Development of RT-PCR based method for genome amplification of human picornaviruses and rescue of viable viruses

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Picornaviruses (family *Picornaviridae*), causing diseases form respiratory illness to life-threatening CNS disease, are among the most common human pathogens. More than 300 human picornavirus types have been currently identified, yet there are no antivirals or vaccines against the common picornavirus types. Picornaviruses are RNA viruses and thus prone to mutations, which alter the virus tropism and pathogenesis. Viral cDNA clones or vectors are essential in the studies of picornaviruses. The standard cDNA clone production methods, however are cumbersome and old fashioned.

The aim of the thesis was to create a streamlined, robust method for amplification of picornaviral RNA genome to enable easy mutagenesis and generation of functional viral vectors.

To amplify viral RNA and cDNA clones with the backbone of coxsackievirus A9 and human parechovirus 1, four RT enzymes and five PCR enzymes, respectively, were used. PCR parameters (primer and nucleotide concentrations, number of cycles, performance based on copy numbers) were assessed to compile an optimal protocol. Further plans were made to follow virus rescue after cell transfection of viral genomes cloned under T7 promoter. Finally, HuTu80 cell line was evaluated for its suitability for parechovirus propagation and for reverse genetics experiments.

Platinum SuperFi II DNA polymerase was the most robust and sensitive PCR enzyme and allowed amplification from 10^2 viral cDNA genome copies. It performed consistently and worked on a wide range of nucleotide and primer concentrations. LunaScript and SuperScript IV produced the best results in RT step and were also the most straightforward in use. Parechovirus growth in HuTu80 cell line was observed based on cytopathic effect, suggesting that HuTu80 cells are suitable for further parechovirus studies.

In all, novel findings were made to develop a sensitive and robust protocol for amplification of full-length picornaviral genomes. Together with further optimization and optimal regulatory primers, it should allow efficient and sensitive viral genome amplification from small RNA amounts. This study paves way for development of modified viruses for pathogenicity studies and for oncolytic virotherapy.

Keywords: picornavirus, RT-PCR, RNA genome amplification, reverse genetic

Table of contents

1. Introduction

Picornaviridae family contains many very common human pathogens, such as enteroviruses and rhinoviruses. Even though these infections are very common, there are practically no vaccines or antiviral therapies against them available, as immunity against them is very much type-dependent and picornaviruses as RNA viruses are very susceptible to mutate.

RT-PCR is widely used tool in picornavirus research. It can be utilized to produce a great amount of cDNA from a relatively small amount of RNA. On top of genome amplification, PCR can also be used to modify the genome that is amplified, by utilizing primers that contain additional coding sequences. This PCR based modification strategy has many advantages, as it makes the process that otherwise requires many steps relatively simple. The method also has less chances for errors to occur, as number of steps decreases.

Development of a streamlined RT-PCR based amplification and modification method of the picornavirus genome could entice studies related to virus tropism and pathogenesis, and aid in the process of developing picornavirus vectors that could be used as backbone in vaccines and in oncolytic cancer therapy.

1.1 Picornavirus taxonomy

Picornaviridae family is a large family of small RNA viruses. At the time of writing (31.10.2022), recognized are 68 genera, containing 158 species and hundreds of virus types, and even more a waiting to be classified (Zell et al., 2017; https://www.picornaviridae.com/). The 68 genera are presented in Table 1. The experimental section of the project deals with coxsackievirus A9 (CVA9) and parechovirus A1 (PeV-A1), members of the enterovirus and parechovirus genera, respectively.

1.1.1 Enteroviruses

The *Enterovirus* genus contains a large variety of virus types, as it includes 15 species: *Enterovirus A* through *L* and *Rhinovirus A* through *C*. CVA9 belongs to the *Enterovirus B* species. In addition to the common enteroviruses and rhinoviruses, the enterovirus genus also includes some notorious names such as polio (*Enterovirus C*) and echoviruses (*Enterovirus B*) (Zell et al., 2017).

1.1.2 Parechoviruses

The *Parechovirus* genus contains 6 species: *Parechovirus A* through *F*. PeV-A1 belongs to the *Parechovirus A* species. Parechoviruses were at first classified to be a part of the enterovirus genus, but were later determined to be different enough to form a genus of their own (Joki-Korpela and Hyypiä, 2001; Zell et al., 2017).

Table 1. The 68 genera of the picornavirus family. Number of species in each genus can be found in the square brackets.

Aalivirus [1]	Ailurivirus [1]	Ampivirus [1]	Anativirus [2]	Aphthovirus [4]
Aquamavirus [1]	Avihepatovirus [1]	Avisivirus [3]	Boosepivirus [3]	Bopivirus [1]
Caecilivirus [1]	Cardiovirus [6]	Cosavirus [5]	Crahelivirus [1]	Crohivirus [2]
Danipivirus [1]	Dicipivirus [2]	Diresapivirus [2]	Enterovirus [15]	Erbovirus [1]
Felipivirus [1]	Fipivirus [6]	Gallivirus [1]	Gruhelivirus [1]	Grusopivirus [3]
Harkavirus [1]	Hemipivirus [1]	Hepatovirus [9]	Hunnivirus [1]	Kobuvirus [6]
Kunsagivirus [3]	Limnipivirus [4]	Livupivirus [1]	Ludopivirus [1]	Malagasivirus [2]
Marsupivirus [1]	Megrivirus [5]	Mischivirus [5]	Mosavirus [2]	Mupivirus [1]
Myrropivirus [1]	Orivirus [1]	Oscivirus [1]	Parabovirus [3]	Parechovirus [6]
Pasivirus [1]	Passerivirus [2]	Pemapivirus [2]	Poecivirus [1]	Potamipivirus [2]
Pygoscepivirus [1]	Rabovirus [4]	Rafivirus [3]	Rajidapivirus [1]	Rohelivirus [1]
Rosavirus [3]	Sakobuvirus [1]	Salivirus [1]	Sapelovirus [2]	Senecavirus [1]
Shanbavirus [1]	Sicinivirus [1]	Symapivirus [1]	Teschovirus [2]	Torchivirus [1]
Tottorivirus [1]	Tremovirus [2]	Tropivirus [2]		

1.2 Virus structure and genome composition

The picornavirus structure is rather simple. Picornavirus particles are built of a single stranded, positive sense RNA genome that is enclosed in an icosahedral protein capsid. The virus particle is between 30 and 32 nm in diameter and has no envelope. For most picornaviruses, the protein capsid contains 4 different viral proteins, VP1-4. The outer shell of the capsid is made of the VP1-3, while VP4 is located on the inner surface of the shell (Fig. 1). For some picornaviruses, such as parechoviruses, the shell is made up of VP0 (the uncleaved precursor of VP2 and VP4), VP1 and VP3. 60 of each VP is needed to construct the capsid. The VPs cluster together and form triangular protomers (Fig. 1, outlined in red). Five of these protomers form a pentamer (Fig. 1, outlined in yellow), 12 of which complete the icosahedral capsid structure (Zell et al., 2017).

Figure 1. Picornavirus particle structure. The picornavirus particle contains the single stranded, positive sense RNA genome encapsulated by the icosahedral protein capsid. The shell of the capsid is made of 60 sets of VP1-3, while 60 units of VP4 line the inner surface of the capsid. The VPs 1-3 form protomers (outlined in red), which again form pentamers (outlined in yellow) that finally form the icosahedral structure of the capsid.
Figure edited from (ViralZone, SIB Swiss Institute of Bioinformatics).

The picornavirus genome is made up of single stranded, positive sense RNA, meaning the genome can essentially function as a messenger RNA. The presence of internal ribosomal entry site (IRES) allows for direct translation from the genomic RNA. Mutations of the genome are common, as the 3D RNA polymerase amplifying the genome is more prone to mistakes than DNA polymerase is. The picornavirus genome is approximately 6500 – 8500 nucleotides in length. At the 5'-end, the genome has a linked peptide VPg that acts as a primer for RNA synthesis and as a control for translation of the genome, while at the 3'-end a poly-A tail terminates the genome, while providing stability to the genome structure. Both ends of the genome also contain UTRs, containing elements associated with RNA secondary structure, and in between there is one singular open reading frame, meaning the whole genome translates into one polyprotein. The genes coding for the virus capsid proteins are located in the P1 area at the 5'-end of genome, while P2 and P3 areas at the 3' end contain genes that code for the proteins involved in the viral replication. The polyprotein is cleaved by viral-encoded proteases, into the final proteins (Fig. 2) (Tuthill et al., 2010; Cifuente and Moratorio, 2019).

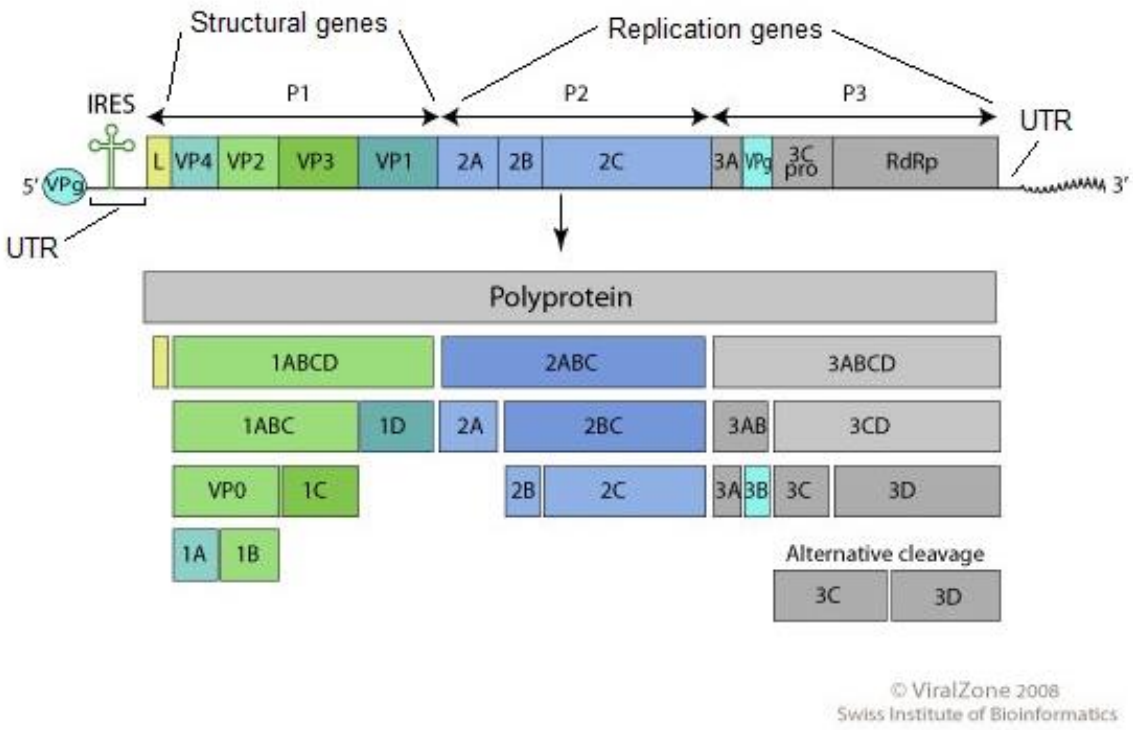

Figure 2. The picornavirus genome structure. The picornavirus genome is made of single stranded, positive sense RNA, which means it can act as a messenger RNA. The genome is approximately 6500-8500 nucleotides in length. The genome starts with a 5' linker peptide VPg and is followed by an UTR. After that come the structural genes and replication genes, in a single open reading frame that produces a single polyprotein. The genome also terminates with a UTR and a poly-A tail. The formed polyprotein gets cleaved into the protein products by viral proteases.
Figure edited from (ViralZone, SIB Swiss Institute of Bioinformatics)

1.3 Picornavirus replication

Picornaviruses replicate in the cytoplasm (Fig. 3). The replication is quite direct, as the RNA genome is essentially mRNA as well. They gain entry to the cell either through creating a pore in the membrane of the host cell or by receptor mediated endocytosis. Picornaviruses can utilize a range of different receptors for their entry, such as integrins, glycoproteins, immunoglobulin like receptors and LDLR family receptors (Tuthill et al., 2010).

In the case of receptor mediated endocytosis, once the virus binds its receptor, an entry vesicle is formed and the virus particle gains access to the cytoplasm. The entry vesicles later fuse with early endosomes that further develop into late endosomes with low pH. The change in pH alters the composition of the virus capsid, allowing for the uncoating of the virus and the RNA to exit from the endosome.

In the pore mediated entry, the virus (e.g., poliovirus) binding to its receptor triggers a change in the conformation of the virus capsid. The conformation change allows for a pore to be formed on the surface of the capsid through the host cell membrane, through which the RNA genome gains entry to the cytoplasm, leading to the uncoating of the virus. At the 5'-end of the genome is a covalently linked viral VPg protein, enabling efficient transcription, making it essential for the infectivity of viral RNA. The translation is dependent on the IRES region of the virus genome assembling ribosomes on the viral genome, as the difference in the 5'-end of the host mRNA and virus genome makes the viral genome unable to utilize host cells ribosomes. Once the polyprotein is translated, it gets cleaved, creating precursors and functional proteins of the viruses own replication machinery and structural components (Whitton et al., 2005; MacLachlan and Dubovi, 2017).

RNA synthesis for the formation of new virus particles is performed in a viral factory, a membrane compartment inside the cytoplasm that shields the process from the host cells defense mechanisms. Picornaviruses produce a heterogeneous pool of viral factories, including double membraned vesicles as well as single membraned spherules and rosettes, likely generated from the Golgi apparatus, endoplasmic reticulum or from autophagosomes (Netherton and Wileman, 2011).

Inside the viral factory the virus encoded 3D RNA polymerase creates a complementary negative strand of the genome. The negative strand is then used as an intermediary template, to obtain RNA genomes with positive sense. New RNAs containing the VPg linker are packaged into capsids, made up of capsid proteins cleaved from the polyprotein. Completed virus particles are then released, leading to lysis of the host cell (Whitton et al., 2005; MacLachlan and Dubovi, 2017).

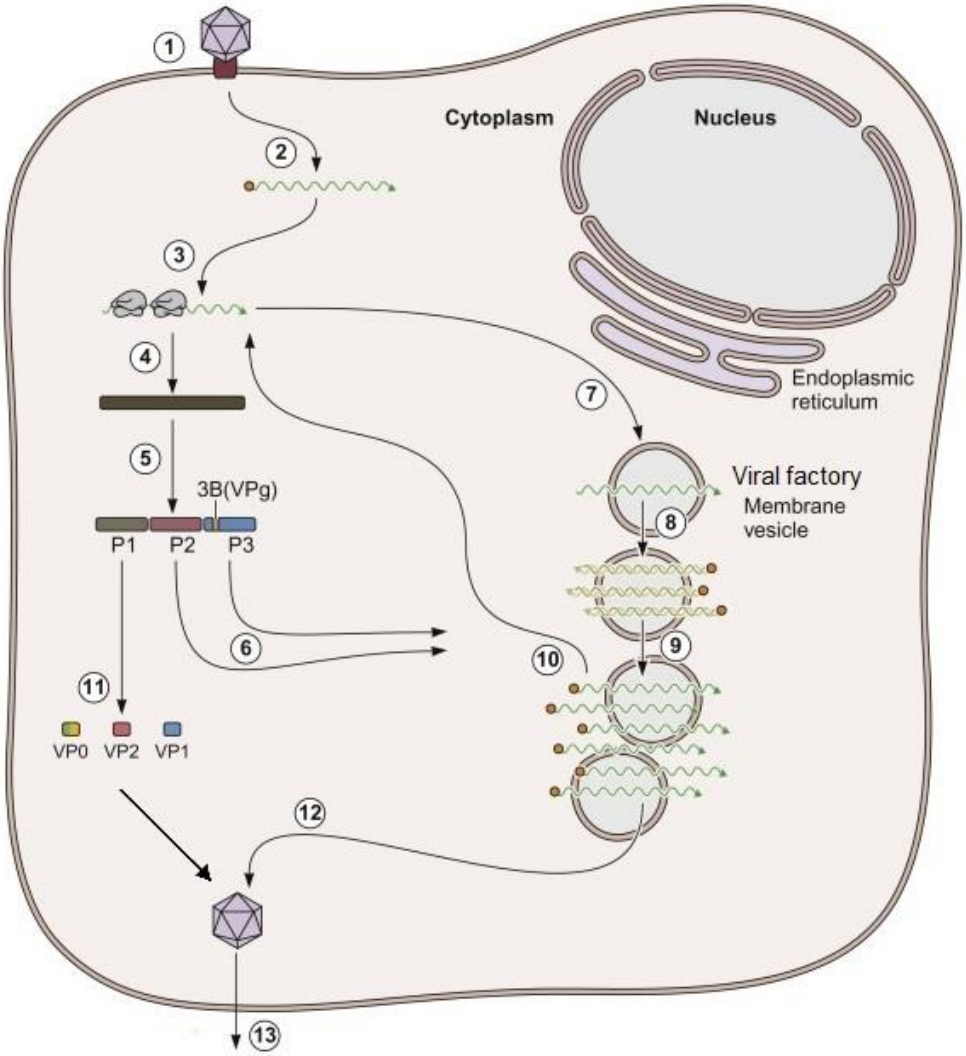

Figure 3. The picornavirus lifecycle, depicted with pore mediated entry route. Virus enters the cell through receptor & pore mediated entry. (1) In the cytoplasm, virus is uncoated and VPg linker protein is cleaved (2), allowing for IRES dependent translation (3) of the RNA genome, producing a polyprotein product (4), which is then cleaved to individual proteins (5). Proteins needed in the synthesis (6) and the genome template (7) are delivered to viral factories, where RNA synthesis takes place. The positive sense genome is used as template for the production of complimentary, negative sense genomes (8), which are again used as templates for the production of genomic, positive sense RNA (9). The produced RNA can be used as a template of translation (10) or it can be packaged into new viral particles (12), made of certain proteins cleaved from the polyprotein (11). Once virus particles are completed, they exit the host while leading to lysis of the cell (13). Figure edited from (Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, 2008).

