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Pseudotyping lentiviral vectors for transduction of *Salmo salar* cells using glycoprotein from infectious salmon anaemia virus

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Genome science

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Abstract

Lentivirus is commonly used as vector for CRISPR applications. The common vector system utilises glycoproteins from the vesicular stomatitis virus (VSV-G). This vector has broad a tropism, including the salmonid species *Oncorhynchus tshawytscha* (Chinook salmon). However, the existing vectors are not able to transfect *Salmo salar* (Atlantic salmon), which is an economically important species.

In this work, the compatibility of lentivirus and *Salmo salar* cells were attempted improved by altering the transduction conditions of VSV-G based lentivirus and by pseudotyping using glycoproteins from the infectious salmon anaemia virus (ISAV). None of the pseudotyped vectors succeeded in transfection of the salmon cells, but the ISAV pseudotyped vector was able to transduce the control human cells, which was unexpected.

As no transduction of *Salmo salar* cells were achieved, an understanding of where in the transduction the vector falls short was attempted. The internalisation patterns were explored by labelling the virus and observing the infection using confocal microscopy. Reverse transcriptase activity was evaluated by qPCR, and translation and GFP expression were tested through electroporation. The results found in this work indicate transduction is terminated after endosomal uptake and before reverse transcriptase, possibly due lack of viral escape leading to the degradation of the vectors.

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Abbreviations

ASK Atlantic salmon kidney cell line

AMP Ampicillin

bp base pair

cDNA complementary Deoxyribonucleic acid

CRISPR Clustered regularly interspaced short palindromic repeats

Ct cycle threshold

DMEM Dulbecco's Modified Eagle Medium

DNA Deoxyribonucleic acid

EGFP Enhanced Green Fluorescence Protein

e.g. For example

FBS fetal bovine serum

F Fusion protein of ISAV

FPV fowl plague virus

GFP green fluorescent protein

HIV human immunodeficiency viruses

HEK Human embryonic kidney

HE Hemagglutinin Esterase

ISAV Infectious Salmon Anaemia Virus

kb Kilo Base

LB (broth) Luria-Bertani

LV Lentivirus

LDL-R low-density lipoprotein receptor

mRNA messenger ribonucleic acid

M2 Matrix protein 2

M1 Matrix protein 1

NK negative control

NA Neuraminidase

ORF open reading frame

PBS Phosphate Buffered Saline

pHE Plasmid with Hemagglutinin Esterase

pF Plasmid with Fusion protein

pSeg8 Plasmid with Segment 8

PCR Polymerase Chain Reaction

qPCR Quantitative Polymer Chain Reaction

P6 Protein 6

P7 Protein 7

RNA ribonucleic acid

RT reverse transcriptase

TAE Tris-Acetate-EDTA

VSV Vesicular Stomatitis Virus

VSV-G Vesicular Stomatitis Virus glycoprotein

Chapter 1. Introduction

1.1 Gene transfer

The transfer of genetic material into cells is an essential technique in modern molecular biology. DNA can be introduced to a cell by transfection (non-viral) using physical methods (e.g. electroporation) or chemical methods (e.g. lipofection) (Wang, Ma & Steinhoff, 2013). Alternatively, DNA can be introduced via transduction (viral) using various types of viruses (transduction) (Prince, 1998). Transient expression is non-permanent expression of transgene. Stable transgene expression usually involves the integration of the transgene into the genome of the organism. Viral and non-viral methods exist to achieve both states of gene expression (Prince, 1998). Stable transgene expression where the transgene is integrated into the host genome is more time-consuming than transient gene expression and usually involves a long process of selection after the initial transfection (Smith, 2013). Viral vectors such as lentiviruses can be highly efficient at delivering transgene, but viral gene transfer methods are further complicated by differences in viral tropism across species, due to variation in glycoprotein structure, host immune responses, and cellular environment (Thomas, Erhardt & Kay, 2003) . In this work, we attempt to alter the viral tropism of lentiviral vectors to infect salmon cells by pseudo typing with glycoproteins from Infectious Salmon Anaemia Virus (ISAV).

1.2 Viral tropism and cell entry.

Viral tropism refers to the virus's ability to infect cells. Viral tropism can be generalized into two categories, receptor dependent and receptor independent. The initial binding and internalization are receptor dependent and is decided by the glycoproteins ability to attach and interact with a recipient host cell receptor (Nomaguchi, Fujita, Miyazaki & Adachi, 2012). Viral glycoproteins are the spike proteins of which a virus bind to and interact with the hosts receptors. This attachment to receptors triggers conformational changes of the viral protein, which trigger internalisation (Dimitrov, 2004). There are two general pathways of which viral

entry occurs (**Figure 1**). The uptake via Endosomal entry/endocytosis (**Figure 1A**) (eg. clathrin mediated endocytosis), or the entry via non-endosomal pathway (**Figure 1B**) (Dimitrov. 2004). Viruses can target a specific host species or even a specific cell type (Ecotopic), or they may have a broad spectrum of potential hosts (Aamelfot, Dale & Falk, 2014). The receptor independent tropism involves the replication steps within the host cell (Nomaguchi Fujita, Miyazaki & Adachi, 2012). All steps of the viral replication are affected by a multitude of factors, both from the environment and the host (Staring, Raaben, & Brummelkamp, 2018).

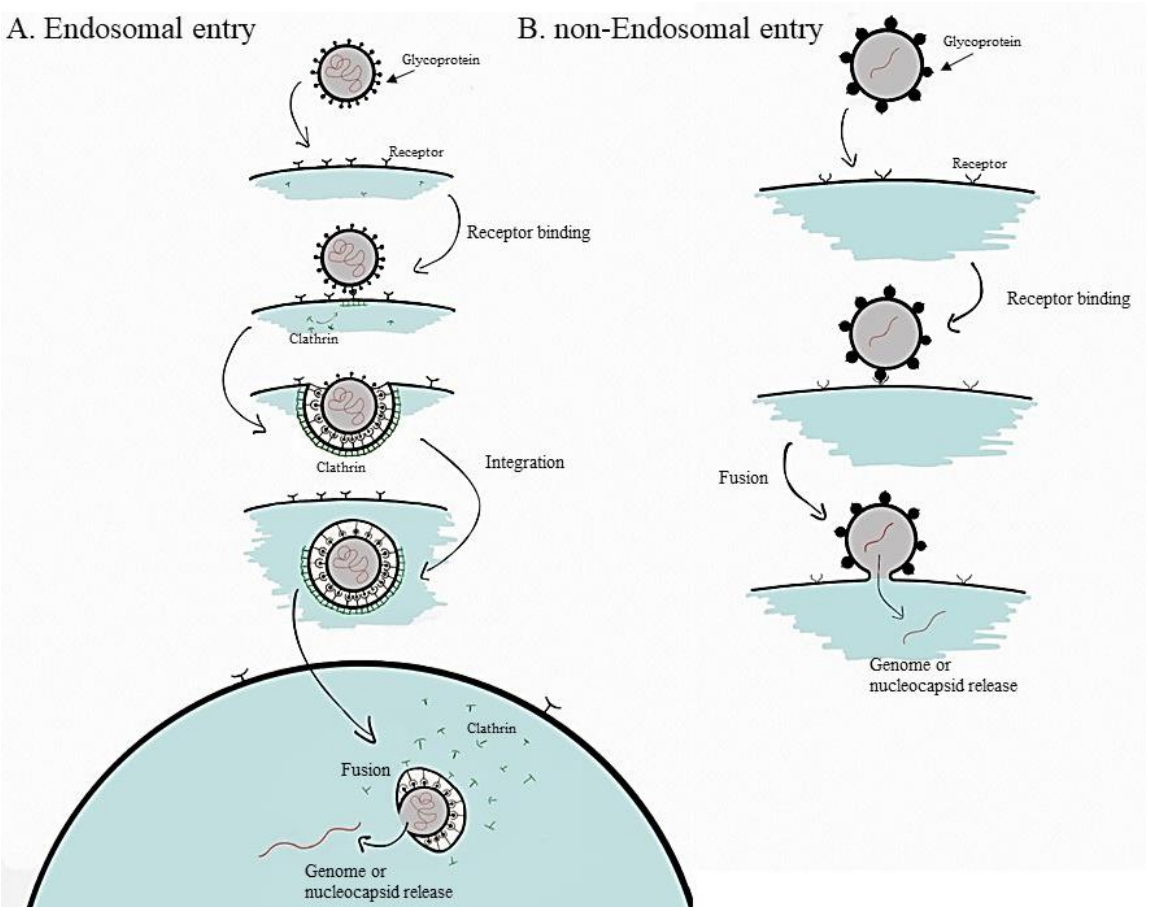

Figure 1: Endosomal entry is visualised in figure 1A, clathrin-mediated endocytosis has been used as example in this figure, but multiple viral methods of entry has been described. The virus binds to the cell surface receptor, which triggers the formation of endosomes. Once inside the endosome the virus uses different methods to exit, often triggered by the lowering of pH. In figure A the virus membrane fuse to the endosomal wall releasing the genome into the cytoplasm in one step, but different mechanisms are used for different viruses. Non-endosomal viral entry is visualized in figure B, where the fusion/penetration occurs at the cell surface at neutral pH. Viruses capable of non-endosomal entry can also enter through the endosomal pathway. Figure 1 is an interpretation of literature from multiple sources, but mostly Dimitrov (2004)

1.3 Lentivirus

Lentiviruses (LV) is in the genus retroviridae (reverse transcribing viruses) (Baltimore classification VII). Their genome is diploid and consists of two positive-sense single stranded RNA (Collins, Reuter, Rush & Villano, 2017). The Natural tropism of HIV is the CD4 cell (Klatzmann et al., 1984; Collins et al., 2017;). After receptor binding the viral particles fuse directly with the cell membrane, releasing the capsid into the cytoplasm (Wilen, Tilton & Doms. 2011). After internalization, viral RNA is reverse transcribed (RT) into DNA and integrated into the hosts genome, which cause persistent infection (Cockrell & Kafir. 2007). Where in the host cell the reverse transcription occurs has recently become the topic of debate. For a long time, it was believed that the virus was uncoated in the cytoplasm and only proviral DNA were transported into the cell nucleus, however increasing evidence have demonstrated intact or nearly intact HIV-1 capsid entering the nucleus (Selyutina et al., 2020; Shen et al., 2021).

1.3.1 Lentiviral vector

Most lentivirus-based vector system use the Human Immunodeficiency Virus (HIV) 1 and 2 reverse transcriptase and integration mechanisms (Collins et al., 2017). Lentiviruses are ideal vectors for gene transfer as they can package relatively large gene sequences, of up to 8.5 kB (Naldini et al., 1996; Collins et al., 2017; White et al., 2017). The virus and the vector are capable of infecting non-dividing cells (Lewis, Hensel & Emerman, 1992). When compared to other viral vectors, lentivirus is more efficient at transducing stem cells (Collins et al., 2017). The transgene is integrated permanently into the hosts genome, which provide sustained and heritable transgene expression (Blomer et al., 1997), this can both be positive and negative, depending on what the goal of the transduction is (Prince, 1998). The receptor dependent tropism can be altered by pseudotyping using numerous heterologous envelope glycoproteins (Dalgleish et al., 1984; Joglekar & Sandoval, 2017; Collins et al., 2017). As a tool it is utilised for targeted gene transfer into a wide spectrum of cells including cells not normally infected by the original virus (Cronin, Zhang & Reiser, 2005; McKay et al., 2006; Cockrell & Kafir, 2007). This process that can occur naturally during virus assembly in cells infected by two or more viruses, where a virus is assembled with different glycoproteins, and/or the sequence for the glycoprotein is packaged in the wrong viral particle (Závada, 1982).

The biosafety of the vector has improved through extensive engineering (Klatzmann et al., 1984; Cockrell & Kafri, 2007), and due to alterations the vector does only triggers a low immune response in the host (Naldini et al., 1996; Collins et al., 2017). There are now 3 generations of lentivirus vectors, with different modifications of the lentiviral genome with the goal of increasing the safety of the vector (Collins et al., 2017). In this work the 2nd generation was used, and therefore will be discussed further. In the 2nd generation lentiviral vector system, the genome is separated into three plasmids (Collins et al., 2017) (**Figure 2**), one transfer plasmid, one packaging plasmid, and one envelope plasmid. The transfer plasmid carries the transgene (**Figure 2**) (Collins et al., 2017). The viral promoter region in the 3' Long Terminal Repeat (LTR) of the transgene plasmid contains a deletion, which is during transduction transposed to the 5' LTR. This leaves the integrated genome replication incapable (Zufferey et al., 1998). Genetic elements necessary in the replication cycle, Gag (Group Antigens), Pol (Reverse transcriptase), Rev (Transactivating protein) and Tat (Trans-Activator of Transcription) are altered and separated onto different plasmids (**Figure 2**). Non-essential pathogenic genetic elements producing the proteins vpr, vpx and nef (**Figure 2**) are removed from the vector, these proteins have multiple functions in increasing the efficiency of the infection but are not required for viral reproduction (Cockrell & Kafri, 2007; Collins et al., 2017). The transgene promoter used in this work was the human cytomegalovirus (CMV) immediate- early promoter (Gruh et al., 2008), but other promotors are used to (Cockrell & Kafri, 2007).

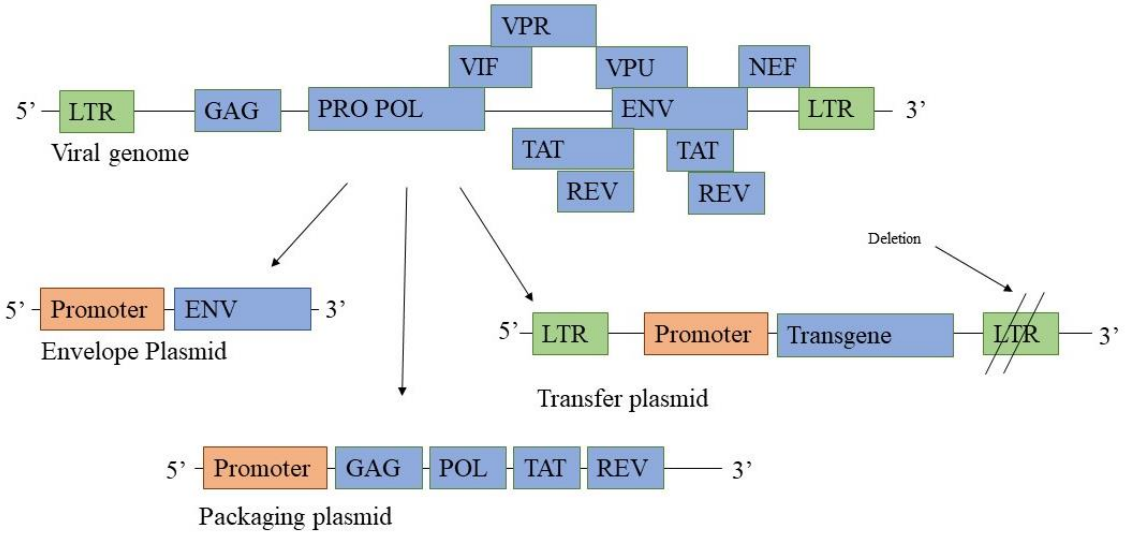

Figure 2: show the lentiviral genome, and the modifications of the 2nd generation lentiviral vector. The sequence is split up in three plasmids where the lentiviral vector contains deletions of the pathogenic genetic elements (vpr, vpx and nef) and the replication dependent genetic elements are separated to two plasmids. The third plasmid holds the transgene. In this wark the promoter was the human cytomegalovirus (CMV) immediate- early promoter. The viral promoter region of the U3 in the 3' Long Terminal Repeat (LTR) contains a deletion, which is during transduction transposed to the 5' LTR. This leaves the integrated genome replication incapable. Figure adapted from addgene lentiviral guide (Addgene, 2022) and Cockrell & Kafri (2007) review.

The 2nd generation lentiviral vector system (**Figure 2**) is pseudotyped by changing the protein coding sequence of the envelope plasmid to the sequence of glycoproteins derived from other viruses. The vector would then possess the natural tropism of the virus of which the glycoprotein was derived. The most common pseudotyped lentivirus used today utilizes the glycoproteins (G) from Vesicular Stomatitis Virus (VSV). It is preferred due to its high particle stability, allowing for concentration by ultracentrifugation (Burns et al., 1993) and broad tropism (Cronin, Zhang & Reiser, 2005).

1.3.1.1 VSV-G lentivirus system

Vesicular Stomatitis Virus (VSV) is a rhabdovirus, which is a rod- or bullet- shaped, enveloped RNA virus, with a single stranded, negative-sense, unsegmented genome (Letchworth, Rodiguez & Cbarrea, 1999). The glycoprotein is called G-protein, this viral glycoprotein is responsible for both attachment and membrane fusion (Sun et al., 2009). There are two proposed methods of VSV cell entry. The clathrin-mediated endosomal pathway (**Figure 1A**).

And the clathrin- associated two step fusion where VSV enters cells by exploiting a specific combination of clathrin- associated proteins and cellular factors (Johannsdottir et al., 2009). The subsequent fusion has been suggested to occurs in two successive steps. The first fusion occurs in the intermediate stage between early and late endosomes, probably releasing the nucleocapsid into the lumen of an intra-endosomal vesicle (Le Blanc et al., 2005; Gruenberg, 2009; Johannsdottir et al., 2009). The nucleocapsid remains hidden there until the late endosomes phase where it is transported into the cytoplasm after back-fusion of internal vesicle with the limiting membrane of late endosomes. The threshold for the conformational changes that triggers the fusion steps is pH 6.2 (Le Blanc et al., 2005; Gruenberg, 2009; Johannsdottir et al., 2009). Vesicular Stomatitis virus (VSV) infect both mammals and insects, and is believed to transmit via close contact (e.g. saliva, skin lesions) and/or via invertebrate vectors. Whether this vector is a mechanical or a biological is not well understood. The virus has been found to infect a broad spectrum of insects (Rozo-Lopez, Drolet & Londoño-Tenteria, 2018). Evidence shows that VSV-G protein binds to the CR domain of low-density lipoprotein receptor (LDL-R) receptors and other members of this family to initiate entry (Jovan et al., 2018; Nikolic et al., 2018). This receptor family have multiple roles in lipid metabolism and are found in both vertebrates and invertebrates (Willnow, 1999). This could explain why the virus and the pseudotyped lentiviral vector have a broad tropism over a range of species and cell types (Cronin, Zhang & Reiser, 2005).

1.3.2 Application of lentiviral vectors

Lentiviral vectors are commonly used for many CRISPR applications as the main delivery method for the CRISPR/Cas9 systems but are promising in clinical gene therapy applications too (Dong & Kantor, 2021). A CRISPR screen is large-scale loss of function experimental approach to identify and evaluate the role of a gene in functions and phenotypes, often in the context of identifying putative disease resistance genes in cell lines. A library of lentiviruses is produced from oligos designed to target a massive number of genes (Netanya, 2019). Lentiviral vectors are necessary in a CRISPR screens for the delivery of the complex system, due to its large loading capacity, and permanent integration of transgene (Dong & Kantor, 2021). CRISPR screens are limited in salmonoids as there lack techniques and tool. For CRISPR screens in salmonoid cells a pseudotyped variant of a lentiviral vector capable of infection of

salmon cells is needed specifically, although this tool would be useful for other gene delivery and editing applications.

1.4 Orthomyxovirus pseudotypes

The family orthomyxovirus, consist of the six generas. The influenza A, B & C virus, Thogotovirus, Quaranjavirus and Isavirus (Abdelwhab & Abdwl-Moneim, 2019), table 1 lists characteristics and compare the different members of the orthomyxovirus family.

Previous studies have successfully pseudotyped retroviruses using the glycoprotein of Fowl Plague Virus (FPV) influenza A, hemagglutinin (HA) (Hatziioannou et al., 1998; Bosch et al., 2001; Sandrin et al., 2002; McKay et al., 2006). The FPV pseudotype was found to infect a broad spectre of hosts, and in some cases enhance the fusion ability of the retroviral particle (Hatziioannou et al., 1998), the vectors produced were efficiently concentrated by ultracentrifugation (Sandrin et al., 2002), and the titers of FPV lentivirus were greatly increased when including all three membrane proteins neuraminidase (NA) and M2 (a proton channel) in the produced vector (McKay et al., 2006) (**Table 1**).

Table 1: Comparison of the members of the Orthomyxovirus. The proteins produced, although named the same, do not have the same sequence, and some of them are just based on limited homology to the infuenza genome. Proteins involved in the internal core: NP= Nucleoprotein, PB2= Polymerase, PB1 & PA= Polymerase acidic. Surface type I membrane glycoproteins: HA= Hemagglutinin, HE= Hemagglutinin esterase, GP involved in attachment, fusion and neutralisation. F= fusion protein is only found in ISAV. Non-glycosylated matrix protein M 1 & 2 function as proton-selective ion channels. Px= Putative protein nr X, NA= *Neuraminidase. Table made based on Orthomyxoviruses, Abdewhab & Abdel-Moneim. (2019).*

Genus	***Influenza A***	***Influenza B***	***Influenza C***	***Influenza D***	***Thogotovirus***	***Quaranjavirus***	***Isavirus***
NR of segments	8	8	7	7	6 or 7	6	8
Genes pr segment	1: PB2 2: PB1 3:PA 4: HA 5:NP 6:NA 7:M1 & M2 8: NS1 & NEP/NS2	1: PB2 2: PB1 3: PA 4: HA 5: NP 6: NA 7:M1 & M2 8: NS1 & NEP/NS2	1: PB2 2: PB1 3: PA 4: HE 5: NP 6: M1/ CM2 7: NS1/ NEP (NS2)	1: PB2 2: PB1 3: P3 4: HE 5: NP 6: P42 7: NS1/ NEP (NS2)	1: PB2 2: PB1 3: PA 4: GP 5: NP 6: M & ML 7: Unknown	1: PB2 2: PB1 3: PA 4: Unknown 5:GP 6: Unknown	1: PB2 2: PB1 3: NP 4: PA 5: F 6: HE 7: P4 & P5- nuclear export proteins 8: P6 & P7- matrix proteins(?)
Main host	Wild Aquatic bird	Humans and seals	Human, swine and dog	Swine and cattle	Ticks, mosquitoes, humans, + other mammal & waterfowl	Ticks and mosquitos	Farmed Atlantic salmon (wild Atlantic salmon and brown trout)
Note: table adapted from Orthomyxovirus, by Abdewhab & Abdel-Moneim. 2019, Chapter 19							

1.4.1 Infectious Salmon Anaemia Virus

ISAV (**Figure 3**) is an enveloped, aquatic, cold adapted, halophile virus which causes severe and slowly developing disease in Atlanic Salmon (*Samlo salar* L.), which appear to be systemic (Rimstad et al., 2011; Aamelfot, Dale & Falk, 2014; Cook, Sultana & Lee, 2017). It has a negative sense single stranded -RNA genome, which is subdivided into eight segments encoding at least ten proteins (Mjaaland et al., 1997; Rimstad et al., 2011). The envelope surface has projections of approximately 10-12nm, which are formed by two glycoproteins; hemagglutinin esterase (HE) and the fusion protein (F) (Falk et al., 1997) (**Figure 3**). The virus prefers lower temperatures, where no replication occurs at or above 25° C (Falk et al., 1997). (Rimstad et al.,2011).

