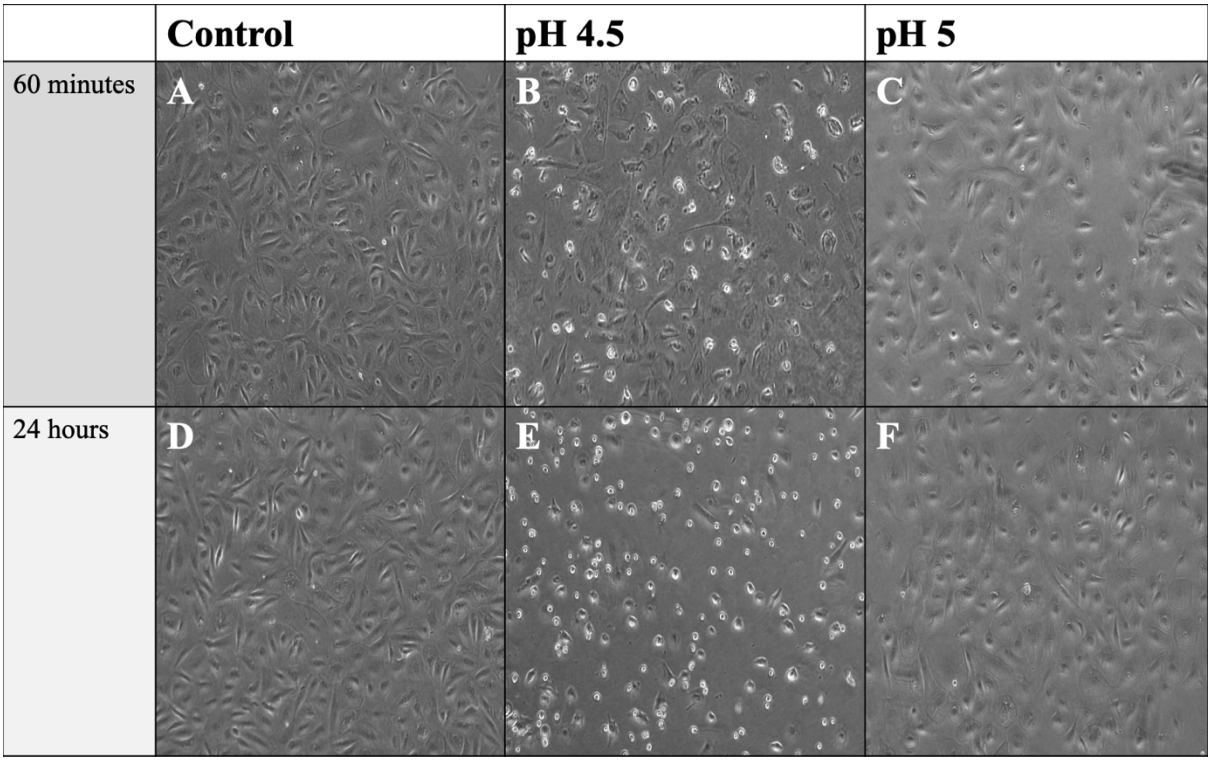


Figure 16: Part 1 of pH experiment. A,B and C) ASK cells after 60 minutes treatment with media holding different pH values. D, E and F) the same treated ASK cells after 24 hours. A and D) cells treated with normal pH media. B and E) cells treated with media holding a pH of 4.5. C and F) cells treated with media holding a pH of 5.

3.5.3.2. Transducing ASK at low pH

The pH of media ASK cells were incubated with during the transduction with VSV-G lentivirus did not influence transduction efficiency. There was no observable GFP expression in any of the samples during the 5 days the cells were observed. The level of cell death in all samples were normal compared to control sample without lentivirus treatment. ASK cells treated with media holding normal pH did not show any GFP expression.

3.5.4. pH experiment HEK

HEK cells were transduced at pH 5 in order to observe the effects of lower pH during lentiviral transduction. The low pH in the endosome environment triggers conformal changes in the VSV glycoprotein which leads to fusion of cellular and viral membranes. Two methods were tried

out, pH was lowered before virus was added, and pH was lowered after virus was added. Day one after transduction, GFP expression in both samples of HEK cells was similar (Figure 17). Day five after transduction, the GFP expression was much higher in HEK sample where pH was lowered before virus was added.

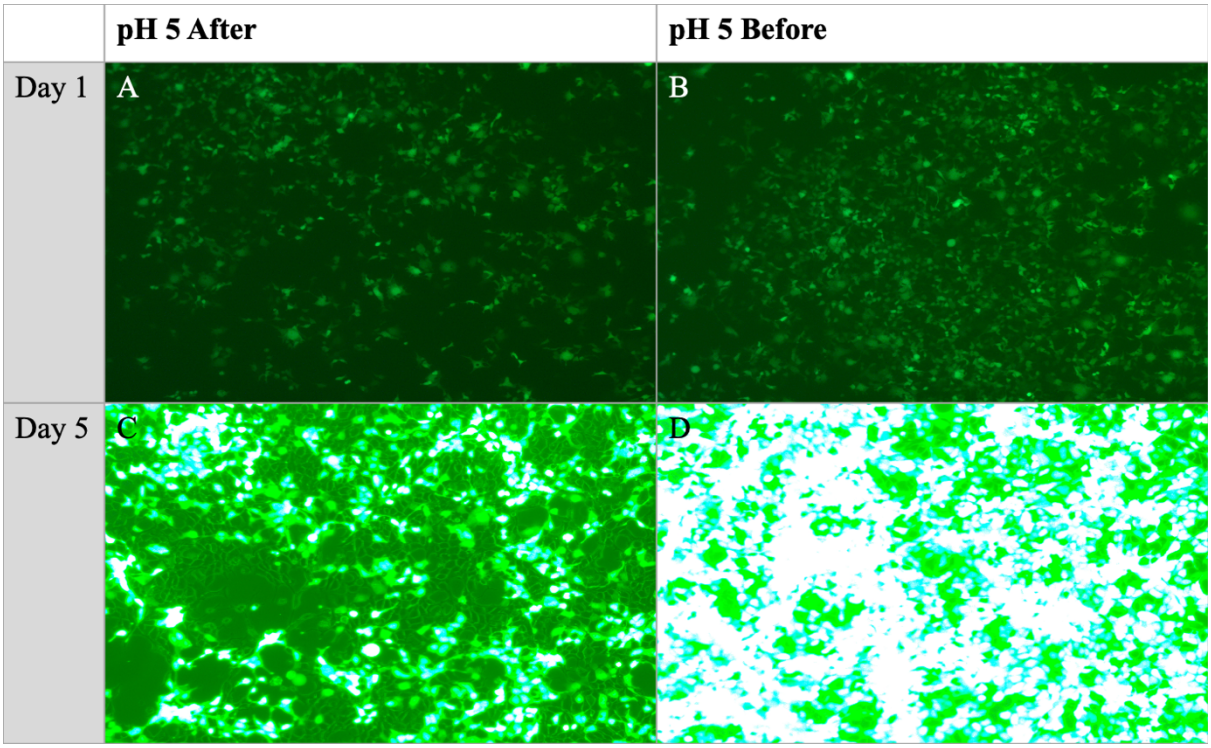


Figure 17: GFP expression in HEK cells after lentiviral transduction at pH 5. 10 x objective, exposure time 366 ms. A) cells day 1 after transduction, where pH was lowered after virus was added. B) cells day 1 after transduction, pH lowered before virus was added. C) cells day 5 after transduction, where pH was lowered after virus was added. D) cells day 5 after transduction, pH lowered before virus was added.