1.4 Disease burden

Picornaviruses cause a wide range of different conditions, from respiratory infections to myocarditis, hepatitis and CNS infections. Most cause mild, subclinical infections while others cause more severe, even fatal infections. Picornavirus transmission primarily happens through the fecal-hand-oral or airborne, respiratory routes (Cifuente and Moratorio, 2019).

Enteroviruses being a genus with multiple species, its many virus types cause a range of different symptoms. Poliovirus is one of the most notorious members of the genus, and it has a history of causing major outbreaks of poliomyelitis and associated paralysis. *Enterovirus* genus also includes rhinoviruses, which are one of the most common pathogens infecting humans. Rhinoviruses cause relatively mild respiratory infections, such as common cold symptoms. Rhinovirus outbreaks are common and they affect a large number of people worldwide, resulting to a large number of days missed from work and school (Cifuente and Moratorio, 2019).

Coxsackieviruses cause mostly mild infections, associated with rash and skin lesions. However they can cause more severe conditions like pleurodynia and myocarditis. CVA9 for example has been known to cause outbreaks of aseptic meningitis (Cui et al., 2010). Hand, foot, and mouth disease and encephalitis can also be caused by members of the *Enterovirus* genus (Jubelt and Lipton, 2014).

Parechoviruses mainly cause mild respiratory conditions and gastrointestinal diseases. There are also cases where they have been associated with diseases similar to echoviruses, such as meningitis and encephalitis, as well as myocarditis. Most parechovirus infections are pediatric, happening in early life (Joki-Korpela and Hyypiä, 2001).

1.5 Combatting picornavirus infections

There are few methods of combatting picornavirus infections available. The polio outbreaks are under control thanks to the polio vaccine, and hepatitis A can also be avoided by getting vaccinated. There is also a vaccine against the Enterovirus A71 that causes Hand, foot, and mouth disease, though it is not approved by either FDA or EMA. Research and development on that front is however continued (Li et al., 2021a). Aside from these mentioned, there are no other vaccines available. Many of the most common picornavirus infections that contribute the majority of the disease burden, remain without prevention methods.

The case is similar when it comes to antivirals. At the time of writing, there are no approved antivirals for the treatment of picornavirus infections. Some potential compounds classes that have been studied include interferons, immunoglobulins, capsid-inhibiting compounds, Enviroxime-like compounds, and protease inhibitors, but no established therapy has been found. Therefore the current treatment is purely supportive and symptom based (Rotbart, 2002).

1.6 Reverse genetics and virus rescue

Reverse genetics is a method of understanding the function of a gene, by looking how the phenotype changes when the genome is mutated. In reverse genetics, function of a gene product, protein, is restored from cloned cDNA. In most cases, this allows functional studies in which the cDNA fragment is mutated and hence the effect of mutation is visualized at protein level. Through this method useful modifications can be identified. Modified viruses can serve many purposes, they can for example be modified into oncolytic viruses that could be used in cancer therapy or they can have potential for vaccine development (Li et al., 2021b). Virus rescue as a technique falls under reverse genetics. Rescue of modified picornaviruses has been successfully performed in prior research, one example being a rescued Senecavirus A, modified to express luciferase and therefore facilitate analysis of its oncolytic potential (Liu et al., 2021).

The process planned for this study was as follows: First a cDNA genomic clone of the virus is constructed, and possibly modified. The different methods of constructing cDNA clones are explored in chapter 1.7. The modifications may include e.g. point mutations or insertion of marker genes. The infectious cDNA clone is then amplified and cloned to a vector plasmid that contains the promoters needed for the cDNA of the virus to function as a virus in cells (reg-cDNA) – to be infective and produce virus proteins. The reg-cDNA is then transcribed back to RNA, either *in vitro* or *in vivo* to introduce the viral RNA (vRNA) into cells. If the vRNA is functional in cells, meaning virus replication takes place producing virus particles, viral rescue from vRNA has been successful.

Infectious RNA can be produced from the reg-cDNA in many ways and there are many options for ways to deliver it into cells. All options have some advantages and limitations, and by testing the different methods, the most efficient method of delivery could be found. One such method would be to transfect both the reg-cDNA product and a plasmid containing T7RNApol encoding gene into cells. The expressed T7 RNA polymerase would transcribe the reg-cDNA back into vRNA inside the cells, producing infectious viral RNA and virus particles. This method can be tricky, as it requires both the reg-cDNA product and the T7RNApol construct to enter the same cell, in order to

produce any RNA form the reg-cDNA. This issue can possibly be avoided by using cells, such as T7-BSR cells that intrinsically produce T7RNA polymerase. In this case, only the reg-cDNA product needs to enter the cell in order for vRNA to be produced. The drawback of this method is however that depending on the virus type and strain, the cell line might not be suitable for propagation. Instead of transfecting the reg-cDNA, a corresponding *in vitro* transcribed RNA can also be transfected. In this case, there is no need to worry about transfecting the RNApol construct or using cells that can provide the polymerase. The challenge with this method lies in whether the delicate RNA will stay intact during the process, and remain functional in the cells.

1.7 Vector development

The potential therapeutic use of picornavirus vectors is one of the main driving forces behind research and construction of picornavirus vectors. Many different picornaviruses have been researched with therapeutic applications in mind, and promising findings have been made especially with enteroviruses, such as coxsackievirus and poliovirus. Out of the coxsackievirus in cancer therapy research, A21 (CVA21) has held a central position. With oncolytic activity detected in a range of cancers, CVA21 has been linked as a possible therapy for multiple cancers, such as breast cancer, melanoma, bladder cancer and multiple myeloma. CVA21 is however quite a common pathogen, meaning many patients could have a prior immunity against the treatment, weakening its effects significantly. Therefore, coxsackievirus with lower immunity rates are beginning to take center stage in the research. When it comes to poliovirus in the research of oncolytic viruses, a Poliovirus and Rhinovirus chimera, PVSRIPO, has demonstrated efficient oncolytic activity against many cancers, such as gliomas, breast cancer and melanoma (McCarthy et al., 2019).

Plasmid vectors, containing the cDNA equivalent of the picornavirus RNA genomes are essentially important for the research, as they are required for efficient handling and study of the picornavirus genome. The current standard methods of producing said vectors however are starting to get a bit old fashioned and they have certain bottlenecks. It is often required to amplify the amount of starting material by growing the virus sample in a cell culture. This procedure can easily take a number of weeks, delaying the start of the actual experiments. It should also be noted that finding a cell line that supports the growth of the picornavirus in question, is not to be taken for granted, as it can actually be quite difficult to find a line that supports efficient growth.

Once enough starting material has been purified from the culture, it is generally amplified by RT-PCR in fragments. The reason for amplifying the genome in fragments stems from the fact that it

can be difficult to obtain it in full, as it is nearly 8000 bp long. The number of fragments can range, but generally between three and five fragments have been used. Once the fragments have been generated, they need to be cloned into the plasmid frame. As there are multiple fragments to incorporate, the process is going to require multiple steps of enzymatic ligation and restriction, making the process inefficient and time consuming. The margin of having errors in the composition of the genome increases as well, as the number of cloning steps increase. The number of cloning steps will increase even further, as in most cases, regulatory regions necessary for the virus to replicate in cells are also cloned into the plasmid, as there is often intent to transfect the plasmids into cells, to see the virus phenotype (Sasaki et al., 2001; Poirier et al., 2012; Qin et al., 2018).

Picornaviruses have been handled in this way in the majority of the prior research that has been conducted. For example, in a study by Deng et al., a cDNA clone of Coxsackievirus A16 was constructed from four separately amplified fragments, in a study by Sasaki et al., a cDNA clone of the Aichi virus was constructed from five separately amplified fragments, in a study by Poirier et al., a cDNA clone of Seneca Valley virus was constructed from three separately amplified fragments, all instances requiring multiple cloning, fragmentation and ligation steps for the full length cDNA clone to be achieved (Deng et al., 2015; Sasaki et al., 2001; Poirier et al., 2012).

The new, improved method of producing the clones would consist of using no fragments, instead just one full length cDNA equivalent of the genome could be cloned into a plasmid in one step, through long homology based cloning methods. Using a very sensitive RT-PCR protocol that can amplify from starting samples with small amounts of template, the culturing and purification step can be bypassed, saving valuable time and resources. By using PCR primers that contain the wanted regulatory regions in the amplification process, the number of cloning steps can be pushed to the minimum.

The PCR is also utilized to create regions of homology that match with the used plasmid frame, at the ends of the genome, allowing for the homology based cloning methods (Fig. 4) to be used, in order to incorporate the cDNA into the plasmid with minimal amount of steps and effort. The homology based cloning relies on the 5’ exonuclease to cleave to regions of homology to single stranded overhangs. Those single stranded, homologous regions then anneal together and DNA polymerase and ligase fill in, and seal any possible gaps (Gibson et al., 2009).

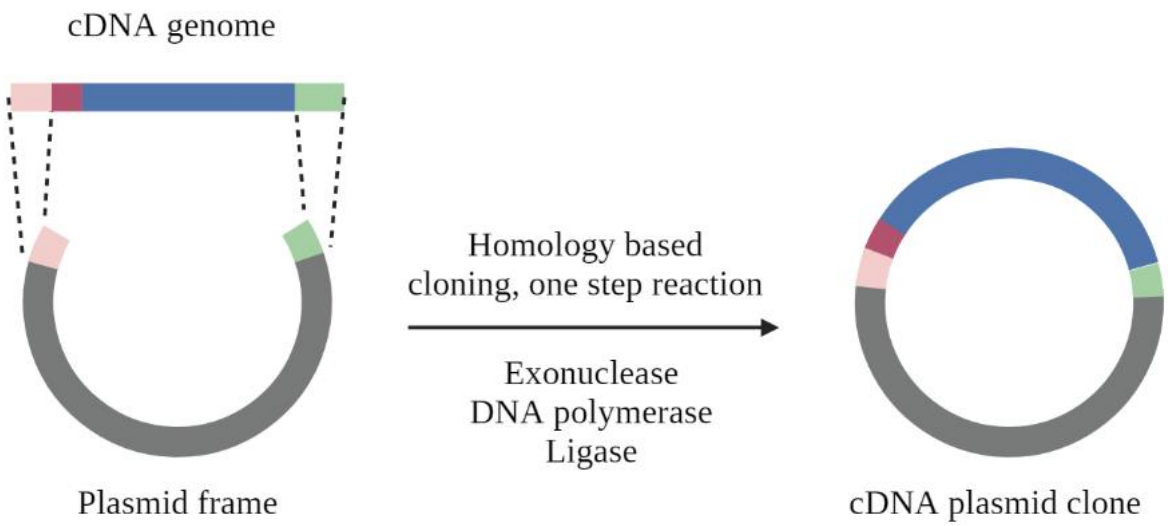

Figure 4. Homology based cloning. The cDNA genome (in blue) containing regulatory regions (in red) and regions of homology (in pink and green) is produced with RT-PCR. The plasmid frame with matching homology, the RT-PCR product and the enzymes associated (5' exonuclease, DNA polymerase and ligase) create the cDNA plasmid clone, in one simple step.
Figure created with biorender.com, by Minna Koskinen.

1.8 Motivations for the study

The disease burden imposed onto humans by viruses is evident, as they can cause major outbreaks and put the health of the population at great risk. Therefore, it is crucial to establish reliable and easy to use protocols for virus research. Such methods allow development of modified viruses, for example for speedy vaccine development or oncolytic cancer therapy or gene therapy.

Streamlined, optimal protocols encourage more studies to be done in the field, as designing the experiments becomes more linear and simpler. Optimization also helps make results more comparable, as the experiments have been performed in a similar fashion. Increase in studies would be welcome, as it means there is a bigger possibility to find modifications that have therapeutic potential or other useful application.

Virus research relies on having plasmid clones to perform experiments on. In order to efficiently generate plasmid clones, there must be means to obtain cDNA versions of virus genomes with ease. Cell culture to amplify virus amounts is time consuming, and in many cases not even feasible, as viruses can have specific requirements for the cells they can propagate in. With a RT-PCR based amplification method, the genomes can be generated much more quickly, easily, and without the restrictions that cell culturing has.

1.9 Aims & Hypotheses

The aim of this project was to create a streamlined, robust method for amplification of the picornavirus RNA genome that can be utilized in studies of mutagenesis, virus tropism and pathogenesis. The genome could also be modified, creating viruses that carry gene inserts useful for visualization or therapy applications. In practice this means devising an optimal RT-PCR protocol that is suitable for amplification of long templates with a low error rate.

It is hypothesized that it is possible to generate functional genomic cDNA clones by using reverse transcriptase and high-fidelity long PCR enzymes, capable of amplifying full-length genomes in one fragment. It is also proposed that the viral genome in the cDNA clone can be converted back to infectious virus in cells by using T7 promoter-tagged PCR primers – T7 promoter drives transcription of the transfected T7-PCR fragment directly in cells into vRNA, which is capable of replication and producing viable virus particles.

1.10 Experimental design

The project began with the development of a PCR protocol that is optimal for amplifying the full length picornavirus genome. For this optimization, coxsackievirus A9 and human parechovirus 1 cDNA clones were used as templates. The effect of different parameters on the yield of the PCR was evaluated, and the optimal picornavirus long PCR protocol was designed based on the results, using the values of each parameter that created the best yield of product. The evaluated parameters included the DNA polymerase enzyme used, number of cycles that the PCR is run for, and the amount of nucleotides and primers in the reaction mix. The PCR performance based on the copy numbers of the starting samples was also evaluated. The available high fidelity and long amplicon production capable DNA polymerase enzymes were cataloged, and the most promising ones were selected and used. The specification that the enzymes must have the ability to produce long amplicons is relevant, as the goal was for the whole genome of approximately 7500bp to be amplified in one go. The low error rate and the ability of high fidelity enzymes to replace misplaced nucleotides is important, as they help protect the integrity of the long genome. References for the values of the parameters are searched from prior literature and manufacturers' instructions.

PCR primers were be designed to be complementary to the target sequences at the ends of the genome, as well as to contain certain regulatory regions that will be incorporated to the genome during the PCR amplification. Once the PCR had been completed, the products were analyzed on

agarose gel and imaged under UV light, to ascertain the optimal parameters, and to find out the copy sensitivity of the long PCR.

Once the long PCR protocol was satisfactory, reverse transcription step was added, in order to use the viral RNA as the template. The available RT enzymes were cataloged, and the most promising ones were selected. For the RT optimization, CVA9 and PeV-A1 viral RNA samples were used.

Optimal protocol can be used to amplify and modify any picornavirus RNA genome. The PCR amplicon can be cloned into form of plasmid, possibly with a marker gene, to create a viral cDNA clone. This can be further modified, turned back to infectious RNA in cells by using T7-tagged primers for virus rescue and further use of the modified virus.

2. Materials and methods

2.1 Plasmid samples

For the testing of PCR enzymes and parameters two different picornavirus genome-containing plasmids were used. The used genomes were of CVA9 and PeV-A1. Detailed information regarding the genomes and plasmids is presented in Table 2.

Table 2. Plasmids used in the testing of PCR enzymes and parameters.

Abbreviation	Genome	GenBank accession	Reference
pCVA9	Coxsackievirus A9	D00627	(Hughes et al., 1995)
pPeV-A1	Human parechovirus 1	L02971	(Nateri et al., 2000)

Concentrations of the plasmid samples were determined through spectrophotometry, using a DeNovix DS11 (DeNovix Inc.). Once concentrations were known, amount of copies in one microliter of the samples were determined through genome equivalent calculation:

$$Y\ plasmid\ copies/\mu l = \frac{plasmid\ concentration\ (g/\mu l)}{length\ of\ plasmid\ (bp)\ x\ 660}\ x\ 6{,}022\ x\ 10^{23}$$

The plasmids were then diluted with Tris buffer (5 mM Tris/HCl, pH 8.5) to obtain a dilution series from 10^9 to 10^2 copies / µl. Later on the series was extended to reach 10^{-1} copies / µl, in order to accurately determine the enzyme sensitivity. To further validate this, the plasmid concentrations were checked again, using a Qubit™ assay kit (Invitrogen by Thermo Fisher Scientific) and a Qubit® 2.0 Fluorometer (Life Technologies by Thermo Fisher Scientific), which provides the concentration more accurately than spectrophotometry.

2.2 PCR

2.2.1 DNA polymerase enzymes

Five different high fidelity, long range DNA polymerase enzymes were tested, to see which one had the best yield of product. The PCR enzymes tested alongside the reaction mixes recommended by the manufacturers' user manuals are presented in Table 3.

Table 3. A. PCR enzymes tested. B. Reaction mixes of PCR enzymes used.