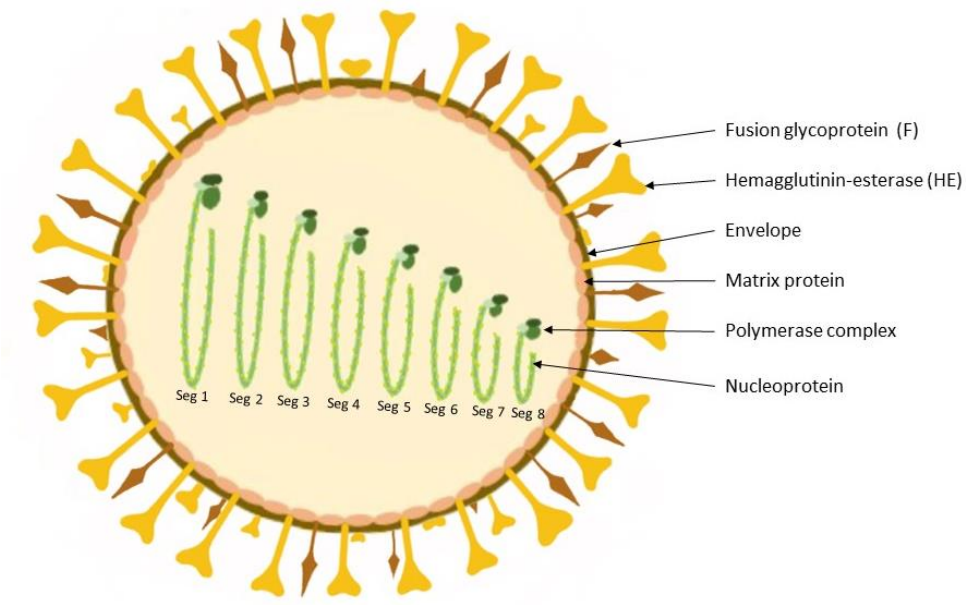

Figure 3: A simplified illustration of the ISAV. The genome is separated into 8 segments, segment 7 and 8 codes for two proteins each and the rest codes for one protein each. The segments are covered in Nucleoprotein. Figure based on existing figures of ISAV, mainly from Cottet et al. 2011's review.

Several findings indicate that the ISAV and the influenza virus are similar, in the structure of the viral particle and replication strategy (Rimstad & Mjaaland, 2002). Organization of the ISAV genome shares some levels of structural homology to influenza viruses (Zhang et al., 2017). Variations are usually related to the differences in the environment where replication occur, and the immune response of their respective animal hosts (Rimstad & Mjaaland, 2002). Since Influenza A has been used for successful pseudotyping of lentivirus (Hatziioannou et al., 1998; Bosch et al., 2001; Sandrin et al., 2002; McKay et al., 2006), ISAV was chosen for the salmonid vector pseudotype.

Although ISAV is closely related to the influenza virus, its mechanisms of cell entry are unique, regarding the hemagglutinin esterase (HE) structure and the fusion protein (F) (Cook, Sultana & Lee, 2017). ISAV are believed to have a M2 proton channel (Cottet et al., 2011), Whether or not ISAV have a M2 proton channel, its potential location in the genome or importance to the virus is however not fully understood, and the literature is lacking. The addition of M2 protein were found to increase the titer of produced virus in the FPV pseudotype (McKay et al., 2006).

1.4.1.1 Hemagglutinin esterase

Hemagglutinin esterase (HE) is encoded by the sixth segment of the eight-segmented ISAV genome (Müller et al. 2010; Aamelfot, Dale & Falk. 2014), (**Figure 3** & **Table 1**) (NCBI accession nr: NC_006499). It is one of the glycoproteins on the envelope surface and has both receptor binding and destroying properties (Müller et al. 2010). HE binds to the 4-O-acetylated sialic acid residues on the hosts cell surface which allows the virus to be endocytosed (Müller et al., 2010; Aamelfot, Dale & Falk, 2014). The HE exhibits receptor-destroying activities by d-O-acetylation of N-acetyl-4-O-acetylneuraminic acid, this activity is important for virus release (Müller et al., 2010). The glycoprotein is mainly hydrophobic, with no salt bridges (Cook, Sultana & Lee, 2017). The overall structure of HE is trimeric. It has three functional domains, the receptor binding domain (RDB), the esterase domain, and the stalk domain with dimensions of ~130x70x70 Å. The genome of ISAV exhibits relatively low recombination rate, though one region of the HE protein is highly polymorphic (Aamelfot et al., 2014), this region encodes for the stalk of the protein, near the transmembrane (Mjaaland et al., 2002: Müller et al., 2010). It has been hypothesized that this diversity is a result of recombination due to template switch (Castro-Nallar et al., 2011), or possible through deletions (Mjaaland et al., 2002). 24 different HE variants had been identified in 2009 (Kibenge et al., 2009). The polymorphic region has been shown to impact the virulence of the virus going from nonvirulent strains as HPR0, to highly virulent deadly strains (Müller et al., 2010; Mjaaland et al., 2002). The HPR0 variant although low- pathogenic is found in healthy Atlantic salmon (wild and farmed) and have been suggested to be the "wild type" form of ISAV (Christiansen et al., 2011).

1.4.1.2 Fusion protein

The other glycoprotein located on the envelope surface is the Fusion protein. It is the only protein encoded by segment 5 (Müller et al., 2010; Aamelfot, Dale & Falk, 2014) (**Figure 3**) (NCBI accession nr: NC_006500.2) and is responsible for the fusion of the endosome wall and viral membrane. The protein is synthesized as F0, a precursor protein, which is proteolytically cleaved to F1 and F2. F1 and F2 are held together by bisulfide bridges. Once cleaved the protein is in a metastable, fusion-activated state (Aspehaug et al., 2005). The fusion protein can be activated (proteolytically cleaved) by low pH (between 5.4 and 5.6), high temperatures, or high concentrations of urea (Aspehaug et al., 2005). ISAV is the only known example of an orthomyxovirus encoding a fusion protein (Aspehaug et al., 2005; Cook, Sultana & Lee, 2017).

Speculations have been made on F protein and HE protein interactions affecting the difference in virulence observed between the ISAV strains (Müller et al., 2011), though the literature is lacking.

1.4.1.3 Segment 8

Not much is known of the genes located in Segment 8 (**Figure 3**) (NCBI accession nr: NC_006497.1). There are two overlapping open reading frames (ORF- 1 & 2). ORF1 encodes the matrix protein (M1), also called Protein 6 (P6). ORF2 encodes an unknown protein also called Protein 7 (P7), possible a protein that binds single- and double stranded RNA and antagonize type 1 interferon response. This segment is thought to correspond with the influenza segment 7, where the ORF1 codes for the matrix protein (M1) and ORF2 encodes the M2 proton channel, there was however no homologue to the influenza virus ion channel protein identified in ISAV found by Olsen et al. (2016). There are multiple sources disagreeing in the topic of segment 8. However, the proton channel (M2) protein's function in influenza is to facilitate the acidification of the virion once inside the endosome (Pielak & Chou, 2010), and McKay et al. (2006) found that when including the influenza M2 protein in the FPV pseudotyped lentivirus, higher tier of vector was produced. Due to similarities, it is hypothesised that if the ISAV has a proton channel, and if it has then it probably will be in segment 8 and that it is probably ORF2, but the literature disagrees on this. This segment is included in this work as a secondary goal to see if the inclusion of Segment 8 genes results in a difference in titer as the addition of segment 7 made a difference in the FPV vector.

1.5 Lentivirus in fish cell lines

Lentiviral vector systems have huge potential, but their application in non-model organisms requires optimisation. The use of lentiviral vectors has been restricted to mainly model organism cell lines, and little is known about its usage in economically important animals like fish, more specifically in Atlantic salmon. The VSV-G lentivirus have been found to transduce, although at a low efficiency (pre optimization), zebrafish, with GFP and the CMV promoter (Fazio et al., 2017). According to Gratacap et al., (2020) some success was found when applying the 2nd generation lentiviral vector system with VSV-G to transduce Chinook salmon (*Oncorhynchus tshwaytcha*) cell lines (CHSE-214), with some optimisation (Gratacap et al.,

2020). The receptors (LDL-R family) (Jovan et al., 2018; Nikolic et al., 2018), is found in Atlantic salmon (Kleveland et al., 2006), but there are no evidence of transduction.

The cell lines chosen for this work was Atlantic salmon kidney (ASK) cells and human embryonic kidney (HEK) cell line 293T. Additionally salmon fibroblasts cells were tested with the pseudotyped lentiviruses. The HEK293T line was chosen due to its ease of grow, transfection and transduction, it is commonly used to grow lentivirus (Cockrell & Kafri, 2007). The HEK cells were used to compare to ASK cells, and also to grow the different lentivirus types produced. ISAV are commonly cultivated in ASK cells (Devold et al., 2000; Aamelfot et al., 2012), and was therefore chosen. The ISAV port of entry is through the gills, skin, eye, and the gastrointestinal tract, however more research is being done on this topic (Aamelfot, Dale & Falk, 2014). The ISAV variant used was not the low-virulence HEP0 variant (Christiansen et al. 2011).

1.6 Aims of study

This project was separated into two main parts. In part 1, were the VSV-G lentivirus was tested in Salmon cells (ASK and Fibroblats cells) in an attempt to understand properties of the VSV-G lentivirus and also to get an idea of what hinder the successful transduction by the VSV-G lentiviral vector in *Salmo salar*. And to achieve transduction by altering transduction conditions. In Part 2, the tropism of lentivirus was altered by pseudotyping lentivirus with glycoproteins from viruses known to infect salmonoids. In this work we used glycoproteins from Infectious Salmon Anaemia Virus (ISAV) to pseudotype lentiviral vecto

Chapter 2. Methods

2.1 Cell Lines

2.1.1 Atlantic Salmon Kidney cell line

The Atlantic Salmon Kidney (ASK) cell line (gifted from VET) were grown in a Leibovitz's L-15 media (L-15) (Gibco) with 20 % fetal bovine serum (FBS) (Sigma) and 1% penicillin streptomycin (Gibco) (mix referred to as L-15 media) in ventilated T75 cell culture flasks (Sarstedt). The cells were incubated in a 20°C, atmospheric condition incubator. The cells were split at about 90% confluency (once a week). When splitting the cells, they were washed with Phosphate Buffered Saline (PBS) (Sigma-Aldrich) (5 mL) twice before 0.05% trypsin (Gibco) -PBS (2 mL) (Sigma-Aldrich) was used to detach the cells, with a 5 min incubation. Fresh L-15 media (Gibco) was added, to neutralise the trypsin (Gibco), and the cells were grown in a total volume of 10 mL media. Media was changed two times a week. Cell work was done inside microbiological safety cabinets (Kojair). When the cells were splitted in a decided concentration, 10 µL of the cell suspension were taken out for cell counting. 10 µL typhan blue dye (Gibco) were mixed in and 10 µL of the dyed cell mix was added into the groove of the cell counter plate (Invitrogen), the slide was then inserted into the Countess cell counter (Invitrogen). The Countess (Invitrogen) would do the concentration calculations and provide how much cell suspension and L-15 media (Gibco) would be needed to get the desired cell concentration.

2.1.2 Salmon Fibroblast cell line

The Salmon Fibroblast cell line, a gift from Prabin. S. Humagain, where grown in a conditioned L-15 media (Gibco) with 20% FBS (Sigma) and 1 % penicillin streptomycin (Gibco) (mix referred to as conditioned L-15 media) in a ventilated T75 cell culture flasks (Sarstedt). The cells were kept in a 20°C incubator with atmospheric conditions. The cells were split at about 90% confluency (once a week). The condition L-15 media (Gibco) was made from 50% old media and 50% fresh media, filtered through a 0.2 µM filter (Sarstedt) using a 5 mL syringe.

When splitting the cells, they were first washed with PBS (5 mL) (Sigma-Aldrich) twice before 0.05% Trypsin (Gibco) -PBS (2mL) (Sigma-Aldrich) was used to detach the cells, with a 5 min incubation at room temperature. Conditioned L-15 cell media were used to neutralise the trypsin, and the cells were grown in a volume of 10 mL Condition L-15 media. Media were changed two times a week. Cell work was done inside microbiological safety cabinets (Kojair). When the cells were splitted in a decided concentration, 10 µL of the cell suspension were taken out for cell counting. 10 µL typhan blue dye (Gibco) were mixed in and 10 µL of the dyed cell mix was added into the groove of the cell counter plate (Invitrogen), the slide was then inserted into the Countess cell counter (Invitrogen). The Countess (Invitrogen) would do the concentration calculations and provide how much cell suspension and L-15 media (Gibco) would be needed to get the desired cell concentration.

2.1.3 Human Embryonic Kidney cell line

The Human Embryonic Kidney cells (HEK293T) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich), with 10% FBS (Sigma), 1% penicillin streptomycin (Gibco) and 1% 1x glutamine (Gibco) (mix referred to as DMEM media) in ventilated T75 cell culture flasks (Sarstedt). The cells were kept in a 37°C, 5% CO_2 incubator. The cells were split at about 70-90% confluency (every 2nd day). When splitting the cells, they were washed with PBS (Sigma-Aldrich) (5 mL) twice before 10% Trypsin (Gibco)-PBS (Sigma-Aldrich) (3 mL) was used to detach the cells, with a 2 min incubation at room temperature. Fresh DMEM media (Sigma-Aldrich) (10% FBS (sigma), 1% PS (Thermofisher) 1% 10X glutamine (Gibco)) was added, which neutralise the trypsin, and the cells were grown in a total volume of 10 mL DMEM (Sigma) media. Cell work was done inside microbiological safety cabinets (Kojair).
When the cells were split in a decided concentration, 10 µL of the cell suspension were taken out for cell counting. 10 µL typhan blue dye (Gibco) were mixed in with the cells. 10 µL of the dyed cell mix was added into the groove of the cell counter plate (Bio-Rad), the slide was then inserted into the TC-20 Automated cell counter (Bio-Rad). Calculations were then done to identify the needed volume of cell suspension to separate it from the bulk suspension. This was done to achieve the desired number of cells.

Formula used: $C1 \cdot V1 = C2 \cdot V2$

2.2 Part 1: VSV-G lentivirus

2.2.1 Lentivirus temperature transduction experiment

To identify the Lowest temperature HEK cells would express GFP after CMV GFP lentivirus transduction a temperature experiment was conducted. The day prior to the experiment, cells were split (as described in 2.1.3) and seeded in two, six welled plates (Sarstedt) (plates called for now: HEK37 and HEK22), $5x10^5$ cells pr well were seeded in 2 mL DMEN media (Sigma-Aldrich). The day of transduction the media in both plates was changed to fresh DMEM media (Sigma-Aldrich) (2 mL) with 8 µg/mL Polybrene (Sigma-Aldrich). Before Lentivirus was introduced, the HEK22 plate was cooled down to 20° C. The virus was added in volumes of 0 µL, 25 µL, 50 µL, 100 µL and 200 µL to both plates. HEK22 was incubated at 22° C (due to restrictions with the incubator 20° C was not an option) and HEK37 was incubated at 37° C, both with 5% CO_2. The temperature of HEK22 plate were then increased with 2° C, every ~24 h, up to 36° C. To see if temperature affected the GFP expression both experimental plate and control plate were both moved to 22° C for 60 h. Changes in GFP were looked for with a fluorescent microscope (Microscope: EVOS M5000 (Invitrogen), objective: 10X (EVOS_AMEP4981), Light source intensity: GFP: 12.586, Trans: 100, emission wavelength: GFP 510nm, Trans 447nm, Exposure time 20ms, Contrast 0.33, brightness 0.5 (contras and brightness were also adjusted during image editing)). Cell work and lentivirus handling was done inside microbiological safety cabinets (Kojair).

2.2.2 Lentivirus labelling experiment

The virus and cells were labelled, and the infection pattern were observed in a confocal microscope as follows.

2.2.2.1 Optimization and testing of labelling protocol: HEK

2.2.2.1.1 Virus labelling protocol:

The virus was stained with SYTO 82 fluorescent stain (Invitrogen), to see the nucleic acid of the virus. The supernatant was removed by centrifugation through a 50 kD cutoff column (Amicon) at 3000 xg, 15-30 min. For washing, 4 mL HBSS (Gibco) were used to resuspend the particles,

the virus was spun down at 3000 xg, 15-30 min, and the flowthrough was discarded. The washing was repeated two times. After three washes HBSS (Gibco) were used to adjust the total volume to 1 mL and the viral particles were resuspended. 10µM (2 µL) SYTO82 stain (Invitrogen) was used, and the solutions were mixed well by pipetting, then incubated 15 min protected from light at room temperature. After incubation the supernatant was centrifuged through the 50kD cutoff column (Amicon) to remove the stain, then the washing step were repeated twice. Virus resuspended in 4 mL HBSS (Gibco), centrifuged through the 50kD cutoff column (Amicon) at 3000 xg, 15-30 min, and flowthrough discarded. This was done to remove all SYTO 82 stain (Invitrogen). After washing the stained virus could be stored at 4° C overnight. Lentivirus handling was done inside microbiological safety cabinets (Kojair).

2.2.2.1.2 Cell labelling protocol:

The cells were stained with different staining solutions, Hoechst (Invitrogen) (4 µg/mL) which stained the nucleus, CFDA (Invitrogen) (10 µM) which stained the cytoplasm, and CellMask (Invitrogen) (2.5 µL) that stained the cell membrane. Hoechst (Invitrogen) and CFDA (Invitrogen) were tested but excluded from all further experiments due to Hoechst (Invitrogen) weak stain and CFDA (Invitrogen) interfering with the GFP signals. The staining solution was made from 1000x stock solution and diluted in PBS (Sigma-Aldrich), 500 µL pr dish (35mm, Sarstedt) to be stained. Old media was removed from the cells, washed 3x times with cell media (DMEM or L-15) before 500 µL staining solution were added and the plates incubated for 10 min protected from light. The staining solution was then removed. The cells were washed 3x times with cell media, before 2.3 µL cell media were added to the cell dish (35mm, Sarstedt).

2.2.2.1.3 Detection protocol:

For the detection of labelled cells and viral vector a confocal microscope (Carl Zeiss) was used. The lasers used for CellMask (Invitrogen) detection had a wavelength of 637nm, for the detection of SYTO82 (Invitrogen) the wavelength was 532nm, and GFP were detected with a laser at 488nm.

2.2.2.1.4 Labelling Experiment: HEK

HEK cells were split and 5 dishes with $3.5x10^7$ (in 2 mL) cells were seeded in 35 mm dishes (Sarstedt) the day before and incubated at 37° C, 5% CO_2. Virus was stained according to the Virus labelling protocol (section: 2.2.2.1.1). Cells were labelled with Hoechst (Invitrogen) and

CFDA (Invitrogen) according to the cell labelling protocol (section 2.2.2.1.2). The cells were studied in a confocal microscope (Carl Zeiss) before introduction of stained lentivirus. 50 µL virus were added to the 35 mm dish (Sarstedt) and again studied under the confocal microscope (Carl Zeiss) according to the detection protocol (section 2.2.2.1.3).

2.2.2.2 Labelling experiment: HEK and ASK

HEK cells were split and 0.7 $x10^6$ cells (in 2 mL) were seeded to 35 mm dishes (Sarstedt), 5 dishes were prepared. ASK cells were split and $2.52x10^5$ cells in 2 mL were seeded to 35 mm (Sarstedt), two dishes (35 mm, Sarstedt) were prepared. The cells were incubated at optimal conditions overnight. The virus was stained according to the virus labelling protocol (section 2.2.2.1.1). The cell staining solution were made with Hoechst (Invitrogen) and CellMask (Invitrogen), and the staining were preformed according to the cell labelling protocol (section 2.2.2.1.2). The HEK cells were studied in a confocal microscope before introduction of stained lentivirus. 50 µL virus was added to the dish (35 mm, Sarstedt) and again studied under the confocal microscope (detection protocol in section 2.2.2.1.3). Then the ASK cells were stained with the same staining solution (double amount of stain) and studied in a confocal microscope before introduction of stained lentivirus. 50 µL virus were added to the dish (35 mm, Sarstedt) and again studied under the confocal microscope (Carl Zeiss) (detection protocol in section 2.2.2.1.3). Both the HEK cells and the ASK cells were fixated using 3% formaldehyde (Sigma-Aldrich).
Formaldehyde fixations were preformed where cells were washed with tris-buffered-saline (TBS) (Sigma-Aldrich), then 1mL of 3% paraformaldehyde (PFA) (Sigma-Aldrich) were added. The plates were incubated for 5 min, washed with TBS (Sigma-Aldrich). A mounting medium (Southern Biotech) with coverslip (Menzel) were used to seal the samples in the 35 mm dish (Sarstedt), and it were left to dry for 2h.