3.5.5. Temperature and pH experiment

ASK cells and fibroblasts were transduced at pH 5 and incubated at increasing temperatures in order to investigate if low temperature is hindering transduction of salmon cells. The pH was lowered during transduction in an attempt to trigger fusion of viral and cellular membranes. No GFP expression was observed during the four days the cells were observed, with the temperature increased from 24°C to 30°C.

3.5.6. Lentiviral transduction with pseudotyped virus

3.5.6.1. Transduction with pseudotyped lentivirus packaged at 37°C

Transduction by IHNV-G and VHSV-G pseudotyped lentivirus produced at 37°C was unsuccessful for HEK, ASK and fibroblast cells (Figure 18). Positive control VSV-G lentivirus transduced HEK cells, and cells produced GFP after 24 hours. Fluorescence was highest after 3 days.

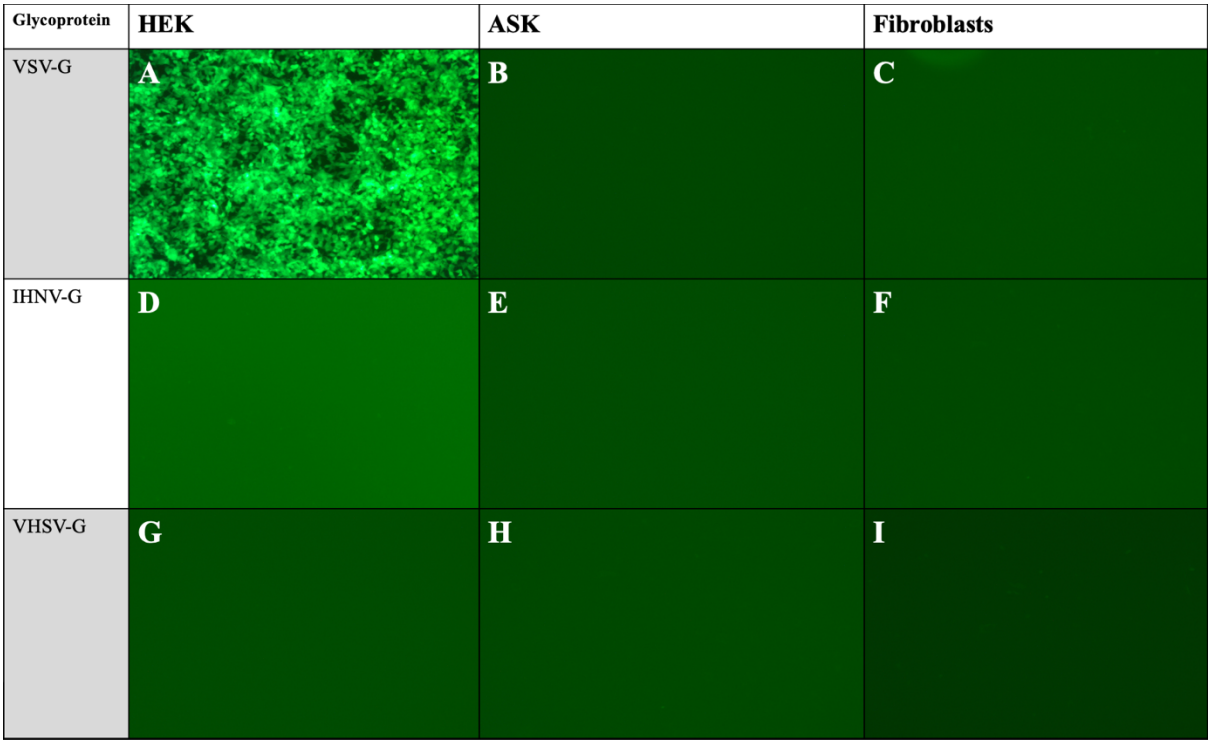


Figure 18: HEK, ASK and fibroblast cells three days after transduction. Pictures are taken at 10X magnification, 366 ms exposure. A, D, and G) HEK cells transduced with VSV-G lentivirus, IHNV-G lentivirus, and VHSV-G lentivirus, respectively. B, E, and H) ASK cells transduced with VSV-G lentivirus, IHNV-G lentivirus, and VHSV-G lentivirus, respectively. C, F, and I) fibroblasts transduced with VSV-G lentivirus, IHNV-G lentivirus, and VHSV-G lentivirus, respectively.

3.5.6.2. Transduction with pseudotyped lentivirus packaged at 22°C

All pseudotyped lentivirus versions packaged at 22°C were tested on ASK and HEK cells. All transductions of ASK cells were unsuccessful. There was no visible fluorescence. For HEK cells, there was one fluorescent cell in the transduction by IHNV-G(tm)–VSV-G pseudotyped lentivirus (Figure 19G). There was also one or two fluorescent cells in the transduction by VHSV-G(tm)–VSV-G pseudotyped lentivirus (Figure 19K). The positive control of VSV-G lentivirus successfully transduced HEK cells (Figure 19A).