A

DNA polymerase	Company	CAT #	Amplification	Reference
Q5® Hot Start High-Fidelity DNA polymerase	New England Biolabs	M0493S	10 kb gDNA	(Tan et al., 2016)
Phusion Hot Start II DNA polymerase	Thermo Fisher Scientific	F549S	7.5 kb gDNA	(Rai et al., 2015)
KAPA HiFi HotStart PCR Kit	Kapa Biosystems	7958889001	11 kb gDNA	(Wehbe et al., 2016)
PrimeSTAR GXL DNA polymerase	Takara Bio	R050A	up to 30 kb gDNA	(Isaacs et al., 2018)
Platinum SuperFi II DNA polymerase	Thermo Fisher Scientific	12361010	20 – 40 kb gDNA	(Xi et al., 2021)

B

Reagent	Q5		Phusion		KAPA		PrimeStar		Platinum	
	V (µl)	C (µM)	V (µl)	C (µM)	V (µl)	C (µM)	V (µl)	C (µM)	V (µl)	C (µM)
Nuclease-Free water	12,4		12,4		12,8		13		12,2	
5X reaction buffer	4		4		4		4		4	
dNTP Mix (10mM each)	0,4	200 each	0,4	200 each	0,6	300 each	0,4	200 each	0,4	200 each
F primer (10µM)	1	0,5	1	0,5	0,6	0,3	0,6	0,3	1	0,5
R primer (10µM)	1	0,5	1	0,5	0,6	0,3	0,6	0,3	1	0,5
PCR Enzyme	0,2		0,2		0,4		0,4		0,4	
Template DNA	1		1		1		1		1	
Total V	20 µl		20 µl		20 µl		20 µl		20 µl	

All PCR reactions were performed in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems™ by Thermo Fisher Scientific). Presented in Table 4, program A was used with Phusion, KAPA, Q5 and Platinum enzymes, and program B was used with PrimeSTAR GXL enzyme, based on the recommendations of the manufacturers' user manuals.

Table 4. A. Cycling program used with Phusion, KAPA, Q5 and Platinum enzymes. B. Cycling program used with PrimeSTAR GXL enzyme.

A

	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Hold
Temperature	98°C	98°C	67°C	72°C	72°C	4°C
Time	60 s	15 s	20 s	4 min	7 min 30 s	
Cycles	1	30-40	30-40	30-40	1	

B

	Denaturation	Annealing	Extension	Hold
Temperature	98°C	60°C	68°C	4°C
Time	10 s	15 s	7 min 30 s	
Cycles	30-40	30-40	30-40	

2.2.2 Primers

T7 promoter region containing forward primers and poly-A tail containing reverse primers were used in the PCR amplifications. The T_m values for each primer were determined using Thermo Fisher Scientific and New England Biolabs online T_m calculators. Detailed information regarding the primers used is presented in Table 5. For PeV-A1, two sets of primers (strains Harris & 14) were tested with an annealing gradient. The Harris primers worked on a wider temperature range, hence those were used in all further PCR testing.

Annealing gradient ranging from 55°C to 70°C were first performed with all enzymes and primers, to assess whether the T_m calculations had landed in the correct range. Based on that, annealing temperatures were decided to be 67°C.

Table 5. Primers used for PCR. Complementary section underlined. F= forward R= reverse

Primer	Sequence	T_m range
CVA9gen1 T7 F	5'-TAATACGACTCACTATAGGG TTTAAAACAGCCTGTGGGTTGTTCCC-3'	68,8 – 70 °C
CVA9gen1 R	5'-TTTTTTTTTTTTTTTTTTT CCTCCGCACCGAATGCGG-3'	68,7 – 73 °C
PeV-A1 Harris T7 F	5′-TAATACGACTCACTATAGGG TTTGAAAGGGGTCTCCTAGAGAG-3'	64 – 65 °C
PeV-A1 Harris R	5′-TTTTTTTTTTT GTCATGTCCAATGTTCC-3'	54,6 – 57 °C
PeVA-1 14 T7 F	5′-TAATACGACTCACTATAGGG CCAAGGTTTRACAGACCCTTTA-3'	60,6 – 64 °C
PeV-A1 14 R	5'-TTTTTTTTTTTTTTTTTTT GTTATGTCCAATATTCCGAAT-3'	56,8 – 57,4 °C

2.2.3 Parameter optimization

The optimal amount of nucleotides was tested by omitting the dNTP mix from the PCR master mix and instead making the different nucleotide concentrations the variable in the reactions. The following dNTP concentrations were tested: 20 µM, 100 µM, 200 µM, 500 µM, 1000 µM.

The optimal amount of primers was tested in a similar fashion. The following primer concentrations were tested: 0.02 µM, 0.1 µM, 0.2 µM, 0.5 µM, 1 µM.

The optimal amount of cycles should be relatively low in order to avoid mutations building up in the genome during the cycling. At the same time, the number of cycles should also be high enough

to achieve sufficient amplification. The effect of the number of cycles was tested at 20, 25, 30, 35, 40 and 45 cycles, by pausing the program and removing a sample as the program had reached each observation point.

The sensitivity of the enzymes was tested by using the samples in a dilution series from 10^8 copies / µl to 10^{-1} copies / µl, to see the amount of starting sample necessary for the PCR amplification to be successful.

2.3 Agarose gel electrophoresis

Success of the PCR reaction and size of the products were checked with agarose gel electrophoresis, using Tris-acetate-EDTA (TAE) buffer and 0,8% agarose gel. The PCR product samples were mixed with loading buffer (6X TriTrack DNA Loading Dye, Thermo Fisher Scientific), and run on 100V until bands had separated appropriately. Midori Green Advance DNA Stain dye (Nippon genetics) was used in the gel to make the DNA visible and GeneRuler DNA Ladder Mix (Thermo Fisher Scientific) (Fig. 5) was used to determine the sizes of the bands. The gels were imagined using Bio Rad Gel Doc XR+ Gel Documentation System (Bio-Rad Laboratories, Inc.) and analyzed using Image Lab (Bio-Rad Laboratories, Inc.) software.

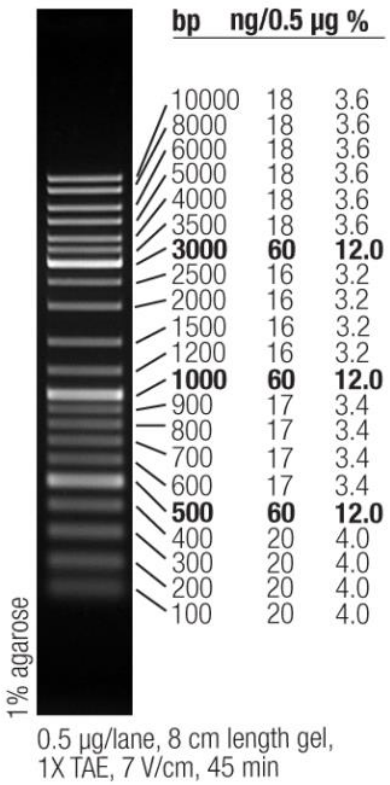

Figure 5. GeneRuler DNA Ladder Mix -molecule weight marker. The marker was used to assess the sizes of the bands formed in the electrophoresis of the PCR reaction product.

2.4 RNA samples

Viral RNA samples of CVA9 and PeV-A1 were isolated using a E.Z.N.A.® Viral RNA Kit (Omega Bio-tek, Inc.). Viral RNA was also extracted from cell culture samples infected with these viruses.

The obtained viral RNAs were used as templates in RT-PCR reactions. A PCR reaction control was also performed with pCVA9 and pPeV-A1, to assess the success of the RNA extraction. Through this control reaction it was noticed that either the extraction had not been successful or there was not enough material to begin with in the case of CVA9. Therefore, prior made *in vitro* transcribed (IVT) RNA samples for both viruses were searched, to find usable samples for RT, in order to obtain results from both test viruses. For these samples the concentrations were known, and copy numbers already calculated. Two samples of both viruses were found, sample dilutions made with PCR grade water, to be used to assess the integrity of the samples and see if they can be used as samples for the RT testing.

After initial testing the only sufficiently intact IVT-vRNA sample was one of PeV-A1, and only that one of the prior made IVT-RNAs was utilizable going forward. A new dilution series from 10^9 to 10^{-1} copies / µl was made with PCR grade water and RiboLock RNase Inhibitor (Thermo Fisher Scientific) in order to combat sample degradation.

As the prior made IVT-RNAs had no usable CVA9 samples, to obtain results from both test viruses and to try and further combat the sample degradation, new samples of CVA9 and PeV-A1 virus RNA were created through IVT, with the hope of fresh samples being more stable and resistant to RNases. T7-CVA9 and T7-PeV-A1 products were produced by PCR, run on gel and purified from the gel using NucleoSpin ® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG,) in order to have a clean starting samples for the IVT reactions. The IVT reactions were performed with the MEGAscript ® Kit (Life Technologies). The produced IVT-RNA was then purified using MEGAclear™ Kit (Life Technologies, AM1908).

Concentrations of the newly made IVT-RNAs were measured with DeNovix DS11 (DeNovix Inc.). The number of RNA molecules in one microliter was determined through the following calculation:

$$Y\ copies/\mu l = \frac{RNA\ concentration\ (g/\mu l)}{length\ of\ transcript\ (nt)\ x\ 340}\ x\ 6{,}022\ x\ 10^{23}$$

The RNAs were then diluted in PCR grade water with 2U / µl of RiboLock RNase Inhibitor (Thermo Fisher Scientific) creating a dilution series from 10^9 to 10^1 copies / µl, to be used in the testing of RT reactions.

Table 6. vRNAs tested and used as templates for RT reactions.

vRNA sample	Obtained through	Source
CVA9	E.Z.N.A kit	Newly made
PeV-A1	E.Z.N.A kit	Newly made
PeV-A1	IVT	Prior made
CVA9	IVT	Newly made from PCR reaction product
PeV-A1	IVT	Newly made from PCR reaction product
CVA9	IVT	Newly made from gel purified band
PeV-A1	IVT	Newly made from gel purified band

2.5 Reverse Transcription

Four different RT enzymes were tested to find out which one provides the largest yield of product in the PCR reaction. The enzymes used are presented in Table 7. One of the enzymes, SuperScript IV, is a one-step RT-PCR, while the other 3 are two-step applications.

Table 7. Reverse Transcriptase enzymes tested. The listed enzymes were tested in order to see which one would result in the highest yield of end product in a RT-PCR reaction.

Reverse Transcriptase	Company	CAT #	Reference
ImProm-II ™ RT system	Promega Corporation	A3800	(Vicenti et al., 2018)
LunaScript ® RT Master Mix Kit	New England Biolabs	E3025S/L	
RevertAid H Minus RT	Thermo Fisher Scientific	EP0451	(Pinto and Lindblad, 2010)
SuperScript ™ IV One-Step system	Thermo Fisher Scientific	12594025	(Aoki et al., 2021)

The used reaction mixes and incubation protocols of each enzyme are presented in Tables 8 - 11. All reactions were assembled and incubated on a thermocycler (DNA Engine PTC-200, BioRad & Veriti™ 96-Well Fast Thermal Cycler, Thermo Fisher Scientific) according to the instructions of the manufacturers' user manuals.

Table 8. A. ImProm-II RT reaction mix. B. Incubation program for cDNA reaction.

A

Reagent	10 µl reaction	Concentration
ImProm-II 5X Reaction Buffer	2 µl	1X
dNTP Mix (10mM each)	0,5 µl	0,5mM each dNTP
RiboLock RNase Inhibitor (40U/µl)	0,5 µl	2,0 U/µl
$MgCl_2$ (25mM)	1 ml	2,5 mM
ImProm-II Reverse Transcriptase	0,5 µl	
Gene-specific primer (10µM)	0,5 µl	0,5 µM
RNA Template	1 µl	
Nuclease-Free Water	up to 10 µl	

B

Step	Temperature	Time
Denaturation	70°C	5 min
Chill on ice		5 min
Primer Annealing	25°C	5 min
Extension	42°C	60 min
Heat inactivation	70°C	15 min

Table 9. A. LunaScript ® RT Master Mix Kit reaction mix. B. Incubation program for cDNA reaction.

A

Reagent	10 µl reaction	Concentration
LunaScript RT Master Mix (Primer-free) (5X)	2 µl	1X
Gene-specific primer (10µM)	0,5 µl	0,5 µM
RNA Template	1 µl	
Nuclease-Free Water	up to 10 µl	

B

Step	Temperature	Time
cDNA synthesis	55°C	10 min
Heat inactivation	95°C	1 min

Table 10. A. RevertAid H Minus Reverse Transciptase reaction mix. B. Incubation program for cDNA reaction.

A

Reagent	10 µl reaction	Concentration
5X Reaction Buffer	2 µl	1X
dNTP Mix (10mM each)	1 µl	1 mM
RiboLock RNase Inhibitor (40U/µl)	0,25 µl	1 U / µl
RevertAid H Minus Reverse Transcriptase	0,5 µl	10 U / µl
Gene-specific primer (10µM)	0,5 µl	0,5 µM
RNA Template	1 µl	
Nuclease-Free Water	up to 10 µl	

B

Step	Temperature	Time
cDNA synthesis	42°C	60 min
Heat inactivation	70°C	10 min

Table 11. A. SuperScript ™ IV One-Step RT-PCR System reaction mix. B. Cycling program used for one-step RT-PCR.

A

Reagent	20 µl reaction	Concentration
2X Platinum ™ SuperFi ™ RT-PCR Master Mix	10 µl	
Gene-specific F primer (10µM)	1 µl	0,5 µM
Gene-specific R primer (10µM)	1 µl	0,5 µM
SuperScript ™ IV RT Mix	0,2 µl	
Template RNA	2 µl	
Nuclease-free water	up to 20 µl	

B

Step	Temperature	Time	Cycles
Reverse Transcription	50°C	10 min	1
RT inactivation / Initial denaturation	98°C	2 min	
Denaturation	98°C	10 s	35
Annealing	67°C	10 s	
Extension	72°C	4 min	
Final extension	72°C	7 min 30 s	1

For the two-step enzymes, once the cDNA products were obtained, PCR was performed using 2µl of the obtained cDNA as template, otherwise according to section 2.2.1., using the Platinum SuperFi II PCR enzyme. The one-step enzyme has built in Platinum PCR in it, so the product can be analyzed directly.

To analyze the yields, agarose gel electrophoresis of the products was performed according to section 2.3.

2.6 RT-qPCR

As some results obtained during the project hinted at the DNA contamination of the used IVT-RNA samples, the presence of contaminating DNA in the PeV-A1 IVT-RNA samples was screened for by RT-qPCR. The testing was done by having two samples of each RNA source, assembling the reaction for one sample according to the instructions and omitting the RT enzyme from the other duplicate sample. The RT-qPCR reaction was performed with QuantiNova™ SYBR Green RT-PCR Kit (Qiagen, 208152) and Rotor Gene Q thermocycler (Qiagen). The primers used are presented in Table 12. The reaction mix and used cycling program are presented in Table 13.

Table 12. Primers used in RT-qPCR.

Primer	Name	Sequence	Reference
PAR-F	HPeV-F31	5′- CTGGGGCCAAAAGCCA	(Benschop et al., 2008)
PAR-R	HPeV-K30	5′- GGTACCTTCTGGGCATCCTTC	(Oberste et al., 1999)

Table 13. A. QuantiNova™ SYBR Green RT-PCR reaction mix. B. RT-qPCR cycling program used.

A

Reagent	20 µl reaction	Concentration
2x SYBR Green RT-PCR Master Mix	10 µl	1X
PAR-F primer (10µM)	1 µl	0,5 µM
PAR-R primer (10µM)	1 µl	0,5 µM
QN SYBR Green RT-Mix	0,2 µl	
RNA template	2 µl	
Nuclease-Free water	5,8 µl	

B

	Step	Temperature	Time	Cycles
RT	RT	50°C	10 min	1
	RT inactivation	95°C	2 min	
PCR	Denaturation	95°C	15 s	50
	Annealing	65 → 56°C *	20 s	
	Extension, Data acquisition	72°C	10 s	

* Touchdown from 65°C to 56°C during the first 9 cycles.

2.7 Cell culture & Infectivity

The ability of HuTu80 cells (human, *duodenum* adenocarcinoma) to facilitate PeV-A propagation was tested using HuTu80 cells (obtained from ATCC) and a panel of 21 different PeV-A strains. The virus types and strains used for the testing are listed in Table 14.

Table 14. PeV-A strains used to infect HuTu80 cells.

Type	Strain	Type	Strain
PeV-A1	Harris	PeV-A3	88
PeV-A1	452252	PeV-A3	145-8
PeV-A1	19	PeV-A3	A308/99
PeV-A1	22	PeV-A4	FI121236
PeV-A1	103-2	PeV-A4	110402
PeV-A1	125-7	PeV-A4	K251176
PeV-A1	350757	PeV-A5	20552323
PeV-A2	Williamson	PeV-A6	47
PeV-A3	252277	PeV-A6	21152464
PeV-A3	152037	PeV-A6	89
PeV-A3	73		

HuTu80 cells were grown and cultured in the following conditions: Growth medium of EMEM, 10% FBS and 1% Penicillin-Streptomycin. Incubation in 37°C with 5% CO_2. Detachment of cells with 1X Trypsin-EDTA, diluted to PBS.

HuTu80 cells were divided to 96-well tissue culture plates, with approximately 20 000 cells per well. Once the wells had grown confluent, growth medium was removed and replaced by infection medium (EMEM, 2% FBS and 1% Penicillin-Streptomycin).

The cells were infected by introducing 10 µl of virus into a well. After a thorough mix with a pipette, 10µl from the prior well was transferred to the next well. The same process was repeated for one more well, now discarding the 10 µl after mixing, producing 3 wells with 10-fold dilutions of the virus.

The development of infection in the wells was followed for 3 days. The development was screened with light microscopy (PrimoVert, ZEISS), by assessing the morphology of the cells in the infected wells, compared with those of the non-infected control wells and whether cytopathic effect could be detected in the infected wells. Images were obtained using ZEN digital imaging software (ZEISS).

3. Results

3.1 PCR

The PCR optimization was performed using viral cDNA copies of pCVA9 and pPeV-A1 as templates. The effect of nucleotide and primer concentration was tested with the plasmids at copy number of 1E4, PCR cycle number was tested at 1E3, 1E4 and 1E6. Finally, the optimal parameters were used to test the assay sensitivity by diluting plasmid concentrations down to zero. Testing of the PCR parameters was started with 5 enzymes, however during the progress of the study, only PCR enzymes with good performance were used in the subsequent assays.