One dish (35 mm, Sarstedt) of ASK cells and one dish (35 mm, Sarstedt) of HEK cells was infected with 400 µL labelled virus and incubated for three days. The HEK cells were stained with 2.5 ug/mL CellMask (Invitrogen) and the ASK cells were stained with 5 ug/mL CellMask (Invitrogen), then the cells were studied in a confocal microscope (Carl Zeiss). The ASK cells were kept for a week and studied in a confocal microscope (Carl Zeiss) at day 6 post transduction. The HEK cells were fixed at day 3 post transduction using 3% formaldehyde (Sigma-Aldrich) (protocol described above).

2.2.3 Electroporation experiment: GFP lentiviral transfer plasmid

To test if the salmon cells were capable of producing GFP upon a successful infection, ASK and HEK293T cells were transfected by electroporation using the lentiviral transfer plasmid (Addgene #17448). Neon^{TM} Transfection System (Invitrogen) was used according to manufacturer's specifications, the 10 µL cell suspension protocol was used. Three wells per cell line were prepared (six welled plate, Sarstedt), one well with a negative control and two wells of transfected cells, each containing 2 mL with a cell concentration of 2 x10^5. The ASK and HEK cells were spit, and cells moved to a 20 mL tube (Sarstedt) and pelleted by centrifugation at 300 xg for 5 min, supernatant removed. The two cell lines were then resuspended in 1 mL PBS (without Ca^{2+} and Mg^{2+}) (Sigma-Aldrich). The cells were centrifuged again to remove the PBS (Sigma-Aldrich) at 300 xg for 5 min and resuspended in 35 µL Buffer R (Invitrogen, Neon Transfection kit,) (10 µL for each well and 5 µL extra). 7 µg lentiviral GFP expressing transfer plasmid (addgene #17448) was added to the cell suspensions (2 µg pr 10 µL Buffer R). 10 µL cell suspension was transduced for each well. The settings on the neon transfection devise (Invitrogen) were Voltage: 1400V, width: 20ms, Pulses: 2. These settings had been tested and optimised for fish cell lines prior by the other researchers at the CIGENE lab. The cells were seeded and incubated, HEK cells in 37° C, 5% CO_2, ASK cells in 20° C, atmospheric conditions. After 24h the cells were studied in a fluorescent microscope (Microscope: EVOS M5000 (Invitrogen)), Objective: 10X (Evos_AMEP4981), Light source intensity: GFP 12.586, Trans 21.489, Exposure time 20ms, Contrast: 0.333, Emission wavelength: GFP 510nm, Trans: 44nm, Brightness 0.5).

2.2.4 pH and temperature transduction experiment: VSV-G Lentivirus on ASK cells

Due to the VSV-G lentivirus apparent ability to enter the endosomal pathway in ASK cells, higher temperature (Gratacap et al. 2020) was tested as this had been found to increase the transduction rate. The lowering of pH was also tested as fusion is a pH sensitive process.

2.2.4.1 Temperature experiment: ASK

As the results from HEK temperature experiment where no GFP were expressed lower than 26° C, this experiment was repeated using ASK cells. ASK cells were split and $5x10^5$ (in 2 mL) were seeded to three wells in a six welled plate (Sarstedt). First the temperature of ASK cells were tested to see if they would survive at 28° C. A plate of ASK were acclimatised to room temperature (from 20° C), then slowly the temperature were increased up to 26° C, over a period of 1h. The cells were incubated overnight and checked, the temp was increased with 2° C every day until the cells started dying at 32° C. The cells appeared to be fine at 28° C and 30° C. ASK cells and fibroblast cells were spitted and $5x10^5$ cells (in 2 mL) were seeded (three wells each) in a six welled plate (Sarstedt). 300 µL lentivirus were added. The cells were acclimatised to 26° C over a period of 1 h then every 24 h the temperature was increased with 1° C every 24 h, up to 30° C.

2.2.4.2 pH effect on transduction: HEK, ASK and fibroblast

pH effect of HEK, ASK and fibroblast were tested. The pH was lowered to pH 5 during transduction of the cells. This was found to be the lowest pH cells would survive after 1 h incubation. The pH of the media was lowered by mixing media with HCL (Sigma-Aldrich) and NaOH (Sigma-Aldrich) to get to the desired pH. All cell lines had the same experimental upset with 1 negative control, one BL (acidified before lentiviral introduction) well, and one AL (acidified after lentiviral introduction) well. The BL wells were incubated for 1 h with lentivirus and pH 5 media, then the media was changed back to normal media (pH 7). The AF wells were incubated for 1h with lentivirus and normal cell media (pH 7), then the media were changed to pH5 for 1h and back to normal media (pH 7). The next day the cells were checked for GFP expression by fluorescent microscopy (microscope: Imager.Z2 (ZEISS), objective: 10X, exposure:366).

2.2.4.3 Combined effect of pH and temperature on ASK

The combined effect of low pH and higher temperature were tested in ASK and fibroblast cell. $5x10^6$ ASK and fibroblast cells were seeded (in 2 mL) the day before in a 6 welled plate (Sarstedt). The media was changed in one well to pH 5, then 500 µL VSV-G lentivirus were added to the cells, after 1 h of incubation at 20° C the media was changed back to pH 7 and the

cells were incubated at 20° C for 24 h, the cells were monitored for GFP expression and temperature were increased with 2° C, every day up to 30° C. Then cells were moved back down to 20° C.

2.2.5 Reverse transcriptase activity

Reverse transcriptase activity was tested for by a qPCR. Two T25 flasks (Sarstedt) with ASK cells (-virus and +virus) were seeded two days before the experiment (**Figure 4**), with a cell concentration of $6x10^5$ (in 10 mL). Two T25 flasks (Sarstedt) with HEK cells (-virus and +virus) were seeded the day before the experiment, with a concentration of $6x10^5$ (in 10 mL). HEK cells grow faster than the ASK cells therefore they were allowed less growth time. 500 µL GFP carrying Lentivirus were added to one of the ASK Flasks (ASK+) and one of the HEK flasks (HEK+), and then incubated with the virus overnight. The cells were washed three times with 3 mL PBS (Sigma-Aldrich) before each flask were split into two samples, one for DNA extraction and one for RNA extraction (**Figure 4**).

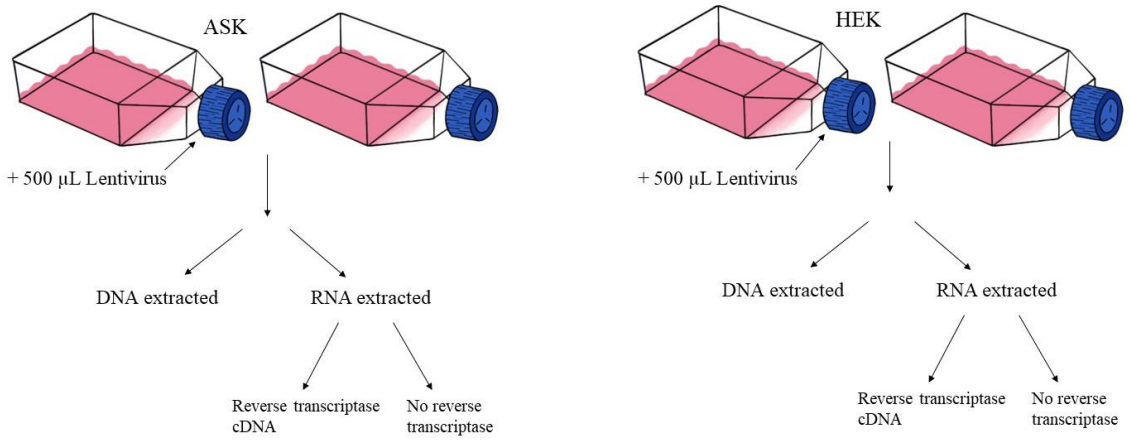

Figure 4: A qPCR was done to look for reverse transcriptase activity, this is an overview of how the samples were treatment. The result was 12 different samples. DNA, RNA and cDNA of ASK cells with and without virus, and DNA, RNA and cDNA samples of HEK with and without virus.

DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen), according to Purification of total DNA from Animal blood or Cells, Spin-Column Protocol. RNA was extracted using the RNeasy Plus Mini Kit (Qiagen), according to the Purification of Total RNA from Animal

Cells, DNase was included to remove potential DNA. The RNA samples were spilt in two, and one of the two RNA samples were used as a template for cDNA with the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's specifications.

The PCR primers were designed in benchling (Benchling.com, 2022) and ordered from Thermofisher and are listed in table 2. These were tested and optimised with a gradient PCR to identify the best annealing temperature. The kit used was SsoAdvansed Universal SYBR Green Supermix (Bio-Rad), according to manufacturer's specifications. The gradient covered 50° C to 65° C (**Table 2**). The primers tested targeted the sequences of GFP and AMP (ampicillin resistance) from the pMD2.G plasmid (addgene #12259), the salmon housekeeping genes EF1a and EF1b, and the human housekeeping gene GAPHD (**Table 2**). A master mix were prepared with 1µM of each primer (forward and reversed), 5 µL SYBR Green supermix (Bio-Rad) and nuclease free water. The template which are listed in table 2 was then added to the respective wells at a concentration of 5 ng/µL in a 10 µL reaction. The thermal cycler (Applied Biosystems) was programmed according to table 2.

Table 2: show the gene targeted by the primers and which sample were used for the optimization. The temperature gradient was chosen based on estimations made from sequence length and how rich C & G the primer was.

Gene	*Sample*	*PCR Gradient*				
EF1A	ASK -virus DNA	50.0° C 51.0° C 53.0° C 55.9° C 59.5° C 62.5° C 64.1° C 65.0° C	98° C 98° C Gradient 72° C 72° C 4° C	30 sec 8 sec 15 sec 25 sec 7.5 sec ∞	Initial denaturation Denaturation Annealing Extension Final extension Hold	X 30
EF1B	ASK -virus DNA					
GFP	HEK +virus DNA					
AMP	Plasmid					
GAPHD	HEK -virus DNA					

The optimal temperatures were determined to be 55.9 ° C for GFP and GAPHD, and 62.5° C for EF1A and AMP, based on the melt curve and the Ct values. EF1B was excluded due to the melting curve having multiple peaks.

Two plates (Bio-Rad) were set up for the different temperatures. The reactions with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was set up according to manufacturer's instructions. A master mix was prepared for each of the primers with 1µM of each primer (forward and reverse), 5 µL SYBR Green supermix and nuclease free water (to adjust the reaction size to 10 µL), this was then added to each well (that were going to have primers). The template was added to the respective wells at a concentration of 5 ng/µL. 4 replicate samples were made for each gene and sample combination, 3 negative controls (no template) for each primer and 3 positive controls for the plasmid were included. Two replicate standard curve for each primer were done, the plate setup and PCR condition is shown in table 3. The plates were sealed (Bio-Rad) and the thermal cycler (Bio-Rad) were programmed according to table 3.

Table 3: There were made two plates. One plate with the annealing temperature of 55.9° C for GFP and GAPDH and one with the annealing temperature of 62.5° C for AMP and EF1A. These temperatures were chosen based on the PCR gradient.

55.9° C	***+ Virus (lentivirus)***						***- Virus (negative control)***						***qPCR conditions***
	RT (cDNA)		No RT (RNA)		DNA		RT (cDNA)		No RT (RNA)		DNA		
GFP HEK													Initial denaturation: 98.0° C 30 sec Denaturation: 98.0° C 8 sec Annealing: 55.9° C 15 sec Extension: 72.0° C 25 sec Final extension: 72.0° C 7.5 sec Hold: 4.0° C ∞ X35
GAPDH HEK													
GFP ASK													
Std curves	HEK DNA GFP std curve						GAPDH std curve						
	HEK DNA GFP std curve						GAPDH std curve						
62.5° C	***+ Virus (lentivirus)***						***- Virus (negative control)***						***qPCR conditions***
	RT (cDNA)		No RT (RNA)		DNA		RT (cDNA)		No RT (RNA)		DNA		
EF1A ASK													Initial denaturation: 98.0° C 30 sec Denaturation: 98.0° C 8 sec Annealing: 62.5° C 15 sec Extension: 72.0° C 25 sec Final extension: 72.0° C 7.5 sec Hold: 4.0° C ∞ X35
AMP HEK							Plenti cmv GFP puro Positive control (plasmid) GFP		Plenti cmv GFP puro Positive control (plasmid) AMP				
							Negative control GFP ASK		Negative control GFP HEK				
AMP ASK							Negative control AMP ASK		Negative control AMP HEK				
							Negative control GAPDH HEK		Negative control EF1A ASK				
Std curves	ASK EF1A std curve						AMP std curve						
	ASK EF1A std curve						AMP std curve						

Delta Ct values were calculated using the reference gene (EF1A and GAPDH) and the target gene (AMP and GFP) according to the formula below.

$$Expression = 2^{Ct(reference) - Ct(target)}$$

The Delta Ct values were then used to produce boxplots using RStudio (R Core Team. 2022). No statistical analysis was done on the results, as the goal were to identify GFP or no GFP.

Table 4: The primers made for the qPCR experiment were made in Benchling (Benchling. 2022) and ordered from Thermofisher.

Primer	*Forward*	*Reverse*
GFP	CAACAGCCACAACGTCTATATCAT	ATGTTGTGGCGGATCTTGAAG
AMP	ACTCGCCTTGATCGTTGGG	GTTGCCATTGCTACAGGCATC
EF1a	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA
EF1b	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG
GAPDH	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTAGTAAA

2.3 Part 2: Lentivirus production

2.3.1 Plasmid production: Restriction cloning

2.3.1.1 Primer design

The PCR primers for restriction cloning were designed in benchling (Benchling. 2022) and ordered from Thermofisher, they are listed in Table 5. A cut site was designed into the PCR primer which correspond with the restriction enzymes PmlI (NEB) and StuI (NEB). The restriction enzyme was chosen on the basis of cutting the plasmid flanking the VSV-G insert, and not within the HE/F/P6/P7 protein sequence. The enzymes identified produced blunt end cuts.

The virus genome was extracted and used as a template for cDNA. Two methods of RNA extractions were tested, Phenol/Chloroform and PureLink^{TM} Viral RNA/DNA Mini Kit (Invitrogen/ ThermoFisher).

2.3.1.2 Phenol/Chloroform protocol for RNA extracted:

The Phenol/Chloroform protocol for RNA was not based on a kit but were based on Toni et al. (2018) ‘s protocol. 1 mL sample to 3 mL QiAzol (Qiagen) were mixed thorough by pipetting up and down, then incubated at room temperature for 5 min. 800 µL chloroform (Sigma-Aldrich) were added to the tube and the mixture vortexed at max setting for 15 sec, then incubated at room temp for 3 more min. The mixture was centrifuged at 12000 xg for 15 min at 4° C, the sample was separated by a yellowish-whiteish interphase band, and the RNA was

in the upper aqueous phase. This were transferred to a new tube with 800 µL fresh chloroform (Sigma-Aldrich). The mix were vortexed at max setting for 15 sec, incubated at room temp, and centrifuged at 12000 xg for 5 min at 4° C. The upper aqueous phase was transferred to a new tube 2 mL (Sarstedt) containing isopropanol (Sigma-Aldrich). The tube was mixed by inversion (10-20 times) and incubated for 10 min at room temp. The RNA was pelleted by centrifugation at 12000 xg for 10 min at 4° C. The supernatant was discarded, carefully not to disturb the pelleted RNA. 4 mL 75% ethanol (Sigma-Aldrich) were added to the pellet and centrifuged at 7500 xg for 5 min at 4° C. Supernatant was discarded, carefully not to disturb the pellet. The pellet was washed two more times with 75% ethanol (Sigma-Aldrich). After supernatant were removed the tube were pulse spun at room temp before the residue were discarded. The pellet was air dried for 3-5 min at room temp, then the uncapped tube was heated to 65° C for 2-5 min on a thermomixer (Eppendorf), no shaking. The pellet was resuspended in water by pipetting up and down, incubated at 65° C for 2-5 min on a thermomixer (Eppendorf), no shaking, and vortexed for 10 sec, placed on ice and a nanodrop (Thermo Scientific) were used to measure the concentration. The RNA was stored at -80° C.

2.3.1.3 cDAN and PCR amplification

cDNA was produced with the iScript™ cDNA Synthesis Kit (Bio-Rad), according to manufacturer's protocol. The cDNA was amplified by PCR with the Phusion™ High Fidelity DNA polymerase kit (Thermofisher) according to the manufacturer's instructions. The PCR was optimized through a gradient of temperatures (**Figure 5**). The thermal Cycler used was Veriti™ 96-Well (Thermofisher) and the samples went through 35 cycles of Denaturation, annealing and extension. PCR primers are listed in Table 5.

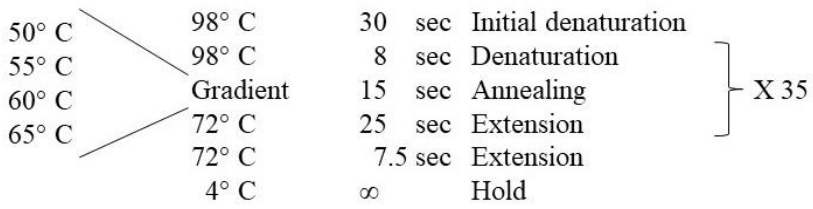

Figure 5: The PCR primers were tested with a gradient to identify the optimal annealing temperature across the genes. The temperatures were chosen based on predictions made with the C/G content and length of the primers, primers are listed in table 5.

Table 5: The PCR primers design for the restriction cloning. The restriction enzyme cut sites were inserted on the flanking side indicated by red letters (CAC/ /GTG the cut site of StuI, AGG//CCT the cut site of PmlI).

PCR Primers	*Forward* 5' end ⟶ 3'end	*Reverse* 3' end ⟶ 5'end
Hemagglutinin-esterase (HE)	CGGTCACGTGATGGCACGATTCATAATTTTATTCCTACTG	GTCGAGGCCTTCAAGCAACAGACAGATTTGCAGG
Fusion protein (F)	AGATCACGTGATGGCTTTTCTAACAATTTTAGTCTTG	CGCTAGGCCTTCACCTTCTAAGACATCCCCATAG
P6	AGAGCACGTG ATGCATGAGAGAAGCAAAC	GCTAAGGCCTTTATTGTACAGAGTCTTCCAATTTGTCG
P7	CGGACACGTGTGAGAGAAGCAAACCCAAAACCAC	TCGAAGGCCTTTACTTCAGGTACCCCAGAAGCAC

2.3.1.4 Restriction and clean up

The PCR products were run through a 1% agarose gel prepared using 1g Agarose (Sigma-Aldrich) in 100 mL TAE (ThermoScience) and 1 µL red safe (Chembio), a wide comb was used for the wells. The ladder used was Gene Ruler 1 kb (ThermoScience) and 5 µL were loaded to the first well. The loading dye used was purple (6x) (NEB) B70245, and 1 µL loading dye (NEB) were mixed with every 5 µL sample. The gel was run on a volage of 100V for 50 min (electrophoresis machine: Bio-Rad) bands could be identified at the expected locations when molecular imager were used to take pictures (Bio-Rad). The desired size fragments were cut out of the gel and purified using the QlAquick® Gel Extraction Kit (Qaigen).

The plasmids and insert DNA (PCR product) was restricted using the restriction enzyme PmlI (NEB) and StuI (NEB) in 100µ reactions. The reactions were set up on ice. About 200 ng DNA, 10 µL rCutSmart Buffer (NEB) and 1 U/mL of each enzyme were mixed with nuclease free water to adjust the reaction volume to 100 µL. For the plasmid restriction, 10 µg plasmid, 10 µL rCutSmart Buffer and 2 U/mL of each enzyme were mixed with nuclease free water to adjust the reaction volume to 100 µL. Both reactions were incubated overnight at 37° C, and heat inactivated at 65° C for 20 min. Incubation were performed on a thermal mixer, no shake (Eppendorf).

The digested DNA were purified by QIAquik PCR purification Kit (Qaigen), according to manufactures specifications. The empty backbone was dephosphorylated by Antarctic phosphatase (NEB). 500 ng/µL empty backbone were mixed with 4 µL Antarctic Phosphate Reaction Buffer (NEB) and 0.5 µL Antarctic phosphatase (NEB) on ice. Nuclease free water were used to adjust the reaction volume to 40 µL. The mix were incubated at 37° C for 30 min,

and reaction were heat-inactivated at 80° C for 2 min. Incubation were performed on a thermal mixer (Eppendorf).

2.3.1.5 Plasmid assembly

The empty backbone and the insert were ligated together with the ratios 1:1, 1:3 and 1:5, Backbone: Insert. The amounts of insert needed for 20 ng backbone in the determined ratios were calculated with NEBioCalculator (New England BioLab Inc. (a.s)). T4 DNA Ligase (NEB) were used for the ligation, and the 20 µL reactions were prepared on ice. 2 µL T4 DNA ligase buffer (NEB), 20 ng empty backbone and insert DNA according to ratio calculations ((New England BioLab Inc. (a.s)) were mixed with T4 DNA ligase 10x (NEB), gently mixed by pipetting and microfuge briefly. The mix were incubated at 16° C overnight in a thermal mixer (Eppendorf, no shake), and heat activated at 65° C for 10 min in the thermal mixer (Eppendorf, no shake).