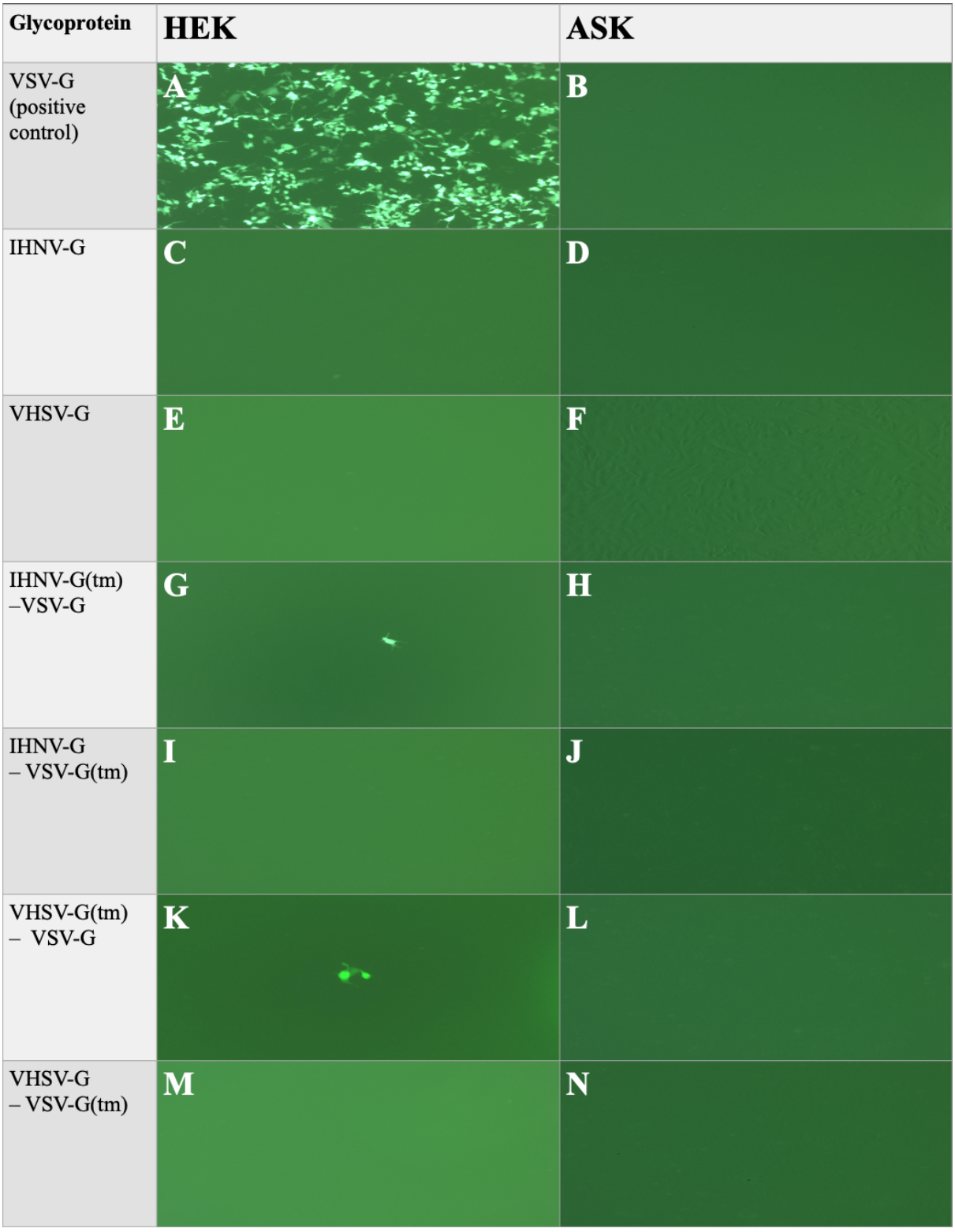


Figure 19: HEK and ASK cells transduced with pseudotyped lentivirus. All pictures are taken three days after transduction, except picture k which is taken 5 days after. Pictures are taken at 10X magnification, except picture k which is taken at 20X magnification. 366 ms exposure. A, C, E, G, I, K, and M) HEK cells transduced with VSV-G virus, IHNV-G virus, VHSV-G virus, IHNV-G(tm)–VSV-G virus, IHNV-G–VSV-G(tm) virus, VHVS-G(tm)–VSV-G virus, and VHSV-G–VSV-G(tm) virus respectively. B, D, F, H, J, L and N) ASK cells transduced with the same viruses.

3.6 qPCR experiment

DNA and RNA was extracted from ASK and HEK cells which had been infected with lentivirus. This was done to find out GFP gene sequence is reverse transcribed from lentiviral transfer plasmid. Control samples without lentivirus were also made for both cell types.

To detect the presence of GFP gene in ASK and HEK cells, primers were chosen for GFP gene sequence. GFP detected in DNA samples would mean virus can enter cells and integrate GFP sequence in cell genome. This is expected of HEK samples transduced with lentivirus, but not from ASK cells. Since RNA cannot be detected by qPCR, a positive result from RNA samples would indicate contamination of plasmid DNA. The presence of GFP sequence in cDNA samples would indicate there is GFP RNA in cells. GFP RNA would only be there if mRNA is being transcribed from GFP sequence integrated in genome.

Primers for ampicillin resistance gene sequence were also used to be able to detect plasmid DNA contamination in the samples. Primers were designed for salmon housekeeping genes $EF1A_a$ and $EF1A_b$, plus human housekeeping genes GAPDH to account for differences in expression.

The cycle threshold (Ct) values of reference genes were used to normalize the expression of target gene GFP. Using the method of relative quantification normalized to a reference gene, ΔCt values were calculated for all samples (Figure 20). For HEK cells, DNA samples are positive for GFP gene (Figure 20B). There is a great difference between samples treated with lentivirus and those untreated. For HEK cDNA samples, the difference is higher between treated and untreated samples with regards to ΔCt values, as seen in graph A. The higher values for cDNA samples could indicate there is mRNA production, and the reason we see such high levels is because of the amplification reaction that happens when cDNA is synthesized.

For ASK DNA samples in graph D, the ΔCt values are higher for the untreated samples than for the ones treated with lentivirus. This could indicate contamination of plasmid DNA. For ASK cDNA samples in graph C, there is not a large difference between the treated and the untreated samples. 0 or virus. The average ΔCt values for ampicillin resistance gene DNA for samples treated with lentivirus were 0.05 for HEK and 0.03 for ASK, while cDNA samples

were 20.55 for HEK and 3.73 for ASK. HEK GFP cDNA is much higher than cDNA for ampicillin resistance, indicating there is more mRNA production than there is plasmid or virus contamination. For ASK cDNA, ΔCt values are only slightly higher for ampicillin target than for GFP.

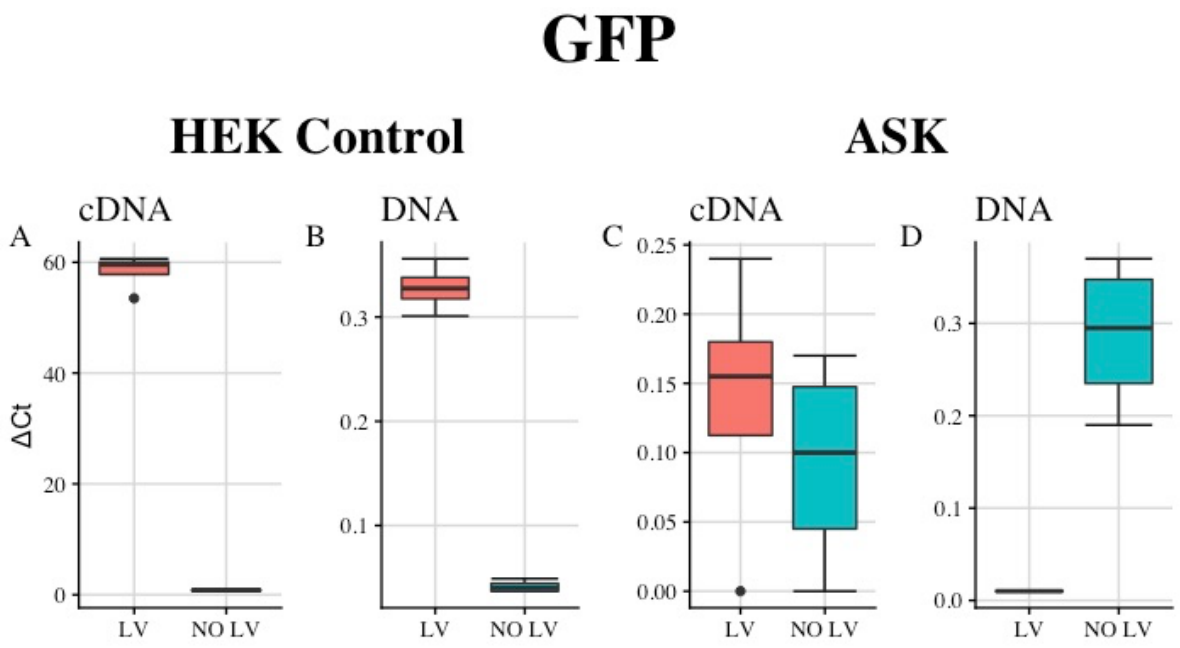


Figure 20: qPCR results for GFP sequence in HEK and ASK cells. Graphs have different scales and show ΔCt values for the different samples. Red bars illustrate samples treated with lentivirus, abbreviated to "LV". Blue bars illustrate samples not treated with lentivirus, abbreviated to "NO LV". A) HEK cDNA samples. B) HEK DNA samples. C) ASK cDNA samples. D) ASK DNA samples.