3.1.1 Effect of nucleotide concentration on viral genome amplification

Nucleotide concentration required for optimal amplification of pCVA9 genome by Q5® Hot Start High-Fidelity, Phusion Hot Start II, KAPA HiFi HotStart, Platinum SuperFi II DNA and PrimeSTAR GXL DNA polymerase enzymes was the first PCR parameter to be evaluated.

The concentration range was selected based on the manufacturers' recommendations for each enzyme, according to Table 3 section B. Clear differences in robustness were observed between enzymes. Both Platinum SuperFi II and PrimeSTAR GLX DNA polymerase enzymes showcased a wide range of functionality, as they were able to amplify the genome with multiple different nucleotide concentrations (Fig. 6 & 7B). KAPA HiFi however was only effective at few concentrations (Fig. 6). Q5 HS HiFi and Phusion HS II enzymes did not produce reliable results when tested, hence no results are shown.

PrimeSTAR GXL DNA polymerase was additionally tested with pPeV-A1 as template, which produced quite different results as the pCVA9 template (Fig. 7). Only faint amplification was observed on the pPeV-A1 template, with the nucleotide concentration of 200 µM producing the most prominent of bands (Fig. 7A). The enzyme however worked well with the pCVA9 template, where nucleotide concentrations between 100 – 500 µM all produced prominent bands (Fig. 7B).

For Platinum SuperFi II, KAPA HiFi HS and PrimeSTAR GXL enzymes 200 µM seems to be the optimal concentration of nucleotides, producing most prominent bands reliably, across all enzymes. The result is quite well in line with the manufacturers' recommendations, which ranged between 200 – 300 µM. In conclusion, Platinum SuperFi II and PrimeSTAR GXL were the most robust enzymes in respect of nucleotide concentration.

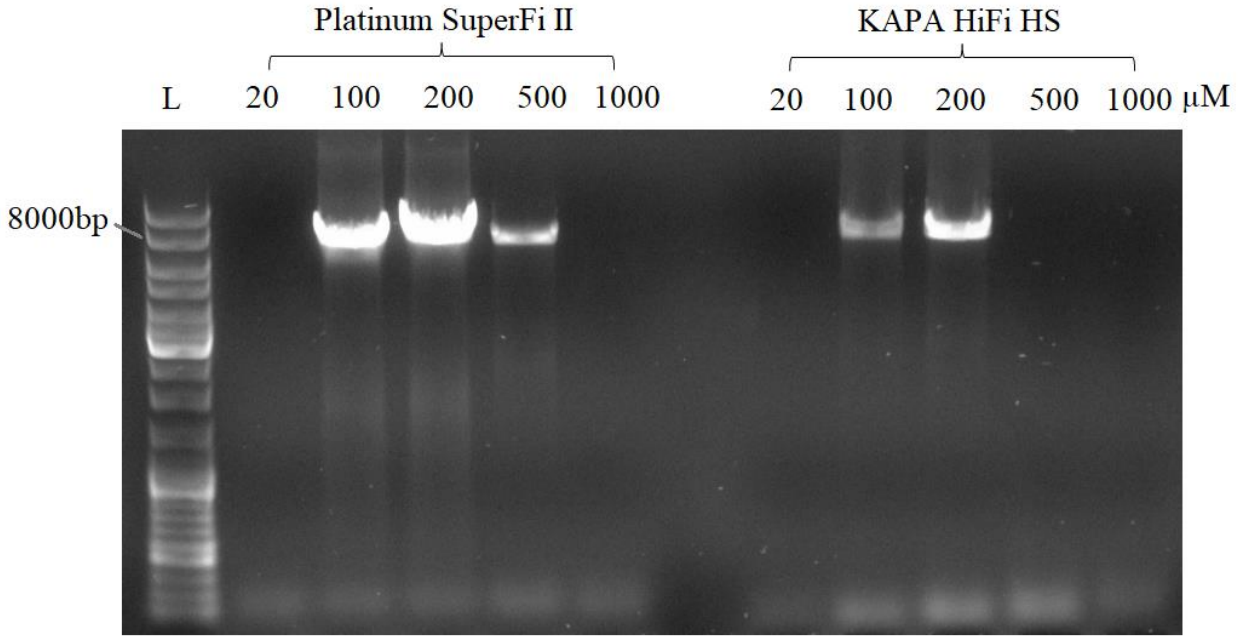

Figure 6. Effect of nucleotide concentration on viral genome amplification tested with Platinum SuperFi II and KAPA HiFi HS enzymes, with pCVA9 as template. L = GeneRuler DNA Ladder Mix -molecule weight marker.

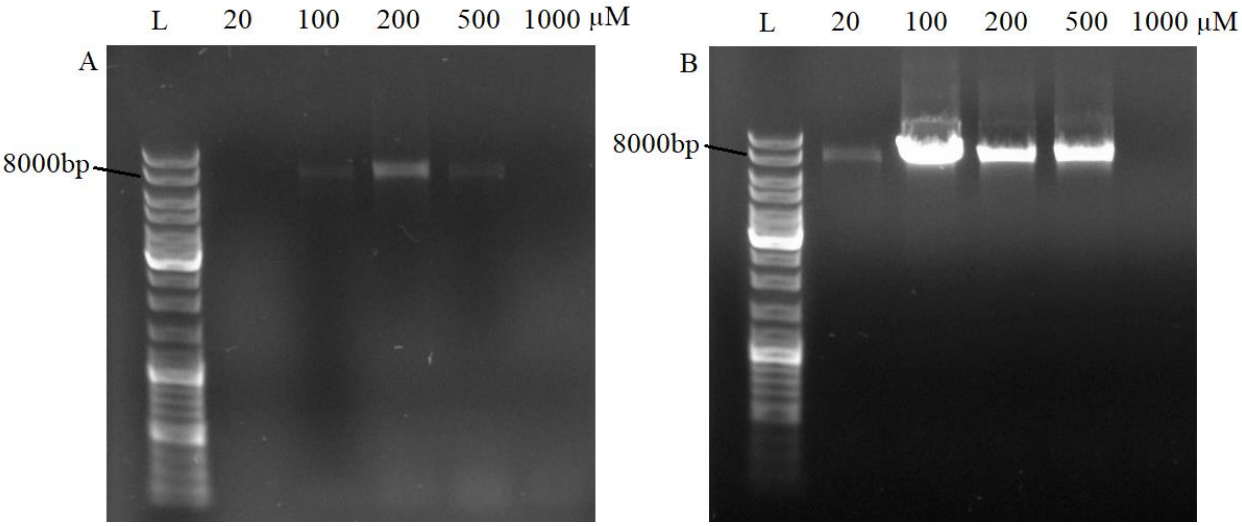

Figure 7. Effect of nucleotide concentration on viral genome amplification tested with PrimeSTAR GXL DNA polymerase enzyme. A. pCVA9 as template. B. pPeV-A1 as template. L = GeneRuler DNA Ladder Mix -molecule weight marker.

3.1.2 Effect of primer concentration on viral genome amplification

Primer concentration required in the reaction mix for optimal amplification of pCVA9 and pPeV-A1 genomes by KAPA HiFi HotStart, Platinum SuperFi II DNA and PrimeSTAR GXL DNA polymerase enzymes was evaluated as the second PCR parameter. Phusion Hot Start II and Q5® Hot Start High-Fidelity polymerase enzymes were excluded from subsequent analyses, as their performance was weak compared to the other three PCR enzymes.

Platinum SuperFi II enzyme had equally good performance between 0,2 – 1 µM primer concentrations, while KAPA HiFi HS enzyme failed to produce any results when tested (Fig. 8).

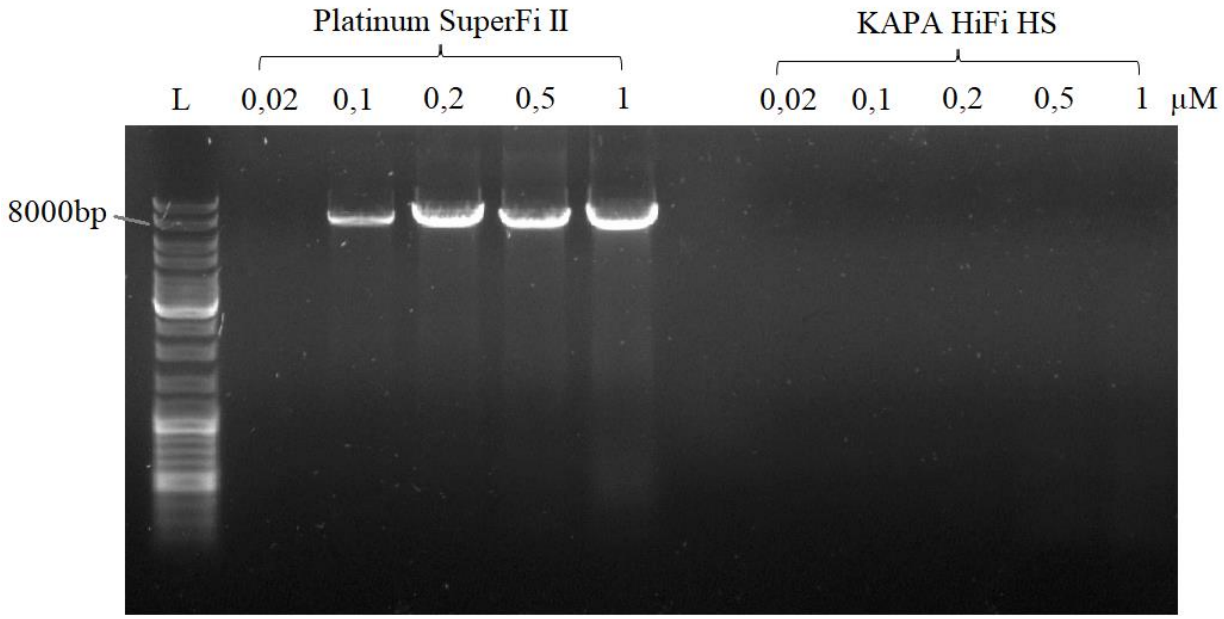

Figure 8. Effect of primer concentration on viral genome amplification tested with Platinum SuperFi II and KAPA HiFi HS, with pPeV-A1 as template. L = GeneRuler DNA Ladder Mix - molecule weight marker.

PrimeSTAR GXL enzyme once again performs well with the pPeV-A1 template, on primer concentrations between 0,1 – 1 µM. The same range of concentrations produces amplification with the pCVA9 template as well, with a greatly decreased performance however, when compared with the pPeV-A1 template (Fig. 9).

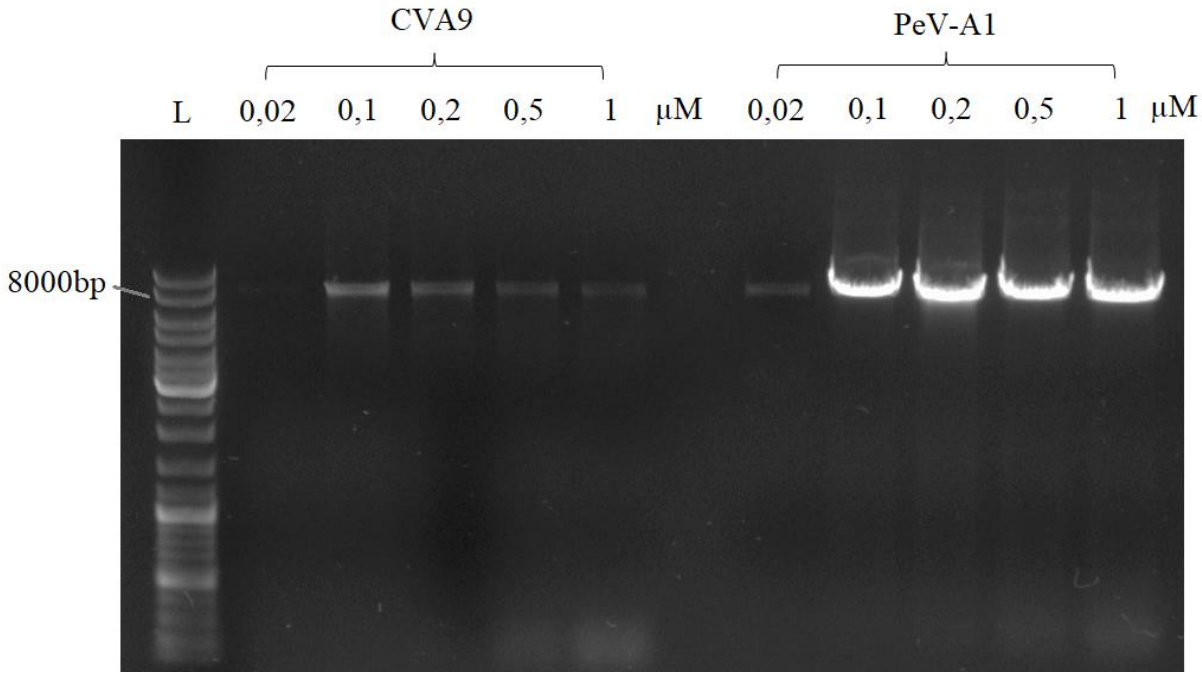

Figure 9. Effect of primer concentration on viral genome amplification tested with PrimeSTAR GXL DNA polymerase, with both pCVA9 and pPeV-A1 templates. L = GeneRuler DNA Ladder Mix -molecule weight marker.

In conclusion, primer concentrations between 0.1 and 1 µM all allow for similar, prominent amplification, across both pCVA9 and pPeV-A1 templates and Platinum SuperFi II DNA and PrimeSTAR GXL DNA polymerase enzymes.

3.1.3 Effect of PCR cycle number on viral genome amplification

Based on the results obtained from nucleotide and primer concentration analyses, two enzymes, Platinum SuperFi II DNA and PrimeSTAR GXL DNA polymerase, were further analyzed for the effect of PCR cycle number on genome amplification.

The number of cycles in the PCR program clearly affects the genome amplification, as a fewer number of cycles have passed, the yield of product is considerably less than it is when a greater number of cycling has taken place. For Platinum SuperFi II, the optimal number of cycles falls between 35 – 45 cycles, as the greatest yield of product can be observed there. When tested with the sample with more template (10^6 copies), there was not much difference in the yield at that range, but when tested with a slightly more diluted template (10^4 copies), the difference of 5 cycles starts to make a difference in the yield (Fig. 10).

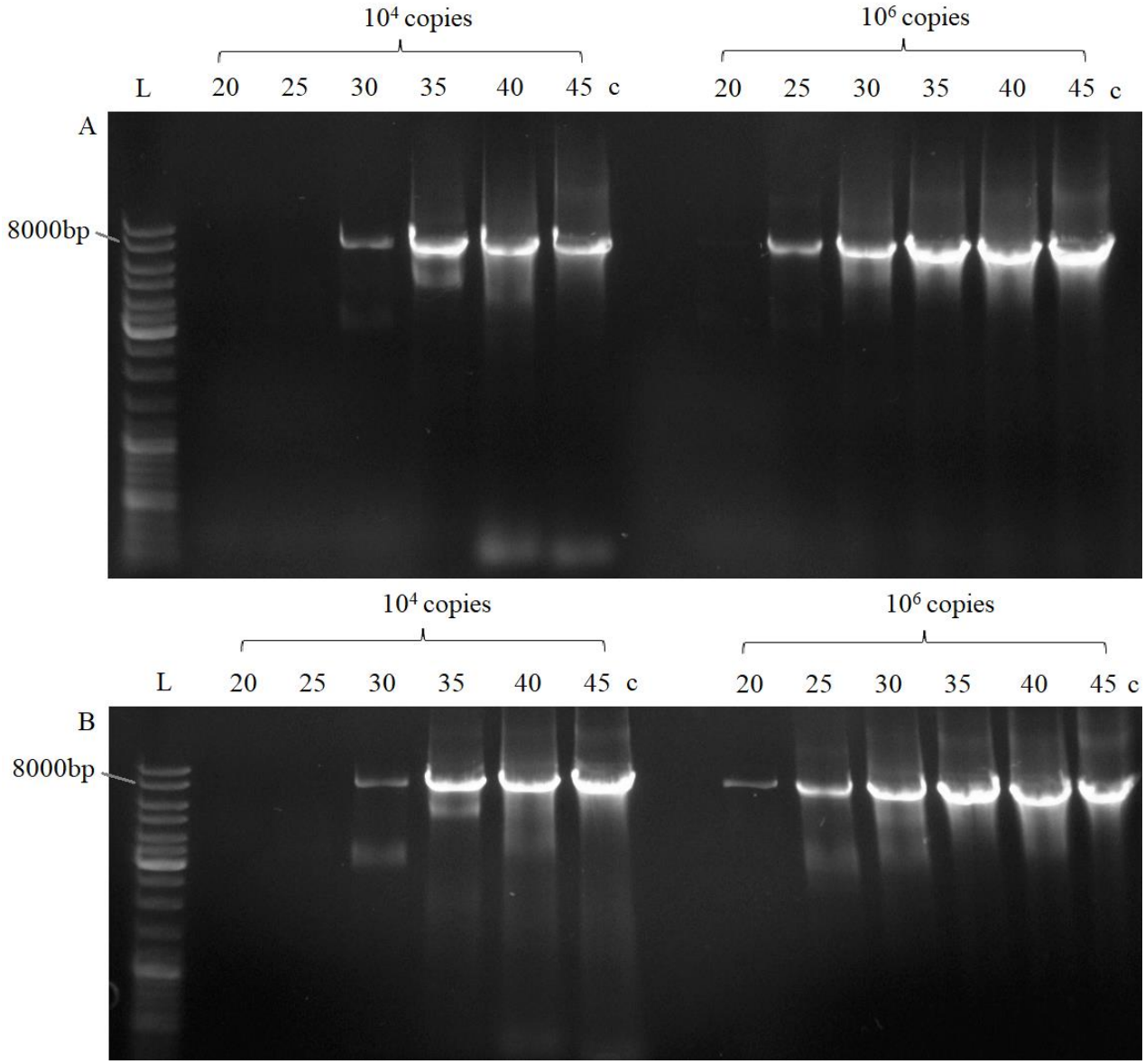

Figure 10. Effect of PCR cycle number on viral genome amplification, tested with Platinum Super Fi II DNA polymerase enzyme. A. pPeV-A1 as template. B. pCVA9 as template. L = GeneRuler DNA Ladder Mix -molecule weight marker. c = cycles.