2.3.1.6 Transform Stbl3 Chemically competent E.coli

Stbl3 Chemically competent E. coli (Invitrogen) were transformed using the different ratio of assembled plasmids, one vile for each plasmid. The *E.coli* were thawed on ice for 10 min, 5 µL plasmid were added to each vile, then the vials were incubated for 30 min on ice. Heat-shock was utilized to make the membrane permeable for the plasmids, this was done in a thermomixer (Eppendorf, no shake) at 42° C for 45 sec, the *E.coli* was incubate on ice for 2 min. 259 µL pre-warmed (37° C) S.O.C medium (Invitrogen) were added to the vials and incubated for 1h at 37° C, shaken horizontally at 225 rpm (Orbital Shaker incubator, BioSan). The *E.coli* were then grown on selective LB broth agar (Sigma-Aldrich) plates. Plates were made with 17.6 g LB Broth with agar (Sigma-Aldrich), 500 mL nuclease free water and 500 µL Ampicillin (Gibco) (1:1000 LB broth). Colonies for HE, P6 and P7 were observe and sampled, but no colonies grew of the pF transfected *E.coli*, after multiple attempts. The plasmids were extracted with ZymoPURE Plasmid Miniprep (Zymo Research), and the plasmids were restricted according to the restrictions described in section 2.3.1.4, this was a analytical restriction to identify plasmid positive colonies. If the plasmid were cut at the expected location, then the plasmid was sequenced by Eurofins genomics. The primers used for sequencing were the same flanking primers listed in table 6. The resulting sequence was aligned in Benching (Benchlin. 2022)

2.3.1.7 RNA extraction with kit

Once RNA was used up and no F colonies were produced, more RNA was extracted using PureLink^{TM} Viral RNA/DNA Mini Kit (Invitrogen/ Thermofisher) according to the manufacture's specifications. cDNA and PCR reactions were performed as described in section 2.3.1.3. The resulting gel indicated contamination in the sample and the no template control. Troubleshooting where initiated where the leftover "old" cDNA was used as a positive control, the new cDNA was used at different concentrations (10 ng/µL and 40 ng/µL), and new reagents were mixed. The PCR were repeated, contamination of negative controls kept being a problem, and the positive control samples did not produce bands. Therefore, due to problems with the RNA extracted, no colonies aligning to the ISAV genome and no cloning of F protein, the decision was made to synthesise the two spike proteins and the entire segment 8. Gibson cloning were to be used for plasmid assembly.

2.3.2 Gibson cloning

2.3.2.1 DNA and primer design

The sequence for the two glycoproteins (HE accession nr: NC_006499.1 and F accession nr: NC_006500.2) and the whole segment 8 (accession nr: NC_006497.1) were imported from the sequence gene bank in NCBI into the online research tool Benchling (Benchling. 2022), as a fasta file with annotation. The HE sequences chosen was aligned to the HPR0 variant in Benchling (2022) to ensure it was not low virulent. The sequences were modified (**Figure 6**). Were the non-coding regions on each side of the VSV-G (which will be cut out) were copied and added to the sides of the insert sequence, this included the cut sits for StuI and PmlI (NEB) (purple in **figure 6**), a backbone homologous sequence after the cut sites (orange in the **figure 6**), and a noncoding buffer sequence (blue in **figure 6**), which will be important during the Gibson assembly. The HE and F sequence were human codon optimized using Integrated DNA Technologies codon optimization tool (Integrated DNA Technologies. s.a), due to the virus being produced in HEK293T cells. Sequence 8 was not changed as it is two overlapping protein coding sequences. Any restriction sites were changed and removed by silent mutations. The synthesised genes were ordered from ThermoFisher's GeneArt String service (ThermoFisher) all gene sequences had the same modified ends.

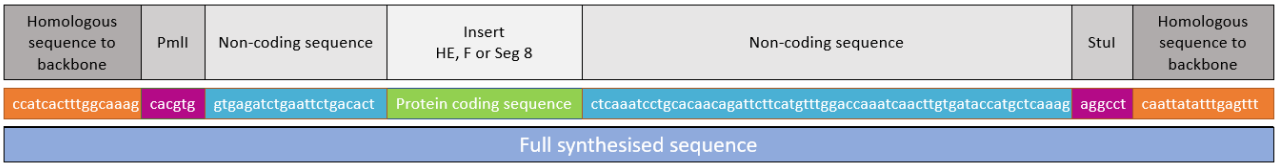

Figure 6: To the gene sequences (green), there was added a backbone homologous (orange), cut sites PmlI and StuI (NEB) (purple) and non-coding sequence (turquoise) taken from the VSV-G plasmid from between the restriction site and the VSV-G coding sequence. All three sequences were synthesised with this construct by GeneArt String (ThermoFisher). (Size not representative of the actual sequence, where the insert is much longer than the added sequence)

Primers for sequencing were designed using benchling (Benchling. 2022) and are listed in table 6. One primer pair, flanking the insert, were used on all three plasmids. For the longer sequences (HE and F) a second pair of overlapping primers were design, these were placed in the middle of the segment to ensure the whole insert could be sequenced. The primers were ordered from Thermofisher.

Table 6: The primers used throughout the Gibson cloning process to identify the successful transformations.

Primers		*Forward*	*Reverse*
Sequencing Primers	Flanking primer	GTGCTGGCCCATCACTTTGGC	GCACTGGTGGGGTGAATTCCG
	HE mid	GCGCACCGTTGCGATTTGAC	GAGGAGCTGCGAAGTATGACACAC
	F mid	CTTGGCTTCGCACGAACCTCAAG	CTTCCCCCGGAAACCCTCTCAG
PCR of the synthesised genes	HE	CCATCACTTTGGCAAAGCACG	GCTCAAAGAGGCCTCAATTATATTTGAGTTT

2.3.2.2 Cloning vector assembly

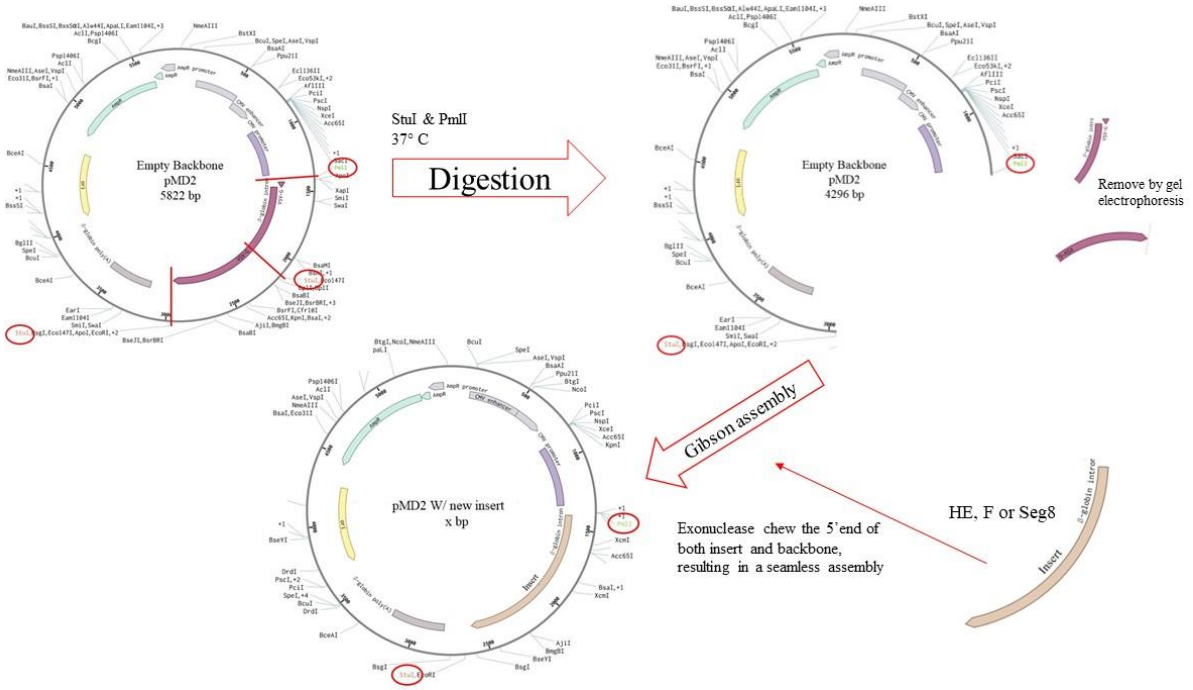

Figure 7: Workflow of the Gibson cloning and the desired outcome. The VSV-G sequence were removed by digestion using enzymes StuI and PmlI (indicated by red circles) at 37° C. The Digests was separated by gel electrophoresis and purified. During Assembly the 5'end of both the insert and backbone were chewed up by exonuclease, leading to sticky ends that attach seamlessly and in the desired orientation.

The cloning vector was the MD2.G plasmid (addgene #12259), which codes for VSV Glycoprotein (**Figure 7**). Before ISAV proteins could be inserted the VSV-G protein sequence had to be cut out, and the plasmid linearized (**Figure 7**). This was done with the enzymes PmlI (NEB) and StuI (NEB). There should be three fragments after digestion as StuI cuts at two location (one within the VSV-G fragment), (**Figure 7**). The enzymes were both blunt end cutters. 5 µg plasmids, 5 Units of the enzymes and 5 µL rCutSmart Buffer (NEB) were used in a 50 µL reaction, where nuclease free H_2O were used to adjust the volume up to 50 µL. A negative control was made using 0.5µg plasmid, no enzymes and 1 µL rCutSmart Buffer (NEB) in a total volume of 10 µL. The reactions were incubated in a thermal mixer (Eppendorf) at 37°C for 2 h, no shake. Heat inactivation was done at 65°C for 20 min.

The linearized plasmid fragments were separated by gel electrophoresis (Bio-Rad). The expected sizes were 4296 bp (backbone), 722 bp and 904 bp. A 1% agarose gel was prepared using 1g Agarose (Sigma-Aldrich) in 100 mL TAE (ThermoScience) and 1 µL red safe

(Chembio), a wide comb was used for the wells. The ladder used was Gene Ruler 1 kb (ThermoScience) and 5 µL were loaded to the first well. The loading dye used was purple (6x) (NEB) B70245, and 1 µL loading dye (NEB) were mixed with every 5 µL sample. The gel was run on a volage of 70V for 90 min. The wanted fragment was cut out of the gel and stored in a 1.5 mL microcentrifuge tube, 0.9g gel containing the empty backbone was retrieved. Picture was taken of a small sample with a molecular imager (Bio-Rad). The empty backbone was extracted from the gel using QIAquick Gel extraction Kit (Qiagen) according to manufacturer's specifications.

Three plasmids were assembled for cloning, one for each gene (called pMD2.HE, pMD2.F & pMD2.S8), using 10 µL (2x) Gibson assembly Master Mix (NEB). A molar ratio of 1 vector to 3 inserts were recommended, and NEBioCalculator (New England BioLab Inc. (a.s)) was used to calculate the ng insert needed for the 1:3 molar ratio when 70 ng empty backbone was used. For pMD2.HE, 65.8ng synthesised HE sequence was used, for pMD2.F, 73.32ng synthesised F sequence was used and for pMD2.S8, 43.34 ng synthesised Segment 8 sequence was used. Nuclease free H_2O were used to adjust the volume to a 20 µL reaction mix, this was then incubated at 50°C for 1 h and stored at -20°C until transformation.

2.3.2.3 Transforming Stbl3 Chemically competent E.coli

The plasmids were cloned in Stbl3 Chemically competent E. coli (Invitrogen). *E.coli* was transfected with one of the three plasmids (pMD2.HE, pMD2.F & pMD2.S8) and a negative control with just the empty backbone (4 samples in all). The transformation was done according to protocol described in section 2.3.1.6, with minor changes: 3 µL plasmid were used instead of 5 µL. Two types of selective LB broth agar (Sigma-Aldrich) plates were made, as described in section 2.3.1.6. One plate type using ampicillin (Gibco) (1: 1000 LB agar (Sigma-Aldrich)) and the other plate type used Carbenicillin (CB) (Gibco) (1: 1000 LB agar broth (Invitrogen)) for selection. The whole tube of transformed *E.coli* were seeded on one selective plate, due to low transformation rate.

The plates were grown at room temperature for two days, and colonies picked and transferred to 13 mL Bacterial culture tubes (Sarastedt) and grown in 5 mL LB broth base (Invitrogen) with carbenicillin (Gibco) (1:1000 LB broth). The tubes were grown for two days on a shaker

incubator (BioSan) at 250 rpm in room temperature. The plasmids were extracted with a miniprep kit (Zymo Research) according to the manufacturer's specifications.

An analytic restriction digestion was performed on the colonies using StuI (NEB) & PmlI (NEB) on F positive clones, and PmlI & BasI (NEB) on HE positive clones, rCutSmartBuffer (NEB) was used and restrictions were preformed according to the method described in section 2.3.1.1.4. The positive colonies that produced the best bands in a gel (Gel made and photographed as described in section 2.3.1.4) were sent to be sequenced by Eurofins Genomics (premixed sample, and primer (listed in **table 6**)) according to their specifications. The resulting sequences were imported and aligned in Benchling (Benchling. 2022).

2.3.2 Lentivirus packaging

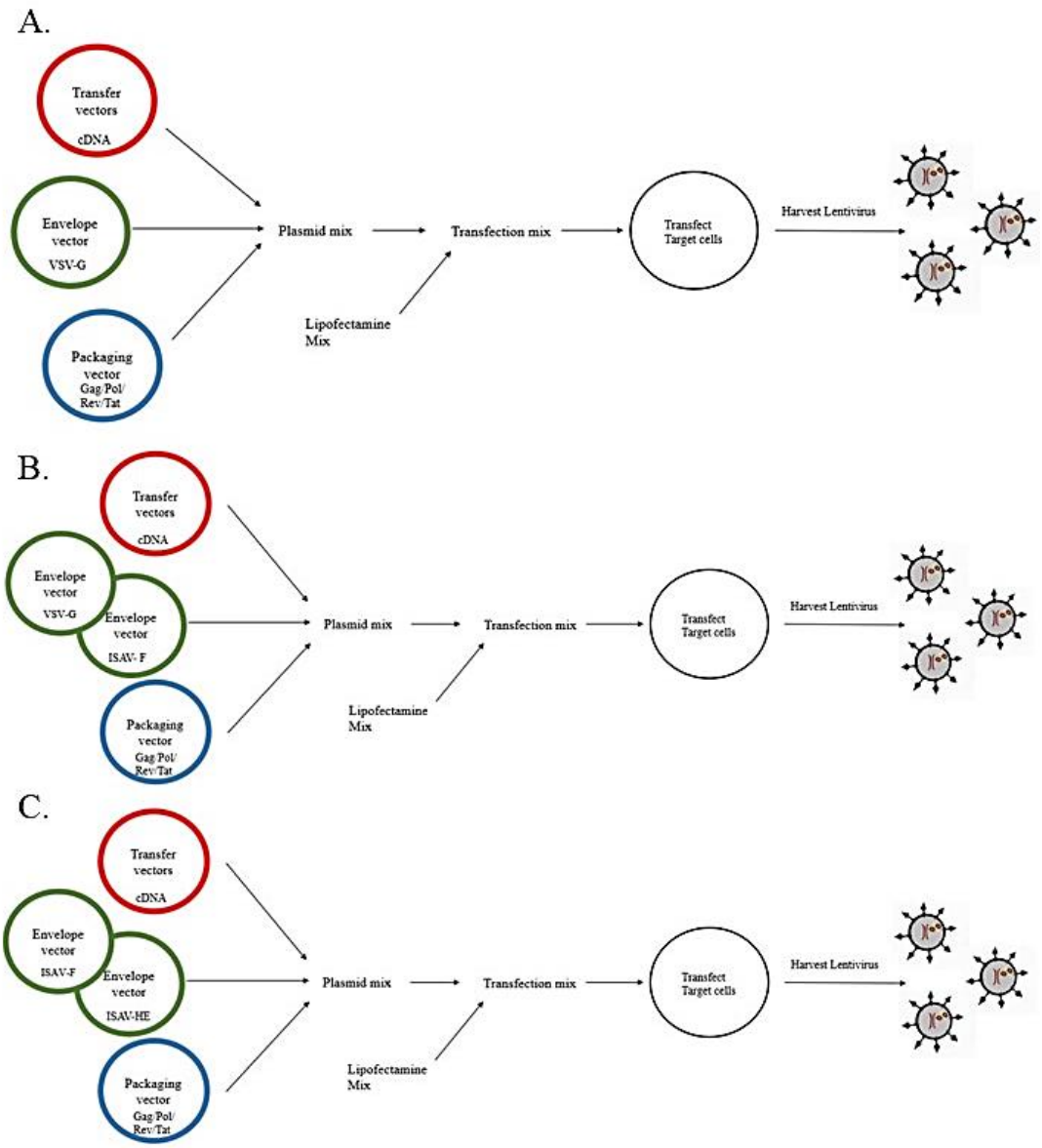

Figure 8: The production scheme of all three pseudotyped lentiviruses produced in this work. First the different plasmid mixes were prepared, then lipofectamine was added, making the transfection mix, the target cells were transfected, and 2- 4 days later (depending on growth temperature) the virus was harvested. When making a multi glycoprotein two envelope plasmids were included.

The workflow was the same for all the different lentiviruses produced (**Figure 8**). First a plasmid mix were made, then a lipofectamine mix. The two mixes were then mixed to a transfection mix, which were used to transfect the target cells. All packaging plates included two negative controls.

2.3.2.1 Plasmid mix:

There were made three different pseudotyped lentiviral vectors. VSV-G vector was used as a positive control and had a plasmid mix ratio of 1 plenti-CMV-GFP-Puro: 0.75 psPAX2: 0.5 pMD2-G (**Table 7**). One VSV-G & ISAV-F hybrid vector were made, the plasmid mix had the ratio of 1 plenti-CMV-GFP-Puro: 0.75 psPAX2: 0.5 pMD2-G: 0.5 pMD2-F (**Table 7**). And lastly a ISAV-HE & F pseudotype were made with a plasmid mix of ratio of 1 plenti-CMV-GFP-Puro: 0.75 psPAX2: 0.5 pMD2-HE: 0.5 pMD2-F (**Table 7**). The plasmid mixes were diluted in 250 µL OptiMEM (Thermofisher)

Table 7: The plasmids type and amount (ng) used for the different pseudotyped lentivirus (LV).

Plasmids	VSV-G LV	VSV-G; ISAV-F LV	ISAV-HE; F LV
plenti-CMV-GFP-Puro	1000 ng	1000 ng	100 ng
psPAX2	750 ng	750 ng	750 ng
pMD2-G	500 ng	500 ng	X
pMD2-F	X	500 ng	500 ng
pMD2-HE	X	X	500 ng
OptiMEM	250 µL	250 µL	250 µL

2.3.2.2 Lipofectamine mix:

The liptofectamine mix were made by gently mixing 10 µL liptofectamine 2000 (Thermofisher) and 250 µL OptiMEM (Thermofisher), pr packaging, and incubating it for 5 min in room temp. The plasmid mixes where then added to the lipofectamine mix and incubated at room temperature for 20 min making the transfection mix. Which were added to the cells dropwise.

2.3.3.1 VSV-G and ISAV-F lipofectamine

Due to the results from the lentivirus labelling experiment it was decided to test a VSV-G and ISAV-F hybrid vector. The plasmids were packaged in HEK293T cells. HEK cells were split and seeded in a 6 welled plate, $7.0x10^5$ cells pr well, the day before the packaging. Two lentiviruses were packaged one VSV-G and one VSV-G & ISAV-F hybrid. Plasmid mixes were made according to table 7, lipofectamine mix were made according to the protocol above.

HEK cells were split and seeded in a 6 welled plate (Sarstedt) the day before the packaging (transfection), with a concentration of $7x10^5$ in 2 mL DMEM (Sigma-Aldrich). The day of transfection the media were changed to fresh media, before 500 µL transfection mix were added to the cells dropwise (**Figure 8A & 8B)**, the cells were then incubated at 37° C with 5% CO_2 for 4 h, after 4 h the media were changed. The next day the cells were checked for GFP, indicating transfection. Day 2 post transfection, the supernatant was extracted with a 5 mL syringe and filtered through a 0.45µm syringe filter (Sarstedt). The lentivirus was stored in -80° C. This was repeated at 22° C. Efforts were made to keep the temperature of the cabinet below 25° C.

2.3.3.1.1 Testing VSV-G: ISAV-F

The lentivirus was tested in ASK and HEK cells (as control). The lentivirus tested was both the 37° C and the 22° C batch. $2.88x10^4$ cells were split and seeded (in 2 mL) the day before the Transduction. Before lentivirus were introduced the cell media were changed and 8µg/mL polybrene (Sigma-Aldrich) were included for both cell types. 500 µL lentivirus were added to the cell wells. The cells were then incubated in their respective environment. 24h post transduction was the cells checked for GFP and the ASK cells temperature were slowly moved up to 26° C (over 1h), the temp was then increased with 1° C every 24h up to 30° C. After 24h at 30° C, they were moved back to 20° C.

2.3.3.2 Packaging via electroporation: VSV-G: ISAV-F

ASK and HEK cells were grown to a high confluency, washed and split. $2x10^6$ cells were taken out of the suspension ($8x10^5$ pr reaction, prepared for 2.5 reactions). The cells were washed with Mg^{2+} and Ca^{2+} free PBS (Sigma-Aldrich), twice, with centrifugation at 150 xg for 5 min to pellet the cells between the washings. The Neon Transfection system, with the 100 µL cell suspension protocol (ThermoFisher). The pellets were resuspended in 250 µL Buffer R (Invitrogen, Neon system kit). The plasmids were added to the resuspended cells, VSV-G plasmid mix had the ratio of 1 transfer: 0.75 psPAX2: 0.5 pMD2-G. The VSV-G & ISAV-F plasmid mix had the ratio of 1 transfer: 0.75 psPAX2: 0.5 pMD2-G: 0.5 pMD2-F. The electroporation settings were for ASK: voltage: 1400V, Width: 20ms, Pulses: 2. And for HEK:

Voltage: 1100V, Width: 20ms, Pulses: 2. The 100 µL were then added to 6 welled plates (Sarstedt) with 2 mL cell media. The plates were incubated in the cell specific environment, 24 h post transfection, GFP expression was evaluated by fluorescent microscopy (microscope: Imager.Z2 (ZEISS), objrctive: 10X (), exposure:366). After 2 days incubation the supernatant was extracted with a 5 mL syringe and filtered through a 0.45µm syringe filter (Sarstedt). The lentivirus was stored in -80° C.