4. Discussion

4.1. Transduction using VSV-G pseudotyped virus

The most commonly used glycoprotein used to pseudotype lentivirus is VSV-G mainly due to its promiscuous tropism. Lentiviral vectors pseudotyped with VSV-G have so far only had limited success in transducing salmonid cells, with only one reported successful transduction in Chinook salmon embryo cells (Gratacap et al., 2020) and no successful reports in Atlantic salmon. In this study we varied the conditions at which Atlantic salmon cells were transduced in an attempt to enable lentiviral transduction. The goal was to identify the step in the transduction process that prevents transduction.

4.1.1. Interaction of VSV-G pseudotyped virus with salmon cells

The first step in viral transduction is the recognition and attachment to receptors. Staining of cells and virus shows virus attaching to the surface of ASK cells, in a similar way as for HEK cells (

Figure ***12***C and D). This could indicate that VSV-G can recognize and bind to receptors on the surface of ASK cells. The low-density lipoprotein receptor (LDLR) is the main entry port for VSV-G (Finkelshtein et al., 2013). Other receptors in the LDL family can also serve as alternate receptors (Nikolic et al., 2018). This LDL receptor is also present in Atlantic salmon (Betancor et al., 2014)., and VSV-G is likely binding this cellular receptor.

The virus seems to be taken up in endosomes, which are still visible on day 3 for both cell types (

Figure ***12***E AND F). Endosome like structures had a similar appearance in both HEK and ASK cells. The appearance of these structures could suggest that the virus is able to bind receptors leading to the formation of endosomes, even though it does not lead to a successful transduction. These structures were still present in HEK cells day three where we also had GFP expression. Therefore, these structures could still be present after transduction.

For the staining experiment virus was stained with fluorescent nucleic acid stain Syto82. This stain visualized the nucleic acids, but we were not able to see if the virus was uncoated and if the RNA was released from the envelope. For future research we suggest doing a labelling of both nucleic acid and lipids of virus, to better observe cell entry. For this experiment, we also suggest adding a negative control where stain is added to media and washed in the same way as virus samples. By doing this we could observe if residual stain is attaching to the cell surface

in a similar matter to the virus samples, and possibly exclude the chance that what we are seeing is residual stain.

Based on results from qPCR, we could not see any evidence that GFP sequence was reverse transcribed and integrated into ASK cells. There was a slightly positive result in cDNA samples, but this could be plasmid contamination. Comparing the results for ASK with HEK results which are successfully transduced we see a great difference. The ΔCt value for cDNA samples for HEK were elevated, and almost three times as high as for ampicillin samples (Figure 20). The results were congruent of mRNA production in HEK cells, with some contamination of plasmid DNA. As contaminant plasmid DNA is amplified together with cDNA in qPCR it can lead to false positive results. One method proposed for avoiding this issue is tagging the 5'-end of mRNA during reverse transcription using specific primers (Shuldiner et al., 1990). Then, using primers which targeted to cDNA and the sequence tag tail in qPCR reaction, exact levels of cDNA can be detected and the false positives are virtually eliminated (Shuldiner et al., 1990).

For ASK samples, there was no great difference in ΔCt values for GFP target in samples treated with lentivirus compared to untreated samples (Figure 20). This tells us that either there is no reverse transcription happening in these cells or the level of reverse transcription is below the limit of detection. ΔCt values for ampicillin target in ASK samples were positive but only slightly. This points to the slightly positive results we see for GFP in ASK is a result of plasmid contamination. From these results there is a possibility the reverse transcriptase activity was occurring at a low level in Atlantic salmon cells but is masked by plasmid DNA contamination. As to better illustrate plasmid contamination in all samples, the test should have included samples not treated with lentivirus for ampicillin target. Plasmid DNA could possibly be transferred from gloves or through the air.

4.1.2. The effect of temperature and pH on lentiviral transduction

The pH experiment done in HEK cells shows increased transduction efficiency when transducing cells at pH 5 compared to control. There is clearly more fluorescence in cells transduced where pH was lowered before addition of virus (Figure 17). Conformal changes of the VSV glycoprotein leading to fusion is pH dependent, and the optimal pH Is between 5 and 6 (Rucker et al., 2012). It follows that the transduction has higher efficiency in a low pH

environment. This is also congruent with other studies with transient low pH treatment during lentiviral transduction with pseudotyped with glycoproteins which initiate fusion at low pH (Morizono et al., 2006). Our results indicate that adding virus to a low pH environment does not lead to premature conformal changes that would hinder glycoprotein-receptor interactions. The same experiment performed with ASK cells showed no difference in ASK cells transduced under normal conditions, with low pH before virus is added, or when lowering pH after addition of virus. Although transducing HEK cells in a low pH environment leads to increased transduction efficiency, it had no impact on ASK cells.

The lentiviral vector is derived from HIV-1 which has human cells as main hosts. These cells thrive at 37℃. As the improved lentiviral transduction protocol for Chinook salmon cells included raising the incubation temperature from 17℃ to 22℃ during transduction to increase efficiency, it would indicate temperature is crucial for one or more steps in the transduction process (Gratacap et al., 2020). Temperature can influence the stability of RNA structure. Low temperatures have been shown to stabilize retroviral RNA secondary structures, and therefore limit enzyme activities (Chursov et al., 2013; Klasens et al., 1998). The single stranded positive sense RNA in retroviruses have secondary structures, which have been demonstrated capable of pausing reverse transcriptase activity (Harrison et al., 1998). Therefore, it could be the case that the low incubation temperature of Atlantic salmon cells is inhibiting reverse transcriptase activity by stabilizing secondary structures of lentiviral RNA.

HEK cells transduced at lower temperatures did not start expressing GFP until reaching 26℃ (Figure 15). It was unclear if this result is caused by the stress imposed on HEK cells at lower temperatures reducing the rate at which GFP was expressed. HEK cells have previously been studied for changes in heterologous protein expression and cell growth under hypothermic conditions. HEK cells have been shown to arrest growth when subjected to temperatures below 35℃ (Lin et al., 2015). Interestingly, HEK cells show a 1.5-fold increase in heterologous protein expression when temperatures are lowered to 33℃, in a mechanism which results of arrest in cell cycle (Fox et al., 2005; Lin et al., 2015). The increased protein expression required an overnight incubation at 37℃ following transfection in order for the cells to recover. Without this overnight recovery, expression levels decreased (Lin et al., 2015). Therefore, we can assume the low temperature could cause HEK cells transduced at lower temperatures to express limited amounts of GFP. This in combination with low temperature possibly inhibiting reverse

transcriptase activity in lentiviral transduction could explain the results of HEK cells not producing GFP before incubation temperature reached 26℃.

ASK cells and fibroblasts were transduced at pH 5 and with temperatures increasing gradually from 24℃ to 30℃ over four days. This had no influence on transduction efficiency, as no GFP was expressed in any samples. Shown earlier, transducing HEK cells at pH 5 increased transduction efficiency, and should elevate the chances of producing a successful transduction in salmon cells. As previously illustrated in HEK cells, lentiviral transductions is possible at 26℃. Since increasing the incubation temperature did not have any visible effects on transduction efficiency, it is reasonable to believe that temperature is not the main constraint for transduction of Atlantic salmon cells, but it could still be a factor.