For PrimeSTAR GXL enzyme, there were once again issues with the pCVA9 template, as it produced no bands (Fig. 11B). The optimal number of cycles for PrimeSTAR GXL enzyme seems to also be in the 35 – 45 cycles range, especially with the more diluted sample (10^3 copies). For a more concentrated sample (10^6 copies), even 30 cycles produced a yield seemingly as good as 45 cycles (Fig. 11A).

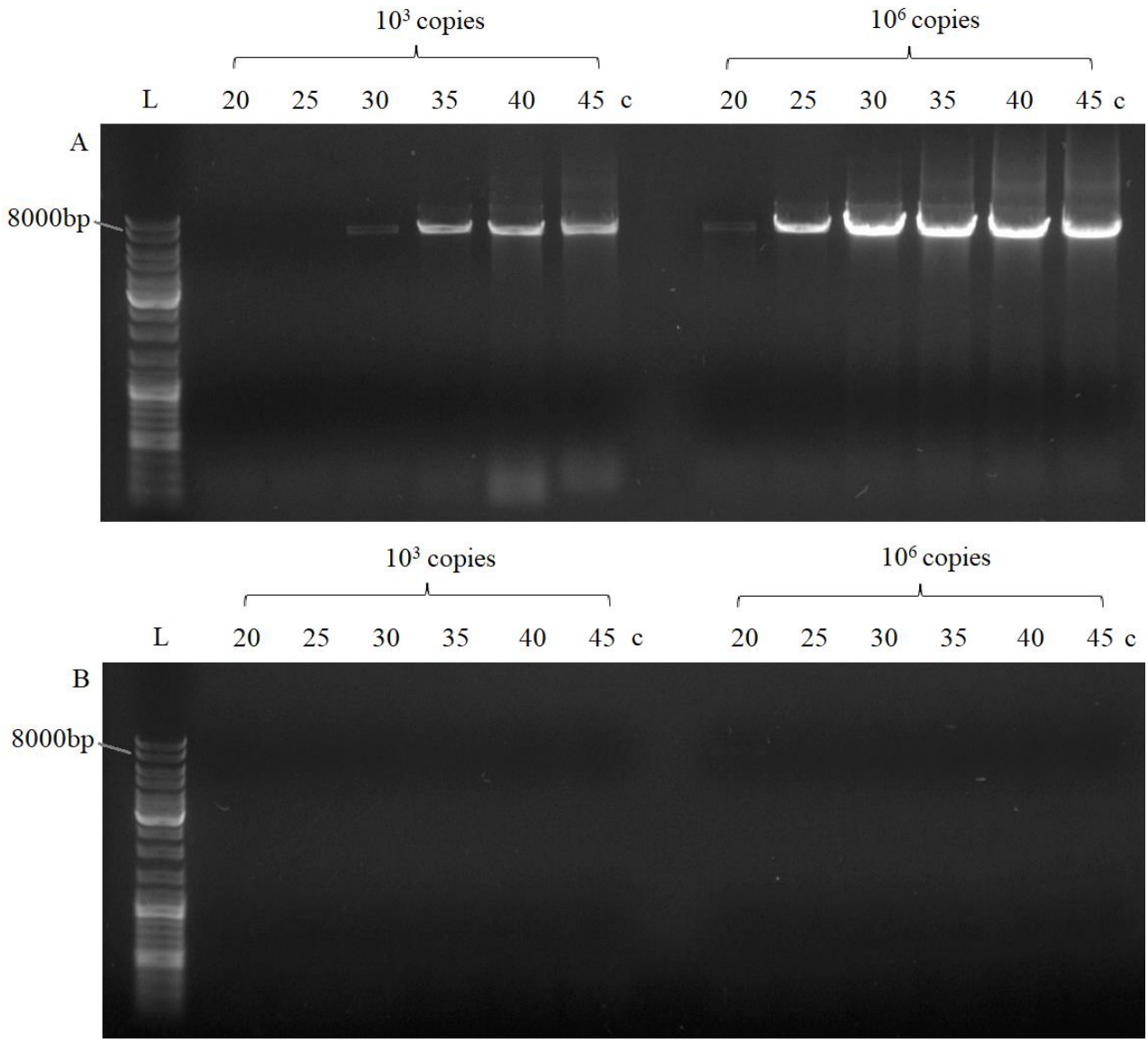

Figure 11. Effect of PCR cycle number on viral genome amplification, tested with PrimeSTAR GXL DNA polymerase enzyme. A. pPeV-A1 as template. B. pCVA9 as template. L = GeneRuler DNA Ladder Mix -molecule weight marker. c = cycles.

In conclusion, for both Platinum SuperFi II DNA and PrimeSTAR GXL DNA polymerase enzymes, the optimal number of cycles with respect to genome amplification falls between 35 and 45 cycles.

3.1.4 PCR performance based on copy numbers

The enzyme copy sensitivity was tested with Platinum SuperFi II DNA and PrimeSTAR GXL DNA polymerase enzymes, using parameters that were deemed optimal based on the earlier experiments

and the manufacturers' user manuals recommendations. For Platinum SuperFi II this means nucleotide concentration of 200 µM each, primer concentration of 0.5 µM and 40 cycles, while for PrimeSTAR GXL nucleotide concentration of 200 µM each, primer concentration of 0.3 µM and 40 cycles is optimal.

Both Platinum SuperFi II and PrimeStar GXL DNA polymerase enzymes were able to produce detectable bands from as low as 10^2 copies using pPeV-A1 as the template (Fig. 12 & 13B). Platinum SuperFi II enzyme however performed more reliably, since PrimeSTAR GXL enzyme once again was not able to produce any bands with the pCVA9 template (Fig. 13A).

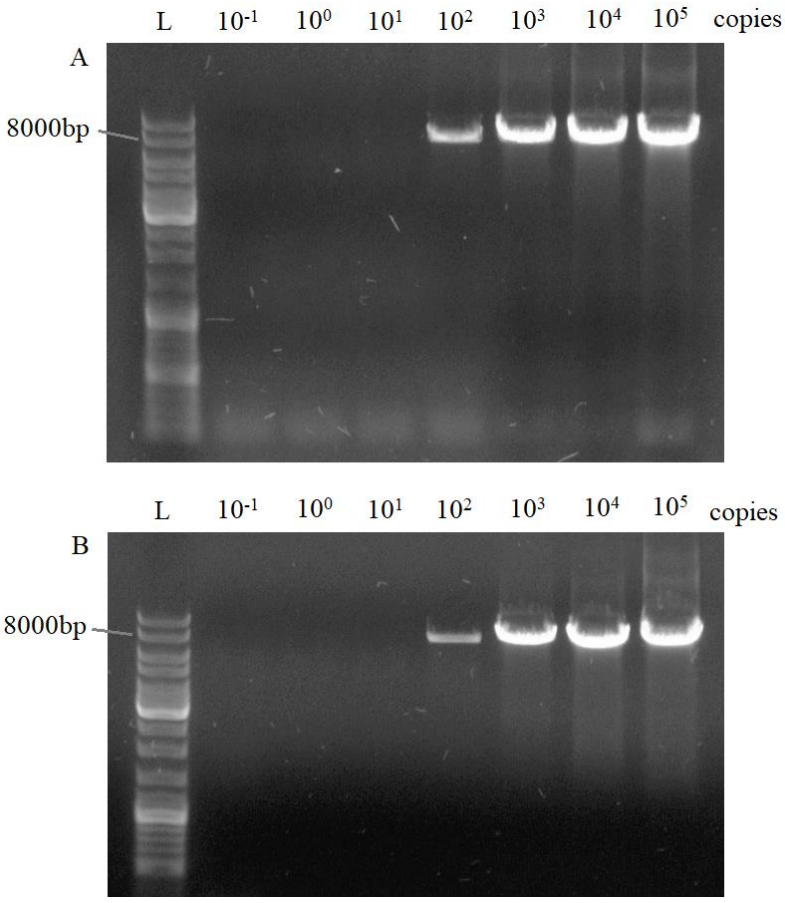

Figure 12. PCR performance based on sample copy sensitivity, tested with Platinum Super Fi II DNA polymerase enzyme. A. pCVA9 as template. B. pPeV-A1 as template. L = GeneRuler DNA Ladder Mix -molecule weight marker.

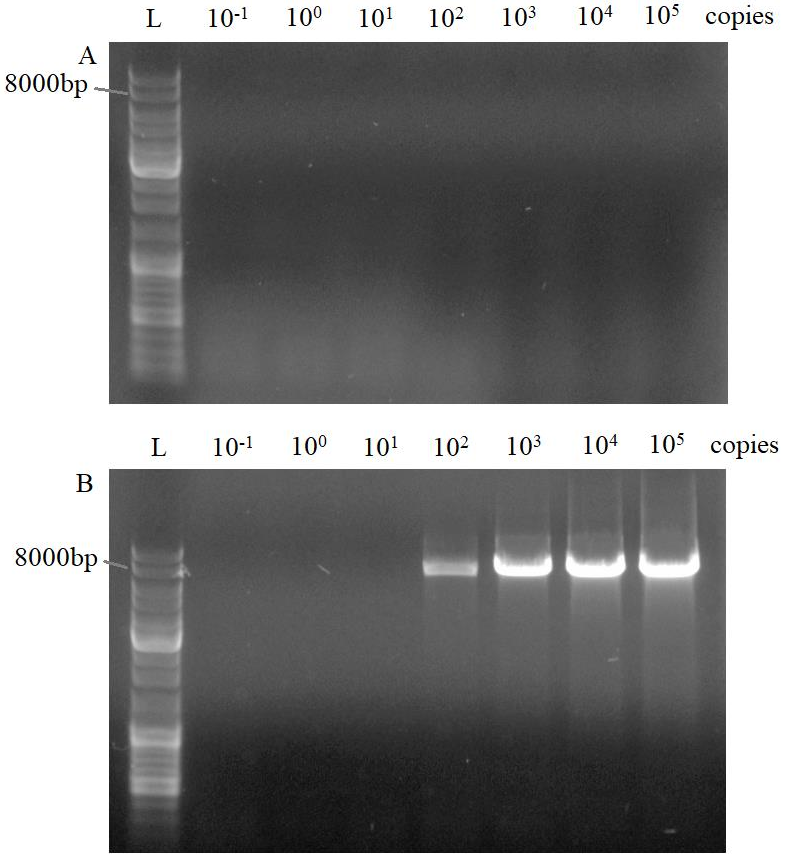

Figure 13. PCR performance based on sample copy sensitivity, tested with PrimeSTAR GXL DNA polymerase enzyme. A. pCVA9 as template. B. pPeV-A1 as template. L = GeneRuler DNA Ladder Mix -molecule weight marker.

All in all, Phusion Hot Start II DNA polymerase enzyme had the worst performance out of the five tested enzymes. It produced appropriately sized bands only in one out of the six runs performed, and even within that one run the performance was not unified for all the samples.
Both KAPA HiFi Hot Start and Q5 Hot Start HiFi DNA polymerase enzymes had quite unreliable performance, producing bands in some of the runs, but not in all of them. PrimeSTAR GXL DNA polymerase enzyme also had reliability issues when it came to performance. It performed well on one of the templates, pPeV-A1, but poorly on pCVA9. Apart from that issue, the bands were appropriately sized, and the range for suitable nucleotide and primer concentrations was wide. Platinum SuperFi II DNA polymerase had the best performance out of the enzymes tested. It produced appropriately sized bands in all the runs, and was reliable and consistent between templates. It was also functional on a wide range of nucleotide and primer concentrations. Therefore, Platinum SuperFi II enzyme was chosen to be used as the PCR enzyme in the upcoming RT-PCR testing.

The optimal PCR protocol would be the following: Using the Platinum SuperFi II DNA polymerase, dNTP concentration of 200 µM, primer concentrations between 0,2 and 0,5 µM, cycling for 40 rounds.

3.2 Reverse transcription

In total, four different reverse transcription enzymes were tested, in order to see which enzyme would lead to the best yield of product at the end of the RT-PCR. Three of the enzymes, ImProm-II ™ RT, LunaScript ® RT and RevertAid H Minus RT, are two-step enzymes, meaning they were first used to perform the RT reaction, the product of which was then amplified with the optimal PCR enzyme, Platinum SuperFi II. The final enzyme, SuperScript ™ IV One-Step, is a one-step RT-PCR system, which has the same Platinum SuperFi DNA polymerase built in one reaction mix, eliminating the need to perform separate, back-to-back reactions.

The CVA9 and PeV-A1 vRNA samples used in the testing of the RT enzymes included E.Z.N.A kit extracted samples, prior made in vitro transcribed samples and newly made in vitro transcribed samples – from both PCR reaction product mix and from a gel purified, specific band (Table 6).

All four RT enzymes were first tested using the newly made, E.Z.N.A extracted CVA9 and PeV-A1 vRNA samples. With all of the tested RT enzymes appropriately sized bands were produced (Fig. 14B) through PCR, which means the enzymes were able to create a cDNA of the 7,5 kb viral RNA. The control lanes are clear, which indicates the amplification to be truly from the target. Yield of product looks to be the best with LunaScript RT, RevertAid H Minus and SuperScript IV, while ImProm-II seems to struggle with the degradation of the sample. The results also showed that the E.Z.N.A extraction of CVA9 vRNA had not been successful, as only the control plasmid had been amplified through PCR (Fig. 14A), meaning no results with CVA9 vRNA as template were obtained.

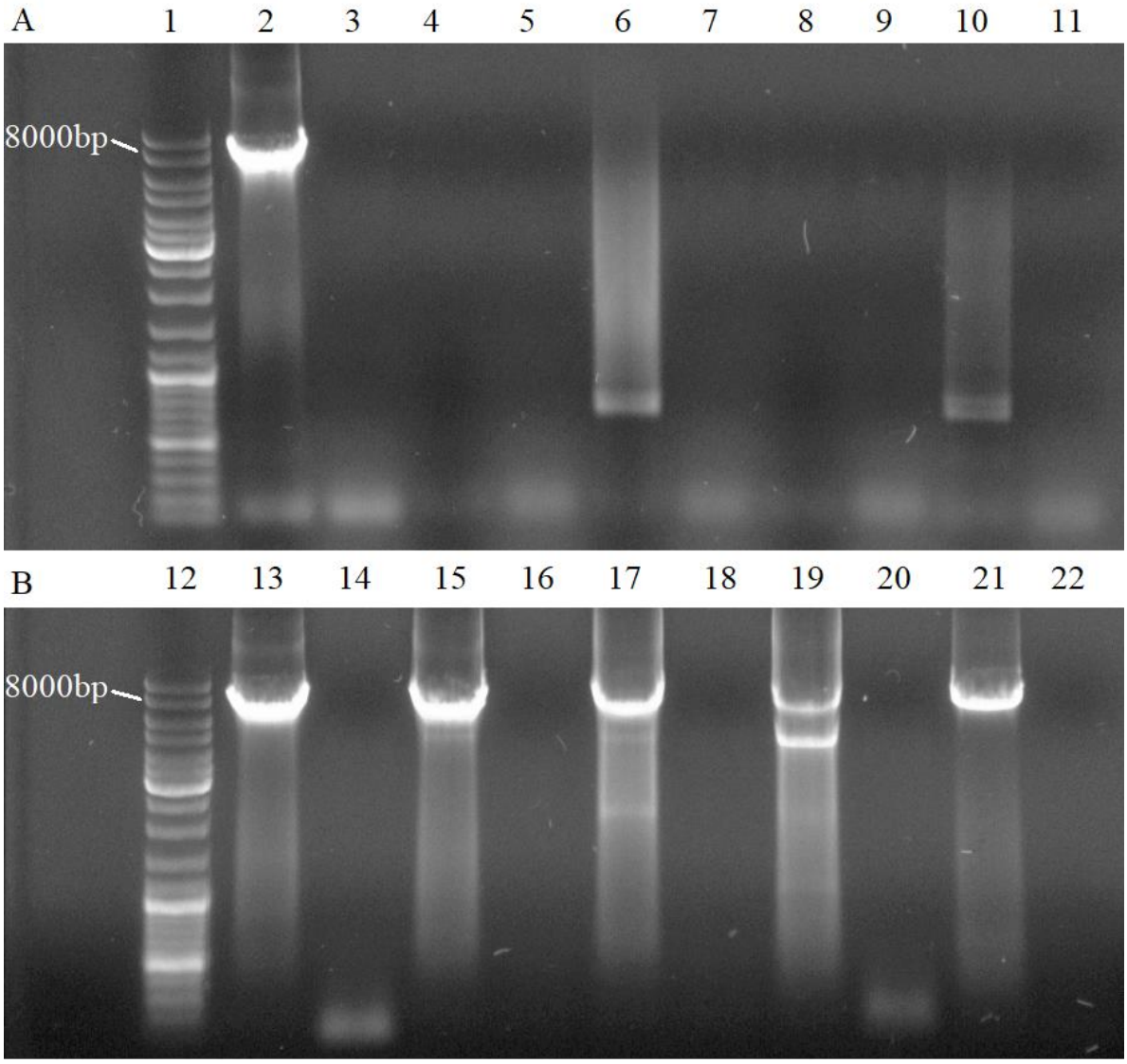

Figure 14. RT-PCR with CVA9 vRNA samples (A) and PeV-A1 vRNA samples (B), with the four tested RT enzymes.
1,12 = GeneRuler DNA Ladder Mix -molecule weight marker. 2,13 = PCR plasmid control 3,14 = PCR H_2O control. 4,15 = LunaScript vRNA. 5,16 = LunaScript H_2O control. 6,17 = RevertAid H Minus vRNA. 7,18 = RevertAid H Minus H_2O control. 8,19 = ImProm-II vRNA. 9,20 = ImProm-II H_2O control. 10,21 = SuperScript IV vRNA. 11,22 = SuperScript IV H_2O control.