2.3.3.2.1 Testing electroporation packaged VSV-G: ISAV-F virus

The lentivirus was tested in ASK and HEK cells. $3x10^4$ cells were split and seeded (in 2 mL) in six welled plates (Sarstedt) the day before the experiment. Before lentivirus were introduced the cell media were changed and 8µg/mL polybrene (Sigma-Aldrich) included. 500 µL lentivirus was introduced dropwise to the cell wells. The cells were then incubated in their respective environment. After 24h transduction the cells were checked for GFP and the ASK cells temperature were slowly moved up to 26° C (over 1h), the temperature was then increased with 1° C every 24h up to 30° C. After 24h at 30° C, they were moved back to 20° C. Fluorescent microscope were used to identify GFP (Microscope: Imager.Z2 (ZEISS), objective: 10X, exposure: 366)

2.3.3.3 ISAV-F & ISAV-HE lipofectamine

The plasmids were packaged in HEK293T cells. HEK cells were split and seeded in two 6 welled plates (Sarstedt), $7.0x10^5$ cells pr well (in 2 mL), the day before the packaging. Two different lentiviruses were packaged, one VSV-G (as a positive control) (**Figure 8A**), and one ISAV-HE & ISAV-F, (**Figure 8C**). The plasmid mixes were made according to table 7, the transfection mixes were made as described previously. 500 µL transfection mix were added to the cells dropwise and incubated at 37° C, 5% CO_2 for 4 h, then the media were changed. Both plates were left in 37° C overnight, to allow the HEK cells to stabilize, then the supernatant (with potential virus) of one plate were removed, the cells washed with PBS (Sigma-Aldrich), given new media, and moved to 22° C, to see if virus were produced at the lower temp. The next day the cells were checked for GFP, indicating transfection. After 4 days incubation 37°

C, 5% CO_2 , and 3 days at 22° C, 5% CO_2 the supernatant was extracted with a syringe and filtered through a 0.45µm syringe filter (Sarstedt). The lentivirus was stored in -80° C.

2.3.3.3.1 Testing ISAV-F & ISAV-HE

The lentivirus was tested in ASK and HEK cells (as positive control). $3x10^5$ of each cell line were split and seeded (in 2 mL) the day before the experiment in 6 welled plates (Sarstedt). Before lentivirus were introduced the cell media were changed and 8µg/mL polybrene (Sigma-Aldrich) were included. 500 µL lentivirus were added to the cell. The cells were then incubated in their respective environment. After 24h the cells were checked for GFP and the ASK cells temp were slowly moved up to 26° C (over 1h), the temp was then increased with 1° C every 24h up to 30° C. After 24h at 30° C, they were moved back to 20° C.

Chapter 3. Results

3.1 Part 1: VSV-G Lentivirus

3.1.1 Lentiviral transduction temperature experiment: VSV-G lentivirus on HEK cells

The lowest temperature HEK cells would express GFP was identified to be 26° C (**Figure 9B**), the observed GFP expression was very faint. The 37° C (5% CO2) positive control cells expressed strong GFP in the wells infected by lentivirus, by the first day (not included in the **figure 9**). No GFP was expressed in the experimental plate at 22° C (day 1) or at 24° C (day 2) (5% CO2) (**Figure 9**). At 26° C (day 3) the cells showed some signs of GFP expression, when the exposure of the microscope was increased (**Figure 9B**). At 28° C (day 4) GFP was expressed in all wells infected by lentivirus (**Figure 9**). After 28° C the GFP intensity increased with the temperature. On 36° C (day 8) there were no difference between the 37° C positive control (not included in **figure 9**) and the experimental plate. The control and experimental plates were moved to 22° C (50% CO2) and after 3 days at 22° C (day 11), both plates had weaker GFP expression (**Figure 9**).

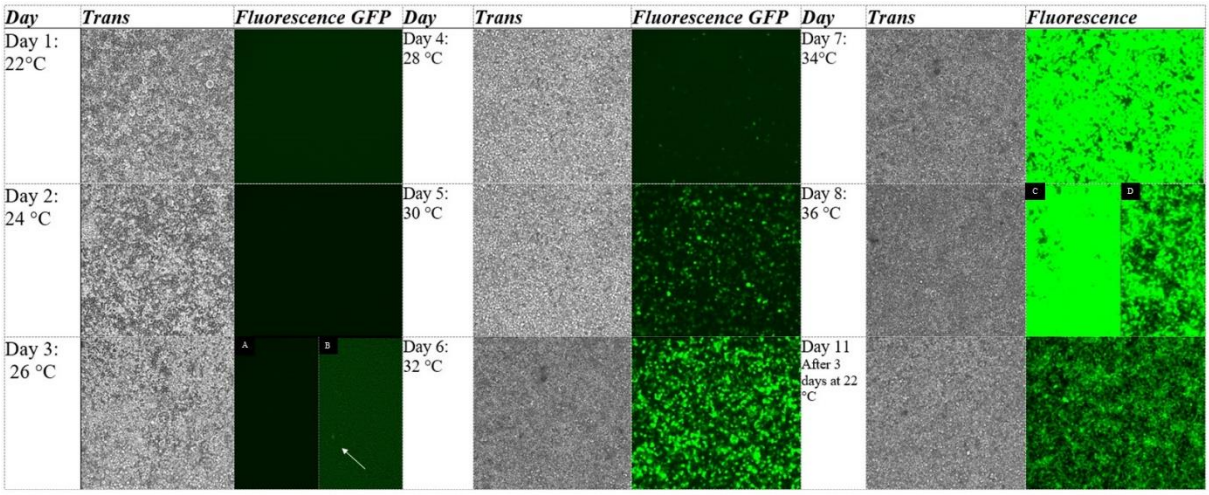

Figure 9: GFP expression in HEK cells at different temperatures. A very faint GFP was observed under higher exposure (picture B) at day 3, 26°C. At day 4, 28°C GFP is visable. Day 8, 36°C, the GFP was so strong the exposure was lowered (picture D). On day 11 (3 days at 22°C) the GFP was decreasing in intensity. Figure 9 was made with the 200 µL lentivirus samples.

3.1.2 Lentivirus labelling experiment: VSV- G Lentivirus

3.1.2.1 Labelling lentivirus and HEK cells

To optimize the protocol and to be familiar with the virus infection pattern by VSV-G in HEK cells, labelled virus was used to transduce stained HEK cells. The HEK cells were infected as expected, right after viral introduction SYTO82 signals was observed outside the cells (not included in **figure 10**), immediately (first minute) the virus signals were observed at the cell surface. After about 5 minutes the SYTO 82 signals were observed and confirmed to be inside the cells close to the membrane (**Figure 10B**) as yellow clusters.

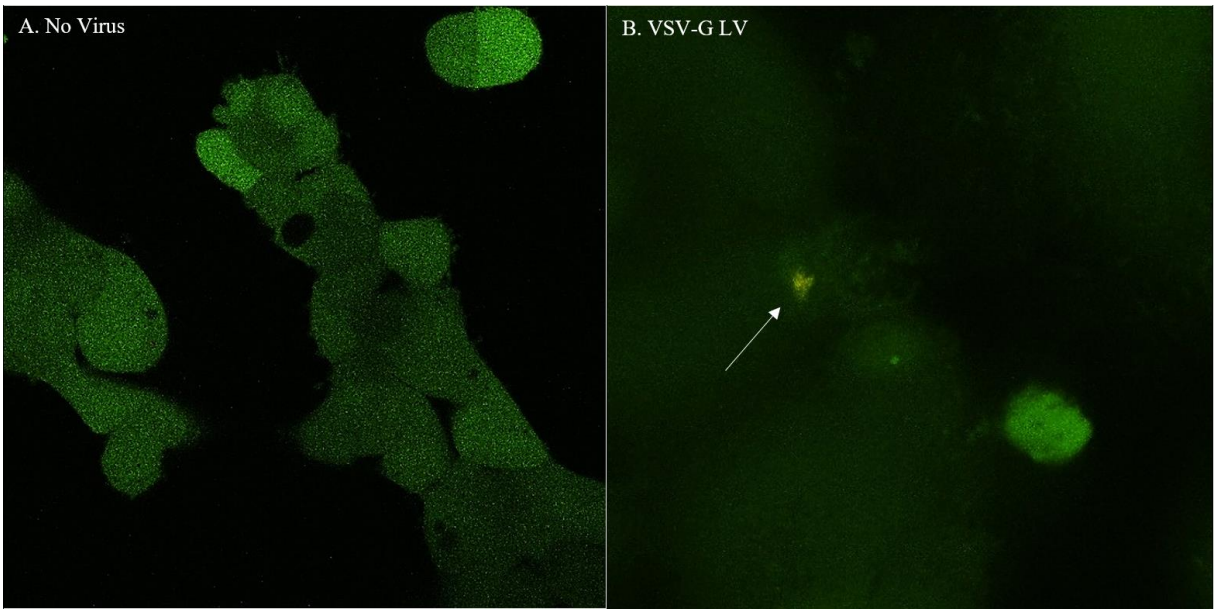

Figure 10: HEK cell cytoplasm was stained with Hoechst (green, not due to GFP), the virus genome was stained with SYTO 82 (yellow). The yellow was believed to be accumulation of labelled virus within the cell, most likely inside endosomes. Picture B. with lentivirus were taken about 5 minutes after virus were introduced to the dish.

3.1.2.2 Labelling lentivirus: HEK and ASK

The experiment was repeated to look for the same pattern in HEK cells, with extended time frame (48h). ASK cells were included to compare with HEK cells. The HEK cells had the same infection pattern as observed in section 3.1.2.1 (**Figure 10**). Two days post transfection did the HEK cells expressed GFP, and virus signals could still be observed on the surface of the cells and in clusters within the cells (**Figure 10**).

When VSV-G lentivirus was introduced to the ASK cells, signals were observed outside the cells. A pattern similar to the HEK cells were observed with signals outside the cells immediately after lentivirus introduction. Then signal covered the cell surface, and within about 5 minutes strong SYTO82 signals were observed and confirmed accumulating within the cells, close to the membrane (**Figure 11D**).

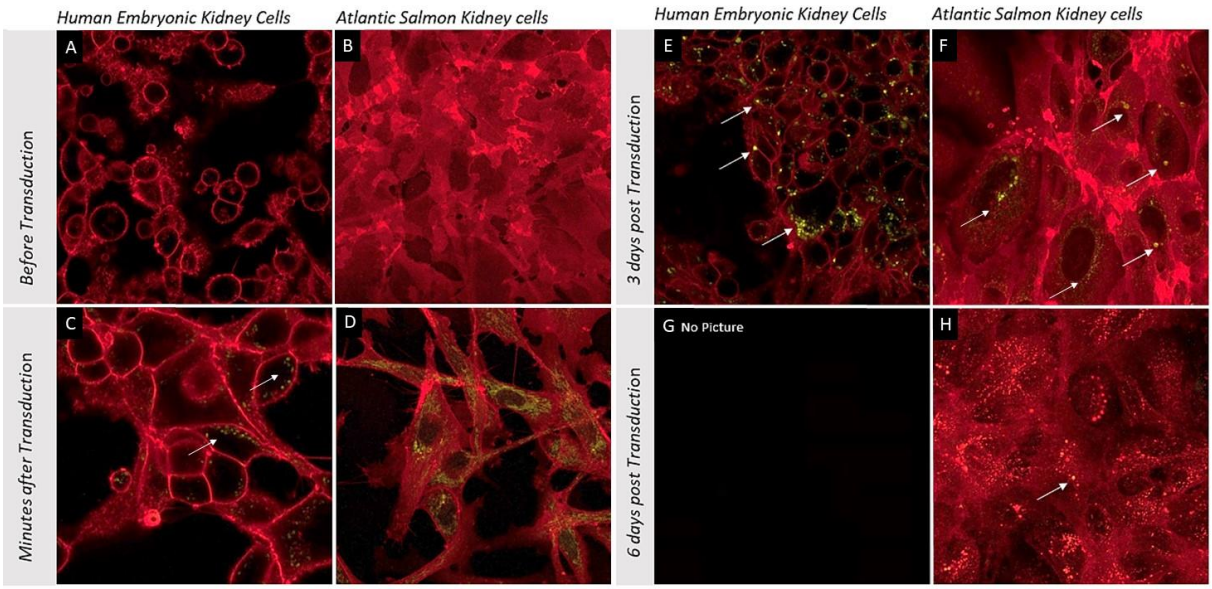

Figure 11: HEK and ASK cells were labelled with CellMask (Red) and Lentivirus was labelled with SYTO82 (yellow). There was no picture taken of HEK day 6 as this plate was attempted fixated at day 3. The ASK cell plate was kept for that long to track the lentivirus signals. The CellMask dye had been largely internalised, and very little SYTO82 signals were observed by day 6.

Three days post viral introduction the accumulations (believed to be the endosomes) indicated GFP expression, which is shown in (**Figure 12**). The accumulations/endosomes had increased in size and gathered closer to the nucleus (**Figure 11**). Six days post viral introduction, almost no virus signals could be observed in the confocal microscope (**Figure 11H**).

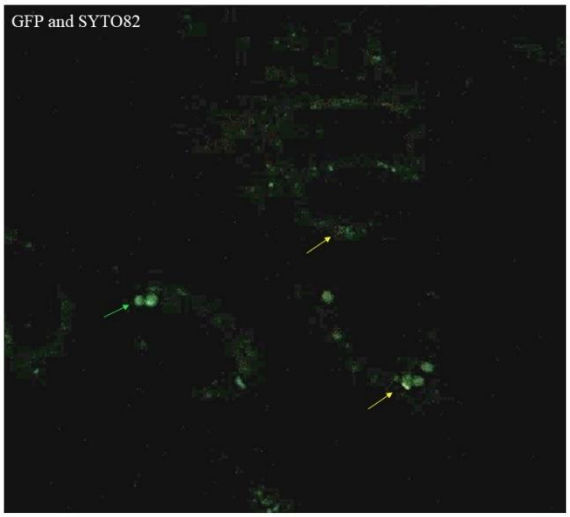

Figure 12: Picture of the virus accumulation (endosomes) inside ASK cells, 3 days post transduction. GFP (green) and virus signal (SYTP 82, yellow) were observed inside the accumulations (endosomes). The signal was faint, and exposure were increased, CellMask signals were removed.

3.1.3 Electroporation experiment: GFP lentiviral transfer plasmid

Due to the apparent VSV-G lentivirus ability to enter the ASK cells (section 3.1.2.2), the question was why no GFP was observed. One reason could be the ASK cells were not translating the GFP sequence of the plasmid, therefore ASK cells was transduces with the GFP transfer plasmid only (**Figure 13**). HEK (positive control) and ASK cells were electroporated. Both HEK cells and ASK cells (**Figure 13**) were able to express GFP post transfection.

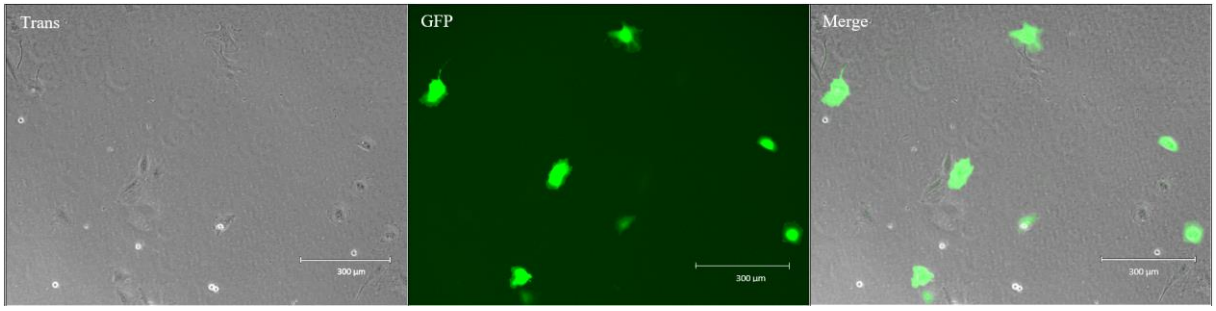

Figure 13: Show electroporated ASK cells 1 day post transfection. Not all live cells expressed GFP. A HEK control was included in the experiment but excluded from the figure. The HEK cells expressed GFP

3.1.4 pH and temperature transduction experiment: VSV-G Lentivirus on salmon cell

Based on the results from prior experiments, methods found to increase transduction rate (lower pH and increased temperature form 20° C to 26 ° C and up) were tested separately and in combination. No GFP were observed in the Salmon cells. HEK cells however did express GFP. When pH lowered from pH 7 to pH 5, the transduction rate of HEK was increased (**Figure 14**).

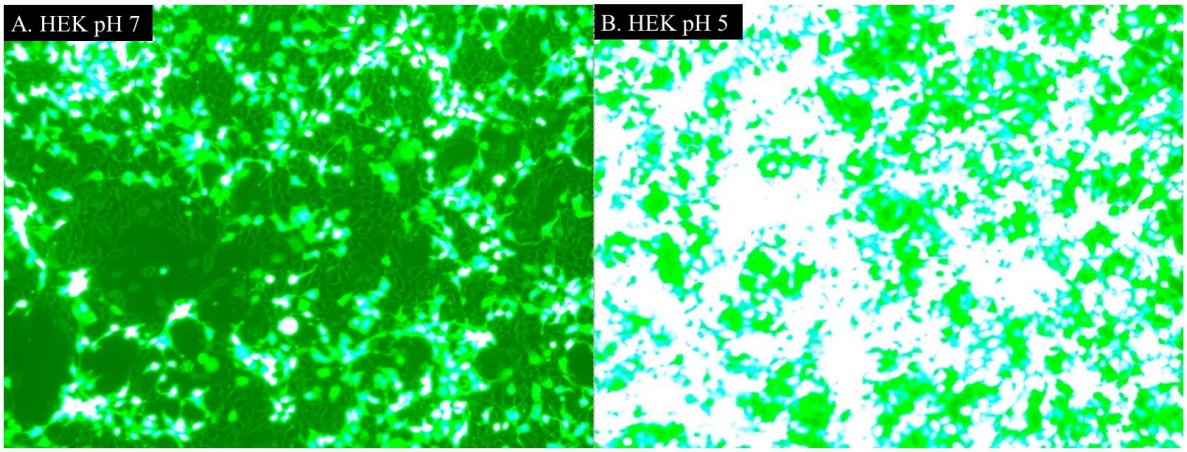

Figure 14: HEK cells treated with normal media pH (pH 7) before lentiviral transduction, picture A, and HEK treated with pH 5 before lentiviral transduction, picture B. The lowering of pH appaired to increase the transduction efficiency.

Prior experiments (section 3.1.1) indicated no transduction, and/or no GFP production below 26° C (**Figure 9**) in HEK cells, this was tested in ASK cell, but no GFP was expressed. When the two factors (lowered pH and increased temperature) were combined on the ASK cells, no GFP was observed either (**Figure 15**)

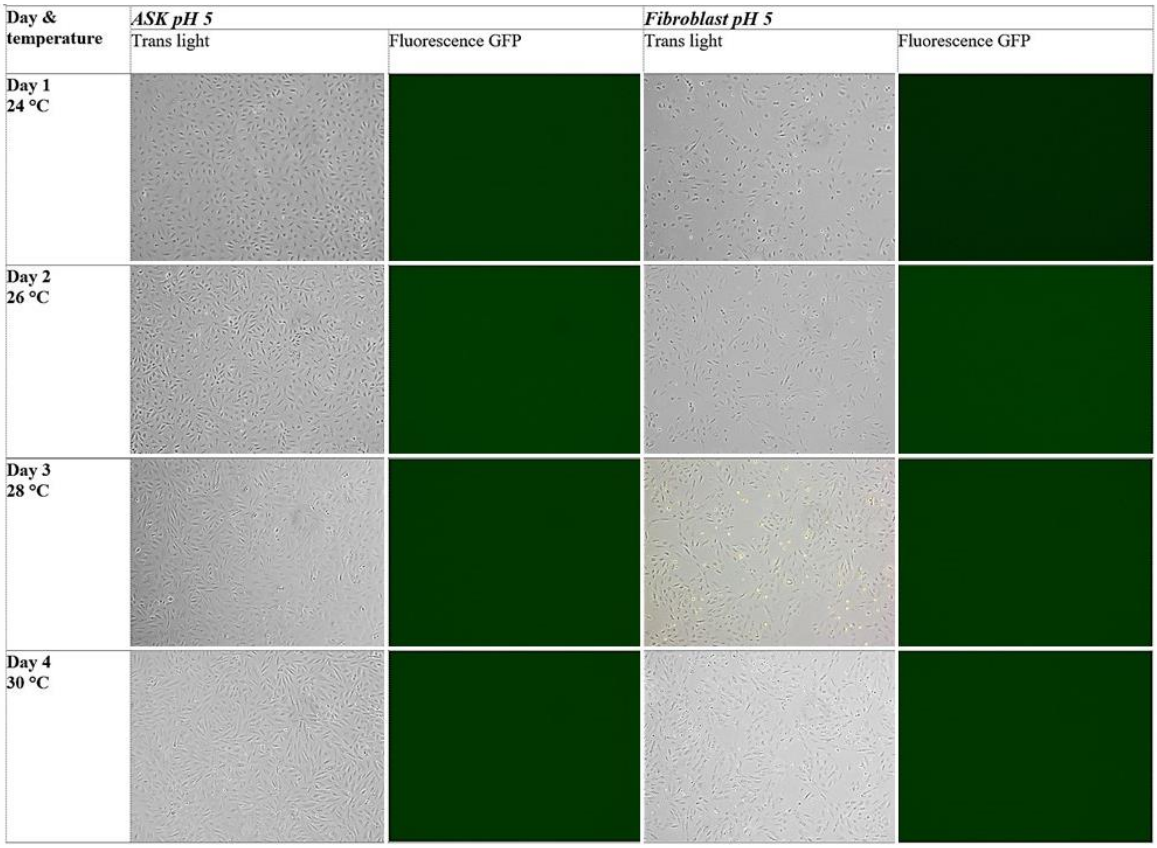

Figure 15: VSV-G lentivirus on ASK cells and fibroblast cells with combined lowered pH and increasing temperature. pH 5 was found to be the lowest pH the cells would survive for 1h, VSV-G lentivirus were tested with a lowered pH media as lentivirus were added. In HEK cells this led to an increase in transduction rate, and 26° C were the lowest temperature GFP were observed on.

3.1.5 Reverse transcriptase activity

To test for reverse transcriptase activity a qPCR was conducted, targeting GFP. The cells (both HEK and ASK) were transduced with VSV-G lentivirus, incubated for 24 h then washed before RNA and DNA were extracted. Some RNA was made into cDNA (**Figure 4**). We hypothesize that if the virus entered the cell, but no reverse transcriptase occurred, then cDNA samples should have amplification and not the DNA sample. If the virus entered the cells and started the reverse transcription of its genome activity, then both cDNA (RNA) and DNA sample should have amplification.