4.1.3. Immune responses to lentiviral vectors

One of the factors limiting the transduction of salmon cells could be immune responses in the cells towards the virus. In order to produce a successful transduction, viral particles have to be able to overcome or evade the cells immune responses. Lentiviral vectors can trigger immune responses in mammalian cells, either in response to viral RNA, integrated reverse transcribed viral DNA, or retroviral proteins (Follenzi et al., 2007). The innate immune system recognizes retroviral components and initiate responses which will hinder viral replication (Sáez-Cirión & Manel, 2018). Just as the host cells have evolved innate immune defense strategies to prevent viral infections, viruses develop adaptive strategies to evade these immune responses (Evans & Desrosiers, 2001). Since this HIV-1 lentiviral vector has not evolved to infect piscine hosts, it is possible that it is unable to escape the cells innate immune response to retroviruses. Novirhabdoviruses with a broad range of aquatic hosts have evolved a non-virion protein which is shown to be expressed in infected host cells at low levels and interfere with host immune responses (Ammayappan & Vakharia, 2011; Kurath & Leong, 1985). It could be of interest to include this non-virion protein as a transgene in lentiviral transfer plasmid and observe effects on transduction of Atlantic salmon cells.

4.1.2. Response to transgene and promoter in salmon cells

The transfection by electroporation of both ASK and HEK gave positive results for both cell types. ASK cells were able to transcribe GFP gene sequence from the CMV promoter, and successfully express GFP (Figure 9). The CMV promoter has also been demonstrated to promote expression in Atlantic salmon smolt (Chang et al., 2014). This indicates that the

problem with transduction of ASK cells was not transcription or translation, but a step earlier in the process.

4.2. Transduction using IHNV-G and VHSV-G pseudotyped virus

Lentivirus was pseudotyped with IHNV and VHSV glycoproteins in both native and chimeric variants in combination with VSV-G domains. This was an attempt to create lentiviral vectors which with high biological activity and the ability to stably transduce Atlantic salmon cells.

The transductions with lentivirus pseudotyped with native and chimeric glycoprotein sequences from IHNV and VHSV were not successful for Atlantic salmon cells (Figure 18 and Figure 19). In HEK cells, IHNVG(tm)-VSVG and VHSVG(tm)-VSVG pseudotyped virus produced GFP.(Figure 19). Both were pseudotyped with the extracellular spike and transmembrane domains of IHNV or VHSV glycoprotein, and the cytoplasmic tail of VSV glycoprotein. The abundant glycoprotein fibronectin of the extracellular matrix is where IHNV and VHVS initially binds in salmonid cells (Bearzotti et al., 1999). This protein has several adhesive functions, and is involved in many interactions in mammalian cells (Proctor, 1987). This protein is highly conserved in vertebrates (Adams et al., 2015). IHNV and VHSV glycoproteins might also bind fibronectin in human cells.

The results of this experiment are similar to results to the study by Carpentier et al. (2012), where the lentivirus pseudotyped with extracellular spike and transmembrane domain from RV glycoprotein, and the cytoplasmic tail of VSVG had the highest biological activity compared to other combinations of domains as well as native RV glycoprotein. Inclusion of VSVG cytoplasmic tail lead to an increase in the number of glycoprotein incorporated in the lentiviral envelope when compared to native RVG, which could be caused by the role of the tail in budding (Carpentier et al., 2012). Inclusion of the transmembrane domain of VSVG reduced biological activity, suggesting interactions between the transmembrane domain and extracellular spike with regards to protein structure and function (Carpentier et al., 2012).

The reason these chimeric glycoproteins showed some transduction success while native glycoproteins from IHNV and VHSV did not, could lie in the cytoplasmic tail which has interactions with the lipid bilayer and matrix proteins during budding from producer cells and cellular entry (Cosson, 1996). These interactions have been suggested to play a role in location

of cholesterol rich domains of host cell membrane which are favored during budding (Carpentier et al., 2012; Sundquist & Krausslich, 2012). Doing a staining of the different pseudotypes to look into binding, endosome forming, and fusion would also provide some insight into the suitability of the different glycoproteins.

The successful transduction of HEK cells by IHNVG(tm)-VSVG and VHSVG(tm)-VSVG pseudotyped virus indicates that we are producing active virus, at least with these two variants. However, we only observed one GFP positive cell under each condition, so the titer of active virus us likely very low. By comparing the sample of HEK cells transduced with VSV-G lentivirus produced at 22℃ (Figure 19A) to the sample of HEK cells transduced with the same virus produced at 37℃ (Figure 18A) it seems the virus has a lower concentration when produced at a lower temperature, based on the number of cells expressing GFP. This could mean we have produced virus capable of infecting ASK cell, but the low concentration combined with the low transduction efficiency leads to no observable transductions. To alleviate this, we propose producing the virus in higher volumes at low temperature and concentrating supernatant by ultracentrifugation or ultrafiltration in order to create higher titer virus.

During production of the pseudotyped lentivirus, the indicator we use for a successful co-transfection of the lentiviral plasmids is GFP expression in packaging cells. This indicator is not a sure signal that virus is being produced, as it only indicates the successful transfection of the transfer plasmid containing the GFP gene sequence. High transduction efficiency of all three lentiviral plasmids is needed for virus production. It would be preferable to do a method of titration such as qPCR of supernatant, in order to monitor the number of viral particles being produced. The total number of lentiviral particles can be determined by a p24 ELISA based assay but does not provide any information on the number active, infectious particles (Bourinbaiar, 1994; Sena-Esteves & Gao, 2018). The ratio of active to defective viral particles can vary within the range of 1:1 to 1:100 (Bourinbaiar, 1994).

Budding of virions from host cells means the phospholipid bilayer composition of virions has similarities to that of the host cell membrane (Quigley et al., 1971). Viruses also make use of host cells post translational modification machinery to improve virulence properties (Kumar et al., 2020). Post-translational glycoprotein glycan modifications are highly variable across species an cell types, even within teleost (Aoki et al., 2021). Changes in glycosylation affect

interactions with receptors on host cells as well as affecting cells immune systems ability to recognize the virus. Therefore, glycosylation patterns can impact infectivity (Vigerust & Shepherd, 2007). It would be of interest to attempt producing lentivirus from salmonid cells as to have a similar membrane composition and post translational modifications and investigate whether this could increase the chances of a successful transduction.

5. Conclusion

The aim of this thesis was to enable lentiviral transduction in Atlantic salmon cells by identifying the inhibitory steps in the process. Results from several experiments point to the problem being either the glycoprotein-receptor interaction not being optimal, leading to problems with fusion and endosomal escape, or other issues with fusion and uncoating. Transduction with fluorescently labeled virus revealed endosomes forming, but no reverse transcription activity was detectable in Atlantic salmon cells by qPCR. Lowering of pH and increasing temperature had no noticeable effects. The production of pseudotyped lentiviral vectors seem to be successful but produced lentivirus was very low titer. Two pseudotypes of chimeric glycoproteins derived from piscine novirhabdoviruses were unexpectedly able to infect human cells, which demonstrates that these viruses are biologically active and have potential for transduction.

6. References

(OEC), T. O. o. E. C. (2022). *Norway*. Available at: https://oec.world/en/profile/country/nor (accessed: 07.08.2022).

Adams, J. C., Chiquet-Ehrismann, R. & Tucker, R. P. (2015). The evolution of tenascins and fibronectin. *Cell Adh Migr*, 9 (1-2): 22-33. doi: 10.4161/19336918.2014.970030.

Aiken, C. (1997). Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. *Journal of Virology*, 71 (8): 5871-5877. doi: 10.1128/jvi.71.8.5871-5877.1997.