In order to obtain results from both viruses, and to have some references to the E.Z.N.A extracted RNA, prior made IVT-vRNA samples for the viruses were searched and tested, to see if usable reference samples could be found. Preliminary testing revealed a usable comparison sample for PeV-A1, but none for CVA9. Therefore, the following few tests were performed with only PeV-A1 vRNA as the template, this time with a range of concentrations from 10^0 – 10^5, in order to assess the sensitivity of the RT enzymes.

As can be seen in Figure 15, all RT enzymes worked again, but the sensitivity had quite a bit of variation. Sample degradation is also largely visible. Worth noting is also the band being produced from the RNA control of the PCR, which is not a desired result (Fig. 15, lane 3). This could be due to contamination or there could be residual plasmid from the IVT process left in the RNA sample despite the use of DNase in the purification protocol. New dilutions with RNase inhibitor were made, and the testing was repeated to confirm the findings.

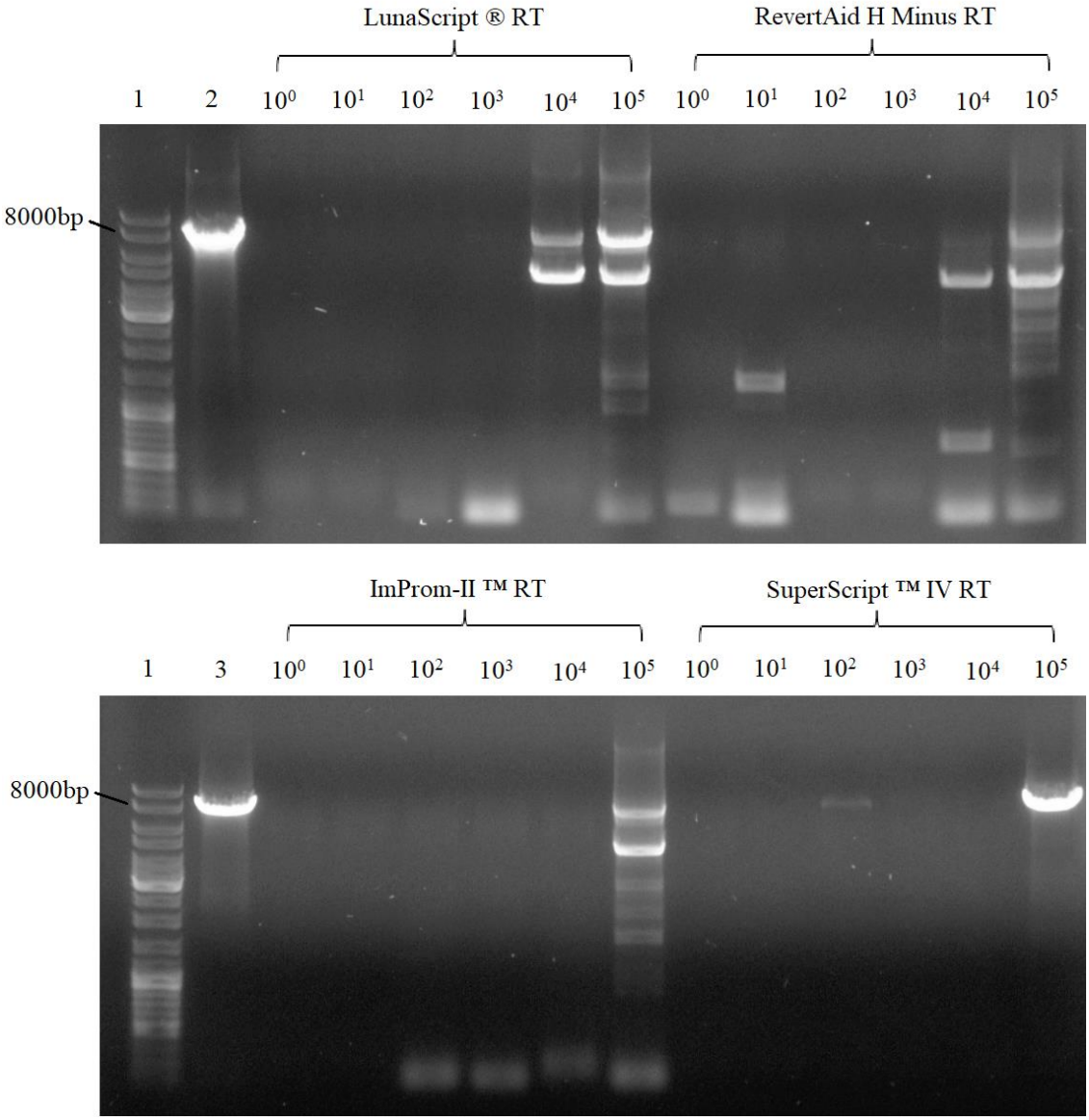

Figure 15. RT enzymes tested with a prior made IVT-PeV-A1 vRNA 10^0 – 10^5 dilution series. 1 = GeneRuler DNA Ladder Mix -molecule weight marker. 2 = PCR plasmid control. 3 = PCR with RNA control.

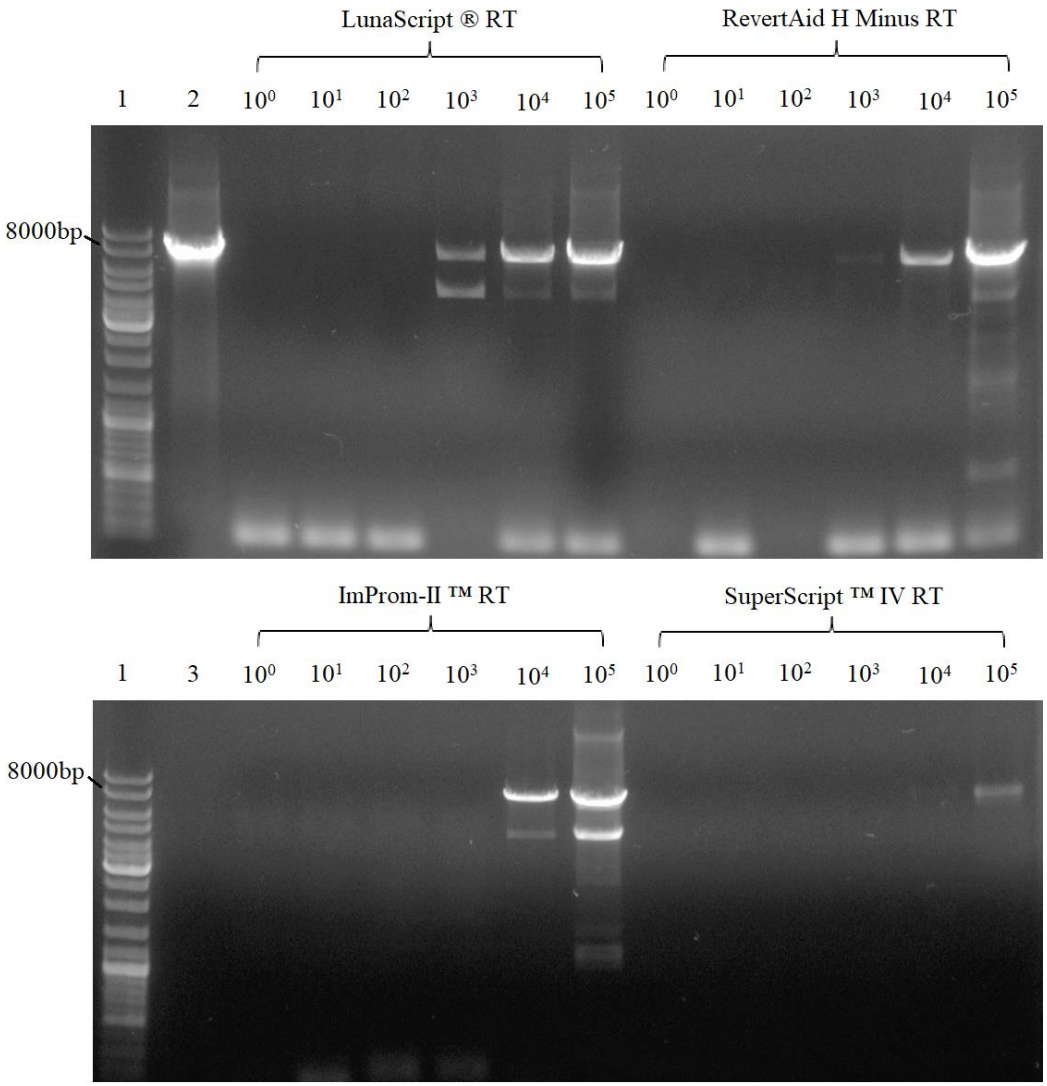

Figure 16. RT enzymes tested with a prior made IVT-PeV-A1 vRNA 10^0 – 10^5 dilution series, with RiboLock RNase Inhibitor. 1 = GeneRuler DNA Ladder Mix -molecule weight marker. 2 = PCR plasmid control. 3 = PCR with RNA control.

After repeating the sensitivity testing with the samples including RNase inhibitor, the results from both tests combined indicated that LunaScript and SuperScript IV were the most sensitive RT enzymes. LunaScript RT was able to produce sufficient bands from 10^3 RNA copies (Fig. 16), and SuperScript IV RT produced faint bands even from 10^2 copies (Fig. 15). Therefore, the two were chosen for further testing. Out of the two LunaScript was more consistent, with its sensitivity landing on 10^3 and 10^4 copies, on the two runs performed. SuperScript IV on the other hand produced quite different results, sensitivity of 10^2 and 10^5 copies, depending on the run.

When the samples were made with the RNase inhibitor, degradation was reduced, yet still clearly visible. In that repeated experiment, the RNA control of the PCR shows up as negative (Fig. 16,

lane 3), which is a desired result, but raises some questions, as it is not in line with what was observed in the first experiment, while the sample is the same in both. New IVT-RNA samples for both viruses were thus made, in order to rule out the possible DNA contamination in the IVT-RNA samples.

A similar sensitivity experiment was once again repeated, with LunaScript and SuperScript IV RT enzymes, using the newly made IVT-RNA samples of CVA9 and PeV-A1 in serial dilutions ranging from 10^2 to 10^6 copies.

As can be seen from figure 17A, only the PCR with plasmid control produced a band, meaning the CVA9 IVT-RNA production was not successful. PeV-A1 IVT-RNA production has been successful, but the sample seems to have a fair amount of substance besides the target in it, indicated by the amplification away from the area of interest, 8000bp. Once again, the PCR control with RNA is positive (Fig. 17B, lane 3), indicating some sort of DNA contamination in the samples that needs to be eliminated.

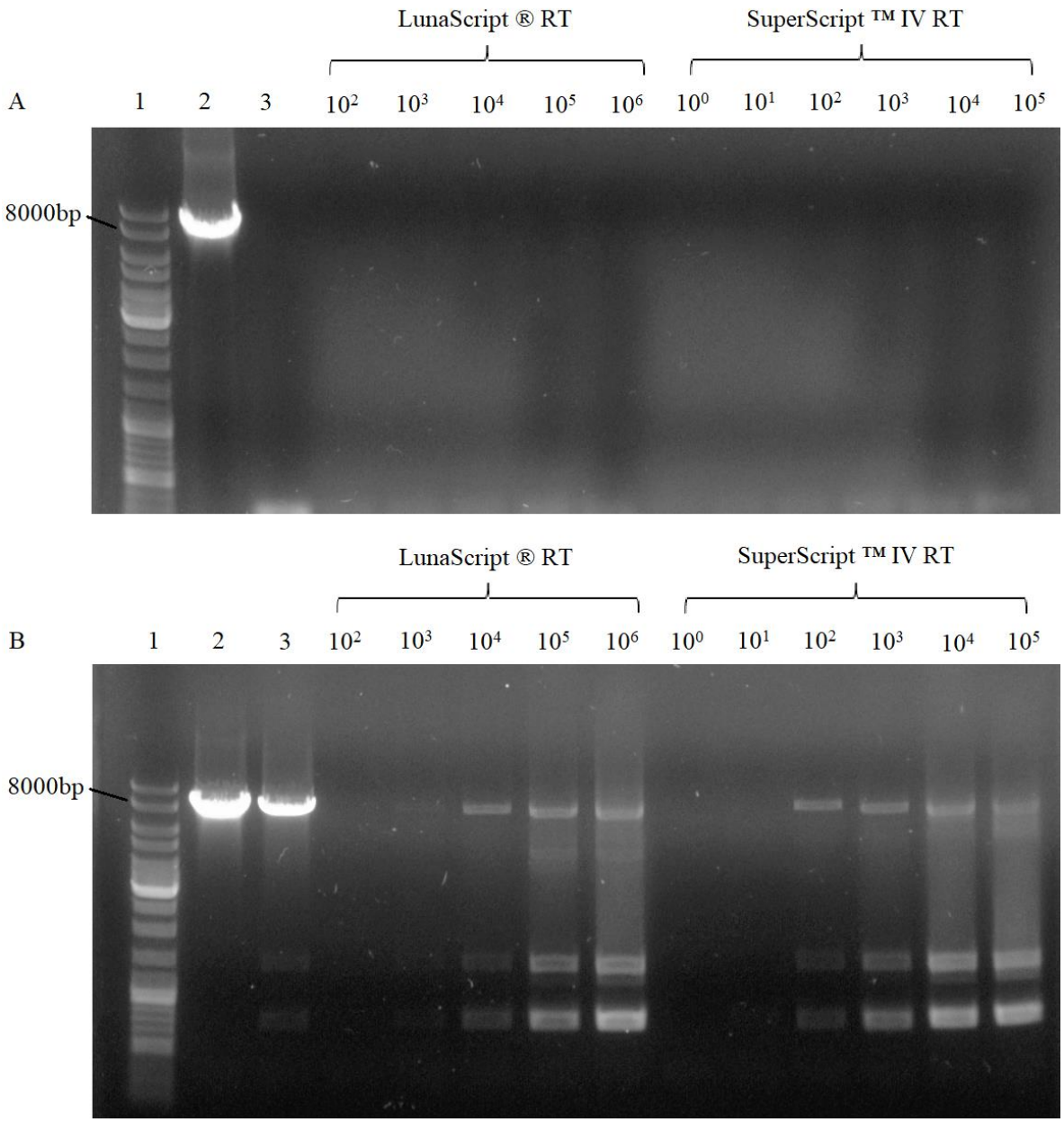

Figure 17. LunaScript RT and SuperScript IV RT tested with newly made IVT-RNAs. A. CVA9 dilutions. B. PeV-A1 dilutions. 1 = GeneRuler DNA Ladder Mix -molecule weight marker. 2 = PCR plasmid control. 3 = PCR with RNA control.

Once again, new IVT-RNA samples were made, this time from gel extraction, to reduce unwanted material in the starting material of the IVT reaction, allowing the IVT-RNA to be purely made from the wanted target. Whether the IVT-RNA samples truly consist of RNA only, and no DNA residue is present, is screened for with RT-qPCR, before any more RT testing is done.

3.3 RT-qPCR

The RT-qPCR performed on the IVT-samples revealed that there was in fact DNA contamination, most likely residual plasmid DNA left from the IVT process, in all the used IVT-RNA samples (Fig. 18, 19, 20). For the IVT-RNA samples that have no RT enzyme in the mix, no cDNA can be formed and therefore no double stranded DNA can be made through PCR and no fluorescence should be produced through SYBR Green and detected. Fluorescence however was detected from all IVT-RNA samples, even when no RT enzyme was in the reaction mix, suggesting that the IVT-RNAs have contaminating DNA in them that can act as the template for PCR.

The contamination however seems to not be too severe. In respect to the amount of RNA in the sample, the amount of DNA is estimated to be very minimal. Signs of this were already showing in Figure 17, where the RT-PCR of the IVT-RNA samples produces bands that are much fainter than the one of PCR plasmid control, meaning those samples have significantly less starting DNA than the plasmid sample, indicating that IVT-RNA is still the main component of the RNA samples. If the DNA contamination was severe, the RT-PCR reactions would also produce bands as prominent as the plasmid control does. The same sentiment can be observed in the RT-qPCR results. Analyzing the difference in Ct-values between the samples with and with out RT enzyme in the mix, a clear difference can be detected with both the newly made IVT-RNAs, indicating them as the more reliable, suitable samples for further testing.