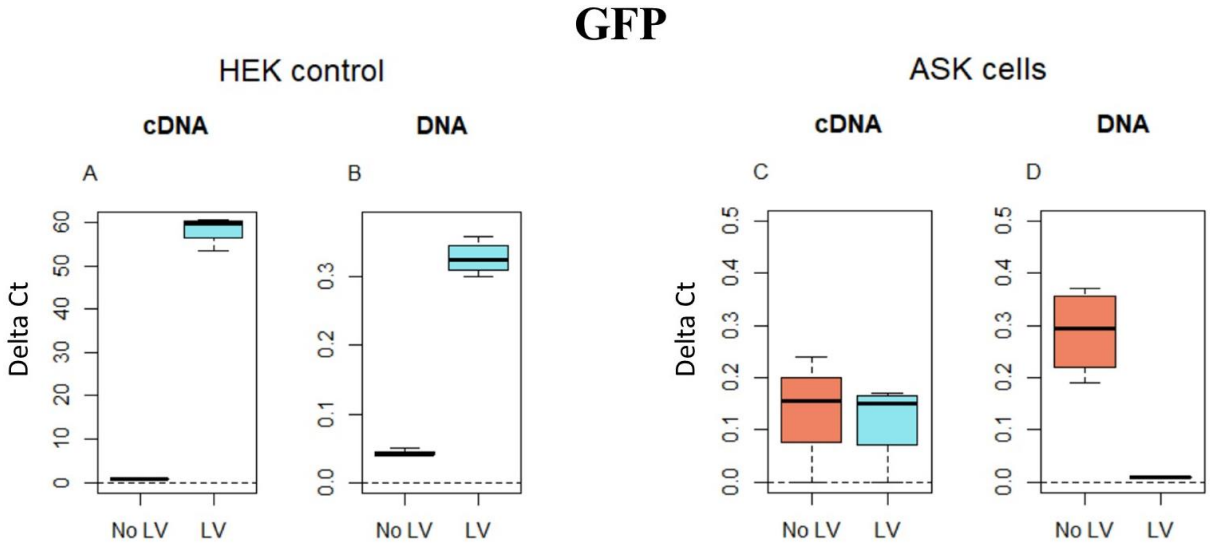

Figure 16: Results from the qPCR when targeting the GFP sequence. The HEK control (A and B) gave high replication of cDNA, indicating high starting concentrations of RNA in the cells. The No lentivirus control had amplification, which indicate contamination. Plot A and B (HEK) do not have the same scale.

The delta Ct values of HEK +Lentivirus samples indicated high amounts of RNA, and some DNA. If the ASK cells had expressed GFP, a pattern similar to the HEK + lentivirus samples could be expected (**Figure 16A & 16B**). In the ASK cells quantification of cDNA was observed (**Figure 16C & 16D**), and some amplification did occur in the DNA sample (**Figure 16D**). These results correspond to the hypothesis of transduction termination post reverse transcription. However, there was GFP quantification in the no lentivirus controls, making the results untrustworthy. Some of the negative controls and the no reverse transcriptase RNA samples had amplification, these should all be negative as there were no readable template there. In the experiment a AMP control were included to identify contamination packaging plasmids (**Figure 17**). AMP and GFP sequence are both located in the transgene plasmid, and AMP sequence can be found in all the lentiviral vector plasmids. AMP should not be packaged into the vector with the transgene as it is there for the selection process during cloning of the plasmids. Amplification was observed in all sample type (**Figure 17**) including the negative controls, but most in the cDNA samples.

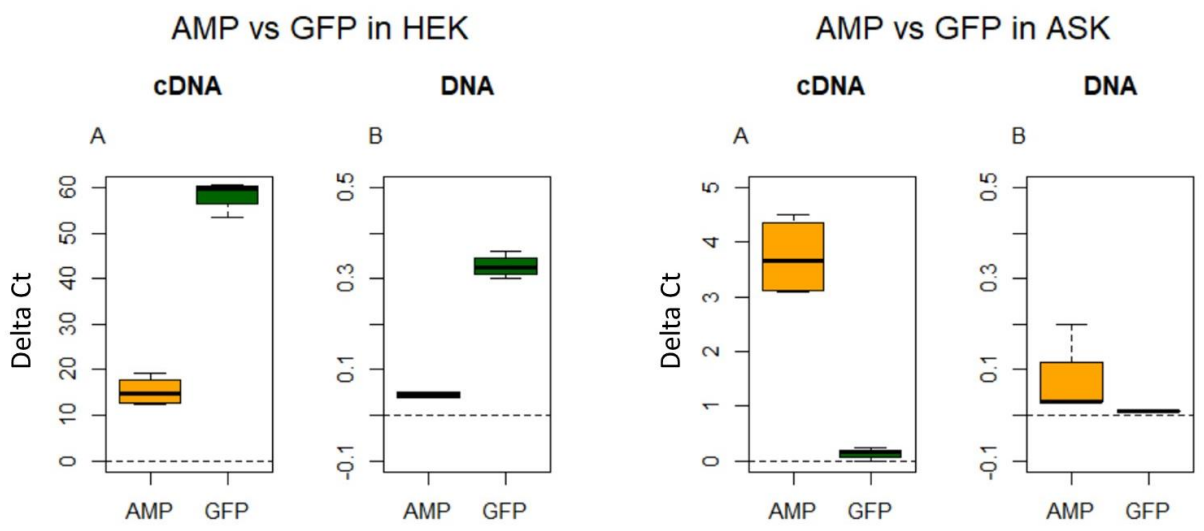

Figure 17: comparison between AMP and GFP in the cells. More AMP were detected in the cDNA samples. In the HEK samples difference between AMP and GFP was larger, indicating that the contamination is not affecting the results too much. The ASK cells indicate more AMP than GFP, probably due to different sequence length. Therefor int can be assumed the results from the ASK cells are more affected by the contamination. The scale of the plots is not the same due to substantial differences in values of the samples.

The impact of the alleged contamination was attempted addressed and boxplots comparing the AMP and GFP Ct value were made (**Figure 17**). For HEK (**Figure 17A**) the AMP amplification is far lower than the GFP amplification, so the GFP signal is likely not solely due to contaminating transfer plasmid. In the ASK cells more AMP was detected than GFP, therefor it is assumed that the observed GFP signal is likely the result of contaminating transfer plasmid (**Figure 17B**).

3.2 Part 2: Pseudotyping

3.2.1 Production of plasmids

3.2.1.1 Restriction cloning

For the pseudotyping, the VSV-G glycoprotein sequence were swapped with one of the ISAV proteins. Before the insertion of the new protein sequence, the MD2.G plasmid had to be linearized (**Figure 18A**), and the VSV-G had to be removed, this was done with the enzyme PmlI and StuI, which produced three fragments (**Figure 18A**). The fragments were all at the expected location at around 4000 bp (backbone), 722bp and 904bp (**Figure 18A**).

The RNA was extracted using two different methods. When extracting with Phenol/Chloroform the RNA concentration was below nanodrops limit of detection. This was used as a template to make cDNA, and a PCR gradient were used to identify the optimal annealing temperature for the primers (**Figure 19A**). The primers for HE was found to work best at 50° C, there were no amplification in the F, and P6 and P7 both had strong bands at all temperatures. Therefore 50° C were used for further applications

A new PCR reaction at 50° C quantified the cDNA, and the desired size were extracted, HE: 1205 bp, F: 1355 bp, P6: 725 bp and P7: 572 bp. The backbone was dephosphorylated, before genes were inserted through ligation. Stbl3 chemically competent *E.coli* were transformed, and after multiple attempts six colonies grew, two HE and three P6, and one P7.

The colonies grown were assessed by an analytical digestion with enzyme PmlI and StuI. This revealed one possible successful transformation (**Figure 18**) of the P6 gene (3rd colony), but the size of the fragment matched that of the P7 expected size (**Figure 18**), of 552bp. There might have happened a mislabelling. Both P7 and P6 colony 3 were sent for sequencing, none of the sequences aligned to the ISAV genome.

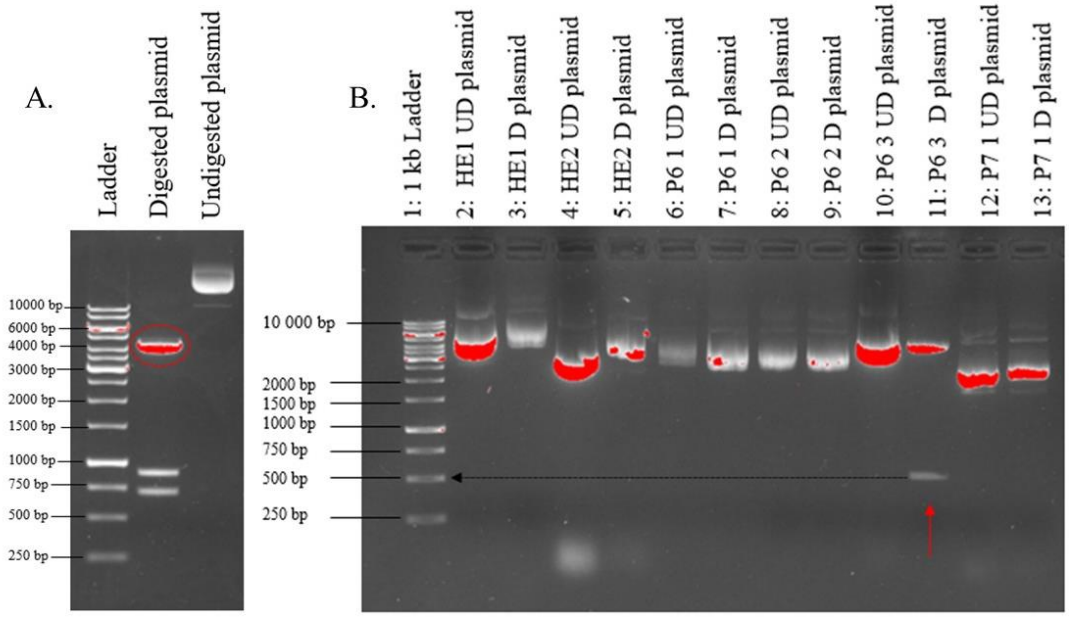

Figure 18: Show gels digested with the restriction enzymes StuI and PmlI. Figure A: show the empty backbone (digested pMD2) and pMD2.G undigested as a reference. After restriction by StuI and PmlI, three fragments were expected. The empty backbone was 4296 bp long (red circle), and the VSV-G fragments were 722bp and 904bp. Everything was located where it was expected so the empty backbone was cut out and extracted from the gel. Figure B: show the results from the restriction check of the restriction cloning, there might be a successful cloning of the P6 3 plasmid, but the expected fragment size is perfect for P7. Both colonies were sequenced but none of them aligned to the ISAV genome. D=Digested, UD= UnDigested. The expected fragment sizes were: HE: 1205 bp, P6: 772 bp and P7: 572 bp

The RNA and cDNA ran out, and due to the poor RNA concentration and lack of F sequence it was decided to use a kit to extract more. The concentration was found to be 42.65 ng/µL, but when the genes were attempted amplified by PCR, the expected fragment size was not observed (**Figure 19B**). And both sample and negative control (no template) indicated contamination of a fragment at about 800bp.

Troubleshooting was done with the little old cDNA left and the new cDNA, new primers and reagents were diluted, and different amounts of genome were used, but none of the expected bands were observed, even the old cDNA which had previously produced nice bands did not (expect P7, old cDNA) (**Figure 19C**).

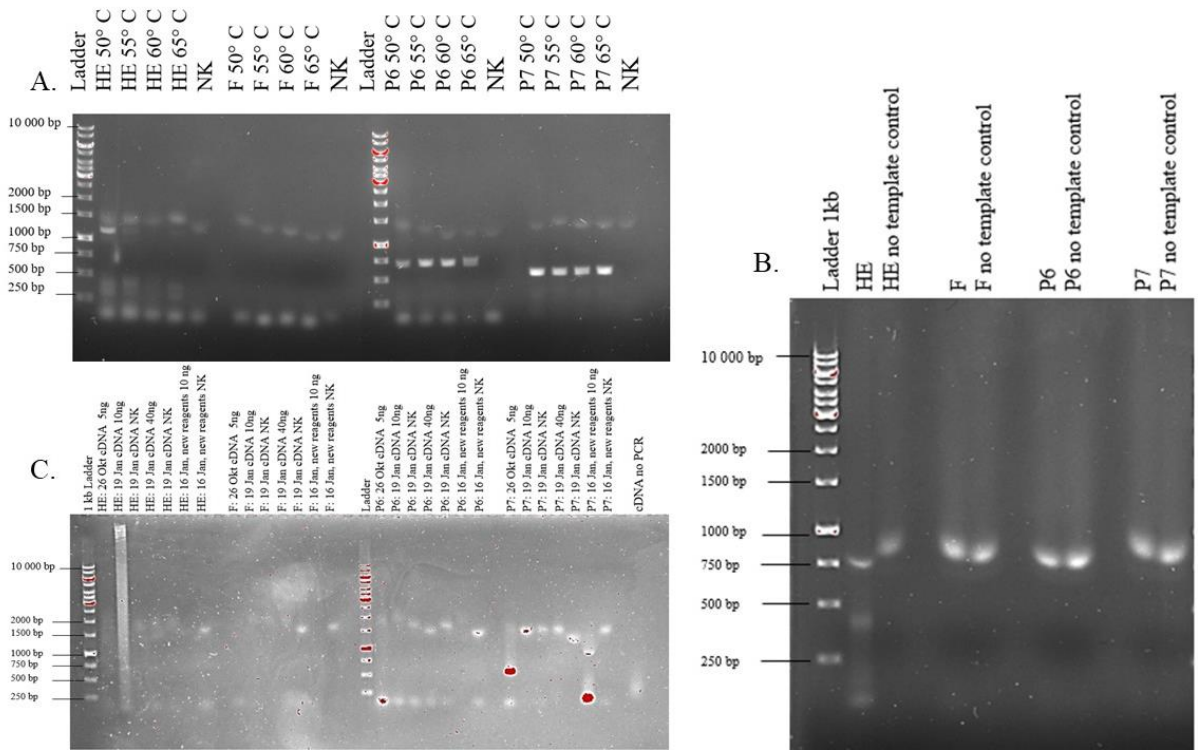

Figure 19: show the PCR amplified ISAV cDNA. Figure A: Show the gradient PCR with the first extraction, which produced bands in HE, P6 and P7 at the expected locations. The temperature producing the best bands overall were 50° C, and therefor this was used for further applications. No bands were observed from F. Figure B: show the PCR of the second extraction, there were contamination in the no template control which seemed to be the same as in the sample. None of the expected bands could be observed. Figure C: show the troubleshooting, where the different cDNA were tested, different amounts, and new reagents were tested, none of the bands were at the expected location, and there was bands in the no template controls. The "positive controls" from the first extraction were epthuy (exeptphenol/chloroform extracted RNA. The expected bands were HE: 1205bp, F: 1355bp, P6: 725bp and P7: 572bp.

3.2.1.2 Gibson cloning

The fusion protein (MD2-F) and Hemagglutinin esterase (MD2-HE) recombinant plasmids were produced from the synthesised sequences. An analytical digestion with the enzyme PmlI & StuI for F and PmlI & Basa HF v2 for HE revealed two possible successful transformations of F (**Figure 20A**) and four possible successful transformations of HE, although only two of them looked good (**Figure 20B**).

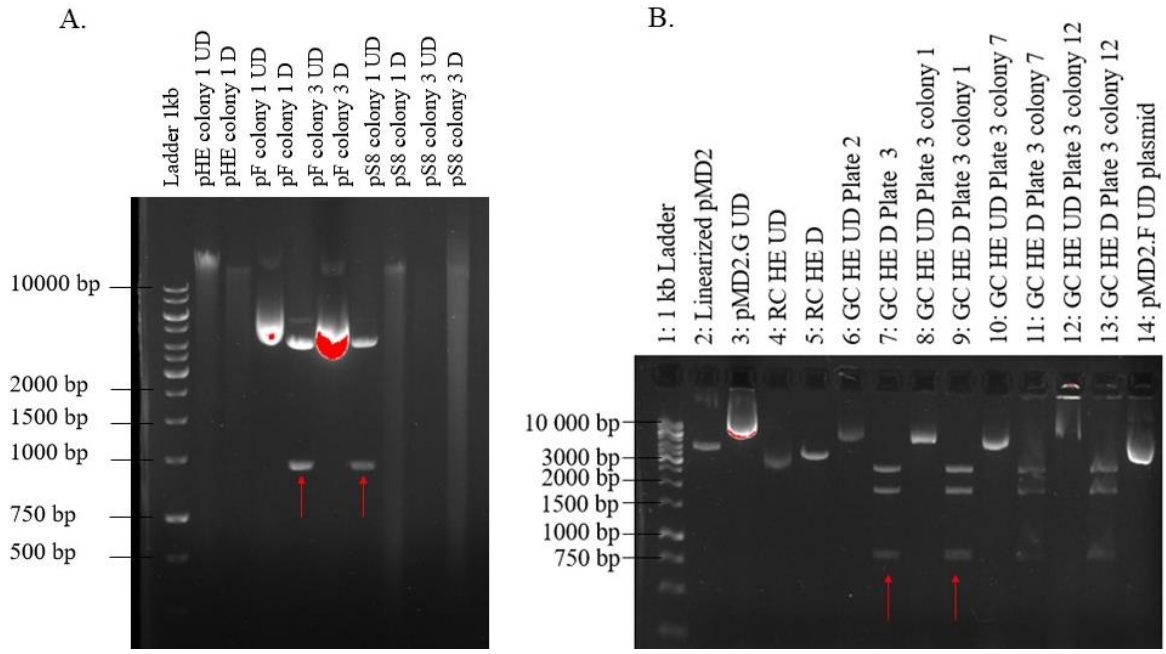

Figure 20: Show the analytical digestion of Fusion colonies (A) and Hemagglutinin esterase (B). A: Both F colonies were cut at expected fragment sizes, and the whole plasmid was located at around 5000 bp, this indicates present plasmid and both samples were sent for sequencing. Expected fragments: HE: 1275bp, F: 1435bp, Seg8: 826bp. B: The analytical digestion of the HE positive colonies indicated positive colonies. The enzymes used was StuI and BsaI HFv2, which would cut the plasmids at three places. The colony of plate 2 and colony 1 of plate 3 were sent for sequencing. D=Digestion, UD=UnDigestion, RC=Restriction Cloning, GC= Gibson Cloning. Expected fragments: 2697 bp, 1930 bp and 844 bp

The identified positive colonies were sequenced and aligned in Benchling (benchling. 2022). Based on the alignment (**Figure 21**), F colony 1 and HE plate 3 colony 1 were chosen for further work.

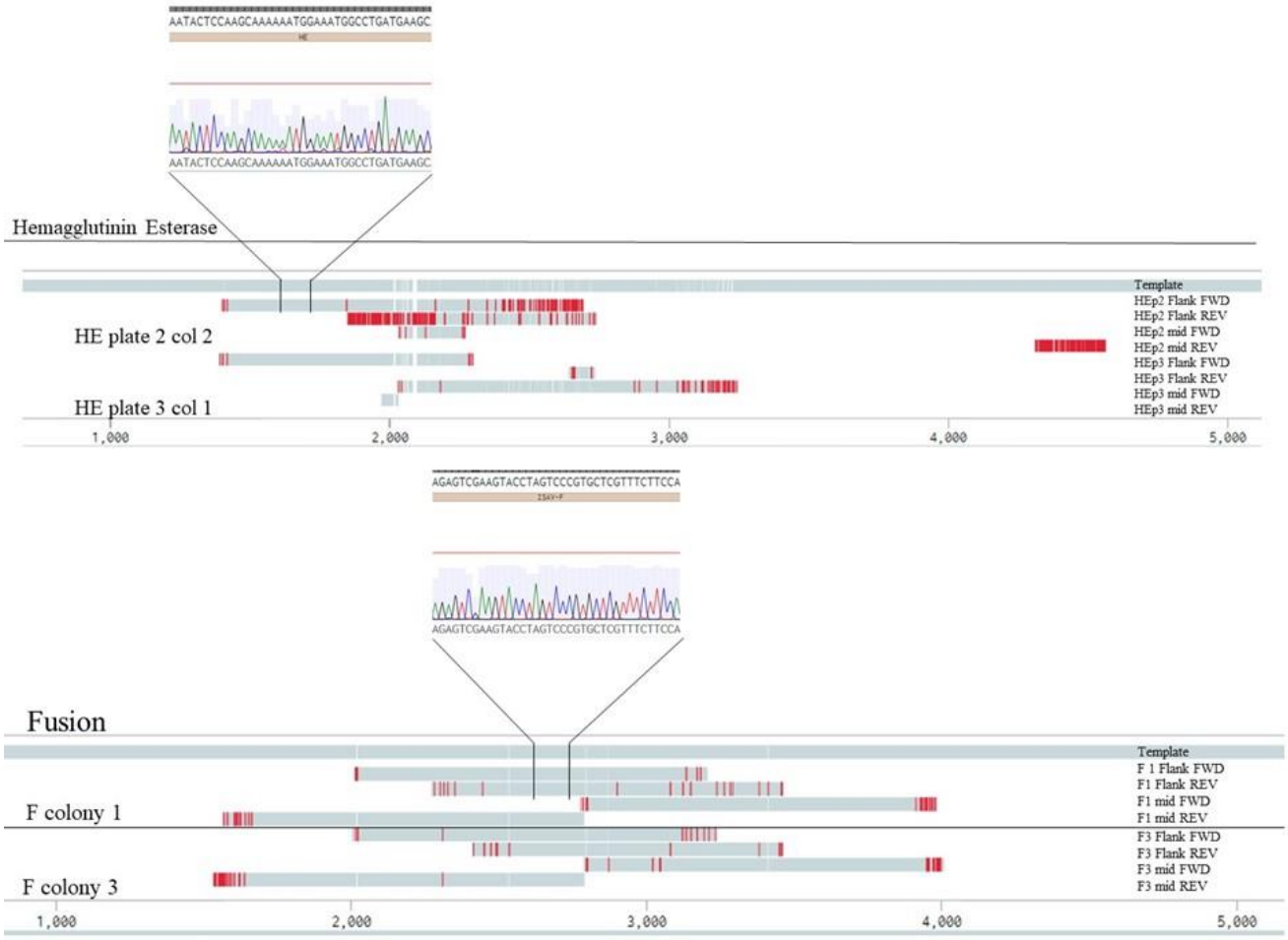

Figure 21: The aligned sequences. Missmaches are indicted by red. The allignment indicated that it was the desired Hemaglutinin and Fusion protein sequence. Colony F1, and Plat 3 colony 1 HE was chosen for rurther use. Alignment made in Benchling (Benschling. 2022).