Akkina, R. K., Walton, R. M., Chen, M. L., Li, Q. X., Planelles, V. & Chen, I. S. (1996). High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J Virol*, 70 (4): 2581-5. doi: 10.1128/jvi.70.4.2581-2585.1996.

Ambrose, Z. & Aiken, C. (2014). HIV-1 uncoating: connection to nuclear entry and regulation by host proteins. *Virology*, 454-455: 371-9. doi: 10.1016/j.virol.2014.02.004.

Ammayappan, A. & Vakharia, V. N. (2011). Nonvirion protein of novirhabdovirus suppresses apoptosis at the early stage of virus infection. *J Virol*, 85 (16): 8393-402. doi: 10.1128/JVI.00597-11.

Aoki, K., Kumagai, T., Ranzinger, R., Bergmann, C., Camus, A. & Tiemeyer, M. (2021). Serum N-Glycome Diversity in Teleost and Chondrostrean Fishes. *Front Mol Biosci*, 8: 778383. doi: 10.3389/fmolb.2021.778383.

Bearzotti, M., Delmas, B., Lamoureux, A., Loustau, A.-M., Chilmonczyk, S. & Bremont, M. (1999). Fish Rhabdovirus Cell Entry Is Mediated by Fibronectin. *Journal of Virology*, 73 (9): 7703-7709. doi: 10.1128/jvi.73.9.7703-7709.1999.

Benchling. (2022). Available at: https://benchling.com/.

Betancor, M. B., McStay, E., Minghetti, M., Migaud, H., Tocher, D. R. & Davie, A. (2014). Daily Rhythms in Expression of Genes of Hepatic Lipid Metabolism in Atlantic

Salmon (Salmo salar L.). *PLoS ONE*, 9 (9): e106739. doi: 10.1371/journal.pone.0106739.

Bootland, L. & Leong, J. (2011). *Infectious haematopoietic necrosis virus*: CABI International.

Bourinbaiar, A. S. (1994). The ratio of defective HIV-1 particles to replication-competent infectious virions. *Acta Virol*, 38 (1): 59-61.

Carpentier, D. C., Vevis, K., Trabalza, A., Georgiadis, C., Ellison, S. M., Asfahani, R. I. & Mazarakis, N. D. (2012). Enhanced pseudotyping efficiency of HIV-1 lentiviral vectors by a rabies/vesicular stomatitis virus chimeric envelope glycoprotein. *Gene Ther*, 19 (7): 761-74. doi: 10.1038/gt.2011.124.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, 263 (5148): 802-5. doi: 10.1126/science.8303295.

Chang, C. J., Robertsen, C., Sun, B. & Robertsen, B. (2014). Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid. *Vaccine*, 32 (36): 4695-702. doi: 10.1016/j.vaccine.2014.05.059.

Chursov, A., Kopetzky, S. J., Bocharov, G., Frishman, D. & Shneider, A. (2013). RNAtips: Analysis of temperature-induced changes of RNA secondary structure. *Nucleic Acids Res*, 41 (Web Server issue): W486-91. doi: 10.1093/nar/gkt486.

Cieslak, M., Wahli, T., Diserens, N., Haenen, O. L. M. & Schütze, H. (2017). Phylogeny of the infectious hematopoietic necrosis virus in European aquaculture. *PLoS One*, 12 (9): e0184490. doi: 10.1371/journal.pone.0184490.

Cockrell, A. S. & Kafri, T. (2007). Gene delivery by lentivirus vectors. *Mol Biotechnol*, 36 (3): 184-204. doi: 10.1007/s12033-007-0010-8.

Coll, J. M. (1995). The glycoprotein G of rhabdoviruses. *Arch Virol*, 140 (5): 827-51. doi: 10.1007/bf01314961.

Cosson, P. (1996). Direct interaction between the envelope and matrix proteins of HIV-1. *Embo j*, 15 (21): 5783-8.

Cronin, J., Zhang, X. Y. & Reiser, J. (2005). Altering the tropism of lentiviral vectors through pseudotyping. *Curr Gene Ther*, 5 (4): 387-98. doi: 10.2174/1566523054546224.

Dietzgen, R. G., Kondo, H., Goodin, M. M., Kurath, G. & Vasilakis, N. (2017). The family Rhabdoviridae: mono- and bipartite negative-sense RNA viruses with diverse genome organization and common evolutionary origins. *Virus Res*, 227: 158-170. doi: 10.1016/j.virusres.2016.10.010.

Doench, J. G. (2018). Am I ready for CRISPR? A user's guide to genetic screens. *Nat Rev Genet*, 19 (2): 67-80. doi: 10.1038/nrg.2017.97.

Doudna, J. A. & Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science*, 346 (6213): 1258096. doi: 10.1126/science.1258096.

Drolet, B. S., J. S. Rohovec, and J. C. Leong. (1994). The route of entry and progression of infectious haematopoietic necrosis virus in Oncorhynchus mykiss (Walbaum): a sequential immunohistochemical study. *Journal of Fish Diseases*. doi: DOI:10.1111/J.1365-2761.1994.TB00229.X.

Einer-Jensen, K., Ahrens, P., Forsberg, R. & Lorenzen, N. (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *Journal of General Virology*, 85 (5): 1167-1179. doi: https://doi.org/10.1099/vir.0.79820-0.

European Commission, D.-G. f. H. a. F. S. (2020). *Document 32020R2002*. Official Journal of the European Union. Available at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32020R2002 (accessed: 14.08.2022).

Evans, D. T. & Desrosiers, R. C. (2001). Immune evasion strategies of the primate lentiviruses. *Immunol Rev*, 183: 141-58. doi: 10.1034/j.1600-065x.2001.1830112.x.

Finkelshtein, D., Werman, A., Novick, D., Barak, S. & Rubinstein, M. (2013). LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. *Proc Natl Acad Sci U S A*, 110 (18): 7306-11. doi: 10.1073/pnas.1214441110.

Follenzi, A., Santambrogio, L. & Annoni, A. (2007). Immune responses to lentiviral vectors. *Curr Gene Ther*, 7 (5): 306-15. doi: 10.2174/156652307782151515.

Fox, S. R., Yap, M. X., Yap, M. G. & Wang, D. I. (2005). Active hypothermic growth: a novel means for increasing total interferon-gamma production by Chinese-hamster ovary cells. *Biotechnol Appl Biochem*, 41 (Pt 3): 265-72. doi: 10.1042/ba20040067.

Gaudin, Y., Ruigrok, R. W., Tuffereau, C., Knossow, M. & Flamand, A. (1992). Rabies virus glycoprotein is a trimer. *Virology*, 187 (2): 627-32. doi: 10.1016/0042-6822(92)90465-2.

Ge, P., Tsao, J., Schein, S., Green, T. J., Luo, M. & Zhou, Z. H. (2010). Cryo-EM model of the bullet-shaped vesicular stomatitis virus. *Science*, 327 (5966): 689-93. doi: 10.1126/science.1181766.

Geneious. (2022). Available at: https://www.geneious.com.

Goldstein, J. L. & Brown, M. S. (2009). The LDL receptor. *Arterioscler Thromb Vasc Biol*, 29 (4): 431-8. doi: 10.1161/atvbaha.108.179564.

Gratacap, R. L., Wargelius, A., Edvardsen, R. B. & Houston, R. D. (2019). Potential of Genome Editing to Improve Aquaculture Breeding and Production. *Trends Genet*, 35 (9): 672-684. doi: 10.1016/j.tig.2019.06.006.