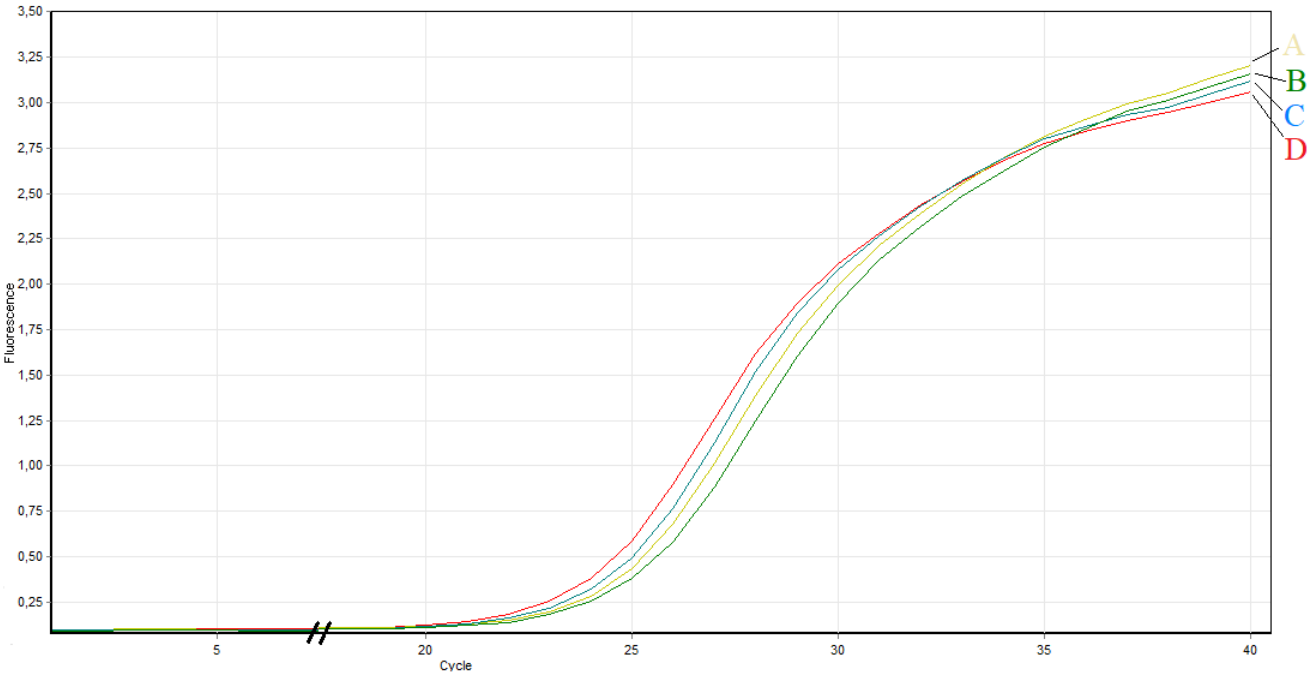

Figure 18. RT-qPCR of prior made PeV-A1 IVT-RNA. A, D = Samples with RT enzyme in the mix. B, C = Samples without RT enzyme in the mix.

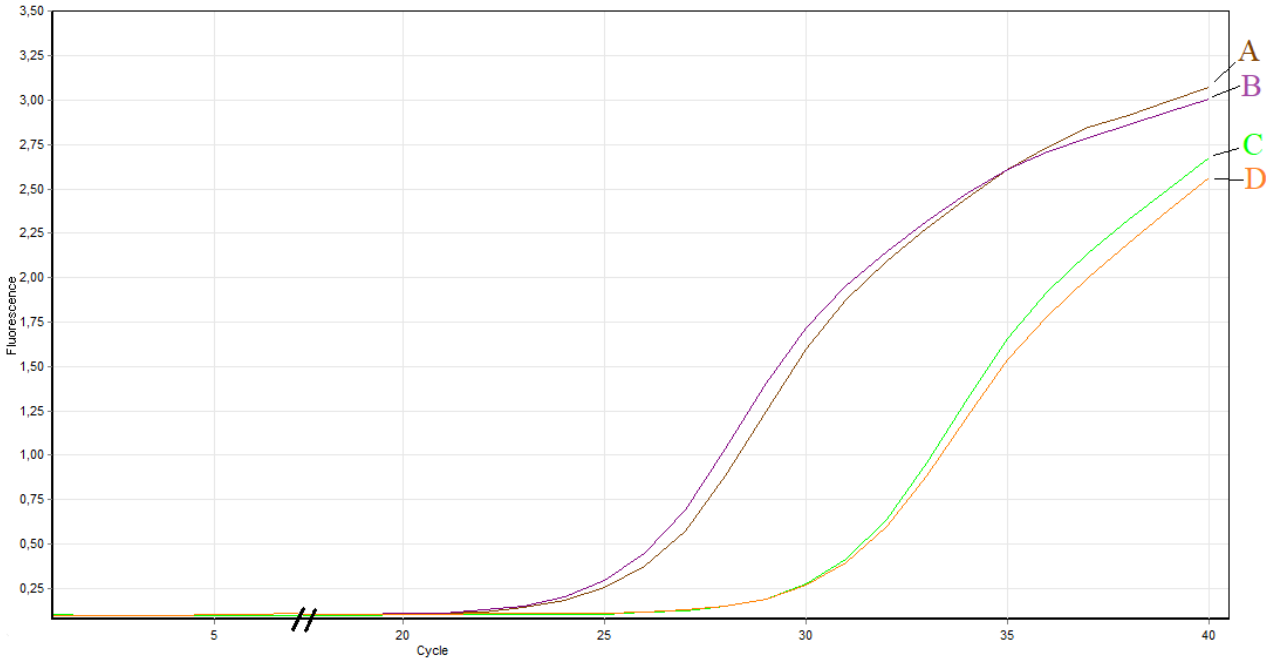

Figure 19. RT-qPCR of PeV-A1 IVT-RNA, made from PCR product mix. A, B = Samples with the RT enzyme in the mix. C, D = Samples without RT enzyme in the mix.

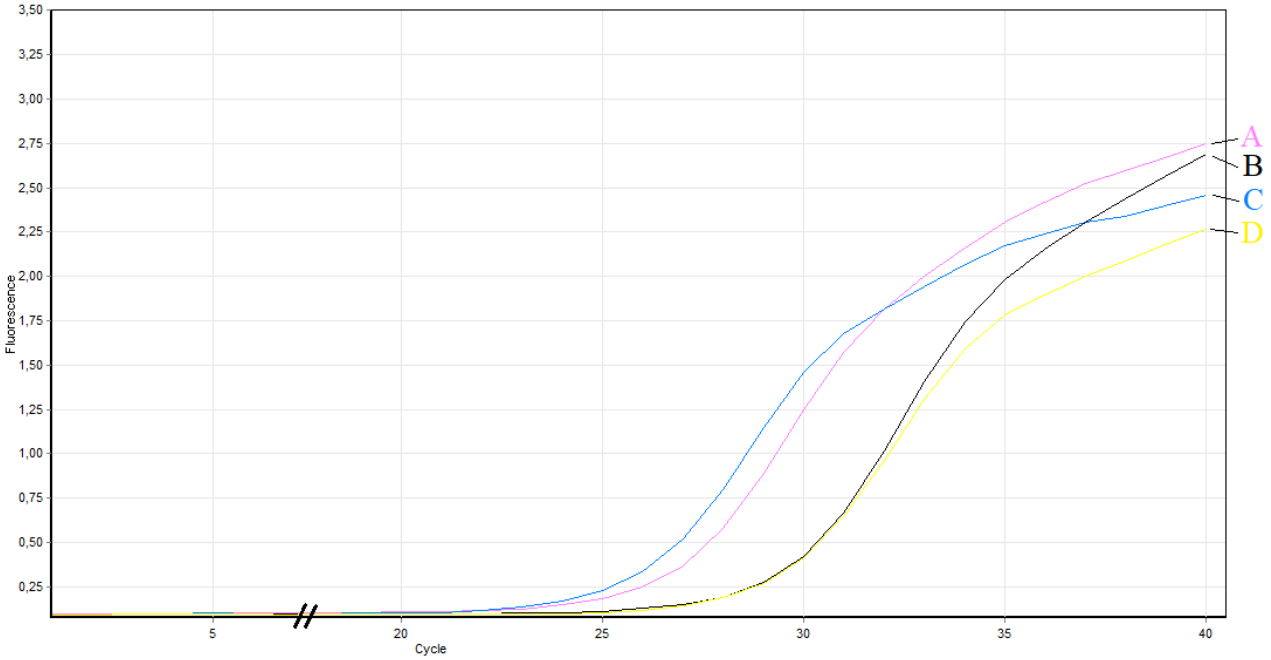

Figure 20. RT-qPCR of PeV-A1 IVT-RNA, made form gel purified target band. A, C = Samples with RT enzyme in the mix. B, D = Samples without RT enzyme in the mix.

3.4 Infectivity of HuTu80 cells with PeV-As

Infectivity of each virus strain tested in relation to DPI is showcased in Table 14. Some of the virus strains showed signs of infection already 1 DPI, while others did not. 2 DPI only one of the virus strains, PeV-A1 125-7, showed no signs of infection, while all other strains showcased varying degrees of infection. 3 DPI however, all viruses had developed some level of infection in the wells.

Table 15. Infectivity of PeV-A viruses in HuTu80 cells. Degree of infection was screened with light microscopy and the wells were graded based on the cytopathic effect (CPE) according to the following:

Not infected	-
Infected, depending on the severity of infection	+ ++ +++ ++++
Uncertain reading	+/-

W1= Well 1, 1:10 dilution W2= Well 2, 1:100 dilution W3= Well 3, 1:1000 dilution

	Virus type and strain		W1	W2	W3		Virus type and strain		W1	W2	W3
2DPI	PeV-A1	Harris	++++	++++	++++	2DPI	PeV-A3	88	++++	++++	++++
3DPI			++++	++++	++++	3DPI			++++	++++	++++
2DPI	PeV-A1	452252	++++	++++	++++	2DPI	PeV-A3	145-8	++	++	++
3DPI			++++	++++	++++	3DPI			+++	+++	+++
2DPI	PeV-A1	19	+/-	+	-	2DPI	PeV-A3	A308/99	++++	++++	++++
3DPI			++	++	+	3DPI			++++	++++	++++
2DPI	PeV-A1	22	++	-	-	2DPI	PeV-A4	FI121236	++++	++++	++++
3DPI			+++	++	+	3DPI			++++	++++	++++
2DPI	PeV-A1	103-2	++++	++++	++	2DPI	PeV-A4	110402	++++	++++	++++
3DPI			++++	++++	++	3DPI			++++	++++	++++
2DPI	PeV-A1	125-7	-	-	-	2DPI	PeV-A4	K251176	++++	+++	++
3DPI			+	+	-	3DPI			++++	+++	+++
2DPI	PeV-A1	350757	++++	++++	++++	2DPI	PeV-A5	20552323	++++	+++	+++
3DPI			++++	++++	++++	3DPI			++++	++++	+++
2DPI	PeV-A2	Williamson	++++	++++	+++	2DPI	PeV-A6	47	++	-	-
3DPI			++++	++++	+++	3DPI			+++	-	-
2DPI	PeV-A3	252277	++	++	+	2DPI	PeV-A6	21152464	+++	+++	+++
3DPI			+++	++	+	3DPI			+++	+++	+++
2DPI	PeV-A3	152037	++++	++	+	2DPI	PeV-A6	89	++++	++	+
3DPI			++++	+++	++	3DPI			++++	+++	+++
2DPI	PeV-A3	73	+++	+++	+						
3DPI			+++	+++	++						

As example, data are shown for PeV-A6 89 at 2DPI (Fig. 21). Figure 21A shows healthy control cells with no signs of disruption. Typical fibroblast-like cell phenotype is clearly visible in the image. Figures 21B-D were infected at different virus dilutions to demonstrate that the CPE is not due to sample cytotoxicity often seen when virus-infected cell lysate samples are used at high concentration. In Figure 21B, the cells are infected with 1:1000 dilution of the virus, leading to a relatively mild infection. The bottom of the well is still mostly coated, and both fibroblast like and roundish cells can be seen. In Figure 21C, at the dilution of 1:100, the well is no longer fully coated with intact cells and fewer fibroblast like cells can be detected, as majority of the cells are rounded

as a characteristic of infected cells. In Figure 21D, the virus dilution is 1:10, leading to a clear, strong infection. The bottom of the well is largely uncoated, and the remaining cells are shrunken and grainy.

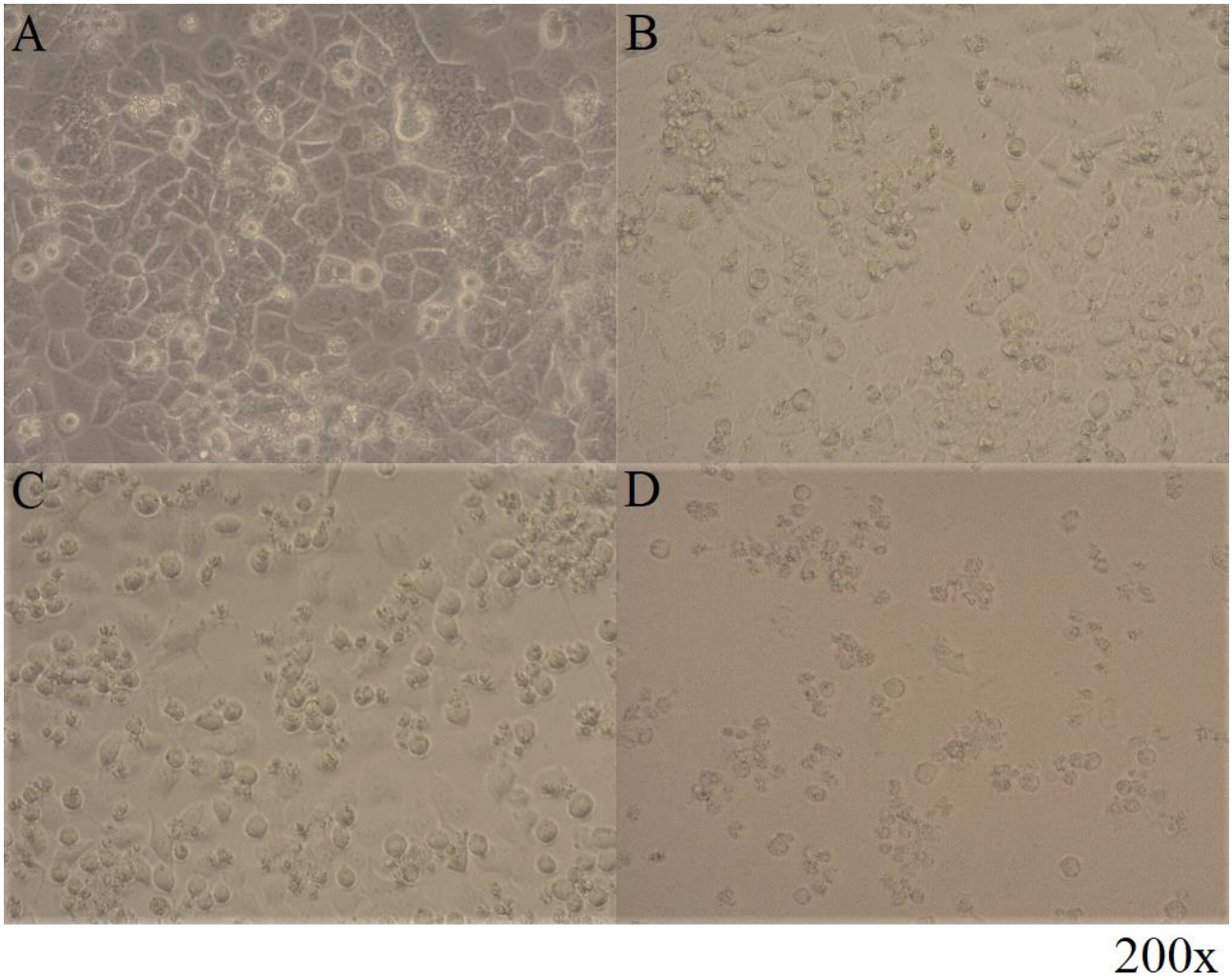

Figure 21. Infection of HuTu80 cells with PeV-A6 89. 2 DPI, 200x magnification. A. HuTu80 cell control. B. 1:1000 dilution of virus. C. 1:100 dilution of virus. D. 1:10 dilution of virus.

Cytopathic effect can be seen clearly in the HuTu80 cells infected with PeV-A3 145-8, as cells are rounded and largely detached from each other (Fig. 22B). HuTu80 cells infected with PeV-A4 FI121236 among others, showcased a severe infection in all dilutions. Most of the cell structure is gone, remaining cells look shrunken, and the leakage of cell contents can be seen as grainy accumulation in the well (Fig. 22C).