None of the proteins encoded by Segment 8 was successfully cloned, and therefore excluded from further experiments.

3.2.2 Lentivirus packaging

3.2.2.1 Lipofectamine VSV-G and ISAV-F glycoproteins

Lentivirus of both VSV-G and VSV-G & ISAV-F hybrid psudotypes was attempted produced (**Figure 22**), by HEK293T cells, at 37° C and 22° C (due to the ISAV being a cold adapted pathogen). 24 h post transfection both variants expressed GFP, indicating production (**Figure 22**). ASK and HEK cells were attempted transduced with the lentivirus variants, and 24 h post transfection both VSV-G and the hybrid lentivirus produced a GFP expression in HEK cells, but no GFP was observed in the ASK cells (**Figure 22**). The 22° C production cells produced

a low vector concentration, indicated by the low transfection and transduction rate of the HEK cells (**Figure 22**). One HEK cells expressed GFP when the 22° C VSV-G lentivirus was tested (**Figure 22**), and two HEK cells expressed GFP when the 22° C VSV-G; ISAV-F lentivirus was tested (**Figure 22**).

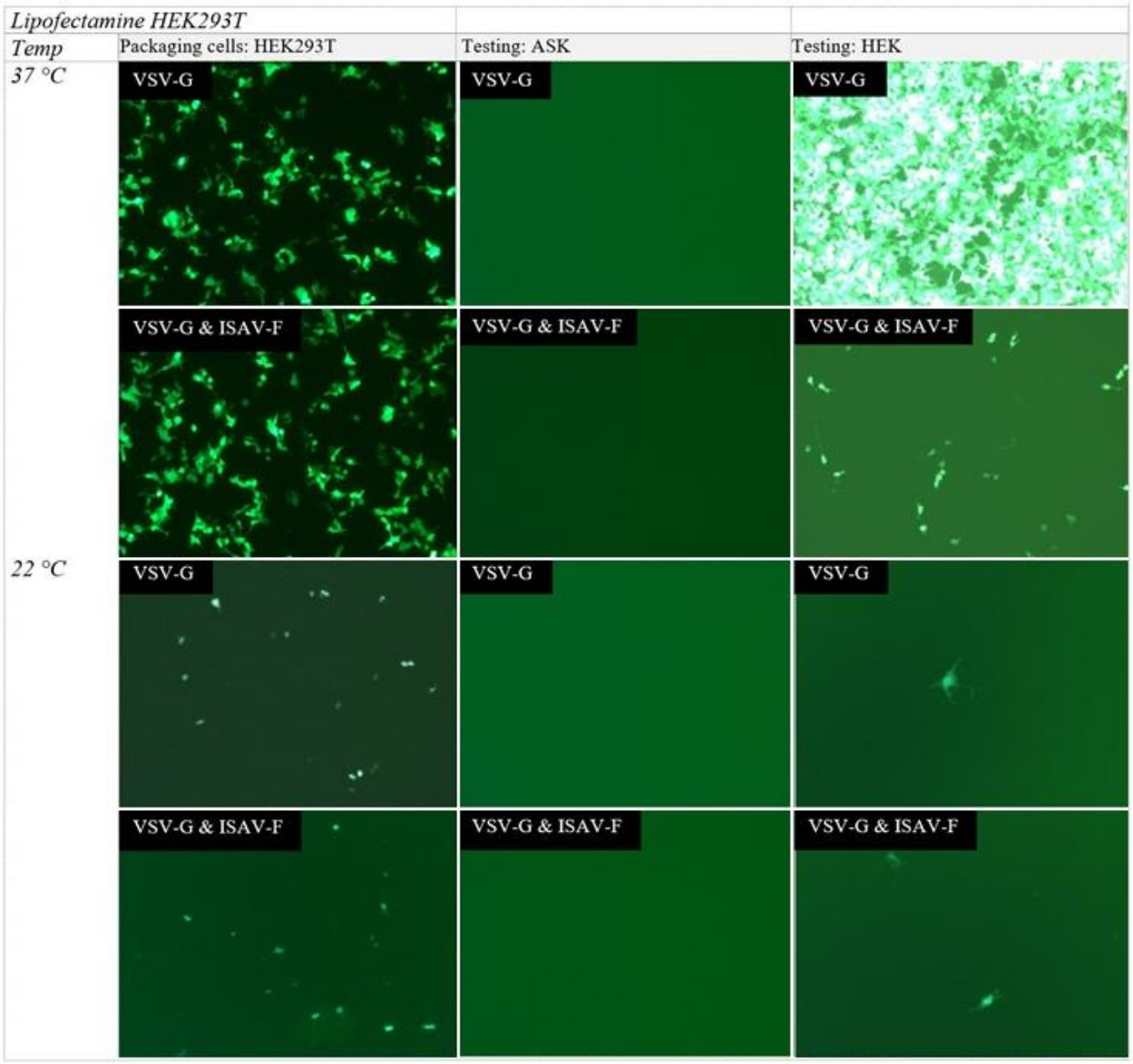

Figure 22: The HEK cells were successfully transduction, both from VSV-G lentivirus and VSV-G & ISAV-F hybrid. No GFP was found in the ASK cells. When the lentivirus was produced at 22 °C there was a very low titer virus produced.

3.2.2.2 Electroporation VSV-G and ISV-F glycoproteins

Due to the low transduction and/or transfection rate of the lentivirus grown at lower temperature (**Figure 22**), lentivirus was attempted grown in ASK cells at 20° C and HEK cells at 37° C (**Figure 23**). Lipofectamine has a low transfection rate in fish cells (Wilberg, 2020) and therefore it was attempted to use electroporation for the plasmids to penetrate the membrane. The resulting lentivirus was not effective, there was high cell death in the production cells, leading to few viruses produced (**Figure 23**).

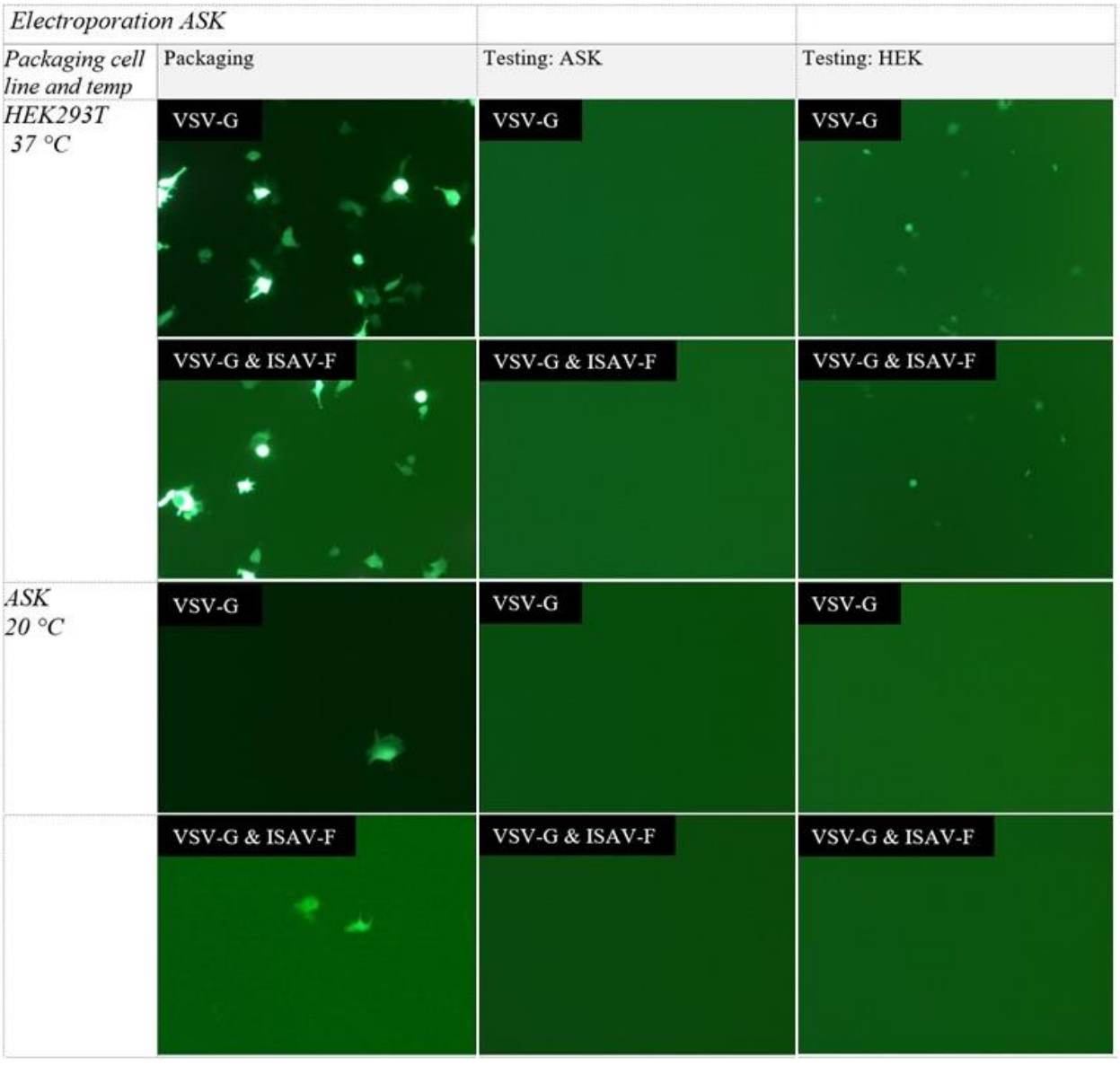

Figure 23: The electroporation had a very low transformation rate, and the produced lentiviruses had a low transduction rate, most cells died in the process.

3.2.2.3 Lipofectamine ISAV-F and HE glycoproteins

Due to the low transfection efficiency of the electroporation the ISAV-HE & F lentivirus were grown in HEK293T at 22° C and 37° C, with some modifications. Lipofectamine was used to deliver the plasmids, but the cells were allowed more time to regenerate, which appaired to increase the cells productiveness (**Figure 24**), even though the first 24h of produced lentivirus were removed. When testing the produced virus in ASK and HEK cells, HEK expressed GFP from all growth conditions and virus type (**Figure 24**). The ASK cells did not express GFP, even after the temperature were increased to 30° C (**Figure 24**). The GFP expression from the ISAV pseudotype in HEK cells was not expected (**Figure 24**), and the experiment should be repeated. Negative controls in the same plate as packaging was included, not shown in the figure, but they were negative.

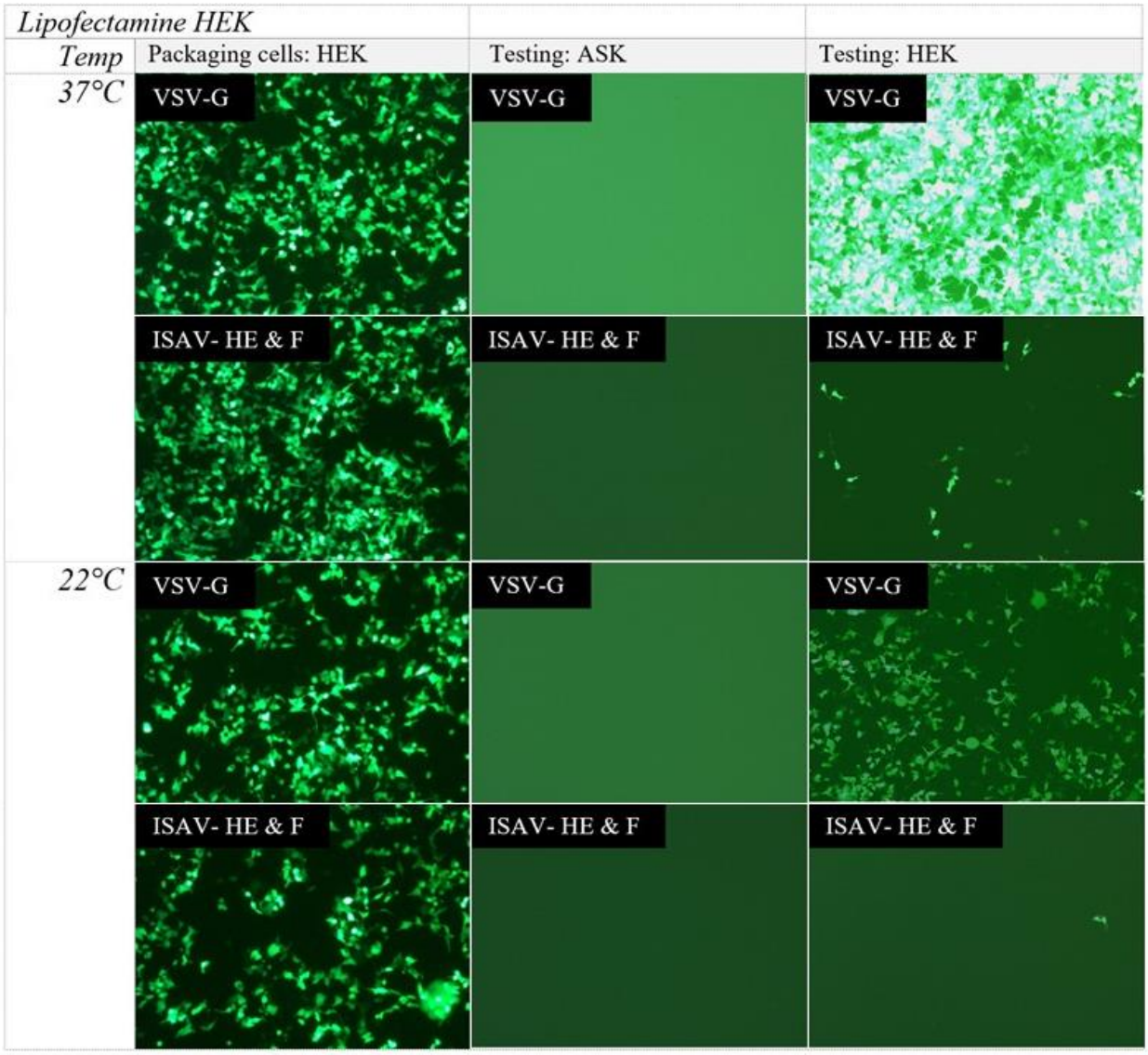

Figure 24: The lentivirus producing HEK293T cells the day after (24h) transfection in column 2. The ASK cells (column 3) and the HEK cells (column 4) 24h post transduction incubated in the cell appropriate incubators. The GFP indicate a successful transfection and that virus was produced. When testing the produced lentivirus, the ASK cells (column 3) showed no signs of GFP, therefore the transduced cells were moved from 22° C to 26°C, 27°C, 28°C, 29°C and 30°C with 24h intervals, but this changed nothing (pictures not in figure). The cells were moved back to 20°C for 24h, no GFP were observed. When the lentivirus was tested in HEK cells, GFP were expressed after 24h, both VSV-G and ISAV- HE& F. The latter was surprising, and the experiment should be repeated.

An overview of what plasmid produced in this work, what lentivirus were produced, under what conditions, the resulting GFP and comments on the produced lentivirus can be found in table 8.

Table 8: An overview of the produced lentivirus, production conditions and observations. Two methods of lentiviral packaging were attempted, lipofectamine and electroporation. Different production temperatures were also tested.

Lipofectamine	*Production cells*	*Conditions*	*Tested in HEK*		*Tested in ASK*		*Comment*
			Entry	GFP	Entry	GFP	
VSV-G	HEK	37° C	Yes	Yes	Inconclusive	No	Normal, High LV concentration
	HEK & ASK	22° C	Yes	Yes	Inconclusive	No	Low LV concentration
VSV-G & ISAV-F	HEK	37° C	Yes	Yes	Inconclusive	No	High LV concentration
	HEK & ASK	22° C	Yes	Yes	Inconclusive	No	Low LV concentration
ISAV-HE & ISAV-F	HEK	37° C	Yes	Yes	Inconclusive	No	Production cells were green, VSVS-G control had high transduction rate of HEK cells
	HEK	22° C	Yes	Yes	Inconclusive	No	Production cells were green, VSV-G control transduced HEK cells
Electroporation	*Production cells*	*Protocol*	*Tested in HEK*		*Test in ASK*		*Comment*
			Entry	GFP	Entry	GFP	
VSV-G	HEK	Voltage: 1100V Width: 20ms Pulses: 2 Growth: 37 ° C	Yes	Yes	Inconclusive	No	Most cells died, low transfection and transduction rate
	ASK	Voltage:1400V Width: 20ms Pulses: 2 Growth: 20° C	Yes	Yes	Inconclusive	No	Most cells died, low transfection and transduction rate
VSV-G & ISAV-F	HEK	Voltage: 1100V Width: 20ms Pulses: 2 Growth: 37 ° C	Yes	Yes	Inconclusive	No	Most cells died, low transfection and transduction rate
	ASK	Voltage:1400V Width: 20ms Pulses: 2 Growth: 20 ° C	Yes	Yes	Inconclusive	No	Most cells died, low transfection and transduction rate

Chapter 4. Discussion

4.1 Summary

Two of the three ISAV proteins were successfully cloned, fusion and hemagglutinin esterase. The transduction of Atlantic salmon cells using lentivirus was not achieved, by altering transduction conditions of VSV-G based lentivirus nor by pseudotyping using glycoproteins from known salmon pathogen ISAV. An understanding of where the transduction of Atlantic salmon cells is terminated has been attempted. Receptor binding and fusion were addressed through pseudotyping. The internalisation patterns were explored by labelling virus and observing the infection using confocal microscopy. Reverse transcriptase activity was evaluated by qPCR, and translation and GFP expression were tested through electroporation. There are multiple factors that could interfere with the transduction of salmon cells.

4.2 The transduction pathway

The lentiviral transduction pathway is complex. The receptor binding and membrane fusion follow the pattern from the glycoprotein donor. Then after internalization the vector uses the same cellular machinery as HIV-1 for nuclear import and integrate into the hosts genome (. Both VSV and ISAV enters a endocytic pathway, and fusion is triggered by the lowering of pH (Aspehaug et al., 2005; Johannsdottir et al., 2009; Gruenberg, 2009; Dale & Falk, 2014;) however the fusion mechanism differs between species. After internalisation the vector rely on HIV systems for integration of the transgene (reverse transcription and integration) (Le Blanc et al., 2005; Gruenberg, 2009; Johannsdottir et al., 2009). (**Figure 25)**.

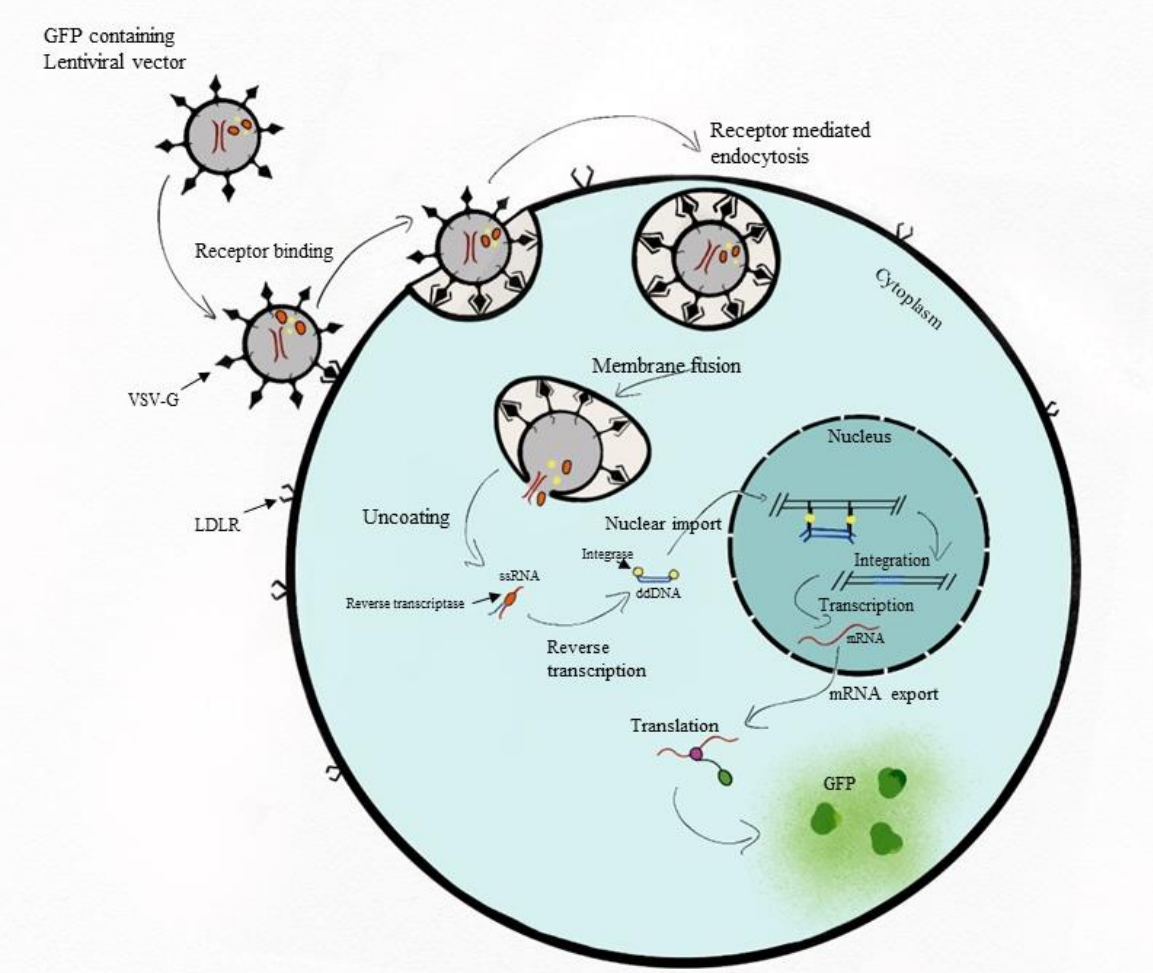

Figure 25: A simplified illustration of the general steps in the lentiviral transduction using the VSV-G pseudotype. There are multiple steps in the transduction of cells, and multiple factors that could affect the success of the transduction. The host have antiviral mechanisms attempting to keep pathogen from replicating. In this work, the receptor binding and membrane fusion, reverse transcription, mRNA export and translation were looked at.

4.2.1 Receptor binding, internalisation, and fusion

The glycoproteins of a virus determine what cells it can interact with. The infectious HIV-1 directly fuse with the cell membrane (Wilen, Tilton & Doms, 2011). But the vectors glycoproteins from both VSV and ISAV trigger the formation of endosomes (**Figure 25**). Mechanisms of which the binding and subsequent fusion occur is different between the glycoproteins (Le Blanc et al., 2005; Gruenberg, 2009; Johannsdottir et al., 2009; Müller et al., 2010; Aamelfot, Dale & Falk, 2014).

Microscopy from section 3.1.2 indicated that the VSV-G is able to interact with both HEK and ASK cell receptors (**Figure 10 & 11**), which led to internalisation, but no GFP could be observed in the ASK cells. Evidence shows that VSV-G protein binds to the CR domain of all LDL-R receptors, initiating entry (Jovan et al., 2018), this receptor is found in Atlantic salmon (Kleveland et al., 2006). Gratacap et al., (2020) were able to transduce chinook salmon cells. This finding indicate that the Atlantic salmon cells have a receptor that allow binding of the VSV-G which initiate endosomal uptake, but for some reason the transduction is not completed. One possible explanation could be that the VSV-G is not able to facilitate fusion in Atlantic salmon cells (no viral escape). Methods known to increase transduction in HEK cells were tested in ASK cells, but no changes were observed in the ASK cells.

The findings of section 3.1.4 indicate that the Glycoproteins of VSV-G are pH sensitive and the low pH inside endosomes initiate the fusion steps. When HEK cells were treated with pH 5 media before transduction, the transduction rate increased (**Figure 14**), this was however not confirmed by literature (Morizono et al., 2006). Based the results found in this section, the lowering of pH was tested as a potential method for increased transduction of ASK cells, but no GFP could be observed (**Figure 15**). Temperature could be a factor for this stage if the receptors are heat or cold sensitive, however the VSV-G glycoprotein have been found to bind to receptors and initiate cell entry at 4° C (Johannsdottir et al., 2009). Additionally, increased temperatures were tested, with no success (**Figure 15**).

Fusion might be the first obstacle, therefore a VSV-G: ISAV-F hybrid (**Figure 22 & 23**) and an ISAV-HE: F (**Figure 24**) lentivirus was produced. VSV-G lentivirus have many great qualities, for example high vector stability which allow for concentration by centrifugation (Burns et al., 1993), the hybrid, if it works could have a different tropism and the entry pathway could possibly be different than a pure ISAV vector. The lentivirus vector producer cells expressed GFP 24h posts transfection, and when testing all the different viruses, the transduced HEK cell expressed GFP 24h post transduction, indicating production of active lentivirus. No GFP were expressed in the ASK or the fibroblast cells when testing the pseudotyped vector, even with increased temperatures (**Figure 22, 23 & 24**). Indicating a more systemic issue.

The ISAV- HE: F lentivirus produced a GFP expression 24h post transduction in HEK cells (**Figure 23**). HE binds to the 4-O-acetylated sialic acid residues. Sialic acids frequently cap glycans on the cell surface of vertebrates, certain invertebrates, and bacteria, where the O-

acetylation at the C-4/7/8/9 positions are the most common (Visser et al., 2021). Less is known of the 4-O- acetylated sialic acid, particularly of its presence and/or function within human tissue (Aamelfot et al., 2014; Visser et al., 2021). One study found no detection of 4-O-acetylation in HEK293 cells with virolectin (Wasik et al., 2017), but traces have been found in erythrocytes (Bulai et al., 2003), and in the intestine (Robbe et al., 2003) when using mass spectrometry. Based on the existing literature, the HEK cell should not be transduced by a ISAV lentivirus, therefore the production of ISAV-HE: F lentivirus should be repeated again, and tested in HEK cell. If this result is real, it shows that the F and HE protein is not heat sensitive and that active pseudotyped virus is being produced during packaging.

When looking at the 3.1.2.2 section (**Figure 11E & 11F**), more of the viral SYTO82 signal have been lost in the ASK during the 3 days incubation compared to HEK cells. In theory, once the virus fuse with the cell and start the reverse transcription the SYTO82 signal would be lost as the RNA is degraded. Some viruses will not achieve fusion so some signals were expected from HEK, but there should be more SYTO82 signals in the ASK cells if no fusion occurs. This could indicate that the vector is degraded before or after fusion of the endosomal wall, pointing towards the innate immune system of cells. There were observed GFP within the endosomes of ASK cell on day 3 post transduction (**Figure 12**). This could possibly be a result of the confocal microscope miss detecting GFP due to crosstalk with SYTO82, the signals observed however were not located in the cross section between GFP and SYTO82. GFP in the endosomes in not likely unless GFP proteins were packaged in vector particle, but maybe the observed GFP were misread autofluorescence from degraded particles, possible lentiviral vectors. Autofluorescence is commonly misread as GFP (Zhang et al., 2022).

4.2.2 Reverse transcription and nuclear import

4.2.2.1 Reverse transcription

The viral RNA is reverse transcribed into cDNA before the genome is integrated into the hosts genome. The reverse transcriptase is packaged in the viral vector during the packaging process, and the process of reverse transcriptase starts after internalization, where in the cell and the state of the nucleocapsid is not fully understood (Arhel, 2010). The proviral DNA synthesis is dependent on two enzymatic activities of reverse transcriptase, a DNA polymerase which can

use RNA and DNA as a template, and a nuclease (ribonuclease H /RNase H) (Hu & Huges, 2012). However, the role of other proteins cannot be ruled out (Arhel, 2010). The first step is the translation of the +sense single stranded RNA to a - sense single stranded DNA, this process is initiated at the 3'end by a tRNA which are bound to the RNA. As the RNA is converted to DNA the RNase H degrade the RNA, once the whole RNA strand has been read the polymerase produces the complementary +DNA strand (Hu & Huges, 2012). This process could possibly be affected by internal (e.g., cells innate immune system (Staring, Raaben, & Brummelkamp, 2018)) and external factors (e.g., temperature).

Findings from section 3.1.1 indicated that GFP is expressed in HEK cells after 26° C and upwards (**Figure 9**). The reverse transcription could be an inhibiting step, caused either by reverse transcription machinery being non-functional at lower temperatures, as a result of e.g. formation of secondary structures in the RNA. The internal stages are reliant of lentivirus mechanisms (Hu & Hughes, 2012). But existing literature indicate that the vector to some degree work at low (20° C) temperatures in other species (Johannsdottir et al., 2009; Gratacap et al., 2020). Reverse transcription mechanisms of HIV-1 have been found to read through stable hair-pin structures at 37° C (Suo & Jhonson, 1997), where it is theorized that the enzyme slows down, awaiting the hair-pin to melt (Suo & Jhonson, 1997), how this is translated to lower temperatures is well documented. Why HEK does not express GFP below 26° C is not fully understood, it could be the vector, but it could also be the HEK cells not producing GFP due to stress. When the plates were moved down to 22° C, photos taken indicate a reduction in GFP in both plates. However, during the first low temperature packaging HEK cells were incubated at 22° C and some GFP was expressed.
To see if the problems with transduction were due to temperature, as the HEK experiment indicated, the ASK cells and salmon fibroblast cells were transduced and incubated at 26° C, 27° C, 28° C, 29° C, and 30° C (**Figure 15)**. No proof of transduction was observed (this was also done with the ISAV pseudotyped variants, and with pH5) (**Figure 15, 21, 22 & 23**).

To determine if the ASK transduction was terminated before or after reverse transcriptase , a qPCR experiment was set up. HEK and ASK cell RNA (made into cDNA) and DNA samples were screened for the GFP coding sequence (**Figure 16, 19, & 20**). Both ASK and HEK cells were transduced by the same amounts of VSV-G lentivirus, then RNA and DNA were extracted. We hypothesize that if the virus entered the cell, but no reverse transcriptase occurred then amplification of the cDNA samples and no amplification in the DNA sample would be

observed, if the virus had reverse transcriptase activity, then amplification in both cDNA (RNA) and DNA sample would be observed. The latter pattern was observed in the HEK samples, and to some degree in the ASK cells, and the results looks to indicate traces of GFP DNA in the ASK sample. There were however observed contaminations, probably plasmid contamination, in most all control samples. The lentiviral packaging plasmids are highly used in the lab environment, these contain an AMP sequence and are important in the plasmid cloning process. The pLenti GFP also holds a GFP sequence. The cDNA samples were seemingly more impacted by the contamination compared to the DNA samples. cDNA samples were handled more (two step reverse transcriptase) which allows for more potential contamination.

The extent of the alleged contamination effect on the results was addressed by a boxplot, where in the HEK cDNA (**Figure 17A**) AMP amplification was far lower than the GFP amplification, therefore most of the GFP signal is likely not due to contaminating plasmids. For the ASK cells more AMP was detected than GFP, therefore it is likely that the observed GFP signal in the samples is the result of contaminating transgene plasmid (**Figure 17B**). The conclusion on this is that in the ASK cells no reverse transcription occurs, but the virus is probably inside the cells. This experiment must be repeated.

4.2.2.2 Nuclear import

Nuclear import is a vital step in the lentiviral replication cycle. Due to lentivirus ability to infect non dividing cells, the genome must be able to cross the nuclear membrane. There are uncertainty of where in the cell reverse transcription occurs, and in this work nuclear import was not directly studied, and will therefore not be discussed in detail. It was believed that the virus was uncoated in the cytoplasm and only proviral DNA was transported into the nucleus, however increasing evidence has demonstrated intact or nearly intact HIV-1 capsid entering the nucleus (Selyutina et al., 2020; Shen et al., 2021). The virus might be capable of both patterns, but more research of the topic is needed. Where in the cell reverse transcriptase occurs would affect what mechanisms could disturb the transduction of Atlantic salmon cells.

4.2.3 Integration

If the transgene is reverse transcribed and successfully transported to the nucleus (or the other way around) (Selyutina et al., 2020; Shen et al., 2021), there the proviral DNA is integrated into the hosts genome. Integrase is the viral enzyme responsible for this, and it is packaged within the viral vectors. It catalyses two reactions, first the viral DNA 3' end processing where two nucleotides are removed, then the hosts genome is nicked and the viral DNA is inserted (Telesnitsky & Goff, 1997). the cell's DNA repair mechanisms is then recruited to rebuild the DNA, and the virus DNA is integrated. In this work integration was not studied directly. There is a possibility that the transduction is stopped during the integration, but there are so many factors that could play a role in this, and it would be speculations.

4.2.4 Transcription, mRNA export, and translation

The integration can take place at various locations in the host cell, but regions of high genome density an transcriptional activity is somehow targeted by the HIV virus (Lusic & Siliciano, 2017), therefore if the promotion region is recognised by the RNA polymerase the gene should be transcribed into mRNA. The transgene is driven by the CMV promoter, which is found to work well in salmon, therefore the RNA polymerase should be able to recognise the promoter region (Bearzotti et al., 1992) In section 3.1.3 mRNA was successfully translated from the transgene plasmid after transfection, indicating that the RNA polymerase does recognise the promotor (**Figure 13**).

After transcription the mRNA is exported out of the nucleus through the nuclear pore complex, to the cytoplasm where it binds to ribosomes for translation, simply speaking (Köhler & Hurt, 2007).

Results from section 3.1.3 show that transient gene expression of GFP (**Figure 13**) was achieved in ASK cells when electroporation was used to transfect the cells with the transfer plasmid. GFP was expressed after 24h incubation at 20° C (**Figure 13**). mRNA is exported around the cytoplasm when transcribed from plasmid DNA, indicating that once the mRNA leaves the nucleus it should be translated into GFP. This could also indicate that the cell in general do not react to the gene sequence after integration.

4.3 Interpretation collectively observations

The results found in this work indicate that Atlantic salmon cell transduction by VSV-G virus is stopped after internalization, but before reverse transcription. Viruses that utilise the endosomal pathway must escape the endosomal compartment before it is recycled back into the extracellular space, or before degradation in the lysosome (Staring, Raaben, & Brummelkamp, 2018). Going into too much detail would merely be speculations, but the results from the experiments done in this work shows VSV-G lentivirus entering ASK cells, and three days later observations of GFP form cellular compartments (or more likely autofluorescence) around the nucleus were made. Possibly form lysosomes (**Figure 11F**) (Andersson et al., 1998). No reverse transcription was detected, which could be explained by the virus never escaping the endosome, and then no reverse transcription would occur. Results also indicated that once the transgene is reverse transcribed it is likely translated. Cellular antiviral systems for viral detection inside endosomes could be the culprit. Cells have multiple systems in place to restrict pathogens access to the cellular interior within the endosomes (Staring, Raaben, & Brummelkamp, 2018) and more are discovered. As the ISAV pseudotype also produced no GFP it could indicate that it is one of the RNA recognising receptors. However, the infection of ISAV lentivirus must be researched more for us to say much.

The proteins of segment 8 was not successfully cloned in this work, the OPR2 appears to be toxic to *E.coli* (Biering et al., 2002) and the findings from this work indicate the same.

4.4 Successful transduction in chinook salmon: Comparing Gratacap et al. and us.

Gratacap et al. (2020) succeeded in transducing Chinook salmon (CHSE-214) cell lines using the VSV-G lentivirus. They reported that increasing the temperature from 17° C to 22° C. increased the transduction rate from 1 to 63%. This is within the temperature *Salmo salar* cells were incubated, however no transduction was observed. The Gratacap (et al., 2020) used heat shock, which were found to increase transduction rate further, this was not attempted in this work. During the transduction the cells were centrifuged at 1000x for 2 h, and an antibiotic selection procedure were used to select for successful transduction over 7 days. This was also not done in this work as an efficient method of transduction was the goal. The transgene used

by Gratacap was EGFP (Enhanced GFP) and RIG-I, in this work GFP was used for transduction identification. Even though the exact protocol was not followed, the presence of one green cell would be “proof of concept”, showing that the cells are transduced but not efficiently. This was not achieved.

4.5 Future perspectives

The packaging of ISAV-HE: F pseudotyped virus should be repeated, as these results could indicate the presence of 4-O-acetilated sialic acids in HEK 293T, which contradict the pre-existing literature (Wasik et al., 2017). The exact method of transduction from Gratacap (et al., 2020) should be tested, with heat shocking, centrifugation, and antibiotic selection. The ISAV HE: F viral vector should be labelled to compare patterns observed with VSV-G. A concentrated pseudotyped virus should be made, as results indicate low titer of virus produced. A study of the salmon innate immune system could also be done, and a thorough comparison between Atlantic salmon, Chinook salmon and other fish shown to be transduced by VSV-G lentivirus (e.g. zebra fish) could be interesting. A possibility could be to knock out antiviral mechanisms in salmon.

4.6 Conclution

The transduction of Atlantic salmon cells using the 2nd generation lentivirus was not achieved, by altering transduction conditions of VSV-G based lentivirus nor by pseudotyping using glycoproteins from known salmon pathogen ISAV. An understanding of where the transduction of Atlantic salmon cells is terminated has been attempted and some of the transduction steps were studied directly. Receptor binding and fusion were addressed through pseudotyping. The internalisation patterns were explored by labelling virus and observing the infection using confocal microscopy. Reverse transcriptase activity was evaluated by qPCR, and translation and GFP expression were tested through electroporation. The results found in this work indicate that HEK cells can be transduced by a ISAV-HE; F pseudotype. Atlantic salmon cell transduction by VSV-G virus is stopped after internalization, but before reverse transcription.

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Product list

Reagents: product, catalogue number and manufacturer

Ampicillin, 11593027, Gibco

Carbenicillin, 10177012, Gibco

Fetal Bovine Serum, 26400044, Gibco

HEPES, 25630-056, Gibco

Leibovitz's L-15 Medium, GlutaMAX Supplement, 31415029, Gibco

Opti-MEM I Reduced Serum medium, 31985070, Gibco

Penicillin-streptomycin, 15140122, Gibco

Trypsin-EDTA, 25200072, Gibco

Typhan Blue solution, 15250061, Gibco

CellMask, C10046, Invitrogen

CFDA, C2925, Invitrogen

Hoechst, H1399, Invitrogen

SYTO82, S11363, Invitrogen

LB Broth base, 12780052, Invitrogen

Neon™ Transfection System 10 µL Kit, MPK1025, Invitrogen

Neon™ Transfection System 100 µL Kit, MPK10025, Invitrogen

One shot Stbl3 competent E. coli cells, C737303, Invitrogen

Platinum II Hot-Start Green PCR Master Mix (2X), 14001012, Invitrogen

PureLink™ Viral RNA/DNA Mini Kit, 12280050, Invitrogen

SOC medium, 15544034, Invitrogen

Phusion™ Hig Fidelity DNA polymerase, F530S, Thermo Scientific

Chloroform, Cas no, 67-66-3, Sigma-Aldrich

Ethanol, 64-17-5, Sigma-Aldrich

Formaldehyd, Sigma-Aldrich, 47608

HCL, 7647-01-0, Sigma-Aldrich

Isopropanol, 563935, Sigma-Aldrich

LB Broth with agar, 1003212151, Sigma-Aldrich

NaOH, 1310-73-2, Sigma-Aldrich

Paraformaldehyde, 30525-894, Sigma-Aldrich

Phosphate Buffered Saline (PBS), pH 7.4, P3813, Sigma-Aldrich

Polybrene, TL-1003-50UL, Sigma-Aldrich

Dulbecco’s Modified Eagle*s Medium- High glucose (DMEM), D6429, Sigma-Aldrich

Tris-buffered-saline (TBS), T5912, Sigma-Aldrich

QIAprep Spin Miniprep Kit, 27106, Qaigen

QIAquick Gel Extraction Kit, 28706X4, Qiagen

QIAquick PCR Purification Kit, 28104, Qaigen

QIAzol Lysis reagent, 79306, Qiagen

ZymoPURE II Plasmid Midiprep Kit, D4201, Zymo Research Crop

ZymoPure II Plasmid miniprep Kit, D4210, Zymo Research Crop

iScript cDNA Synthesis Kit, 1708891, Bio-Rad

SSo Advansed Universal SYBR® Green Supermix, 1725271, Bio-Rad

BasaI-HF v2, R32333, New England BioLab (NEB)

PmlI, R0532S, New England BioLab (NEB)

StuI, R0187S, New England BioLab (NEB)

rCutSmart Buffer, B6004S, New England BioLab (NEB)

Gibson assembly Master Mix M5510A, New England BioLab (NEB)

35mm TC dish, 82.1135.500, Sarstedt

T75 flask, 83.3911.002, Satstedt

50 mL tube, 62.547.255, Sarstedt

15 mL flask, 62.554.502, Sarstedt

e.coli tube, 62.493, Sarstedt

Fluoromount Go, 0100-1, Southern Biotech

Coverslip: Assistant 1000 18x18mm, Menzel

Plasmids

pSPAx3 - Addgene plasmid # 12260

plenti-CMV-GFP-Puro- Addgene plasmid # 17448

pMD2.G- Addgene plasmid # 12259

Equipment: product, catalogue no, manufacturer

Agilent 4150 TapeStation system, G2992AA, Agilent

CFX96 Touch Real-Time PCR detection system, Bio-Rad

CFX96 real-time system, C1000 Touch Thermal Cycler

Consumables:

- Hard-shell PCR Plates, 96-welled, thin wall, HEP9655, Bio-Rad
- Microseal®`B´ Seal, MSB101, Bio-Rad

Veriti™ 96-Well Fast Thermal Cycler, 4375305, Applied Biosystems™

Consumables: PCR tubes

Fluorescence microscope, 3849000909, Carl Zeiss

Confocal microscope, Imager.Z2, AXIO, LSM800, Carl Zeiss

C1562 - Freezing container, Nalgene, Mr. Frosty

NanoDrop 8000 Spectrophotometer, ND-8000-GL, Thermofisher Scientific

Neon Transfection System, MPK5000, Invitrogen

TC-20 Automated cell counter, 1450102, Bio Rad

Consumable: Cell counting slides, 1450011, Bio-Rad

Countess 3 FL, AMQAF2000, Invitrogen

Consumable: Cell counting slides, C10228, Invitrogen

XRS+ System, 1708265, ChemiDoc™

Consumable:

- Agarose, A9539, Sigma-Aldrich
- Tris-Acetate-EDTA (TAE), J63677.K3, Thermo Scientific™
- Loading dye purple (6x), B70245, New England BioLab (NEB)
- Gene Rule 1 kb, SM0311, Thermo Scientific™
- Red safe, 21141, Chembio

Orbital Shaker- Icubator, ES-20, BioSan

Microfuge® 20R Centrifuge, Bacman Coulter

ThermoMixer C, 1.5 mL Eppendorf

Software

Agilent 4150 TapeStation system software

CFX Maestro software, 12004110, Bio-Rad

NanoDrop 8000 V2.1.0 software

Image Lab™ Software, 1

Free software and Online tools

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Nucleotide [internet] Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988]-. Accession NO: NM_ : 006499.1, Infectious Salmon Anemia Hemagglutinin esterase, transcript variant 1, RNA; [cited 2022 08 15] Available from: : https://www.ncbi.nlm.nih.gov/nuccore/NC_006499.1

Nucleotide [internet] Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988]-. Accession NO: NM_ : 006497.1, Infectious Salmon Anemia Segment 8, transcript variant 1, RNA; [cited 2022 08 15] Available from: https://www.ncbi.nlm.nih.gov/nuccore/NC_006497.1

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