Gratacap, R. L., Regan, T., Dehler, C. E., Martin, S. A. M., Boudinot, P., Collet, B. & Houston, R. D. (2020). Efficient CRISPR/Cas9 genome editing in a salmonid fish cell line using a lentivirus delivery system. *BMC Biotechnol*, 20 (1): 35. doi: 10.1186/s12896-020-00626-x.

Hamard-Peron, E. & Muriaux, D. (2011). Retroviral matrix and lipids, the intimate interaction. *Retrovirology*, 8: 15. doi: 10.1186/1742-4690-8-15.

Harrison, G. P., Mayo, M. S., Hunter, E. & Lever, A. M. L. (1998). Pausing of reverse transcriptase on retroviral RNA templates is influenced by secondary structures both 5′ and 3′ of the catalytic site. *Nucleic Acids Research*, 26 (14): 3433-3442. doi: 10.1093/nar/26.14.3433.

Herschhorn, A. & Hizi, A. (2010). Retroviral reverse transcriptases. *Cell Mol Life Sci*, 67 (16): 2717-47. doi: 10.1007/s00018-010-0346-2.

Ikeda, Y., Collins, M. K., Radcliffe, P. A., Mitrophanous, K. A. & Takeuchi, Y. (2002). Gene transduction efficiency in cells of different species by HIV and EIAV vectors. *Gene Ther*, 9 (14): 932-8. doi: 10.1038/sj.gt.3301708.

Jennings, S., Stentiford, G. D., Leocadio, A. M., Jeffery, K. R., Metcalfe, J. D., Katsiadaki, I., Auchterlonie, N. A., Mangi, S. C., Pinnegar, J. K., Ellis, T., et al. (2016). Aquatic food security: insights into challenges and solutions from an analysis of interactions between fisheries, aquaculture, food safety, human health, fish and human welfare, economy and environment. *Fish and Fisheries*, 17 (4): 893-938. doi: 10.1111/faf.12152.

Kim, R. & Faisal, M. (2011). Emergence and resurgence of the viral hemorrhagic septicemia virus (Novirhabdovirus, Rhabdoviridae, Mononegavirales). *Journal of Advanced Research*, 2 (1): 9-23. doi: https://doi.org/10.1016/j.jare.2010.05.007.

Klasens, B. I. F., Das, A. T. & Berkhout, B. (1998). Inhibition of polyadenylation by stable RNA secondary structure. *Nucleic Acids Research*, 26 (8): 1870-1876. doi: 10.1093/nar/26.8.1870.

Kumar, R., Mehta, D., Mishra, N., Nayak, D. & Sunil, S. (2020). Role of Host-Mediated Post-Translational Modifications (PTMs) in RNA Virus Pathogenesis. *International Journal of Molecular Sciences*, 22 (1): 323. doi: 10.3390/ijms22010323.

Kurath, G. & Leong, J. C. (1985). Characterization of infectious hematopoietic necrosis virus mRNA species reveals a nonvirion rhabdovirus protein. *J Virol*, 53 (2): 462-8. doi: 10.1128/jvi.53.2.462-468.1985.

Kurath, G., Garver, K. A., Troyer, R. M., Emmenegger, E. J., Einer-Jensen, K. & Anderson, E. D. (2003). Phylogeography of infectious haematopoietic necrosis virus in North America. *J Gen Virol*, 84 (Pt 4): 803-814. doi: 10.1099/vir.0.18771-0.

Kurath, G., Winton, J. R., Dale, O. B., Purcell, M. K., Falk, K. & Busch, R. A. (2016). Atlantic salmon, Salmo salar L. are broadly susceptible to isolates representing the North American genogroups of infectious hematopoietic necrosis virus. *J Fish Dis*, 39 (1): 55-67. doi: 10.1111/jfd.12323.

Kuzmin, I. V., Novella, I. S., Dietzgen, R. G., Padhi, A. & Rupprecht, C. E. (2009). The rhabdoviruses: biodiversity, phylogenetics, and evolution. *Infect Genet Evol*, 9 (4): 541-53. doi: 10.1016/j.meegid.2009.02.005.

Lapatra, S. E. (1998). Factors Affecting Pathogenicity of Infectious Hematopoietic Necrosis Virus (IHNV) for Salmonid Fish. *Journal of Aquatic Animal Health*, 10 (2): 121-131. doi: 10.1577/1548-8667(1998)010<0121:FAPOIH>2.0.CO;2.

Letchworth, G. J., Rodriguez, L. L. & Del cbarrera, J. (1999). Vesicular stomatitis. *Vet J*, 157 (3): 239-60. doi: 10.1053/tvjl.1998.0303.

Lewis, P., Hensel, M. & Emerman, M. (1992). Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J*, 11 (8): 3053-8. doi: 10.1002/j.1460-2075.1992.tb05376.x.

Lin, C.-Y., Huang, Z., Wen, W., Wu, A., Wang, C. & Niu, L. (2015). Enhancing Protein Expression in HEK-293 Cells by Lowering Culture Temperature. *PLOS ONE*, 10 (4): e0123562. doi: 10.1371/journal.pone.0123562.

Liu, G., Cao, W., Salawudeen, A., Zhu, W., Emeterio, K., Safronetz, D. & Banadyga, L. (2021). Vesicular Stomatitis Virus: From Agricultural Pathogen to Vaccine Vector. *Pathogens*, 10 (9). doi: 10.3390/pathogens10091092.

Meyers, T. R. & Winton, J. R. (1995). Viral hemorrhagic septicemia virus in North America. *Annual Review of Fish Diseases*, 5: 3-24. doi: https://doi.org/10.1016/0959-8030(95)00002-X.

Morizono, K., Ringpis, G. E., Pariente, N., Xie, Y. & Chen, I. S. (2006). Transient low pH treatment enhances infection of lentiviral vector pseudotypes with a targeting Sindbis envelope. *Virology*, 355 (1): 71-81. doi: 10.1016/j.virol.2006.07.015.

Naldini, L., Blömer, U., Gage, F. H., Trono, D. & Verma, I. M. (1996a). Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*, 93 (21): 11382-8. doi: 10.1073/pnas.93.21.11382.

Naldini, L., Blömer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M. & Trono, D. (1996b). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*, 272 (5259): 263-7. doi: 10.1126/science.272.5259.263.

Nikolic, J., Belot, L., Raux, H., Legrand, P., Gaudin, Y. & A. Albertini, A. (2018). Structural basis for the recognition of LDL-receptor family members by VSV glycoprotein. *Nature Communications*, 9 (1). doi: 10.1038/s41467-018-03432-4.

OIE. *Animal Diseases*. World Organisation for Animal Health (WOAH, founded as OIE). Available at: https://www.woah.org/en/what-we-do/animal-health-and-welfare/animal-diseases/.

Olsvik, P. A., Lie, K. K., Jordal, A. E., Nilsen, T. O. & Hordvik, I. (2005). Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Mol Biol*, 6: 21. doi: 10.1186/1471-2199-6-21.

Proctor, R. A. (1987). Fibronectin: a brief overview of its structure, function, and physiology. *Rev Infect Dis*, 9 Suppl 4: S317-21. doi: 10.1093/clinids/9.supplement_4.s317.

Quigley, J. P., Rifkin, D. B. & Reich, E. (1971). Phospholipid composition of Rous sarcoma virus, host cell membranes and other enveloped RNA viruses. *Virology*, 46 (1): 106-16. doi: 10.1016/0042-6822(71)90010-9.