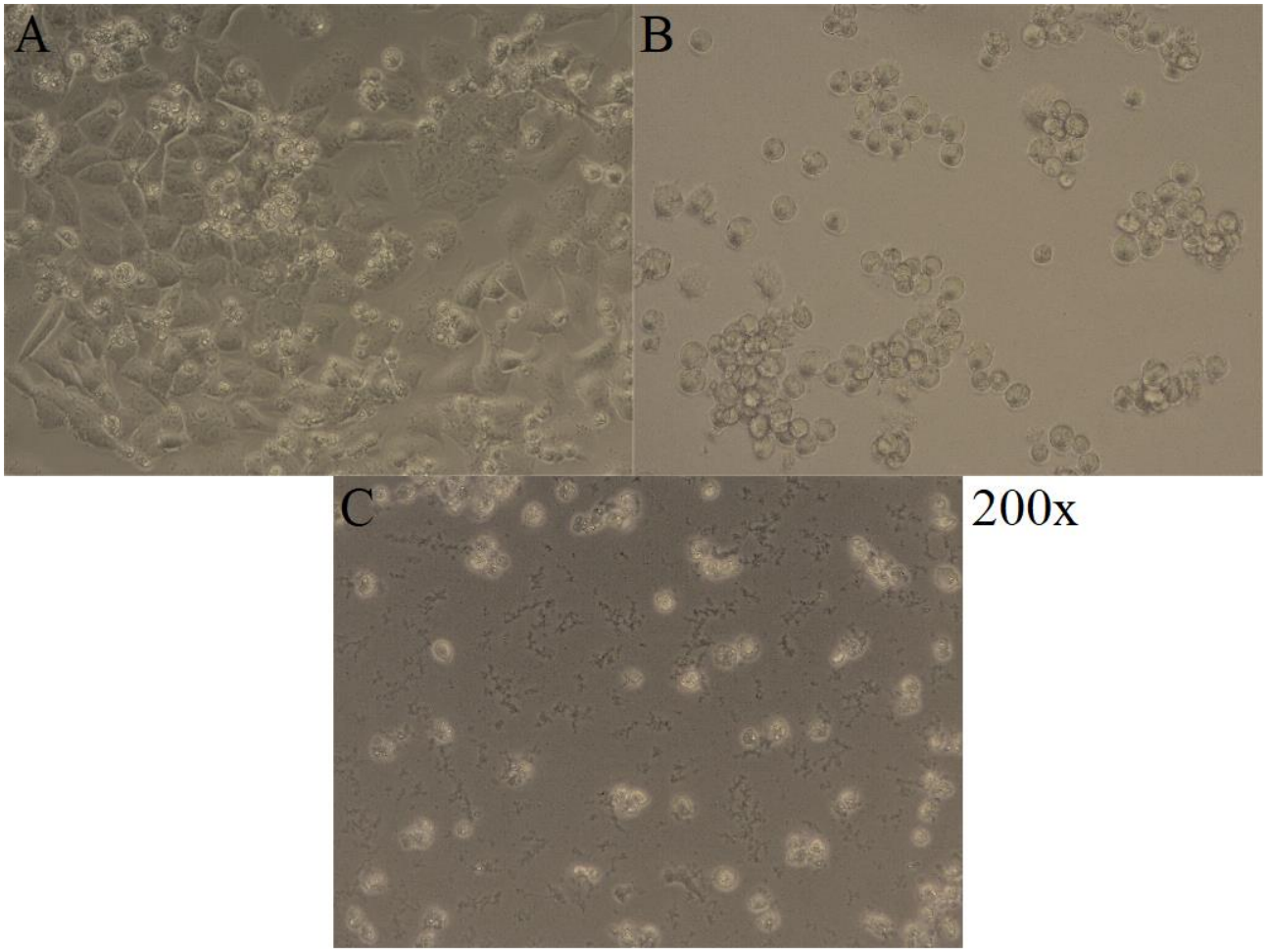

Figure 22. HuTu80 cells 3 DPI, 200x magnification. A. HuTu80 cell control. B. HuTu80 cells infected by PeV-A3 145-8, 1:100 dilution. C. HuTu80 cells infected with of PeV-A4 FI121236, 1:10 dilution.

4. Discussion

4.1 PCR enzymes

In this study, five high-fidelity PCR enzymes were evaluated for their ability to amplify CVA9 and PeV-A1 genomes. While some of the enzymes had optimal performance with high sensitivity, the results somewhat deviated from previous results by others. Both Q5® Hot Start High-Fidelity DNA polymerase and Phusion Hot Start II DNA polymerase that performed poorly in this study, have performed well in prior experiments with picornavirus targets. Q5® Hot Start High-Fidelity DNA polymerase was used to successfully amplify EV-A71 cDNA, resulting in a 7.4 kb product (Tan et al., 2016), and Phusion Hot Start II DNA polymerase was successfully used to amplify modified IRES fragments of Foot-and-Mouth Disease Virus (Rai et al., 2015). KAPA HiFi HotStart PCR enzyme has also performed better in the past than in this study, amplifying the full coxsackievirus B3/28 strain genome of 10.3 kb (Wehbe et al., 2016). The performance of PrimeSTAR GXL DNA polymerase has been variable in prior studies as well, e.g. it was unable to amplify the nearly full length enterovirus genomes when tested (Isaacs et al., 2018), while it successfully amplified shorter, 1550 bp regions of the Aichivirus C genome (Okitsu et al., 2014).

While it is possible that that some of the discrepancies can be explained by user error and relatively low number of repeats, it is also possible that primer compatibility with different target templates contributed to these differences. In addition, the sample type may have had an effect on the performance of PCR enzyme. However, it still does not change the fact that Platinum SuperFi II DNA polymerase had superior performance in these experiments.

When it comes to the parameters tested, nucleotide and primer concentrations producing the best results are quite well in line with what the datasheets suggested. For all tested enzymes, except for KAPA HiFi HotStart, the recommended dNTP concentration of 200 µM each, which was also confirmed to be optimal by the results of this experiment. KAPA HiFi HotStart enzyme datasheet recommends dNTP concentration of 300 µM each, however the 200 µM used in this experiment produced a fair result as well.
Q5® Hot Start High-Fidelity, Phusion Hot Start II and Platinum SuperFi II DNA polymerase enzyme datasheets all recommend a primer concentration of 0.5 µM. KAPA HiFi HotStart enzyme datasheet recommends the primer concentration to be 0.3 µM, and PrimeSTAR GXL DNA polymerase enzyme datasheet recommends the concentration is between 0.2 - 0.3 µM. All of this together is quite well in line with the results obtained in this study. The optimal primer

concentration according to the performed experiments falls between 0,2 and 1 µM. However, it seems safe to say that using 1 µM is excessive, as a similar level of production was obtained with a fraction (1/5) of that concentration.

The number of PCR cycles affected the amount of end product in an expected manner. Up to a certain plateau point, increasing the cycling increases the product yield. When using samples with low numbers of copies, substantial amplification is achieved after 35 cycles. Using samples with higher number of copies, substantial amplification is achieved after 25 cycles. Regardless of the number of copies in the sample, some increase in yield can be observed when 5 cycles are added to the 35 / 25, but beyond that the added cycles seem to have little effect to the final amount of product.
The preferred amount of cycling is also somewhat dependent on the objective behind the amplification. If the goal is to have as much end product as possible, it is justified to use high numbers of cycles (+40), especially when using starting samples with low copy numbers. Then again, if the goal is to obtain the genome with as few mutations as possible, for example for sequencing or to preserve the integrity of the viral genome, it might be wise to use a lower number of cycles (~30). Since high-fidelity enzymes were used in the genome amplification they are unlikely to generate mutations that could alleviate the natural mutation rate of these viruses.

The PCR enzymes tested for copy sensitivity produced surprisingly good results. Both PrimeSTAR GXL DNA polymerase and Platinum SuperFi II DNA polymerase are able to amplify the genome from a sample with as low as 10^2 copies. Adding on the RT step is likely going to push the sensitivity to 10^{3-4} copies, yet this is still approaching on the range of reliable diagnostic RT-PCR (Jokela et al., 2005; Lu et al., 2021). Sensitivity of the protocol is also very advantageous, as it can allow for amplification directly from clinical samples. In these cases one can avoid the process of generating more of the sample in cell culture, and purifying the virus, saving both time and resources.

One of the most puzzling findings is how the performance of PrimeSTAR GXL DNA polymerase seems to be quite different depending on which of the picornavirus templates was used. It produced substantial amplification of the PeV-A1 template, while that was not the case with the CVA9 template. Other enzymes such as Platinum SuperFi II DNA polymerase were able to amplify both templates equally, so the issue does not lie with the quality of the templates. As mentioned before, the performance of PrimeSTAR GXL DNA polymerase in prior research has also been questionable, as it was unable to amplify the nearly full length CVB3 and CVB5 genomes (Isaacs et al., 2018) – interestingly coxsackieviruses proved to be a difficult template for the enzyme in both

studies. Further research on the topic is needed to make any conclusive remarks regarding this finding.

4.2 RT enzymes

All tested RT enzymes were able to convert the full picornavirus RNA genome into cDNA, which is not a small feat, considering the length of the genome is nearly 8000bp. However, the results must be interpreted with a caution since there were some control issues related to IVT-RNA (see chapter 4.3). Nevertheless, sample degradation was observed with all enzymes, whether RNase inhibitor was used in the sample dilutions or not. The issue was most prominent with the ImProm II enzyme, maybe due to the fact that the enzyme required for long incubation times in the RT process.

As expected, SuperScript IV being a one-step RT-PCR system gave it certain advantages over the other tested two-step RT enzymes. SSIV was able to achieve the highest copy sensitivity in regards to the starting sample, 10^2 copies. This is most likely due to its one-step nature, which allows for all the produced cDNA to serve as the template in the following PCR. In the case of the two-step enzymes, it is recommended only 10 – 20 % of the cDNA reaction is used as the template for the following PCR, meaning they essentially have less cDNA to serve as template of the PCR. One of the two-step enzymes, LunaScript, was able to amplify the product from a starting sample with 10^3 copies, while ImProm II and Revert Aid H Minus trailed behind with the sensitivity of 10^4 copies. One-step RT-PCR systems also have a great advantage in the streamlining aspect. One-step reactions require only one tube, less pipetting and all the incubations can be done in the same program, making them much more efficient, user friendly and faster.

The fact that the RT-PCR was able to achieve copy sensitivities no more than a tenfold less than the PCR, and at times even reaching the same copy sensitivity as the PCR, is a promising sign that a robust and sensitive RT-PCR protocol can be developed.

As the results from the RT section ended up being incomplete, the optimal RT-PCR protocol could not be fully completed. More repeats with proper RNA samples are needed in order to obtain conclusive evidence of the performance of the RT enzymes, and complete the protocol. However, the performed experiments and obtained results provide a good starting point from which to further narrow down on the optimal RT enzyme to incorporate to and complete the RT-PCR protocol.

4.3 IVT-RNA production

The IVT-RNA samples used in the experiments were produced with the MEGAscript ® Kit (Life Technologies). The IVT reaction is a useful way of producing RNA samples from DNA templates, but it is by no means a fool proof method. Out of the four reactions performed, only three were successful in producing IVT-RNA. At first it was evident that the used IVT-RNA had DNA contamination that interfered with the interpretation of the results. However, newly generated IVT-RNA treated with DNase had better performance, which was visualized by RT-qPCR control assays (Fig. 19 & 20). In RT-qPCR, PCR amplification resulted in background amplification, which was 100 times less than what was evident from RT-qPCR. This indicated that RT step is essential in producing optimal amplification of these samples.

In the last, optional step of the IVT reaction, a DNase enzyme that is supposed to remove the template DNA from the product mix is used. Unexpectedly, DNase enzyme did not remove all the plasmid DNA from the sample, as the RT-qPCR control test indicated that the IVT-RNA samples still contained residual DNA. It can only be speculated why the enzyme did not work properly. It could be that the enzyme was somehow defective, maybe it had expired or it had been either stored or handled incorrectly. The production was carefully done following the kit instructions, in two instances, and both lead to a similar result.

The unfortunate fact that the IVT-RNA samples had DNA in them, compromises the results regarding the sensitivity of the tested RT enzymes to some degree. It is impossible to know exactly what the ratio of RNA/DNA was in the sample used for the RT reaction, but by comparing the efficacy of RT-PCR and PCR alone with the samples, the contamination can be estimated to be relatively mild, as the RT-PCR produces more amplification than the PCR alone.

The MEGAscript ® Kit also states that at the end of the IVT reaction, template DNA is present with a very low concentration in relation to the RNA concentration, and that for certain applications use of the DNase may not even be necessary. In the light of that fact, at least some broad assumptions or rankings of performance within the same samples can be made that will be helpful in further defining the true sensitivity of the RT enzymes.

4.4 HuTu80 cell line suitability

For virus rescue, it is important to know whether the used cell line supports virus replication. The primary viruses used in this project are CVA9 (enterovirus) and PeV-A1 (parechovirus). While there are several cell lines known to support propagation of CVA9 virus, the situation is not that clear for parechoviruses.

A recent study (Takagi et al., 2021) demonstrated that HuTu80 cell line supports propagation of several parechoviruses including PeV-A3, which is known to be difficult to cultivate. Thus, we aimed to test the suitability of HuTu80 cell line with our parechovirus strains, to ascertain that the results were reproducible. All tested PeV-A types (1 – 6) and all the 21 tested strains showcased infectivity of HuTu80 cells, meaning the line would be suitable for propagation and therefore for reverse genetics experiments. This finding is nicely in line with the findings of the literature we wished to repeat, as in both instances types 1 – 6 all proved suitable for propagation. This is a really positive finding, as there are not too many cell lines that are suitable for the culture of PeVs, as PeV-A3 and PeV-A6 have proved particularly difficult to culture (Westerhuis et al., 2013).

However, it must be noted that these results were obtained through light microscopy and visual assessment only, based on cytopathic effect. Therefore, these results should be verified by immunofluorescence microscopy using parechovirus-specific antibodies, such as Mab-PAR-1 antibodies (Tripathi et al., 2021).

4.5 Future direction of the project

Quite a few of the initially planned experiments had to be excluded from the project due to the restriction of the timeline. Therefore, the future of the project is quite clear and though out. Once the optimal RT enzyme is found and the RT-PCR protocol is fully finished, it still needs to be validated with more picornavirus strains, to make sure that it functions universally within the picornavirus family.

Once the protocol has been established, the testing of different regulatory primers can be implemented. Until now, only the T7 promoter containing primers have been used in the PCR. Having the T7 promoter is important as it allows the PCR product genome to be turned back into functional virus. The T7 promoter is the binding site for T7 RNA polymerase that will initiate the replication of the virus through RNA synthesis. Therefore the product must either be transfected to

cells that have intrinsic T7 RNA polymerase activity, or T7 RNA polymerase producing plasmid can also be co-transfected to cells with the genomic PCR product.

The other types of regulatory primers of interest include T7 termination sequence containing reverse primers and ribozyme sequence containing forward primers, in combinations with the established T7-forward and reverse primers. Adding these regulatory elements to the cDNA genome can improve the replication efficiency once the cDNA has become part of a plasmid and is being transcribed to viral RNA in cells. If no regulatory elements are used, the RNA produced from a circular plasmid can become overly long and unstable, if the RNA polymerase does not have an established termination point. This termination point can be established by incorporating the T7 termination sequence to the reverse primer, behind the coding sequence of the poly-A tail.

It has also been established that extra sequences before the true 5'-end of the RNA genome can have a negative effect on the replication in cells (Herold and Andino, 2000). By using a forward primer that contains both T7 promoter and a ribozyme sequence, in that order, right before the complementary area of the primer, multiple useful functions can be utilized. By the inclusion of T7 promoter, transcription can first be initiated. Once the T7 RNA polymerase has passed the ribozyme sequence and started to transcribe the true 5'-end of the genome, the ribozyme will cut off the extra sequence preceding the true genome. This results in the transcribed RNA having its true 5'-end, while enjoying the boosted transcription provided by the T7 promoter.

Once the PCR products with the added regulatory regions have been transfected to cells, the possible virus rescue and the effect of the regulatory region on the virus function can be observed, by imaging with light and immunofluorescence microscopy. By comparing the difference in performance between the different regulatory regions, their usefulness for different applications can be assessed.

Once the regulatory primers with potential have been identified, all kinds of other modifications on the genome can be made by long PCR, including the construction of the plasmid clone. These modifications could include for example point mutations, the effect of which on the pathogenicity of the virus can be explored.

4.6 Outcomes

The main outcome of this project is that once the protocol is ready, it will streamline the research process of picornaviruses. When the tedious optimization steps do not need to be repeated at the beginning of every experiment, research on the field becomes more accessible. Established protocols make it easier to get into a research that one maybe is not used to, leading the viewpoints and expertise in the field become more diversified.

A protocol with good sensitivity allows for the use of clinical samples as starting material, saving a substantial amount of time and resources, as the virus culture and purification becomes unnecessary. Having a straight forward protocol for the production of the essential cDNA clones and plasmids allows the main focus to be placed on the following steps, where the arguably more important developments lie. Picornaviruses harbor great potential in the development of study tools and therapies. Creation of a picornavirus fusion protein with for example a GFP marker as an intact part of the virus capsid is a potential avenue for developing tools that can be used to follow the transmission of the virus in a cell culture in real time. Another major, interesting direction would be the aided development of viral vectors that could have potential in oncolytic virotherapy.

Picornaviruses hold substantial potential in oncolytic virotherapy, which alone serves to justify base level research relating to picornaviruses. A flawlessly functioning research framework is a great asset that can aid in the successful development of picornavirus based oncolytic virotherapies. Multiple picornaviruses have been researched and developed with oncolytic virotherapy in mind, and one of the few oncolytic virotherapies that have ever made it to clinical use is RIGVIR, an echovirus from the picornavirus family, indicated in treatment of melanoma, meaning the potential they hold is not only theoretical, but very much real (Cao et al., 2020).

Picornaviruses innately possess many attributes that are considered useful in regard with development of viral therapies against cancer. Picornaviruses are small in size, the diameter of the virus particle being only around 30nm, meaning they can penetrate blockages such as the blood brain barrier relatively easily. Picornavirus replication takes place in the cytosol, meaning nothing is incorporated to the genome of the host cells, making them not genotoxic. Viruses in oncolytic therapy can be used as they naturally exist, but often they are modified in some way. Picornaviruses are readily modifiable in their cDNA form (McCarthy et al., 2019). The goal with oncolytic viruses is to get the viruses to replicate in cancer cells, while bypassing healthy cells, and kill the cancer cells as a result. In addition, cancer could be combatted through the immune response that the viral infection triggers. Picornaviruses cause strong immune responses, they have efficient self-

replicating process, leading to a clear cytolytic effect, as the newly formed viruses exit the host cell (Cao et al., 2020).

The fact that many cancers rely on integrins to regulate a large amount of their cellular functions as well as pathways relating to spreading of the cancer (Desgrosellier and Cheresh, 2010) could also be an interesting angle, considering integrins are one of the receptor types picornaviruses also rely on for host entry to the host cells, creating an option of targeting the virotherapy to cancer cells through integrin binding.

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6. List of abbreviations

CVA9	Coxsackievirus A9
CVA21	Coxsackievirus A21
DPI	Days post infection
IVT	*In vitro* transcription
PeV-A	Human parechovirus
RT	Reverse transcription / Reverse transcriptase

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