Rucker, P., Wieninger, S. A., Ullmann, G. M. & Sticht, H. (2012). pH-dependent molecular dynamics of vesicular stomatitis virus glycoprotein G. *Proteins*, 80 (11): 2601-13. doi: 10.1002/prot.24145.

Rupprecht, C., Kuzmin, I. & Meslin, F. (2017). Lyssaviruses and rabies: current conundrums, concerns, contradictions and controversies. *F1000Res*, 6: 184. doi: 10.12688/f1000research.10416.1.

Sáez-Cirión, A. & Manel, N. (2018). Immune Responses to Retroviruses. *Annual Review of Immunology*, 36 (1): 193-220. doi: 10.1146/annurev-immunol-051116-052155.

Sastry, L., Johnson, T., Hobson, M. J., Smucker, B. & Cornetta, K. (2002). Titering lentiviral vectors: comparison of DNA, RNA and marker expression methods. *Gene Ther*, 9 (17): 1155-62. doi: 10.1038/sj.gt.3301731.

Sena-Esteves, M. & Gao, G. (2018). Titration of Lentivirus Vectors. *Cold Spring Harb Protoc*, 2018 (4). doi: 10.1101/pdb.prot095695.

Shalem, O., Sanjana, N. E., Hartenian, E., Shi, X., Scott, D. A., Mikkelson, T., Heckl, D., Ebert, B. L., Root, D. E., Doench, J. G., et al. (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*, 343 (6166): 84-87. doi: 10.1126/science.1247005.

Shuldiner, A. R., Nirula, A. & Roth, J. (1990). RNA template-specific polymerase chain reaction (RS-PCR): a novel strategy to reduce dramatically false positives. *Gene*, 91 (1): 139-142. doi: 10.1016/0378-1119(90)90176-r.

Skall, H. F., Olesen, N. J. & Mellergaard, S. (2005). Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming--a review. *J Fish Dis*, 28 (9): 509-29. doi: 10.1111/j.1365-2761.2005.00654.x.

Stein, B. S., Gowda, S. D., Lifson, J. D., Penhallow, R. C., Bensch, K. G. & Engleman, E. G. (1987). pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell*, 49 (5): 659-68. doi: 10.1016/0092-8674(87)90542-3.

Stothard, P. ((2000)). *The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences.* Available at: https://www.genscript.com/.

Sun, X., Yau, V. K., Briggs, B. J. & Whittaker, G. R. (2005). Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. *Virology*, 338 (1): 53-60. doi: 10.1016/j.virol.2005.05.006.

Sundquist, W. I. & Krausslich, H. G. (2012). HIV-1 Assembly, Budding, and Maturation. *Cold Spring Harbor Perspectives in Medicine*, 2 (7): a006924-a006924. doi: 10.1101/cshperspect.a006924.

Tang, H., Kuhen, K. L. & Wong-Staal, F. (1999). Lentivirus replication and regulation. *Annu Rev Genet*, 33: 133-70. doi: 10.1146/annurev.genet.33.1.133.

Thoulouze, M. I., Bouguyon, E., Carpentier, C. & Bremont, M. (2004). Essential role of the NV protein of Novirhabdovirus for pathogenicity in rainbow trout. *J Virol*, 78 (8): 4098-107. doi: 10.1128/jvi.78.8.4098-4107.2004.

Verschoor, C. P., Dorrington, M. G., Novakowski, K. E., Kaiser, J., Radford, K., Nair, P., Anipindi, V., Kaushic, C., Surette, M. G. & Bowdish, D. M. E. (2014). MicroRNA-155 Is Required for Clearance of Streptococcus pneumoniae from the Nasopharynx. *Infection and Immunity*, 82 (11): 4824-4833. doi: 10.1128/iai.02251-14.

Vigerust, D. J. & Shepherd, V. L. (2007). Virus glycosylation: role in virulence and immune interactions. *Trends in Microbiology*, 15 (5): 211-218. doi: 10.1016/j.tim.2007.03.003.

Walker, P. J., Dietzgen, R. G., Joubert, D. A. & Blasdell, K. R. (2011). Rhabdovirus accessory genes. *Virus Res*, 162 (1-2): 110-25. doi: 10.1016/j.virusres.2011.09.004.

Wills, J. W. & Craven, R. C. (1991). Form, function, and use of retroviral gag proteins. *Aids*, 5 (6): 639-54. doi: 10.1097/00002030-199106000-00002.

Závada, J. (1982). The pseudotypic paradox. *J Gen Virol*, 63 (Pt 1): 15-24. doi: 10.1099/0022-1317-63-1-15.

Zenger, K. R., Khatkar, M. S., Jones, D. B., Khalilisamani, N., Jerry, D. R. & Raadsma, H. W. (2018). Genomic Selection in Aquaculture: Application, Limitations and Opportunities With Special Reference to Marine Shrimp and Pearl Oysters. *Front Genet*, 9: 693. doi: 10.3389/fgene.2018.00693.

Zhu, Z. H., Chen, S. S. & Huang, A. S. (1990). Phenotypic mixing between human immunodeficiency virus and vesicular stomatitis virus or herpes simplex virus. *J Acquir Immune Defic Syndr (1988)*, 3 (3): 215-9.

7. Kits and reagents used

Bio-Rad

- 1725270 Sso Advanced Universal SYBR Green Supermix
- 1708890 iScript cDNA Synthesis Kit

Gibco

- 31415029 Leibovitz's L-15 Medium, GlutaMAX Supplement
- 25200072 Trypsin-EDTA (0.25%), Phenol red
- 14175095 HBSS, no calcium, no magnesium, no phenol red
- 11058021 Opti-MEM Reduced Serum Medium, no phenol red

iNtRON Biotechnology

- RedSafe Nucleic Acid Staining Solution

Invitrogen, Thermo Fisher Scientific

- MRK1025 Neon Transfection system 10 µL Kit
- C737303 One Shot Stbl3 Chemically Competent E. coli
- SM0311 GeneRuler 1 kb DNA Ladder
- S11363 SYTO 82 Orange Fluorescent Nucleic Acid Stain
- C10046 CellMask Deep Red Plasma Membrane Stain
- F530S Phusion High-Fidelity DNA Polymerase
- MAN0012393 Phusion High-Fidelity DNA Polymerase Manual
- 11668019 Lipofectamine™ 2000 Transfection Reagent

New England Biolabs

- B6004 10X rCutsmart
- R3101 EcoRI-HF
- M0289 Antarctic Phosphatase
- B0289 10X Antarctic Phosphatase Reaction Buffer
- B0202 10X T4 Ligase Reaction Buffer
- M0202 T4 Ligase
- R3104 HindII-HF

- R0187 StuI
- R3138 SalI-HF
- E2611 Gibson Assembly Master Mix

Qiagen

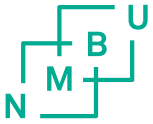
- 27104 QIAprep Spin Miniprep Kit
- 28704 QIAquick Gel Extraction Kit
- 74104 RNeasy Mini Kit
- 79254 RNase-Free DNase Set
- 69504 DNeasy Blood and Tissue Kit

ZYMO Research

- D4201 - ZymoPURE Plasmid Midiprep Kit
- D4210 - ZymoPURE Plasmid Miniprep Kit

Sigma-Aldrich

- D6429 Dulbecco's Modified Eagle's Medium - High glucose
- P3813 Phosphate Buffered Saline
- C1562 Freezing container, Nalgene, Mr. Frosty
- TR1003 Polybrene Infection/Transfection Reagent



Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway