Virus safety of xenotransplantation and porcine endogenous retroviruses (PERVs)

Master's Thesis

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Science works on the frontier between knowledge and ignorance. We're not afraid to admit what we don't know. There's no shame in that. The only shame is to pretend that we have all the answers.

Neil deGrasse Tyson

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Abstract

Introduction The shortage of organs for patients is an issue that needs addressing. Xenotransplantation using pig cells or organs is the most favored option. However, pigs contain porcine endogenous retroviruses (PERV) in their genome which infect human cells. Like all retroviruses PERVs have immunosuppressive properties and may induce tumors. The mechanism of immunosuppression is unclear, but in the transmembrane envelope protein p15E a so-called immunosupppressive (isu) domain was identified. To prevent PERV transmission, a vaccine based on neutralizing antibodies may be useful. Previous results showed that immunization with TM proteins with mutations in the isu domain induced higher titers of antibodies compared with the wild-type.

Methods and results To develop an improved vaccine against PERVs, mutations were introduced in the isu domain after sub-cloning of env of PERV-A(42). Since the molecular clone PERV-A(42) was found not replication-competent, the mutants could not be tested in vivo . Although mutations in the env gene could not be detected, Western blot and immunofluorescence analysis, as well es transmission electron microscopy demonstrated the release of so-called Gag particles. To circumvent these shortcomings a PERV-A/B hybrid was generated, introducing the env of PERV-A(42) into a non-functional PERV-B(33). In addition, vectors were generated expressing the wild-type and mutated Env proteins. To analyze the influence of PERV on human immune cells, a virus preparation of recombinant PERV-A/C was added to human PBMCs and the release of IL-10 was analyzed. A low production of IL-10 was observed. Although the virus preparation was minimally contaminated with endotoxin, also able to induce IL-10, an inhibitor of the receptor of endotoxin, TAK-242, did not inhibit the whole activity.

Discussion The produced recombinant wild-type and mutant p15E will allow testing for the activity to induce IL-10 and other cytokines and will be used for immunization. Since PERV-A(42) was not replication-competent, other strategies have to be followed to obtain viruses with and without mutations.

1. Introduction

1.1. Background and clinical relevance

Organ transplantation is an important issue today. In 2013, 11233 people in Germany alone were registered on the Eurotransplant waiting list. At the same time, only 4511 organs were available through German donors [1]. It is therefore of importance to find a way to close this gap. Many promising ideas to develop novel techniques to overcome this problem are currently under investigation. One approach is to grow new organs from stem cells. This field of research however remains highly controversial and so far only offers limited success. It is for those ethical dilemmas that research groups around the world are trying to produce induced pluripotent stem cells which can then be used to generate organs. The nature of those therapies, as promising as they may sound, however includes serious set backs. Heart tissue generated from stem cells usually does not conduct electric current and is therefore non-functional. In other cases, even harmful effects can arise from these approaches. Cases are reported that generated heart cells will perform contractile movement counteracting the remaining hearts tissue [2].

Another method is to utilize organs from donor species other than human, to perfrom Xenotransplantations (xenos, from the Greek: *foreign*).

Here, cells or tissues are harvested from species other than the recipient species and transplanted into the host to replace the damaged tissue. Various problems arise from this technique. Like in human-human transplants immunological compatibility is an issue. As all cells present self-protein via major histocompatibility complex I (MHC I) to natural killer cells (NK cells) as well as to cytotoxic T-cells, cells from a different donor than oneself present a different set of proteins via a slightly different type of MHC I. In the case of immune rejection, NK cells as well as cytotoxic T-cells trigger rejection of the donor organ. Other mechanisms of rejection include the recognition of foreign antigen.

In pigs galactose-alpha-1,3-galactose (αGal) is such an antigen [3]. As it is not produced by primates, antigen presenting cells can activate B-cells, which will produce antibodies, specifically targeting αGal leading to the activation of the complement system and in the destruction of the donor tissue.

1.2. Retroviridae

Retroviruses are (+)RNA viruses that carry a reverse transcriptase (an RNA dependent DNA polymerase), which allows the virus to rewrite its genome into a coding strand of DNA, which is in turn integrated via an integrase into the hosts genome (Figure 1.1)[4]. *Retrovidae* are comprised of two subfamilies: *orthoretroviridae* and *spuma retroviridae*. Seven genera can be found in *orthoretroviridae*: *Alpharetroviruses*, *Betaretroviruses, Deltaretroviruses, Gammmaretroviruses, Epsilonretroviruses* and *Lentiviruses*. *Spumaviridae* on the other hand is soley comprised of the *Foamyvirus*-genus. Whether or not a virus is considered a complex retrovirus depends on if it carries accessory proteins, which are encoded by alternatively spliced RNA transcripts [5]. When budded from the host cell, the virus particle is still immature and undergoes a maturation process via protease activity (Figure 1.4). The virion itself, like in all retroviruses measures about 100-150 nm in diameter. The mushroom like structures protruding from the particle are comprised of the surface unit (SU), which is attached to the lipid bilayer of the viral envelope via the transmembrane (TM) domain. The viral core , which contains the viral genome as well as reverse transcriptases, integrases, and nucleocapsid proteins.

When the germline of an organism becomes infected with a retrovirus, all resulting cell from mitosis now contain endogenous retroviruses (ERVs). Porcine endogenous retroviruses can be found in all pigs and can form infectious particles[7, 8].

1.3. Genome organization of porcine endogenous retroviruses

The approximately 8 kb genome consists of two (+)RNA flanked by long terminal repeats (LTRs) which contain U5 (unique), R (redundant) and U3 elements (Figure 1.3). These elements are the site of promoters and enhancers, as well as they mark the site of Poly(A)

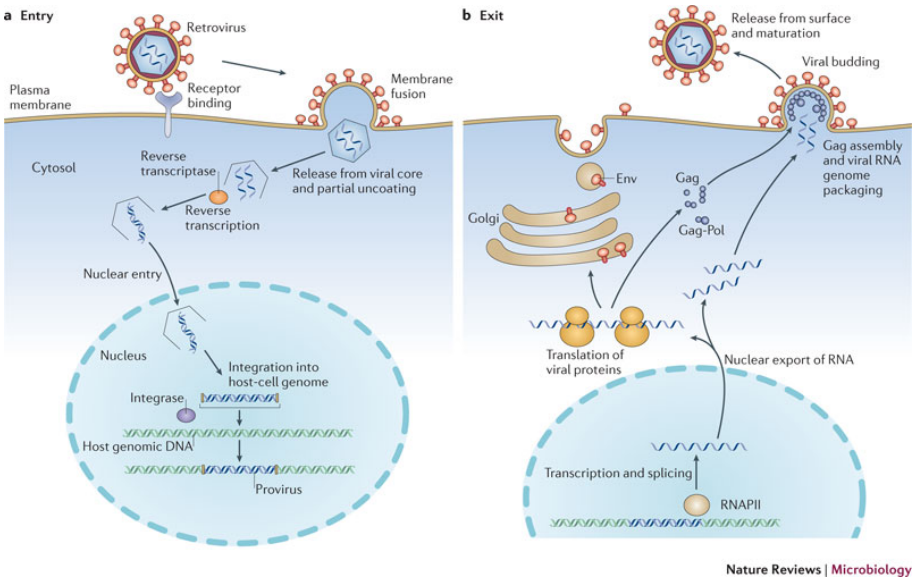


Figure 1.1.: An illustration of different events in the life cycle of a retrovirus. **a)** Entry: Viral particle needs a specific receptor on cell surface to bind leads to fusion of membranes (omitted here). The viral core uncoates and reverse transcriptase generates DNA from genomic +RNA. After transit through cytoplasm and nuclear entry, viral DNA integrates into the host's genome resulting in a provirus. **b)** Viral exit: RNA polymerase II transcribes provirus and viral RNA is processed and exported out of the nucleus. Viral proteins are translated from RNA template and Gag assembly and RNA packaging take place. The virus then buds through the cellular membrane and matures when it has already exited the cell [6].

addition. For reverse transcription to occur, a primer binding site (PBS) is located at the U5 element which binds to an intracellular $tRNA_{Gly}$ to initiate reverse transcription [10]. Through splicing of the genome two variants arise. One spliced variant comprises the full length sequence which code for Gag and Pol. The other variant codes for Env and is about 3 kb in size. Viral protease, matrix, capsid, and nucleocapsid proteins are encodde by the *gag* gene, while *pol* codes for reverse transcriptase and integrase (compare Figures 1.3 & 1.4).

1.4. Safety

Other dangers might arise from the donor's tissue itself. Pigs, as well as a humans, and all other mammals, have retroviral DNA encoded within their own genome. These retroviruses are therefore termed; endogenous retroviruses.PERVs unlike **h**uman **e**ndogenous **r**etro**v**iruses (HERVs) are able to form particles which are still exiting the cells in which

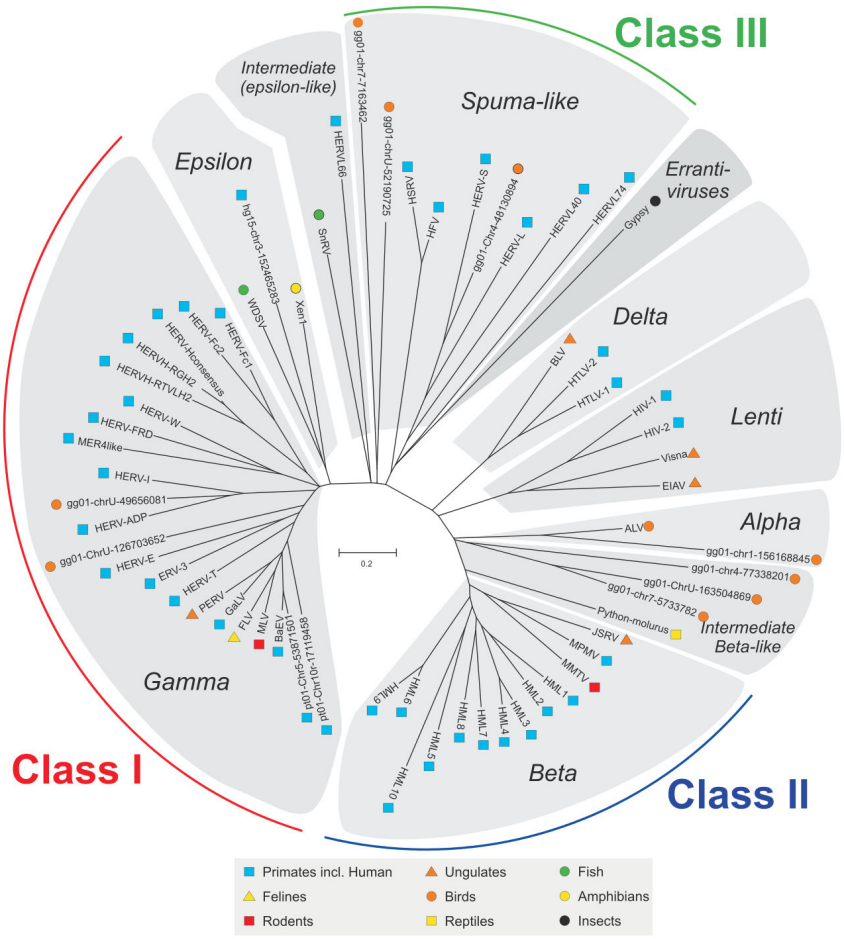


Figure 1.2.: Dendrogram of virus classes of endogenous retroviruses. Unrooted Pool neighbor joining denrogram of seven retroviral genera. Represented are, alpha-, beta-, gamma-, delta-, epsilon-, lenti- and spuma-like retroviruses. Endogenous retroviral classes which are more loosely defined are indicated on the periphery. Host species are described by symbols adjacent to the taxonomic unit. New sequences are named according to their position in their host chromosome (e.g. hg15 and 16: Human genome; gg01: Chicken genome and pt01: chimpanzee genome). Both pt01 sequnces were only observed in chimpanzees [9].

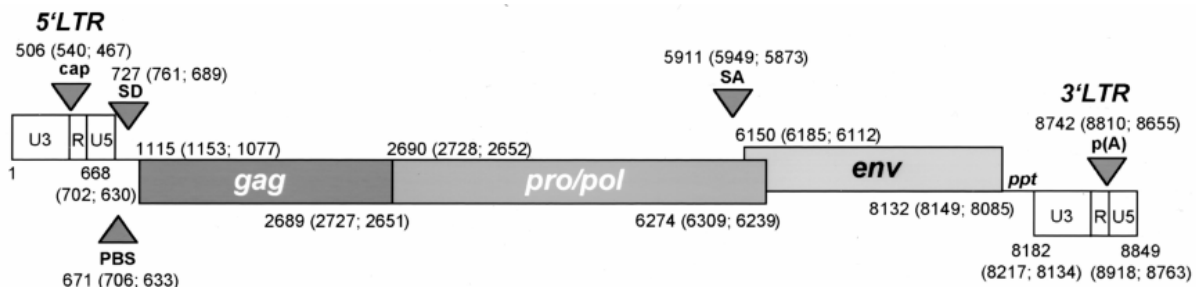


Figure 1.3.: Proviral sequences of 293-PERV-A(42), PK15-PERV-A(58) and PK15-PERV-B(213) are 8,849, 8,918, and 8,763 bp in length, respectively.A.11[11]

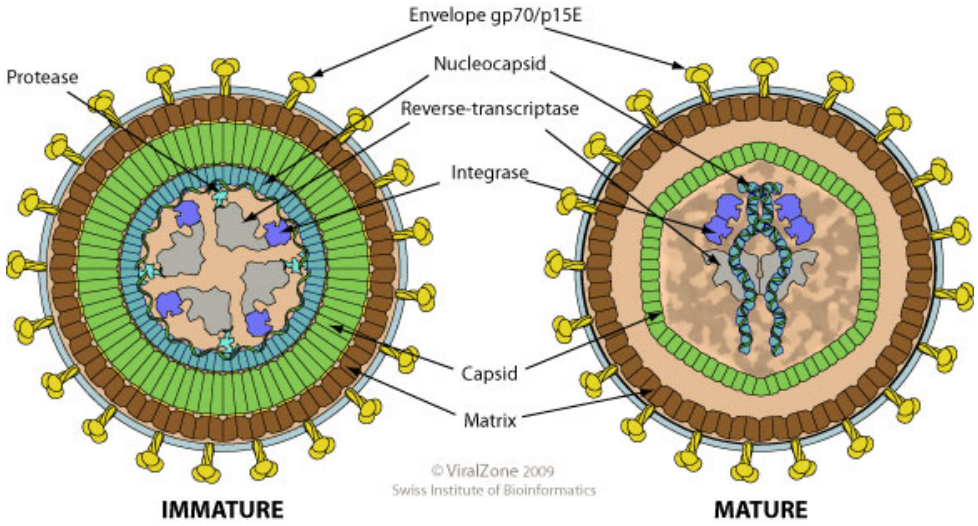


Figure 1.4.: Illustration of the immature (left) and mature (right) virion of a retrovirus.[12]

they are produced and therefore reach the circulation of the organism in which they are present. Despite an enormous increase in knowledge concerning PERVs, the overall safety analysis of pigs which may be used for xenotransplantation is limited. The infectious risks caused by the presence of numerous PERVs in the pig genome and the possible impact of PERVs on the health of the recipient is badly studied due to the lack of an appropriate animal model.All pigs harbour PERVs as an integral part of their genome [13] and PERVs are known to infect human cells in vitro [7, 8]. Although no transmission of PERV has so far been observed in non-clinical and clinical xenotransplantation [14], strategies should be developed preventing virus transmission. However, there is evidence for adaptation of PERVs to human cells [15, 16] and for immunosuppressive properties [17, 18]. Because of the transspecies transmission of the human immunodeficiency viruses (HIVs) which induces fatal immunodeficiencies in the infected individuals, the risk that PERVs induce immunodeficiencies and/or tumours in the transplant recipient cannot be excluded. Furthermore, closely related retroviruses such as the murine leukaemia virus (MuLV), feline leukaemia virus (FeLV) and the Koala retrovirus (KoRV) induce tumours and immunodeficiencies in the infected hosts [19]. Moreover, α1,3-galactosyltransferase (α1,3GT) gene knockout pigs [20–22] may present a new risk for xenotransplantation, because PERV released from these animals lack α1,3-galactose residues in the viral envelope and therefore could escape the complement cascade [23].

Another risk relates to recombinant PERVs. Recombination between human-tropic PERV-A and ecotropic PERV-C has been observed in pigs under natural conditions. Co-

cultivation of PERV-A and PERV-C in pigs has shown to yield recombinant human-tropic replication competent PERV-A/C [24, 25], some of which integrate into the genome of certain pig cells, but not the germ line, behaving like exogenous viruses [26]. PERV-A/Cs infect human cells and adapt to form high-titer viruses [16, 27–29]. Over millions of years host organisms have developed protective mechanisms to avoid retroviral infection and limit expression of integrated viruses. These are mediated by intracellular proteins termed 'restriction factors', which are components of the innate immune system that act at different stages in the retroviral replication cycle. Some families and factors, such as APOBEC, Fv1, tripartite motif (TRIM)5α, TRIM28, ZAP and tetherin provide a barrier against γretroviral and lentiviral infections, including HIV[30–32].

To detect PERVs, direct and indirect detection methods were developed by this lab [14]. Direct methods include: detection of proviral DNA by PCR, quantitative real time PCR, or Southern blot; detection of viral RNA or mRNA expression by RT-PCR or real time RT-PCR; detection of spliced mRNA as criteria for env expression, detection of protein expression by immunofluorescence. Indirect methods empoloyed are either ELISA or Western blot analyses.

1.5. Aim of study

The final aim of the project is to induce an improved preventive vaccine against infection by PERVs as one possible strategy to prevent virus transmission. PERVs are integrated in the genome of all pigs, they are released from normal pig cells, and infect human cells. Like most retroviruses, PERVs may possibly induce immunodeficiencies and promote the establishment of tumors in the infected host.

How retroviruses induce immunosuppression is still unclear. However, it was shown that viral preparations of different retroviruses, their recombinant TM proteins and synthetic peptides corresponding to the so-called immunosuppressive domain of the retroviral TM proteins were able to modulate cytokine release and gene expression in normal peripheral blood mononuclear cells [33–36]. Il-10, IL-6 and IL-8 were upregulated, IL-2 and some genes involved in innate immune responses were down regulated, suggesting that the immunosuppressive domain of the transmembrane protein may be involved.

Neutralizing antibodies were induced by immunisation with the PERV transmembrane

and surface envelope protein [37, 38]. It was shown that mutations in the immunosuppressive domain of the TM protein of different retroviruses including HIV-1 abolishes their immunosuppressive properties *in vivo* and immunization with the mutated TM protein induces higher antibody responses in the immunised animals when compared with the wild-type TM protein [33–36].

Therefore, immunization with mutated p15E of PERV may induce higher titers of neutralizing antibodies than with the wild type. To investigate this, a replication-competent clone should be characterized in its wild-type form and mutated to observe and measure its effect on cytokine release when it interacts with human peripheral blood mononucleated cells (PBMC). Furthermore, mutated and non-mutated versions of *env* in an expression vector (pTarget)for protein expression should be established. Additional efforts were undertaken to produce PERV-A/B hybrid clones, which include the PERV-A(42) Env cloned into an Env-deficient PERV-B clone, to restore the virus' infectivity.

2. Methods

2.1. Virus production and purification

PERV producing HEK293T cells were grown using standard cell culture medium (A.8) and grown to confluency. Supernatant was harvested and through a series of centrifugation steps (using Heraeus Centrifuge, Appendix A.6) separated from remaining cells, as well as cellular debris. The steps include centrifugation at 1200 rpm for 10 min followed by another 10 min interval at 4000 rpm. To remove further debris from the sample, a cell strainer (Appendix A.6) is used. The supernatant then undergoes ultracentrifugation (A.6) at 34000 rpm for 1^{30}h to pellet the produced virus using rotor SW41. This pellet is then re-suspended in a scraping motion with PBS and used for purification using a 20% sucrose solution and a lighter phase material (here PBS). The resuspended pellet ins injected into the phase between the two layers and centrifuged at 38000rpm in the ultracentrifuge (Rotor: SW50.1) to obtain the virus pellet in the sucrose solution. After careful removal of supernatant, the pellet contained in the bottom phase is re-suspended in PBS to obtain the desired concentration from measured from original amount of supernatant harvested and processed (Equation 2.1).
Example:

$$100\,mL\,Supernatant \quad \Rightarrow \quad resupsended\,in\,100\,\mu L \;=\; 1000\times \tag{2.1}$$

2.2. Characterization of the PERV-A(42) clone

A clone, which was provided by Ralf Tönjes from the Paul Ehrlich Institute, Langen, was transformed into chemically competent cells (JM109, A.3) using the standard protocol provided by the vendor (Zymo Research, A.3). An isolated colony was picked and grown overnight in LB+AMP medium (A.8) at 37 °C in a shaking incubator (A.6) at 180 rpm. To confirm the identity of the clone, a restriction digest using, PstI and HindIII. Digest was set up as described in table 2.1.

Table 2.1.: Set up for restriction digest to characterize PERV-A(42) clone. Appendix A.4 for more information.

Component	Amount (in µL)
10× Fast Digest Buffer	1
Restriction enzyme	1
DNA sample (total of ≈ 200 ng)	2
H_2O (fill up to final vol. here 10 µL)	6
Total	10

The resulting fragments have been confirmed on a 1% agarose gel as illustrated in figure 3.1.

2.3. Polymerase chain reaction

Polymerase chain reaction (PCR) performed using several different DNA dependent DNA polymerases. In general, a Taq polymerase (A.4) was used for short fragments and colony PCR as well as for TA- cloning, due to the template independent 3' T overhang it produces. Taq polymerase has a much higher error rate as compared to Phusion polymerases (Hot start flex A.4) which I used to perform site-directed mutagensis as well as for several cloning applications which included the amplification of long terminal repeats of viruses.

Table 2.2.: **a)** Setup for GoTaq-Master Mix and **b)**Phusion Hot Start Flex

(a) Setup of 50 µL Reaction

Component	Amount (µL)
GoTaq Green Master Mix	25
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
DNA Template (≈100ng)	x
Nuclease Free H_2O	x
Total	50

(b) Setup of 50 µL Reaction

Component	Amount (µL)
5× Phusion HF or GC Buffer	10
10 mM dNTPs	1
Forward Primer (10 µM)	2.5
Reverse Primer (10 µM)	2.5
DNA Template (<250 ng)	x
Nuclease Free H_2O	x
Phusion Hot Star Polymerase	0.5
Total	50

Table 2.3.: Thermocycler settings for PCR amplification (30-40 cycles)

		GoTaq Green Master Mix	Phusion Hot Start Flex
Phase	Temperature (in °C)	Time (seconds)	
Denaturation	95	120	300
Annealing	T_m - 5	30	15
Extension	72-74	$(\frac{1\,kb}{min})$	$(\frac{2\,kb}{min})$
Final extension	72-74	300	
Storage	4	∞	

2.4. Cloning

Previously obtained PERV-A clone (accession №AJ133817) was transformed into competent cells (JM109, A.3) using the standard protocol provided by the vendor (Zymo Research, A.3). An isolated colony was picked and grown overnight in LB+AMP medium (A.8) at 37 °C in a shaking incubator (A.6) at 180 rpm. The plasmids were isolated and the Env region containing p15E, was amplified using GoTaq® Green Master Mix in PCR reaction (table 2.4 for primers). Exemplary thermocycler settings can be found in table 2.3.

Table 2.4.: Primers for *env* amplification

Name	Sequence	T_m
Forward *env*- Primer	5'-TTTGTTCTCTAGGCTCAAGGCG-3'	66.5 °C
Reverse *env*- Primer	5'-TTCTAATCTTAGAACTGGGAAGG-3'	59.3 °C

After PCR, fragment length was determined using 1% agarose gel, as seen in figure 3.8 The remaining PCR product was purified using Invisorb™ DNA CleanUp (A.5, concentration: ≈350 $\frac{ng}{\mu L}$) and was cloned into the pGEM®-T Vector using the protocol provided by the vendor (A.10).

The resulting construct was then transformed into JM109 cells using the previously described protocol. Plasmids were isolated from bacterial culture (using Invisorb®: Appendix A.5) Spin Plasmid Mini Two) after an 8 hour growth period in 4 mL of bacterial medium (A.8) and confirmed via restriction digest with NotI and consecutive sequencing. The isolated plasmid construct served as the basis for site-directed mutagenesis (2.6).

2.5. Transfection

Target cells (293T cells, A.3) were transfected with wild type (WT) PERV-A(42) to elucidate its ability to form particles as well as various mutated forms for virion harvest and purification. Previously, 293T cells were seeded onto 6 well cell culture dish and grown to ≈ 80 % confluency at 37 °C in CO_2 incubator. 250 µL of cell culture medium without antibiotics (A.8) and 2.5 µg of DNA are mixed and 7.5 µL of transfection reagent (A.8) are added and mixed via short vortexing. The solution was incubated at room temperature (RT) for 20 min. After incubation the solution is added drop-wise to each well and incubated at 37 °C in a CO_2incubator for 24-48 hours. Virus particles can now be harvested and purified by harvesting supernatant and cell lysate can be analysed for transfected material as well as viral proteins. For further details and variations of the protocol the vendors manual can be consulted (A.8).

2.6. Site-directed mutagenesis

Site-directed mutagenesis was performed by "back to back" PCR of the PERV-A(42) clone-DNA. Here, two primers anneal at the site of the desired mutations on the coding as well as template strand of the template DNA, at in consecutive order (2.2). The primers are therefore attached to the template in a back to back fashion and synthesize the complete template strand including the desired mutations (Figure 2.1). The template was digested via *DpnI* digest. This a linear fragment of DNA, is ligated (2.15) to form a circular piece of plasmid DNA and transformed into competent cells (2.2).

	Postition													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
PERV-A WT	L	Q	N	R	R	G	L	D	L	L	F	L	K	E
Mut1	A	Q	N	R	R	G	L	D	L	L	F	L	K	E
Mut2	L	A	N	R	R	G	L	D	L	L	F	L	K	E
Mut3	L	Q	A	R	R	G	L	D	L	L	F	L	K	E
Mut4	L	Q	N	A	R	G	L	D	L	L	F	L	K	E
Mut5	L	Q	N	R	R	G	L	A	L	L	F	L	K	E
Mut6	L	Q	N	R	R	G	L	D	L	L	F	L	K	A
Mut7	L	Q	A	R	I	L	A	V	E	R	Y	L	K	D

Figure 2.1.: Illustration of mutations to be induced from WT PERV-A(42) infectious clone (A.11). Mutations are introduced into construct shown in figure 3.8 by site-directed mutagenesis (2.6).

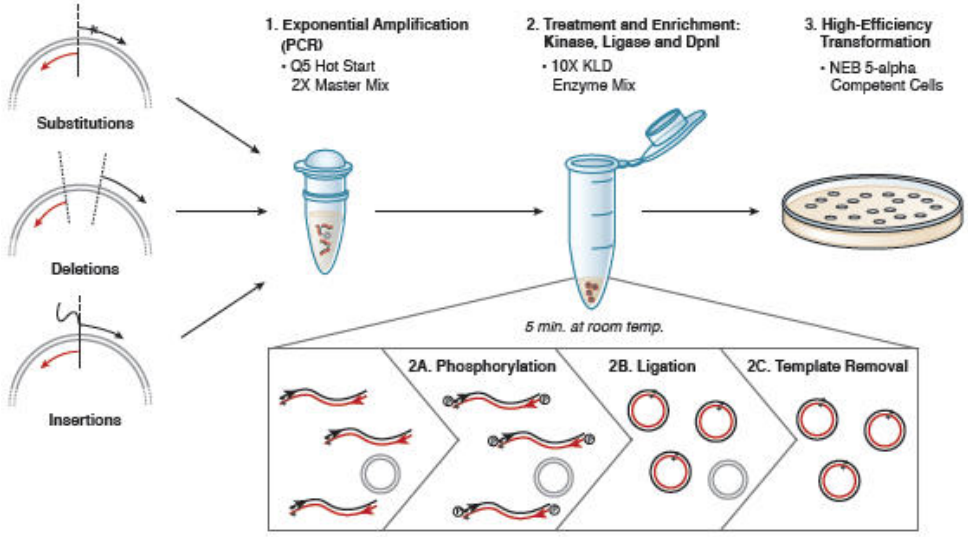


Figure 2.2.: Diagram of the work-flow of site-directed mutagenesis. **1)** original clone (PERV-A(42)) with phosphorylated primers (mutations indicated with red crosses) annealed. **2)** linear amplicon containing mutations. **3)** Clone after ligation reaction with T4-Ligase. Ligated Clone contains desired mutations.

2.7. Generation of clones for protein expression

Protein expression clones were created by producing amplicons from clones created via site-directed mutagensis as well as wild type PERV-A(42) using primers (trunc-env-Forward, trunc-env-Forward-kozak, and trunc-env-reverse-close, A.9)(Figure 3.14). PCR reaction set up and thermocycler settings were adjusted according to GoTaq Green Master mix protocol (Tables 2.2 and 2.3). Primers were designed to truncate Env before its transmembrane domain, as illustrated in figure 2.3.

Amplicons were isolated from a 1 % agarose gel using Invisorb® Spin DNA Extraction Kit (A.5) and quantified. Those fragments were used for cloning using the pTarget™ -

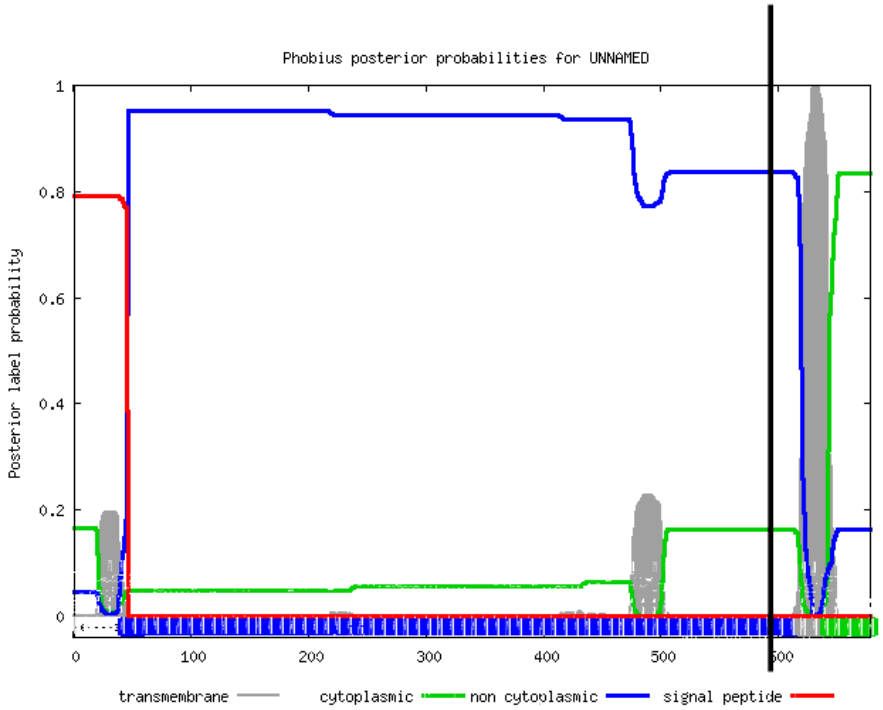


Figure 2.3.: Prediction of location of transmembrane domain in Env. Black vertical line represents cut-off at 583 aa as used in generation of Env-expression-clones.Diagram was created using Phobius from the bioinformatics centre Stockholm.

Vector System as described in section 2.4. The resulting clones were transformed according to protocol in section 2.4 and plasmid DNA was isolated using Invisorb® Spin Plasmid Mini Two (A.5). KpnI digests were performed according to protocol described in table 2.1. The resulting fragments can be seen in figure 3.17. After verification of directionality of inserts, sequencing as described in section 2.8 was carried out to confirm the presence of the mutations as well as the partly modified transcription start site through addition of Kozak sequence (Figure 3.18).

2.8. Sequencing

All sequencing reactions were performed at the *Robert Koch Institute* is an advanced chain-terminator method, which employs ABI BidDye 3.1v cycle sequencing. Sequencing PCR was performed using thermocycler settings in table 2.5

Table 2.5.: Sequencing PCR setup

(a) Reaction setup

Component	Amount (µL)
DNA template	200 ng
Primer (10 µM	0.5
BigDye-Mix 3.1	1
5× ABI-buffer	1.5
Nuclease free H_2O	up to 10
Total	10

(b) Thermocycler settings

Phase	Temparature (in °C)	Time (seconds)
Initial denaturation	96	120
Denaturation	96	10
Annealing	55	5
Extension	60	240
Storage	4	∞

2.9. Cell lysis

Cell culture cells are collected at $\approx \frac{10^8 cells}{mL\ lysis\ buffer}$ and washed three times with cold PBS. Supernatant is removed and cells are resuspended in 2 × NP40 lysis buffer (A.2) + cOmplete (A.8) and incubated for 30 min on ice, while vortexing every 10 min. Cellular debris is pelleted at 13,000 rpm for 10 min and clear supernatant is collected in new centrifuge tube and can now be stored at - 80 °C for use in SDS-PAGE.

2.10. SDS PAGE

A 12 % sodium dodecyl sulfate (SDS) polyacrylamide separation-gel with a 4 % stacking-gel was used to detect proteins within samples. The composition of the gel and its buffers can be seen in table 2.6.

Table 2.6.: SDS-PAGE according to Jagow and Schägger [39].

Stock solution for SDS-PAGE

Buffer	Tris (M)	Tricine (M)	pH	SDS (%)
Annode Buffer	0.2	∅	8.9[a]	∅
Cathode Buffer	0.1	0.1	8.25[b]	0.1
Gel Buffer	3.0	∅	8.45[a]	0.3

[a] Adjusted with HCL

[b] No correction of the pH, which is around 8.25.

10 µL of samples were with 10 µL Novex® Tris-Glycine SDS Sample Buffer (A.8 (2×) with 5 % beta-mercaptoethanol to reduce intra or intermolecular disulfide bonds. The resulting 1× solution was denatured at 95 °C for 10 min and then loaded onto the previously prepared gel for analysis. Voltage settings for stacking gel were 80 V for ≈20 min and 110 V for separation gel for ≈70 min. Times are approximated due to the varia-

tion in gel-fraction-size. The resulting gel can now be used for Western blot analysis or Coomassie blue staning.

2.11. Western Blot analysis

The transfer chamber is prepared using one layer of extra thick blotting paper, a matching piece of Optitran BA-S 83 (GE) membrane on which the gel is placed. The gel is covered with one piece of extra thick blotting paper. All pieces of this assembly are previously soaked in transfer buffer (A.2). To improve the transfer, potential air bubbles are pushed out by a rolling and pushing after the the transfer is assembled. To cover the full spectrum of proteins of interest, a 1 hour transfer at 20 V is sufficient.
After the transfer, the recovered membrane is blocked over night at 4 °C or for 1h at RT in 6 % milk in PBST (PBS supplemented with 0.1 % Tween) can be probed with several antibodies. The Antibodies used in my experiments were derived from earlier PERV immunization studies conducted with goats. It was determined that these serum-Antibodies are to be used at a 1:500 dilution in 6 % milk in PBST for 1h at RT. The process was followed by three consecutive 5 min washing steps in PBST on a shaking platform. The secondary antibody (A.1) was added in a 1:10000 dilution in 6 % milk in PBST for 1h. The membrane was developed using ECL Western Blotting Detection Reagents Kit (A.5) according to the protocol provided. Visualization was achieved using CHEMOCAM Imager 3.2 (A.6)

2.12. Coomassie blue stain

SDS-PAGE gel is washed with lukewarm ddH_2O three consecutive times for 5 minutes each on a shaking platform. Coomassie blue R250 (A.2) is added and covered with a lid on a rocking platform for ≈15 minutes. After staining, Coomassie blue solution is removed and the gel is destained using 50 % destaining solution (A.2) and 50 % ddH_2O overnight. Visualization was achieved using CHEMOCAM Imager 3.2 (A.6) including a back-light apparatus to enhance the contrast between gel and bands.

2.13. Immunofluorescence

Previously cells were seeded out to grow to ≈ 50 % confluency and were transfected according to section 2.5. Newly transfected cells were fixed with 2 % paraformaldehyde for 20 min at 37 °C. Cells are washed twice with PBS and and placed on ultra adhesive microscope slide and dried at 37 °C until liquid has evaporated. Permiabilization was performed with a 0.2 % Saponin solution for 30 min and blocked with blocking buffer for 2 hours at RT (A.2). Primary antibodies (A.1) used are diluted in antibody-dilution buffer (A.2) and applied at various concentrations directly on top of the sample for 1 hour. Cells are washed three times using IF- wash buffer (A.2) and incubated with secondary antibody (A.1) for 45 min. The secondary antibody is conjugated with fluorophores resulting in the signal observed (3.5). As those fluorophores are light-degradable, incubation has to take place in the dark. Finally, cells are washed three times with wash buffer and covered with glass slides using DuolinkII mounting medium (containing DAPI). It is imperative to conduct all incubation steps in a wet chamber to prevent the samples from falling dry.

2.14. Gel analysis

Coomassie blue stained bands and resulting concentrations were determined using ImageJ by comparison of a BSA dilution series with known concentrations. The resulting data points serve as a basis to produce a line of best fit-equation, which can be used to estimate the concentration of the virus in the sample.

2.15. Ligation

Amplicons received from mutagenesis PCR reaction (2.6) were previously phosphorylated (Table 2.7) on the 5' end to allow for ligation reaction to occur. The T4 ligase covalently links the 5'- phosphate with the 3'-hydroxy group, which results in a cirular stretch of DNA (reaction mixutre in table 2.7). The reaction mixture was incubated at room temperature for 1h.

Table 2.7.: **a)** Phosphorylation reaction setup as performed for mutagensis reaction. **b)** Ligation reaction setup, for circularization of linear plasmid amplicon from mutagenesis PCR.

(a) Phophorylation reaction

Primer (100 µM)	0.9 µL
10× T4 PNK buffer A	0.9 µL
10 mM ATP	0.9 µL
T4 PNK	0.8 µL
ddH_2O	5.5 µL
Total	10 µL

(b) Ligation reaction

DNA	10-20 ng
10× ligase buffer	2 µL
T4 ligase (5 U/µL)	1 µL
ddH_2O	x µL
Total	20 µL

2.16. Endotoxin assay

To ensure that the virus sample tested in the IL-10 assay is endotoxin free, a reporter cell line (THP-Xblue cells, A.3) was employed. The essay is based on the activation of NF-κB by pattern recognition receptors (here, toll like receptor 4 (TLR4)). Upon activation of the NF-κB pathway, the inducible gene *Secreted embryonic alkaline phosphatase* (SEAP) is activated. SEAP is a truncated version of the human placental alkaline phosphatase (PLAP) which was modified by removing the GPI (Glycophosphatidylinositol) anchor, rendering it extremely heat stable and unaffected by inhibitor L-homoarginine. Upon TLR4 triggering, SEAP is produced and secreted into the cellular supernatant from where it can be harvested for testing.

THP-Xblue cells were thawed quickly at 37 °C in a water bath and resuspended in 15 mL of THP-growth medium (A.8). To remove the excess of DMSO from the freezing media, cells are centrifuged at 200-300 rcf for 5 min and old medium is aspirated. Cells are resuspended in 1 mL of growth medium and added to a 25 cm^2 cell culture flask containing an extra 5 mL of growth medium. Cells are growth at 37 °C in a CO_2 incubator until they have recovered and grow. Selection process was initiated by the addition of 200 $\frac{\mu g}{mL}$ of Zeocin ™ to THP-growth medium. Once under selective pressure, cells need to be passaged every three days at 7×10^5 $\frac{cells}{mL}$, not to exceed 2×10^6 $\frac{cells}{mL}$. Cells are used at 1×10^6 $\frac{cells}{mL}$ for testing the samples resuspended in growth medium. In a flat-bottom 96 well plate setup, 20 µL of sample are added to each well, followed by 180 µL of THP-cell-suspension. The plate is incubated for 18-24 h at 37 °C in a CO_2 incubator. After incubation, cells are centrifuged to the bottom of the wells at 200-300 rcf for 5 min and 20 µL of supernatant is added onto previously prepared 96-well-plate containing 180 µL of QUANTI-Blue™. The

plate is incubated for 1-8 h at 37 °C and can is read out on a photometer at 620-655 nm.

2.17. Interleukin-10 assay

Interleukin-10 (IL-10) or human cytokine synthesis inhibitory factor (CSIF) is a an anti-inflammatory cytokine encoded by the *IL-10* gene, which is found downstream of several cell signal pathways (Figure 2.4) mainly from patter recognition receptors. However, there is also crosstalk between several signal transduction pathways. Mitogen activated protein kinase pathway (MAPK) signaling pathway is also capable to induce *IL-10* production as it signals over *RAF* which is capable to phosporylate *MEK*.

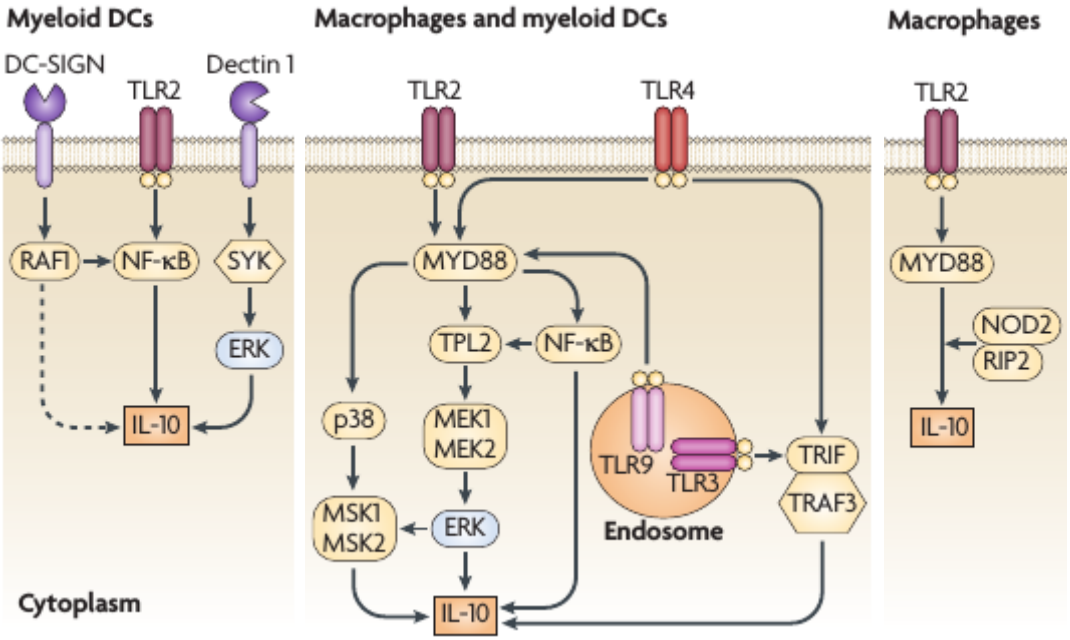


Figure 2.4.:
Interleukin- 10 can be induced by Toll-like receptors (TLR) or other pathways in macrophages and myeloid dendritic cells (DCs). Via the activation of TLRs and their adapter moleculres, myeloid differentiation primary-repsonse protein 88 (MYD88) and TRIF result in the activation of ERK1 and ERK2 (here: ERK), p38 and NF-κB pathways. The activation of these pathways leads to IL-10 response of cells [40].

PERV-A/C, which binds to the human PERV-A receptor is also said to be an inducer of an IL-10 response by peripheral blood mononucleated cells (PBMCs). These human PBMCs were previously harvested from donors from the lab and have either been frozen and thawed before use, or were freshly isolated and isolated using Ficoll-Paque centrifugation in accordance with the protocol established by the manufacturer (A.8).

Samples were previously analyzed using SDS polyacrylamide gels Coomassie blue staining to determine the size the amount of viral particles within the supernatant concentrate using ImageJ. To inactivate and to make the viral isu-domain more accessible, to the PMBCs, the samples undergo a 5 × freeze-thaw process, in which the viral particles are disrupted [41]. A dilution series of duplicate samples was applied to observe the effect of IL-10 induction of the samples. Additionally another set of identical duplicates was set up with the addition of TAK-242 (A.8), a small-molecule inhibitor, which binds selectively to TLR4, to silence the effects of residual LPS in the sample. TAK-242 was used at 5 µM to completely circumvent activation of TLR4 [42].

On a 96-well-plate format, 3×10^6 PBMCs are seeded out and the sample is added at the previously calculated concentration. The final volume of each well is 200 µL. The plate is incubated at 37 °C in a CO_2 incubator for 18-24 h.

A 96 well plate is prepared simultaneously by adding 100 µL of capture Antibody (A.1) per well. The plate is sealed and incubated at 4 °C overnight.

After incubation, the wells are washed three times with washing buffer (A.2), the plate is blocked for 1 hour at RT with assay diluent solution and then removed as previously described. Samples have been added (100 µL) and incubated for 2 hours at RT. After incubation period, samples are removed by washing five times. After the addition of working detector (Detection antibody + SAv-HRP reagent), the plate is sealed and incubated for 1 hour at room temperature. A final wash of seven times is applied and 100 µL of substrate solution (A.2) is added. After a final incubation of 30 min shielded from light, the reaction is stopped by the addition of stop solution (A.2) and the plate is read at 450 nm and 570 nm.

2.18. Gibson assembly for Generation of PERV-A/B hybrid

Gibson assembly is a molecular cloning technique which relies on homology of the endpoints of backbone and insert. T5 exonuclease degrade the ends of the amplicon on one strand, producing sticky ends. When homology is present, the ends of both, backbone and insert, will be compelmentary to each other allowing for annealing. A possible resulting gap is closed by polymerase activity and the nick is sealed via a T4 ligase. For the

purpose of this study Gibson Assembly® Master Mix (A.4) by New England Biolabs was utilized. The master mix contains the above mentioned components. Additionally, chemically competent cells are included (5-α *E.coli*) which have been used for transformation according to the vendor-specific protocol.

I have identified flanking regions of PERV-A(42)-Env and PERV-B(33)-Env which showed sufficient homology for Gibson assembly. These flanking regions serve as a template for primer design. Env-amplifying primers are used on PERV-A(42) Env. Their reverse complement serves as the primer pair for PERV-B backbone amplification. Due to the large size of the PERV-B(33) genome, additional primer pairs were palced inside the genome to minimize the amount of error (A.9). Intact and verified Env from PERV-A(42) was amplified using Phusion Hot Start flex polymerase (A.4). Additionally, PERV-B(33) backbone was amplified using primers described above. The resulting fragments were verified (Figure 3.20) on a 1 % agarose gel and eluted with stratec gel extraction kit (A.5).

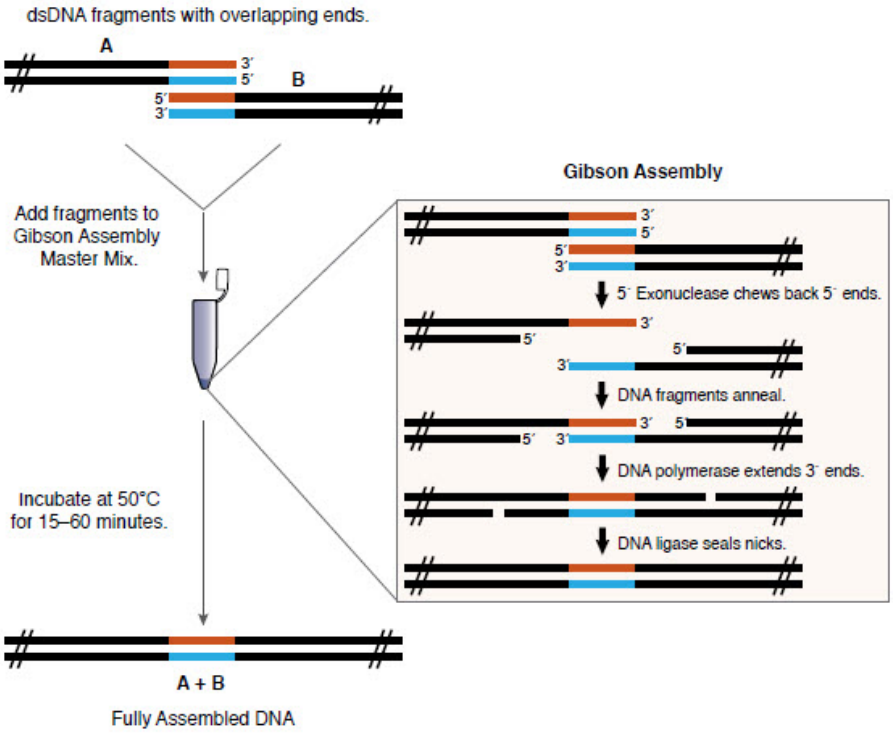


Figure 2.5.: Outline of Gibson Assembly [43]

3. Results

3.1. Characterization of PERV-A(42)

To develop higher titers of neutralizing antibodies by mutating the immunosuppressive domain of PERV, site-directed mutagenesis, using the molecular clone PERV-A(42, (kindly supplied by Prof. Dr. Ralf Tönjes, *Paul Ehrlich Institut*), was performed. This clone served as the basis for the entire work and had to be verified for authenticity first. Several steps were undertaken to verify the clone.

3.1.1. Characterization of the PERV-A(42) genome

First, the clone was analyzed by performing multiple restriction digests. The fragments obtained were used for comparison with fragments from *in silico* digests of the clone's sequence (A.11). As the molecular clone contains a vector backbone, which may result in extra bands, only fragments, which matched sizes within the 5' and 3' region of the clone were used for analysis. By performing a restriction digest we showed that regions of the clone, which are not part of the backbone vector can be verified using PstI and HindIII (Figure 3.1).

Following this analysis, the clone was used for amplification of *env* using primers shown in table 2.4 and PCR conditions as shown in table 2.2. The resulting amplicons had a size of ≈2300 bp which is in accordance with the expected size (Figure 3.2). The resulting fragment was used for amplification of *env* as previously described in section 2.4 using primers as shown in table 2.4. The resulting apmlicon shows strong bands in the range of ≈2300 bp as expected.

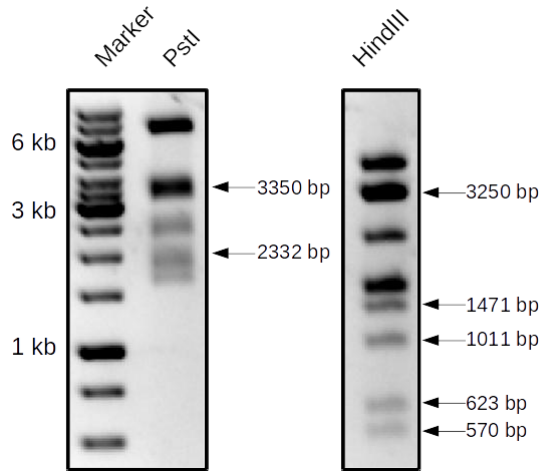


Figure 3.1.: Electrophoretic analysis of fragments after digestion with PstI and HindIII showed fragments not flanked by 3' and 5' regions including ≈3350 bp and ≈2332 bp fragments for PstI as well as ≈570 bp, ≈623 bp, ≈1011 bp, ≈1471 bp and ≈3250 bp for HindIII. Additional bands are the result of cutting of cloning vector backbone.

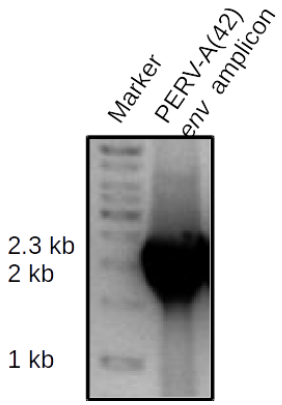


Figure 3.2.: Amplification of the entire *env* of PERV-A(42) including two StuI sites on the 5' and 3' end. Marker in lane 1 (GeneRuler 1 kb DNA Ladder, A.8 and A.7) indicates clear fragment (Lane 2) at expected size (≈2.3 kb)

Part of the assessment included sequence analysis and comparison of the original PERV-A(42) sequence as published under accession number (AJ133817) with sequence data which was obtained from the Institute's sequencing facility.

The published amino acid sequence showed no difference when compared with our sequenced data (Figure 3.3). No differences in the nucleotide sequence were identified. However the last ten N-terminal amino acids were not sequenced. From the total length of Env (660 amino acids), 650 amino acids were successfully sequenced, leading to a 98.48 % identity of the published sequence to provided molecular clone. It can be concluded that both, the published sequence as well as the experimentally determined sequence are identical.

Figure 3.3.: Sequence-comparison between the published PERV-A(42) sequence (*accession №: AJ133817*) and a consensus sequence (consensus depicted by gray bars on top with sequence shown underneath) derived from multiple alignment from multiple sequencing steps to cover the entire Env of the molecular clone. Although amino acids 650-660 were not sequenced, the entire Env showed complete homology. Colors depicted serve to illustrate homology between amino acids.

3.1.2. Evidence that viral particles were produced

To verify if particles were produced by PERV-A(42), wild-type PERV/5° (a PERV-A/C recombinant form [24]) (positive control), supernatant from uninfected cells (negative control), and 293T transfected cells was continuously harvested and used for Western blot analysis. PERV-A(42), produced proteins after transfection of 293T cells with the same molecular weight as Gag (compare PERV-A/C), as shown by using Gag specific antibodies (lane 7, Figure 3.4). Also PERV-A(42) transfected cells show clear signals for Gag specific serum antibodies.

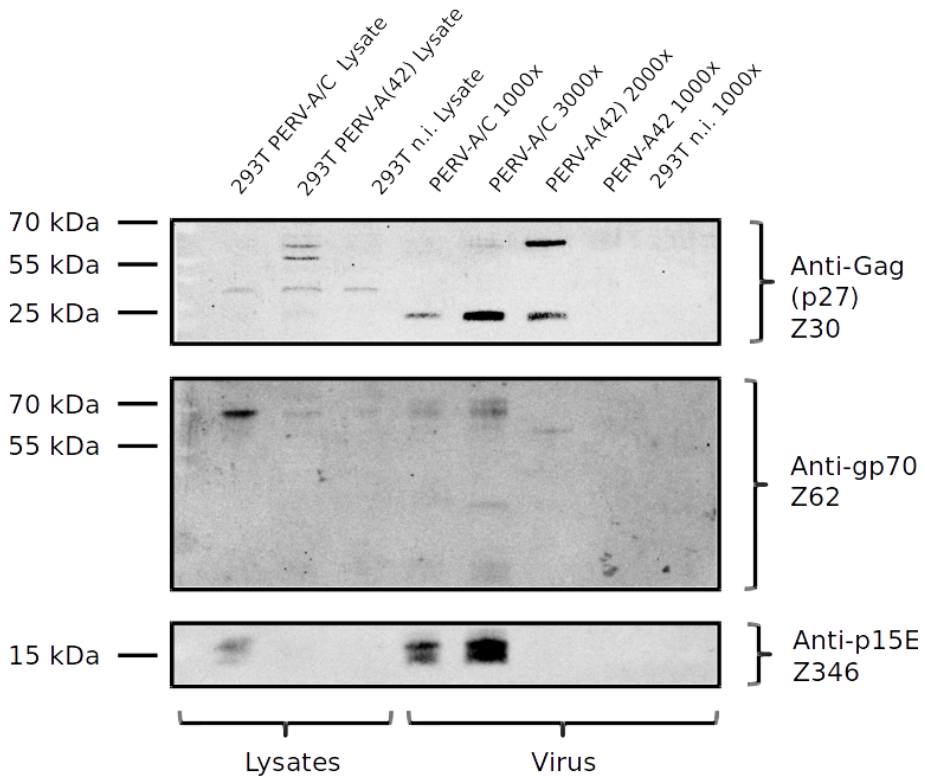


Figure 3.4.: Western blot analysis, using anti-Gag/p27 (goat-serum# 30), anti-gp70 (goat-serum# 62), and anti-p15E (goat-serum# 346). Sample PERV-A(42) 2000× is a pooled sample constituted of supernatant of at least 3 consecutive weeks. Most recently obtained PERV-A(42) was pelleted > 1 month after transfection (Lane 7). No bands can be observed anymore. No bands for gp70 as well as for p15E can be seen, when compared to positive control (PERV-A/C). 293 T n.i. represents cellular supernatant harvested from non infected 293T cell line and serves as a negative control. Denotations of 1000×, etc. refers to factor by which supernatant was concentrated.

Western blot analysis showed Gag specific bands for PERV-A(42) which is in accordance to the literature [11]. Other viral proteins like p15E and gp70 which are essential for infectious particles were however not detected in the virus sample. Gp70 was however present in the cell lysates. Interestingly, also the Gag signal disappeared analogously to how many times cells were split. The viral particles were therefore not able to sustain infection within 293T cells.

3.1.3. Detection of viral proteins by immunofluorescence

To reinforce and confirm the current findings of the Western blot analysis, *in situ* immunofluorescence was used to track Env protein within a freshly transfected 293T cell. Three different samples were analyzed. Firstly, transfection was carried out using PERV-A(42). Additionally, PERV-B has shown in other Western blot analysis (not shown) clear bands when probed with Gag specific antibodies (goat-serum # 30) and serves as a positives control. Negative control was provided by non-infected 293T cells. No signal was detected from PERV-A(42) in immunofluorescent staining. The positive control (PERV-B) clone demonstrated a clear signal, which was not observed in non-infected cells. The results mirror previously obtained data from Western blot analysis in terms of Env expression (Figure 3.5).

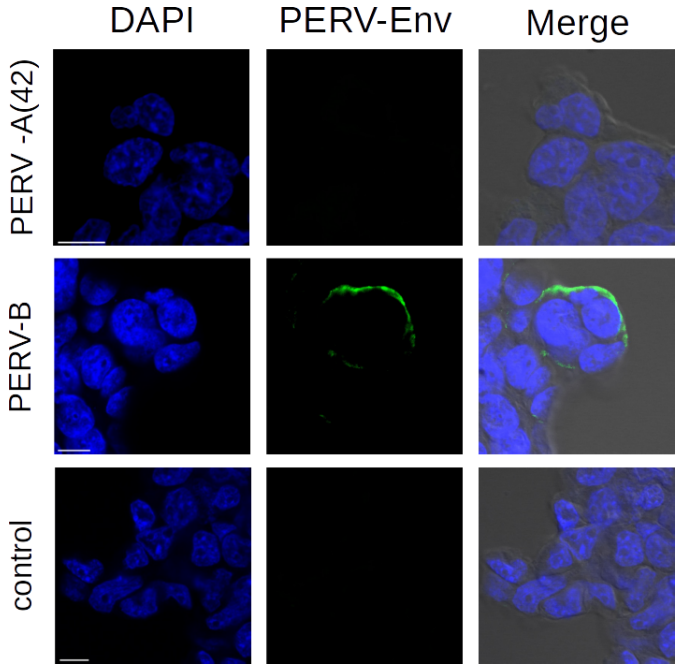


Figure 3.5.: Immunofluorescence analysis of PERV-A and PERV-B Env. Nuclei of the cells have been stained using DAPI. The Env specific antibody is identical with the antibody used for Western blot analysis. No signal can be detected for PERV-A(42). PERV-B showed clear signals and therefore production of Env. The right column shows a merged version of both, DAPI and Env-specific immunofluorescence.

3.1.4. Detection of viral particles by electron microscopy

Furthermore, transmission electron microscopy was performed to evaluate if newly PERV-A(42)-transfected 293T cells produced particles. Confirming the missing of Env by earlier Western blot analyses, no spikes protruding the viral envelope were seen on the virus particle (Figure 3.6).

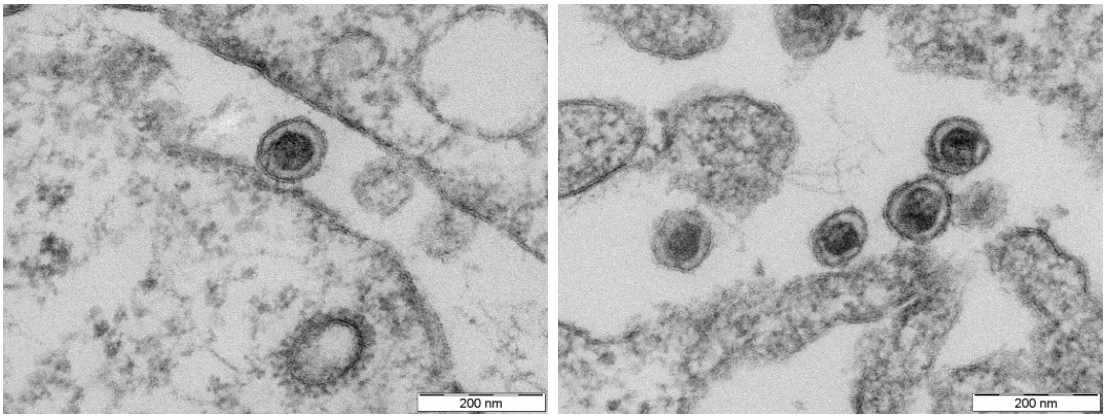


Figure 3.6.: Transmission electron microscopy photograph showing 293T cells with produced vial core-particles. No spikes were seen protruding through the viral envelope.

3.1.5. Determination of the amount of protein

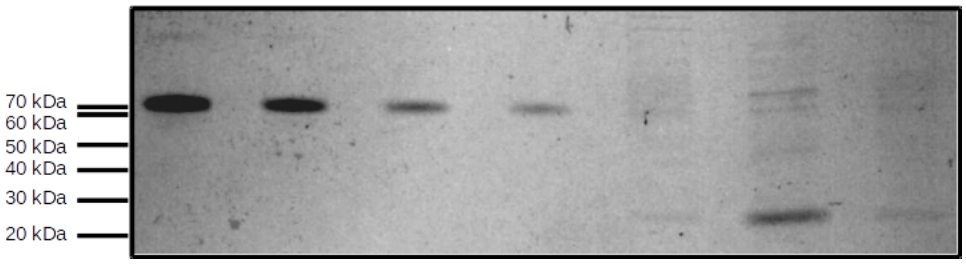
To measure the amount of produced viral proteins, purified virus samples were evaluated via Coomassie blue stain. The amount of viral particles was estimated via the amount of p27Gag found in the virus samples. A line of best fit was determined using a standard BSA dilution series yielded equation 3.1. The amounts of p27Gag was estimated, leading to the results shown in table 3.1. As the average number of Gag required to form a viral particle can be estimated as only 40 % of the 5000 Gag-proteins that are the considered maximum [44]. The amount of viral particles per µL was therefore determined applying equation 3.2. The resulting number of viral particles is shown in table 3.1.

$$f(x) = 2.62 \cdot 10^{-4}x - 1.53 \times 10^{-1} \tag{3.1}$$

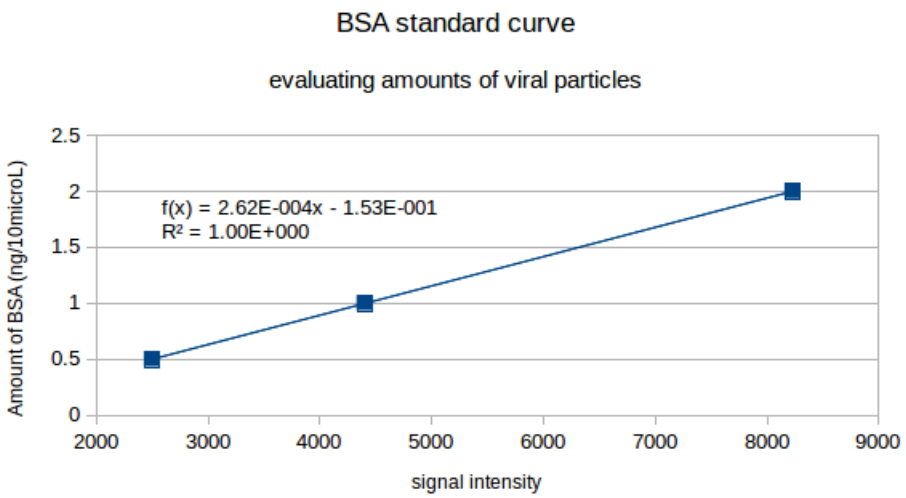
$$\frac{\#\,of\,particles}{\mu L} = \left(\frac{x\,ng}{10\mu L}\right)\left(\frac{1kDa}{1.66 \times 10^{-12}\,ng}\right)\left(\frac{1\,particle}{27\,kDa}\right)\left(\frac{1}{\sim 2000}\right) \tag{3.2}$$

Table 3.1.: Determination of the amount of virus within sample determined on the basis of Coomassie blue staining with a BSA standard. Average number of particles per µL is based on 2000 Gag proteins necessary to form a viral capsid [44].

Lane	Sample	Signal	Corresponding amount in $\frac{ng}{10\mu L}$	$\frac{Number\ of\ particles}{\mu L}$ (on Average)
5	PERV-A/C (1000 ×)	771.355	0.05	5.48×10^4
6	PERV-B (1000 ×)	5105.154	1.18	1.32×10^6
7	PERV-A/C (1000 ×)	793.113	0.05	6.11×10^4



(a)



(b)

Figure 3.7.: **a)** Coomassie blue staining of the BSA standard at 4 ng in lane 1, 2 ng in lane 2, 1 ng in lane 3, and 0.5 ng in lane 4. 10 µL PERV-A/C (1000× concentrated) samples from two different dates were running in lanes 5 and 7. Lane 6 contained 10 µL of PERV-B sample (1000× concentrated). **b)** Determining the amount of viral particles based on BSA plot, carried out at 4 ng, 2 ng, 1 ng, and 0.5 ng per lane. 4 ng reading was excluded because of obvious saturation effects which distorted the signal strength.

Although PERV-A(42) was described in the literature as a fully infectious clone [18], these findings were not confirmed here. The digestion pattern matches the supposed pattern determined by virtual digests of the published sequence, the clone failed to replicate

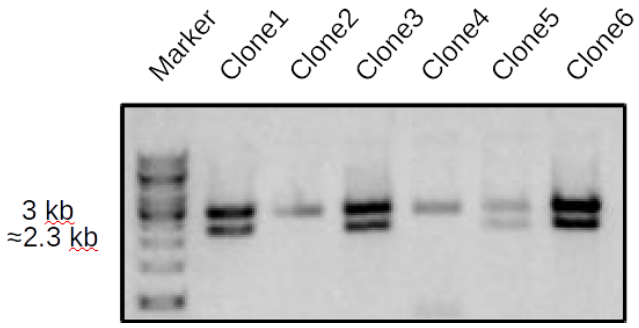
in cell culture, as ascertained by several Western blot analyses (Figure 3.4). Sequencing of *env* did not show changes in the amino acid sequence of the used clone. As however *env* seems fully intact, it can still be utilized for site-directed mutagenesis and the generation of protein expression clones.

3.2. Generation of PERV mutants

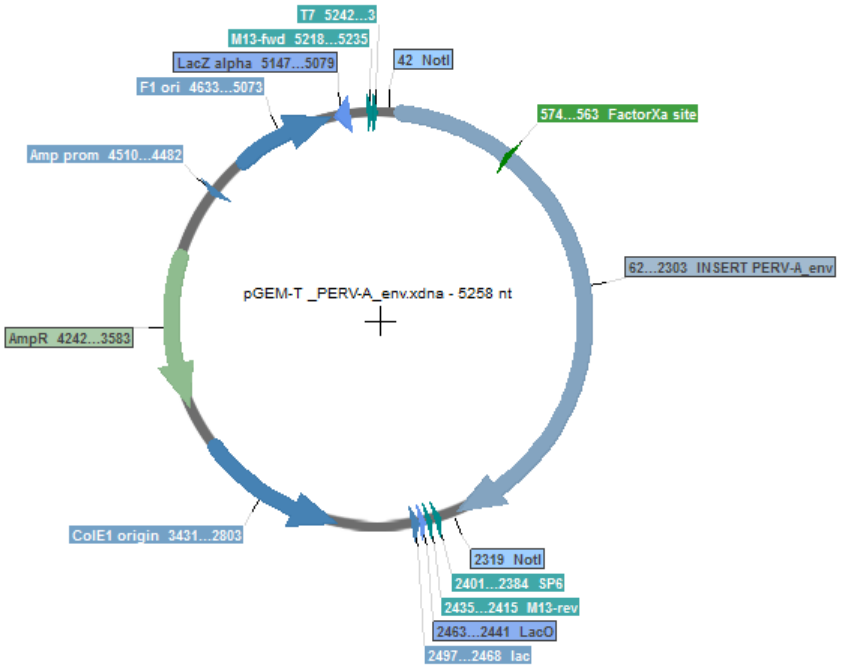
To study the effects of mutations on the *isu*-domain in terms of cytokine release and the production of neutralizing antibodies against PERV, several mutants were created applying a back to back site-directed mutagenesis. Despite its inability to infect cells *in vitro*, PERV-A(42) still serves as a good basis to apply such site-directed mutagenesis, as its *env* gene was verified to be entirely intact.

3.2.1. Cloning of the env gene

The *env* fragment of PERV-A(42) was amplified using a Taq polymerase and cloned into the pGEM®-T Vector system for growth in *E.coli* to obtain larger amounts for site-directed mutagenesis. The Taq amplified *env*-fragment was cloned into the pGEM®-T Vector (A.10), and the resulting construct was cleaved using restriction enzyme NotI after successful transformation and isolation of plasmid DNA (for setup refer to table 2.1). Electrophoretic analysis has confirmed the successful incorporation of the fragment which was used for site-directed mutagenesis (Figure 3.8). The size of the amplified fragment as well as the the fragment detected via restriction enzyme digest match the size of the *in silico* cloning with its annotated NotI restriction digest site, which predicts a fragment size of 2277 bp.



(a)



(b)

Figure 3.8.: **a)** Restriction digest and elecrophoretic analysis of Digest- pGEM®-T Vector + env shows two fragments at 3000 bp (vector backbone) as well as at 2300 bp (insert) (Compare with construct in figure 3.8. Clones 1 (lane 2), 4 (lane 4), 5 (lane 6), and 7 (lane 8) show the desired fragment and were sequenced for further verification. **b)** Construct of pGEM®-T Vector including the previously amplified *env* fragment (labelled: 62...2303 INSERT PERV-A_env) showing features of the backbone vector as well as NotI restriction sites, which were used for verification-digests

3.2.2. Site-directed mutagenesis of the env gene

In order to create mutant versions of *env* that contain mutations known to abrogate cytokine release from PBMCs [45], site-directed mutagenesis was performed using back to back PCR, with primers containing the desired mutations (Figure 3.9). Performing PCR reaction yielded linear fragments containing the desired mutations. These DNA fragments were isolated from the 1 % agarose gel using DNA extraction kit (Figure 3.9). To improve the efficacy of the following transformation, a KpnI- digest was performed prior to gel electrophoresis to reduce the number of unmutated template.

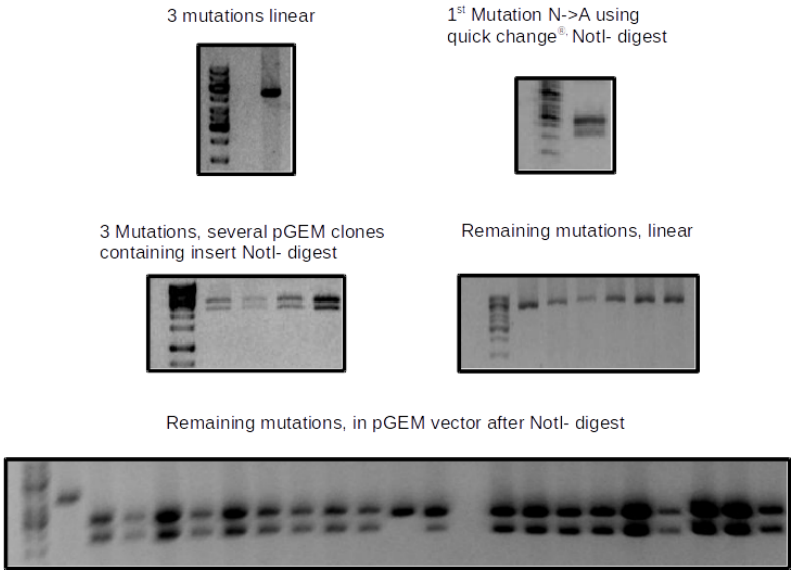
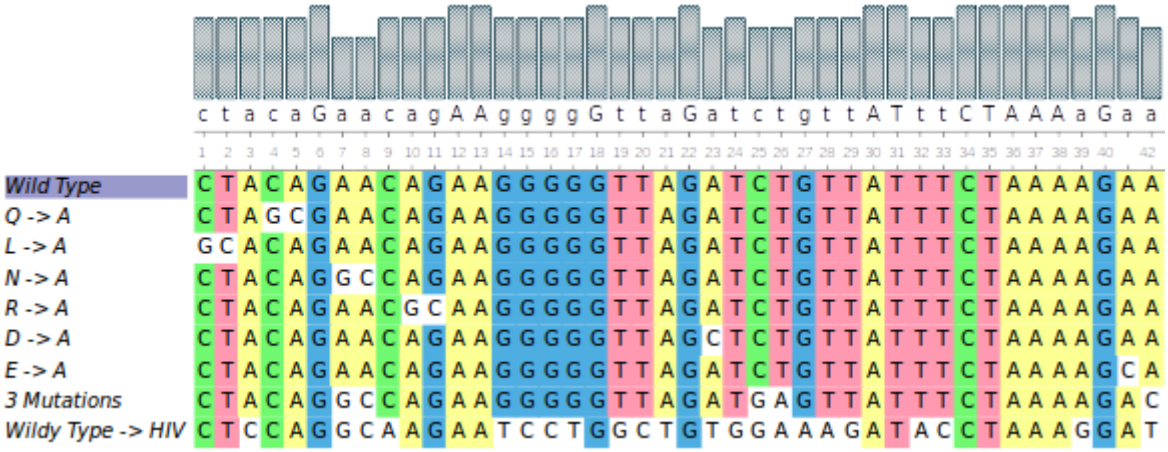
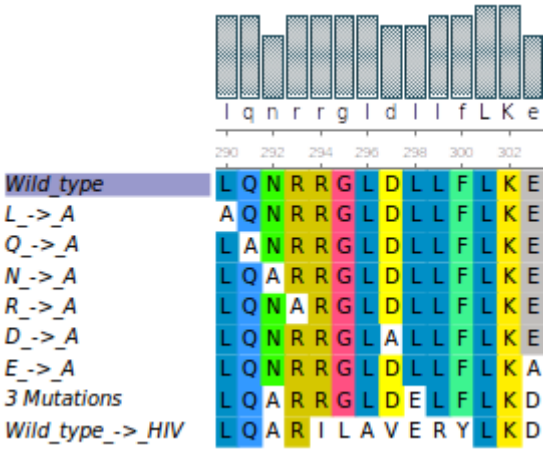


Figure 3.9.: Gel electrophoresis showing back to back site-directed mutagensis PCR with their linear products for 3 mutations induced (Figure 3.10) as well as for the remaining mutations (Figures 2.1 and 3.10). NotI cuts at multiple cloning site in pGEM vector, effectively separating backbone and insert. Initial N$\Longrightarrow$A mutations was performed using the quick change site-directed mutagenesis kit (A.5) and shows the same band pattern after NotI-digest.

The ligated products were transformed into Z-cells and grown overnight on LB-Amp agar plates at 37 °C. Several colonies were picked the next day for inoculation of 4 mL LB+Amp broth. Due to no alteration in insert size or alteration of the insert itself, no colony PCR was performed on the arising colonies. Plasmid DNA was recovered using Invisorb® Spin Plasmid Mini Two (A.5) and quantified via Nanodrop (A.6). The resulting isolated DNA was sequenced and analyzed for induced mutations, as well as for randomly induced single base changes, due to amplification. This was accomplished using the UGENE sequence analysis tool, published under the Gnu Public License (GPL). Sequence analysis showed that all desired mutations were induced(Figure 3.10).



(a)



(b)

Figure 3.10.: Mutations induced by back to back site-directed mutagenesis. Sequence analysis was performed using UGENE. **a)** Reference amino acid sequence is shown as first entry. Colored amino acids show homology to reference sequence. Amino acid changes are indicated by **initial amino acid** $\Longrightarrow$ **amino acid resulting from mutation**. Three amino acids were changed in the case of the 3 mutations. Colors depicted serve to illustrate homology between nucleotides. Non-homologous nucleotides are depicted in white compared to the reference sequence, which is shaded gray. A consensus sequence is depicted on the top and nucleotide homology is shown by gray bars. **b)** The translated sequence of the base nucleotide sequence shown above and the resulting mutations it contains on the amino acid level.

Several PERV-A(42)-*env* mutants were produced. Those mutants may serve as the basis for further experiments and will ultimately be used in the production of higher titer antibodies for the development of an improved PERV vaccine.

3.3. Influence of PERV on cytokine release of human PBMCs

In order to investigate the immunosuppressive properties of PERVs on human PBMCs an IL-10 assay was performed. As IL-10 is a common immunosuppressive and anti-inflammatory cytokine, which is usually produced by $CD4^+$ cells (T_H2 cells) as well as macrophages and dendritic cells [46, 47]. IL-10 along with other cytokines, such as IL-4, and IL-13 are known to downregulate microbicidal activities of macrophages, as well as to reduce costimulation and downregulate the expression of major histocompatibility complex (MHC) class II [47]. The exposure to human PBMCs is therefore a good tool to estimate the immunosuppressive effect PERVs can have on the organism.

3.3.1. Characterization of virus sample

Since endotoxins can trigger TLR4 and result in the release of IL-10, prepared virus samples needed to be tested for endotoxin contamination to avoid false positives. Samples were tested for endotoxin contamination via THP-Xblue cells (Engineered Human Reporter Monocytes) which carry a NF-κ-B inducible SEAP system to analyze activation of toll like receptors. Previously virus samples were prepared via ultracentrifugation and 20 % sucrose-filtration. The resulting pellet was analyzed by via SDS-PAGE as well as Western blot analysis (Figure 3.11). Additionally the amount of viral particles was determined using equation 3.2. The resulting number of particles per µL is mentioned in table 3.1.

Samples tested and used for the PBMC-based IL-10 assay did not show significant contamination with endotoxin after adjusting the standard (Figure 3.12). The sample was measured at three different time points. Similar results were obtained at all three different time points (1h, 2h, and ≈16h post addition of QUANTI blue).

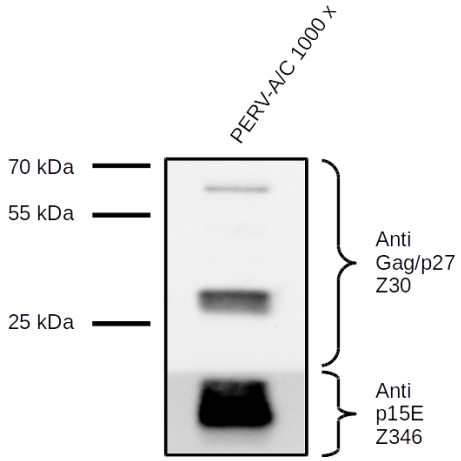


Figure 3.11.: PERV-A/C used for endotoxin assay with THP-Xblue cells, as well as for IL-10 release with PBMCs. Top portion show signal from anti Gag/p27 goat-serum (# 30) antibodies. Bottom portion shows signal from anti p15E goat-serum (# 346)antibodies.

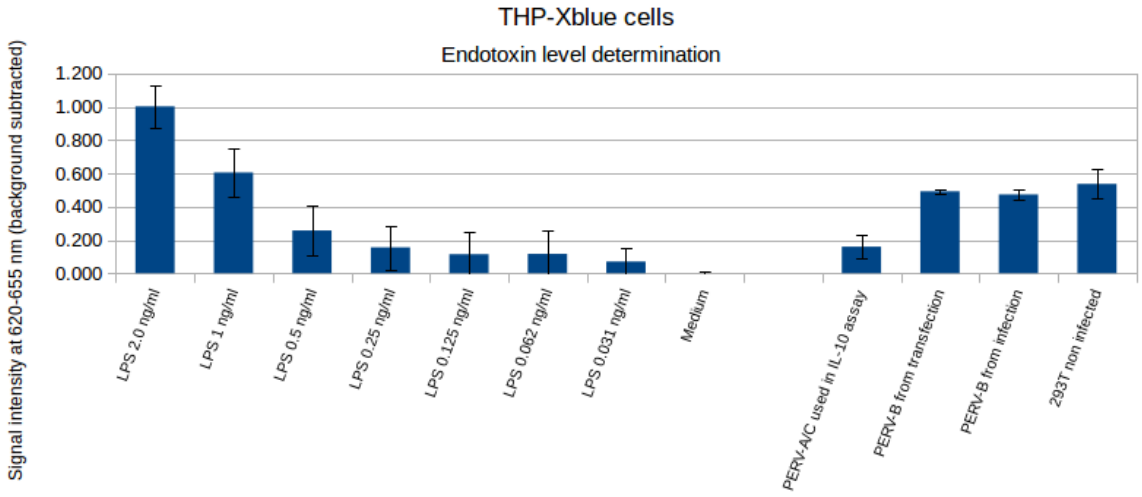


Figure 3.12.: Endotoxin assay employing THP-Xblue cells. The endotoxin standard is shown on the left side. Standard was diluted in a 2-fold serial dilution. PERV samples showed very low amounts of endotoxin. The PERV-A/C pool was utilized for IL-10 assay. Error bars show ±1 standard deviation [48].

3.3.2. Influence of PERV on IL-10 release

Effects of PERV-A/C on human PBMCs were evaluated using human PBMCs. The anti-inflammatory cytokine interleukin-10 expression was measured by incubating PBMCs with virus samples as described. To inhibit the activity of putative endotoxin induced IL-10 expression, TAK-242, a TLR4 inhibitor was added (5 µM).

A very strong activation of IL-10 expression for samples not treated with TAK-242 inhibitor (blue) was observed. With decreasing amount of virus used, the effect decreased

accordingly. TAK-242-inhibited samples showed IL-10 release for the highest concentration of virus-particles used. Here, with decreasing number of viral particles no more effect on PBMCs was observed in terms of detectable IL-10 production (Figure 3.13). The

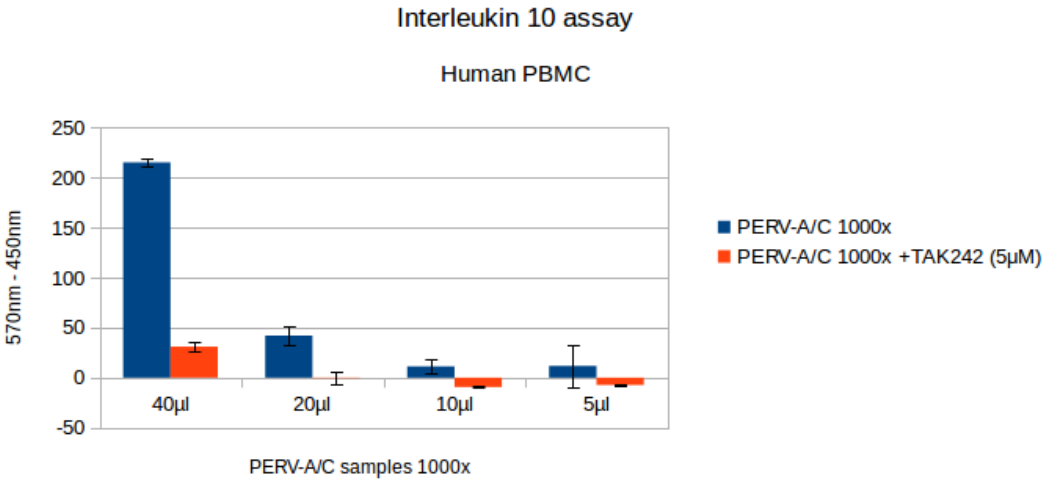


Figure 3.13.: PBMCs after addition of PERV-A/C. The amount of virus was estimated (Table 3.1). Strong decrease in IL-10 release was observed for TAK-inhibited samples.

difference in IL-10 release between the absence and presence of TAK-242 still needs to be addressed in other experiments. What triggers the IL-10 release still observed in the TAK-inhibited sample still also requires continued testing and further investigation. Furthermore, the effect of TAK-242 as well as the time point at which it was added to the samples could play a role.

3.4. Expression of wild-type and mutated Env protein

To circumvent the problem of having a non infectious molecular clone of PERV-A, a second strategy was to measure Env protein effects on human PBMCs. For this purpose amplified truncated fragments of *env* containing the desired mutations were generated. To improve upon expression efficiency, an additional Kozak sequence was added via primer modification to the 5' end of the amplicon produced for cloning. The necessity to truncate the protein arose from it's transmembrane domain. Transmembrane domains are hydrophobic regions within a protein, which hinder the secretion of the protein. Primers used were designed to truncate the protein before it's transmembrane domain (Figure 2.3).

3.4.1. Production of truncated env amplicon

To produces truncated versions of *env*, additional primer sets were used to amplify the previously produced mutated *env* of PERV-A(42) to produce a truncated Env. Two sets of forward primers were used to produce a truncated *env* with a Kozak sequence and one was used to produce a truncated Env without a Kozak sequence. The resulting amplicons are indistinguishable in size on a 1 % agarose gel (Figure 3.14).

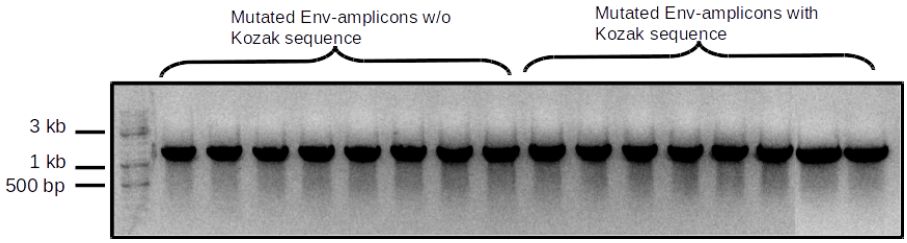


Figure 3.14.: 1 % agarose gel analysis of truncated mutated *env*. Left side shows amplicons without Kozak sequence. Right side shows amplicons with Kozak sequence. Differences in sizes cannot be distinguished on agarose gel.

Additionally, we prepared a positive control of unmutated truncated Env with and without Kozak sequence. Primers chosen are identical to those used for the amplification of mutated Env-fragments (Figure 3.15).

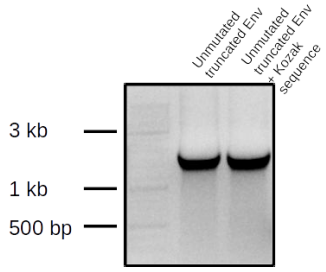


Figure 3.15.: 1 % agarose gel of truncated Env. Left lane shows amplicons without Kozak sequence. Right lane shows amplicons with Kozak sequence. Different amplicon sizes cannot be distinguished on agarose gel.

3.4.2. Cloning of amplicons into expression vector

To obtain a protein expression clone, fragments were cloned into pTarget™ -Vector System (2.4). After transformation into *E.coli* (A.3) and overnight incubation, resulting colonies were tested for successful insert via colony PCR (Figure 3.16). Positive clones were used

for inoculation for plasmid isolation. To verify if the desired insertion was incorporated in the right direction, additional probing via restriction enzyme digest was imperative.

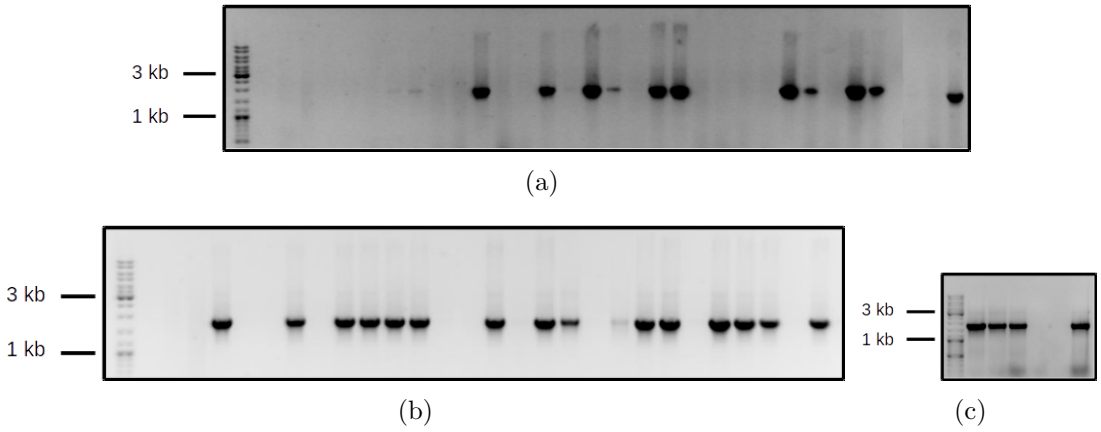


Figure 3.16.: **a)** Colony PCR of fragments containing Kozak sequence. **b)** and **c)** Colony-PCR of fragments not containing Kozak sequence. Primers selected are identical to primers selected for generation of truncated *env* fragment.

3.4.3. Verification of directional insertion of amplicons

To verify the proper insertion of the amplicon into the pTarget vector system, a restriction enzyme digest was performed. Depending on the direction in which the fragment is inserted, it will result in different size fragments from digestion, as the position of the restriction enzyme site will change with respect to the backbone vector. Electrophoretic analysis on a 1 % agarose gel has revealed several clones that show inserts in the right orientation (Figure 3.17).

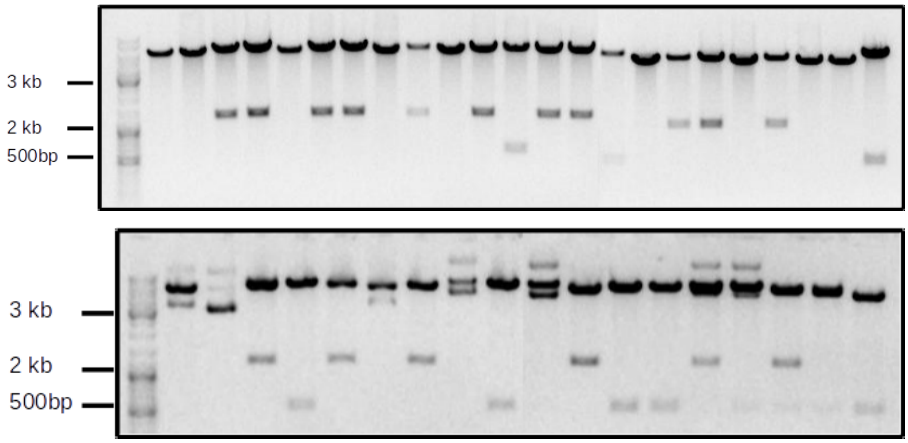


Figure 3.17.: Electrophoretic analysis of KpnI digest of pTarget-env clones. Direction-independent cloning occurred as a result of TA cloning. Small band ($\approx$ 500 bp) and large band ($\approx$ 6 kb) represent successful orientation of insert.

Mutated as well as unmutated inserts have a KpnI restriction site within their genome located towards their 5' end. The pTarget backbone vector has the same restriction site at it's 3' end of its cloning site. When insertion occurred in the right direction, one small fragment (≈ 500 bp) and one large fragment (≈6 kb) result from a KpnI digest. Several clones carry the insert in the right direction and continued on by verifying their sequences in two positions of their genome.

3.4.4. Characterization of pTarget-env clones

In order to verify if the amplified fragments were incorporated into the expression vector without any additional errors, the immunosuppressive domain as well as the translational start site of each clone was sequenced. Sequence analysis confirmed the successful generation of six protein-expression clones (Figure 3.18).

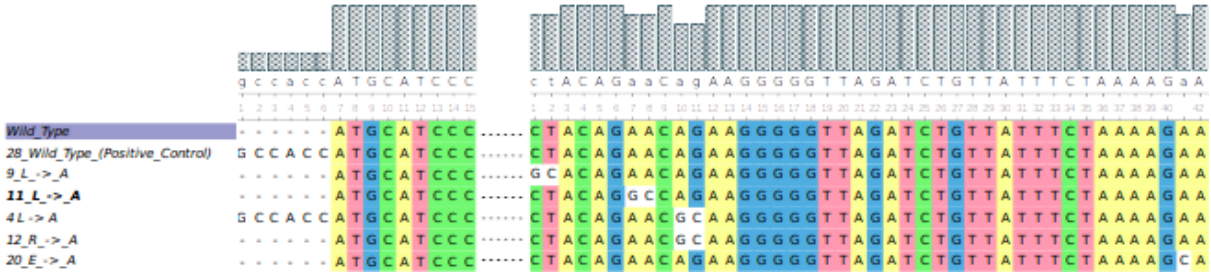


Figure 3.18.: Sequence alignment showing start codon of various clones with amino acid change alluded to in their names. Separated by dashed line mutations base changes are illustrated by non-colored boxes, as compared to reference sequence (wild-type).

Several expression clones were created. Among those clones is also a control construct (28-wild-type(Positive-Control)), which contains unmutated and truncated PERV-A(42)-*env*. Out of the six successfully created expression clones, two carry a Kozak sequence to enhance translation. Amplicons were created with primers not including the transmembrane domain, preventing attachment in the cell membrane and therefore facilitating cell exit. The final assortment of expression clones can be seen in figure 3.19.

At this point it was not verified if and to what amounts these clones are able to produce protein. Hence additional transfections with SDS-PAGE gel electrophoresis will have to be conducted in the future. Also these constructs may prove to offer new insights in terms of cytokine release from human PBMCs.

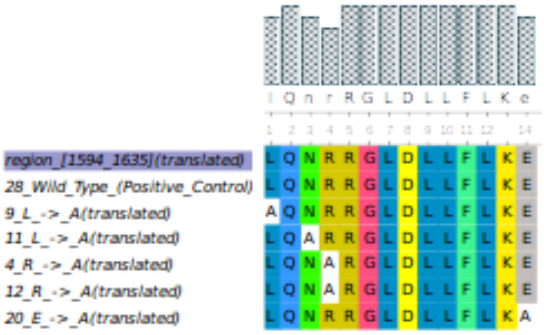


Figure 3.19.: Isu domain of expression clones created with pTarget Vector system.

3.5. Generation of replication-competent hybrid PERV-A/B

To develop an alternative route and to counteract the problem of a non-replicative PERV-A(42), transplanting PERV-A(42)-*env* into replication-incapable PERV-B(33) clone was decided. PERV-B(33) is know to have a defective start codon in *env*, which is the reason for its inability to produce infectious particles [11]. However, by replacing PERV-B(33) *env*, with the full length PERV-A(42) *env* could restore infectious properties. To still be able to use the prepared mutations, the *env* transplant was performed using Gibson assembly. Several fragments were produced using PCR (Figure 3.20). These fragments were used in Gibson assembly, as described in Figure 2.5.

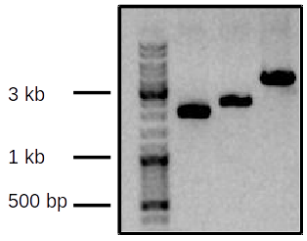


Figure 3.20.: Fragments produced using PCR. Lane 2 shows amplified *env* from PERV-A(42). Lane 3 and 4 show the amplified parts of the PERV-B(33) backbone. All three fragments were used in the Gibson assembly.

Efforts so far were unsuccessful to re-assemble PERV-B(33) backbone while incorporating PERV-A(42)-*env*.

4. Discussion and Outlook

Xenotransplantation is aiming on closing an ever wider-growing gap between the growing demand of donor organs in an aging population and the decreasing number of donors. Porcine organs seem to be a good candidate due to their relative similar size as well as similar physiological conditions in pigs such as blood pressure. Other issues are well known and documented, such as hyperacute rejection. Strategies to overcome these problems are known and applied.

It is therefore becoming more and more important to understand the impact of endogenous factors on the health of a potential organ recipient. Like all retroviruses, porcine endogenous retroviruses could pose a threat to public health. Permanent exposure to released viral particles could lead to immunosuppression. The development of viruses mutated in the *isu*-domain and an improved vaccine based on these mutants was therefore the focus of this study. It has been shown that HIV, HERV-K (human endogenous retrovirus-K) and other retroviruses have immunosuppressive properties that can be abrogated with single amino acid substitutions in several sites in their immunosuppressive domains [18, 33–36, 45].

4.1. PERV-A(42)

The declared goal of this project was to perform an immunization study with mutated forms of the received PERV. For this, the PERV-A(42) clone from Prof. Dr. Ralf Tönjes from the *Paul Ehrlich Institute*, Langen, Germany was used. This clone was supposed to be infectious and therefore able to sustain itself in 293T cells *in vitro*. It was shown in the experiments that this molecular clone was not replication-competent. Figure 3.4 illustrates a previously established positive control consisting of PERV-A/C used in this research group. This positive control is maintained as infectious particles within a 293T cell line and is not available as a molecular clone. It has been generated by co-infectiion

of porcine cells with PERV-A and PERV-C *in vivo*, to create a recombinant PERV-A/C strain [24, 25]. This strain was shown to be highly replication-competent in human 293T cells *in vitro*. This is possible despite the non-human-cell-tropic nature of PERV-C. The interaction partner of PERV-A/C is the human PERV-A receptor (HuPAR) [49].

As described [11], Gag protein could be measured for PERV-A(42)-transfected cells *in vitro* several days after transfection was performed. Those findings was confirmed as part of this study. The effect is however fleeting and vanishes overtime, as cells were split and the viral DNA became more dilute. Finally, no more Gag was detected anymore in the harvested supernatant (Figure 3.4). There was reason to believe that the PERV-A(42) therefore has a defect in it's *env* gene. The possibility of frameshift mutations, random deletions, and nonsense mutations had to be investigated. Possible causes for such events could be subcloning into a sequencing vector or re-transformation and random recombination due to bacterial recombinases. Also, preceeding PCR amplifications, may have caused point mutations leading to a non-infectious clone. As sequencing data revealed, the clone used for transfection of 293T cells shows a sequence identical to its published reference (accession №: AJ133817). Since Western blot analysis did reveal that Gag protein is produced, so called Gag-particles were formed which do not posses the ability to infect 293T cells *in vitro*. To elucidate if any virus particles were formed after transfection, transmission electron microscopy was employed. Photographs show a virus-particle without visible Env protrusions sticking out from the viral envelope.

Another possibility was that faulty cleavage of Env was responsible for the absence of visible spikes of the virus. Such cleavage errors may result in a malformed Env that could render the virus unable to infect new cells and would therefore stop it's progression [11, 50]. While very small amounts of Env were detected by Western blot analysis in cell lysates produced from freshly transfected 293T cells, no Env was detected, as verified by immunofluorescent imaging (Figure 3.5) supporting the findings from the electron microscopy. Therefore other explanations are needed to elucidate how the translation of the *env* region has come to a halt. Yet this scenario can still not be completely ruled out as Env may simply be inaccessible during processing in the Golgi apparatus, and degraded when not cleaved properly. Nevertheless, it is feasible to entertain other ideas. One possible explanation would be an error within the splicing donor or splicing acceptor site. The resulting transcript would therefore only contain a polyprotein of Gag-Pol-Env

[50] that was not shown by Western blot analysis. The polyprotein would not participate in forming infectious particles and is possibly degraded after synthesis. This could result in a particle consisting only of core proteins as well as a non-detectable poly-protein (lysate and supernatant).

4.2. Endotoxin and IL-10 assays

When IL-10 assays were performed, effects on the release of IL-10 in human PBMCs were determined (Figure 3.13). As IL-10 release can be triggered by several stimuli [40], other factors that might activate such pathways need to be investigated further before any conclusions are made. The samples were tested for endotoxin, which is known to be able to activate TLR4 via CD14 binding. At the end of the TLR4 signaling-cascade IL-10 production was induced. The tests showed that samples used for the IL-10 assay were very low in endotoxin. As can be seen in figure 3.12, samples from 1 $\frac{ng}{mL}$ to 0.062 $\frac{ng}{mL}$ show no significant change in endotoxin levels ($significant\,change\ =\ p\ <\ 0.05$) as their difference did not exceed two standard deviations ($2\sigma = 95.45\ \% \approx 0.95$). This is in accordance with findings also published by Rylander, 2002 who states that normal endotoxin concentration in the air in biotechnology industry ranges from 0.1 $\frac{ng}{m^3}$ to 12.8 $\frac{ng}{m^3}$ [48].

Those low endotoxin levels found in the samples were not disregarded (Figure 3.12). To circumvent any endotoxin dependent activation of TLR4, and therefore false positive IL-10 release readings, TAK-242-inhibitor was added to the samples to block the LPS-dependent activation of TLR4 [42](final concentration 5 µM). 2.6×10^6 particles (40 µL of 1000× virus sample) were used for as the highest concentration in the endotoxin assay. Despite the addition of TAK-242, signal could still be confirmed although significantly lower than without the addition of the inhibitor (Figure 3.13). Previously a dose-dependent activation of PBMCs and a subsequent production of IL-10 cytokine was demonstrated [45]. HIV-1, which also contains an isu peptide that shows great homology with other isu peptides, found in retroviruses [35], was used to illicit an IL-10 response by PBMCs in previously performed experiments [45]. In these previous investigations, a strong release of IL-10 was observed when PBMCs were incubated with the isu-peptide [35, 45]. This is however not in accordance with the findings of this study with PERV. While a small variance was found between negative control (standard growth medium)

and virus sample, it does not compare to the findings of the immunosuppressive effect of HIV-1, or HERV-K published earlier [35, 45]. Also, experiments that were conducted simultaneously to the PERV-study, showed that newly prepared wild-type pNL as well as mutated pNL did not induce any release of IL-10 by PBMCs [Data not published].

As TAK-242-inhibitor completely blocks TLR4 signal transduction, the remaining IL-10 production observed in the sample is object to discussion. The so called *isu* domain which has been shown previously to have immunosuppressvie effects [35, 45, 51] could have induced IL-10 production in PBMCs. However, no receptor for the isu-protein was identified to this date.

There has been little evidence that indicates that PERV-A/C was able to induce IL-10 release in human PBMCs. Little release of IL-10 could still be observed after blocking TLR4 using TAK-242 inhibitor. To obtain more reliable results, the remaining levels of endotoxin in the samples need to be lowered. Ideally, no additional TAK-242 inhibitor should be added. Although TAK-242 was used at 5 μM (maximum working concentration) it was not incubated with PBMCs prior to the addition of the samples as suggested by the available literature [52]. Given the data at hand, it can be deduced that any remaining IL-10 release of PBMCs will vanish with longer exposure to the inhibitor.

4.3. Generation of a PERV-A/B hybrid and protein expression

Efforts were undertaken to create a PERV-A/B hybrid by excising the *env* region of PERV-A(42) and cloning it into a PERV-B(33) backbone. This backbone is itself not replication competent as it lacks a functional *env* [11]. It was however demonstrated above that PERV-A(42)-*env* is error free, but not expressed when the viral clone was used for transfection, as shown by *in situ* immunofluorescence (Figure 3.5). However, generating a hybrid clone from two different non-working clones offers several challenges. Firstly, the length of the genome itself represents problems. Producing error free amplicons requires high fidelity polymerases with proof-reading ability. However, complicated templates such as long terminal repeats with GC rich regions that are hard to verify pose an additional burden on the process [53]. First attempts to generate whole-backbone-amplicons for the insertion of PERV-A(42)-*env* were therefore unsuccessful. An alternative strategy was

to amplify the PERV-B(33) backbone in two steps leaving four homologous regions to be ligated during Gibson assembly. Amplification of fragments was successful and clear bands were observed as seen in figure 3.20. However efforts have so far been unsuccessful to assemble the various fragments after gel extraction via Gibson assembly. Possible reasons could be in the degradation of DNA over time, as these fragments were not used immediately. Another reason may be a too short extension period for the long (6 kb) amplicon. This may result in variable-length fragments that could be observed as slightly thicker bands on a 1 % agarose gel (Figure 3.20). At last, genetic material recovered may have been contaminated with salts or other organic compounds due to the elution process from the agarose gel. To circumvent this possibility in the future and to improve cloning efforts, a DpnI digest could be used to digest methylated template DNA only leaving intact amplicons. This is possible, as no extra bands were obtained. The resulting solution could then be cleaned via PCR-clean up kit (Invisorb® DNA CleanUp A.5) to minimize the effect of residual ethanol and salts for downstream applications. It seems that a viable system was found to bypass the problem, with all tools in place to create a replication competent clone, provided the constructs given allow for proper assembly of the virus. Similarly the production of protein expression vectors, wild type and mutated, has revealed additional problems, such as long length amplicons and good efficiency in terms of protein secretion. The employment of a well functioning site-directed mutagenesis technique as well as the proper incorporation of the mutated fragments into the expression vector is crucial for success. Additionally, Env contains a transmembrane domain, which anchors the protein within the membrane of a cell, making it impossible to isolate sufficient amounts of Env from the supernatant of cells in cell culture(Figure 2.3). In order to prevent this from happening the *env* gene needed to be truncated in a way that it's transmembrane domain was cleaved out and it could be readily secreted from the cell. Otherwise only harvesting of cell-lysate, which contains remnants of cellular debris, would have been possible. Such debris (e.g. fibrinogen and heparan sulfate) may be a reason for false readings when conducting IL-10 assays as it can also trigger TLR4 [54, 55].

Several clones containing mutations described in section 3.4.1 were produced. However, not all of the created mutants were incorporated into the expression vector. Directionality of the inserts was reversed in the majority of clones rendering them useless for protein ex-

pression. For future applications, this process could be improved by employing directional cloning techniques such as Gibson assembly as previously described (Section 2.18).

Until now, these clones have not been tested for secretion *in vitro*. Only small amounts of the clones were produced to verify the incorporated changes as well as the translational start-site modifications. It was part of this effort to keep the protein as much in a native form as possible. Hence, attaching affinity-tags was not a viable option, which could have yielded higher results when purifying protein over a metal-matrix-column. Lower amounts are therefore expected to be present after harvested from supernatant and would have to be quantified employing the method described earlier (Section 3.1.5).

4.4. Outlook

Future work should be directed to the generation of replication-competent wild-type and mutated PERV as basis for the development of a better vaccine. This also includes the completion of the PERV-A/B hybrid as well as full characterization of the protein expression clones. Further assays to observe IL-10 release can be conducted to verify the immunosuppressive properties of wild-type PERV-A/C as well as wild-type PERV-A/B. Furthermore, more stringent care needs to be taken to reduce endotoxin contamination. The use of endotoxin-free water, as well as the use of chemicals designated only for IL-10 assays and the production of virus samples should be imperative and could reduce the risk of contamination. Also, the use of air-conditioners which could potentially act as a endotoxin-distribution systems should be considered. Devices with air filters should be maintained regularly and used air-filters should be replaced on a regular bases [48].

An entirely different route could be taken by establishing a molecular clone of the recombinant PERV-A/C virus, which is currently only available in the form of viral particles. The establishment of such a clone would guarantee a more reliable standard as it can more easily be replicated by *de novo* transfection. This would ensure the identity of the clone, as the virus may change over time due to accumulating mutations. Also, efforts could be undertaken to isolate PERV-A/C-*env* via reverse transcription or via amplification of proviral DNA. It is difficult to determine which technique would offer better results in terms of virus infectivity. Reverse transcription of viral RNA would ensure that only genetic material from viruses that actually form infectious particles, was used. However,

it cannot be guaranteed that even those viral particles contain the same genetic material that codes for it's proteins. This is due to the fact that multiple copies of proviral DNA from different *in vitro*-infections are present within the host-cell's genome. Other retroviruses like HIV-1 for instance incorporate an average of 50 copies of proviral DNA into the host-cell's genome ($CD4^+$ Cell) [56]. This also explains why, isolating proviral DNA is not neccessarily going to yield infectious particles. As a comparison, the fraction of infectious particles in HIV ranges from 50 % to as little as 1 % [57]. Similar fractions can probably be expected for PERVs. This would imply that trying to develope a full clone, as well as trying to isolate a functioning *env* would be unsuccessful in most cases.

Lastly wild-type and mutated p15E from both, PERV-A/B hybrid and PERV-A/C will be expressed, purified and tested in immunosppressive properties as well as for immunization experiments. With both PERVs available as a molecular clone it can be accurately determined that the virus is in an unmutated form, as otherwise mutations may accumulate over time in cell culture, due to ongoing replication. Deviations on the effect of antibody titer as well as IL-10 production from PBMCs due to induced mutations are therefore characterized on a more reliable basis.

4.5. Conclusion

To establish a solid basis for future trials and the successful development of an improved vaccine against PERV further testing and the establishment of a replication-competent molecular clone are of importance. Additionally, to reliably demonstrate the immunosuppressive effect of the isu peptide, the produced wild-type and mutated proteins, have to be characterized and tested in IL-10 assays. Those mutants will serve as the basis for immunizations. The produced antibody titers will be quantified and characterized.

A. Appendix

A.1. Antibodies

Name	Additional information
Human IL-10 ELISA Set	Vendor: BD OptEIA™ Capture antibody, detection antibody, streptavidin-HRP-standards, Standard Range 7.8 - 500 $\frac{pg}{mL}$
Rabbit anti goat HRP (detection Ab)	Vendor: Dako, Cat. №: 0449
Rabbit anti goat 488 nm (IF)	Vendro: Abcam, Cat. №: ab96931

In-house serum Antibodies	
Name	Additional information
Anti Gag	In- house produced antibody from Goat (Ziege)30
Anti Env	In- house produced antibody from Goat (Ziege)62
Anti p15E	In- house produced antibody from Goat (Ziege)346

A.2. Buffers and Solutions

Name	Content
Annode buffer	Tris (0.2M), final pH (with HCl): 8.9
Antibodi dilution buffer (IF)	5 % BSA (bovine serum albumin) in PBS
Blocking Buffer (IF)	5 % BSA grade V, 2 % chicken albumin, PBS
Cathode buffer	Tris (0.1M), Tricine (0.1M), 0.1 % SDS, final pH: 8.25
Coomassie R250	45 % Methanol (reagent grade), 10 % Glacial acetic acid 45 % Water, 3 $\frac{g}{L}$ Coomassie Brilliant Blue R250
Destaining solution	50 % H_2O, 40 % methanol, 10 % acetic acid
IL-10 Substrate Solution	Tetramethylbenzidine and Hydrogen Peroxide Alternatively: BD Pharmingen™ TMB substrate reagent set
Mountin Medium	90 % glycerol, 10 % PBS (with 1 % p-phenylenediamine) Cat.№: 555214
NP40- cell lysis buffer (2 ×)	100 mM Tris-HCl, pH 8.0 , NaCl : 300 mM, NP-40 : 2 %, EDTA : 10 mM
IL-10 Assay Diluent	PBS + 10 % Fetal bovine serum (pH 7)
IL-10 Coating Buffer	0.1 M Sodium Carbonate, pH: 9.5 7.13g $NaHCO_3$, 1.59g Na_2CO_3, q.s. to 1 L, pH to 9.5 with 10 N NaOH
IL-10 Stop Solution	1 M H_3PO_4 or 2 N H_2SO_4
SDS-PAGE gel buffer	Tris (3M), 0.3 % SDS, final pH (with HCl): 8.45
Transfer buffer	Tris (48mM), Glycine (39mM), Methanol 20 %, SDS (0.03 %)
ELISA wash buffer	PBS + 0.05 % tween-20
Wash Buffer (IF)	0.1 % Tween®-20 in PBS

A.3. Cells used

Component	Additional information
JM109 (*E.coli*)	Vendor: Zymo Research, Catalog №T3001 & T3002
HEK 293T	Human Embryonic Kidney Cells Contains SV40 Large T-antigen
THP1-XBlue™ Cells	Vendor: Invivogen, NF-κB/AP-1-Reporter Monocytes Cat.№: THPx-sp

A.4. Enzymes

Component	Additional information
Fast Digest 10× Green Buffer	Supplied with Fast digest enzymes, Vendor: Thermo Scientific
Fast Digest HindIII	Vendor: Thermo Scientific Catalogue №FD0504 & FD0505
Fast Digest PstI	Vendor: Thermo Scientific Catalogue №FD2224
Fast Digest EcoRI	Vendor: Thermo Scientific Catalogue №FD0274
Fast Digest KpnI	Vendor: Thermo Scientific Catalogue №FD0524
Fast Digest NotI	Vendor: Thermo Scientific Catalogue №FD0594
Fast Digest StuI	Vendor: Thermo Scientific Catalogue №FD0424
GoTaq® Green Master Mix	Vendor: Promega, Catalogue №M712
Phusion® Hot Start Flex DNA Polymerase	Vendor: New England Biolabs Catalogue №M0535S, Contains also: Phusion® HF Buffer (5×), Phusion® GC Buffer Pack(5×), $MgCl_2$ solution (50 mM), DMSO (100%)

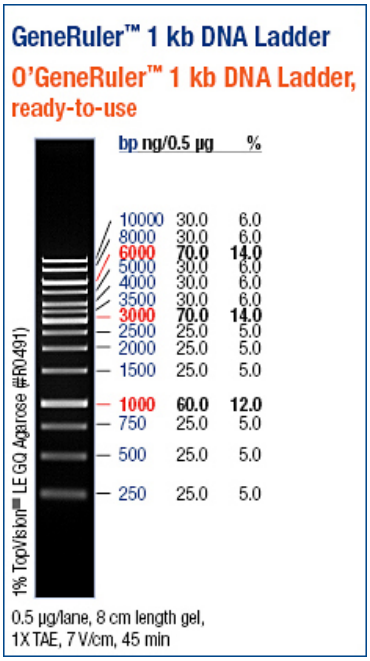
A.5. Kits used

Component	Additional information
BigDye® Direct Cycle Sequencing Kit	Vendor: LifeTechnologies Cat.№: 4458689, 4458687, 4458688
GoTaq® Green Master Mix	Vendor: Promega Cat. №M7121, M7122, M7123
Pierce ECL Western Blotting Substrate	Vendor: Thermo Scientific Cat. №: 32106, 32209, 32109
pGEM®-T Easy Vector System I	Vendor: Promega Cat.№A1360, A1360
TOPO®TA Cloning®Kit for Sequencing	Vendor: Invitrogen Cat.№K4530-20,K4575-J10, K4575-01, K4575-40, K4580-01, K4580-40, K4595-01, K4595-40, K4575-02, 450030
Invisorb® Spin Plasmid Mini Two	Vendor: Stratec, LOT: AG130010
Invisorb® Spin DNA Extraction Kit	Vendor: Stratec, Cat.№1020110300
Invisorb® DNA CleanUp	Vendor: Stratec, Cat.№1020400200
QuikChange II XL Site-Directed Mutagenesis Kit	Vendor: Agilent Cat№200521, 200522

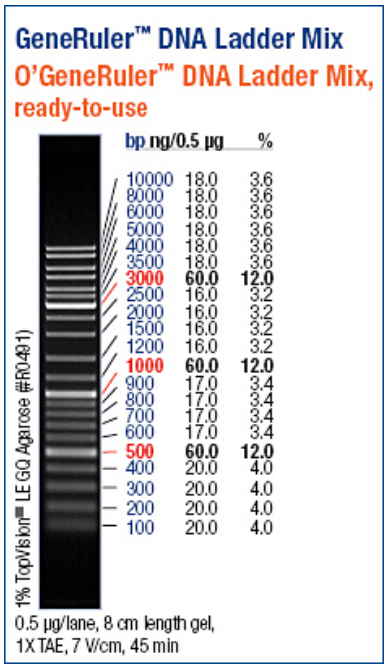
A.6. Lab equipment

Component	Additional information
Centrifuge	Vendor: Eppendorf, Model: 5810 R
Bench top centrifuge	Vendor: Eppendorf, Model: Centrifuge MiniSpin®
High speed Centrifuge	Vendor: Beckman Coulter, Model: Optima L-100K, High speed Centrifuge
Cell Culture Centrifuge	Vendor: Heraeus, Model: Megafuge 1.0R
Imaging system	Vendor: INTAS GmbH, Model CHEMOCAM Imager 3.2
Midiprep System	Vendor: Promega, Cat№: A2490
Cell Strainer	Vendor: Sartorius Stedin, Size: 45 µL
Shaking incubator	Vendor: Infors HT, Model: Ectotron
Thermocylcer	Vendor: Eppendorf, Model: Mastercycler ProS

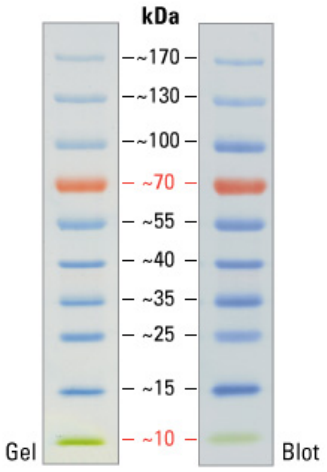
A.7. Markers



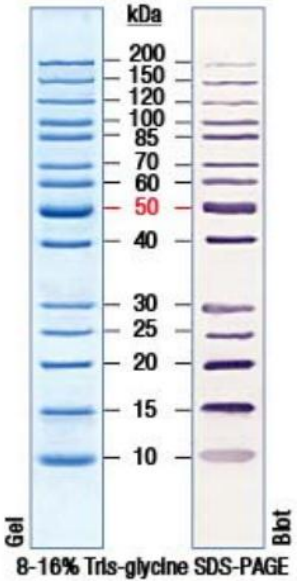
(a) GeneRuler 1 kb DNA Ladder



(b) GeneRuler DNA Ladder Mix- Thermo Scientific



(c) PageRuler Prestained Protein Ladder- 10 to 170 kDa



(d) peqGOLD Protein Marker II - 10 to 200 kDa

Figure A.1.: Markers used for experiments, closer description can be found in the individual captions.

A.8. Media and reagents

Comopnent	Additional information
Standard Medium	450 mL of DMEM (Vendor:) 5 mL Ultraglutamine 50 mL Fetal Calf Serum (FCS) 5 mL Antibiotics (Penicillin 10000 U/mL, Streptomycin 10000 µg/mL) 5 mL Vitamins
Bacterial Medium	Standard LB + Ampicillin (final: 100 $\frac{\mu g}{mL}$)
Ficoll-Paque PLUS	Vendor:formerly Amersham bioscience, General Electric Healthcare, Cat.№: 17-1440-02
cOmplete- protease inhibitor mix	Vendor: Roche, Cat.№: 04693116001
QUANTI-Blue™	Vendor: Invivogen, Cat.№: rep-qb1, rep-qb2
TAK-242 TLR4 inhibitor	Vendor: Invivogen Cat.№: tlrl-cli95
THP-growth medium	RPMI 1640 (2 mM L-glutamine, 1.5 $\frac{g}{L}$ sodium bicarbonate, 4.5 $\frac{g}{L}$ glucose, 10 mM HEPES and 1.0 mM sodium pyruvate) with 10 % heat-inactivated fetal bovine serum (30 min at 56 °C), 100 $\frac{mg}{mL}$ Normocin™, Pen-Strep (50 $\frac{U}{mL}$-50 $\frac{\mu g}{mL}$)
Transfection reagent	TransIT®-293 Transfection reagent Vendor: Mirus, Cat№: MIR 2700, 2704, 2705, 2706
DNA Marker	Name: GeneRuler 1 kb DNA Ladder Vendor: Thermo Scientific
Protein sample loading buffer	Novex® Tris-Glycine SDS Sample Buffer Vendor: Lifetechnologies, Cat№: LC2676

A.9. Primers

Table A.1.: Primers used for mutagenesis listed according to their postion in the Env-sequence. (Position of abrogading mutations are described in figure 2.1)

Position	Name	Sequence	T_m
1	L→A_Reverse	5'-TAACCCCCTTCTGTTCTGTGCAACCACTTCAGATAAGGAGG-3'	80.8 °C
2	Q→A_Reverse	5'-TAACCCCCTTCTGTTCGCTAGAACCACTTCAGATAAGGAGG-3'	79.4 °C
3	N→A Forward	5'-GTTCTACAGGCCAGAAGGGGGTTAG-3'	69.3 °C
	N →A Reverse	5'-CTAACCCCCTTCTGGCCTGTAGAAC-3'	69.3 °C
4	R→A_Reverse	5'-TAACCCCCTTGCGTTCTGTAGAACCACTTCAGATAAGGAGG-3'	79.9 °C
8	D→A_Forward	5'-GCTCTGTTATTTCTAAAAGAAGGAGGGTTATGTGTAGCCTT-3'	73.7 °C
14	E→A_Forward	5'-GATCTGTTATTTCTAAAAGCAGGAGGGTTATGTGTAGCCTT-3'	74.2 °C

Table A.2.: Remaining primers, used in preliminary PCR.

Name	Sequence	T_m
env_forward_unmuated	5'-GATCTGTTATTTCTAAAAGAAGGAGGGTTATGTGTAGCCTT-3'	72.5 °C
env_reverse_unmuated	5'-TAACCCCCTTCTGTTCTGTAGAACCACTTCAGATAAGGAGG-3'	77.5 °C
HIV_complete_Forward-mutated	5'-GTGGAAAGATACCTAAAGGATGGAGGGTTATGTGTAGCCTTAAAAGAGGAA-3'	79.1 °C
HIV_complete_Reverse-mutated	5'-AGCCAGGATTCTTGCCTGGAGAACCACTTCAGATAAGGAGGTTAGGGATTC-3'	84.2 °C

Table A.3.: Protein expression Primers

Name	Sequence	T_m
Trunc-env-Forward	5'-GTGAAAGTCGAAAGAATCCC-3'	54.77
Trunc-env-Kozak-Forward	5'-gccaccATGCATCCCACGTTAAGCCG-3'	76.00
Trunc-env-Reverse	5'-TTCCCTTCGACGCCTCTCTA-3'	60.81

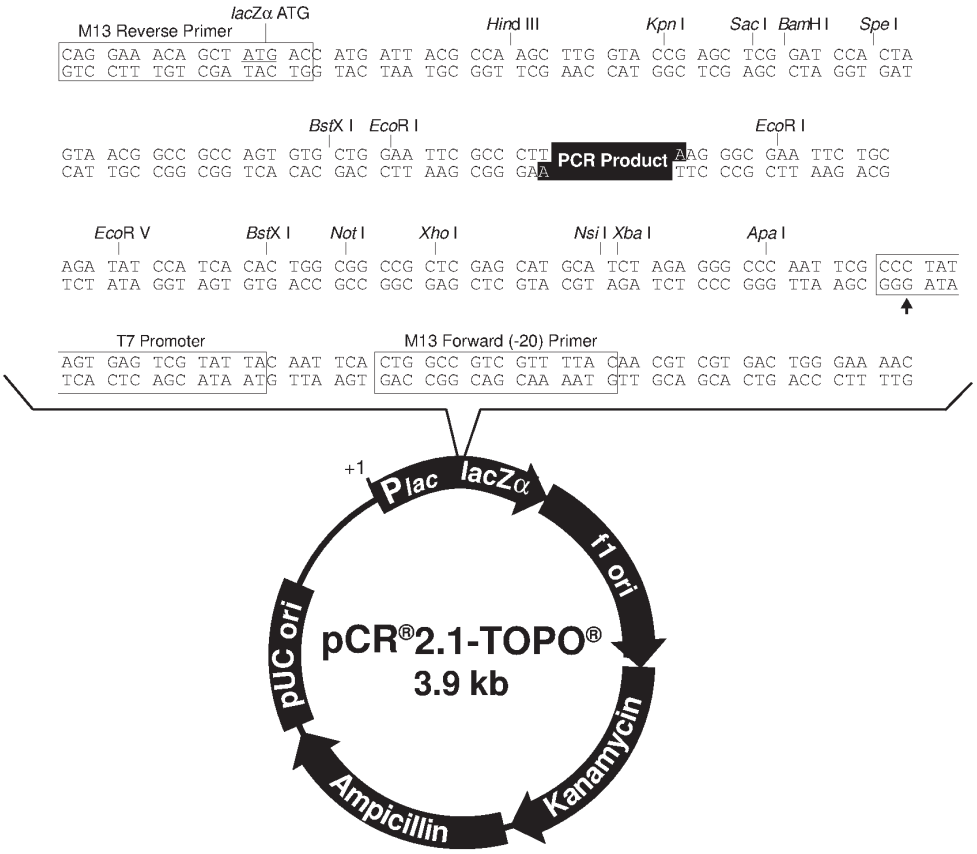
Table A.4.: Primers for PERV-A/B hybrid generation

Name	Sequence	T_m
Forward-backbone-PERV-B	5'-TAGCCTTCCCAGTTCTAAGATTAGAACTATT-3'	64.9
Forward-PERV-B-middle	5'-CAGGAGGCTGAGTTACGTGATCTAGTGAGAGAGGC-3'	76.7
Reverse-PERV-B-middle	5'-GCCTCTCTCACTAGATCACGTAACTCAGCCTCCTG-3'	76.7
Forward-PERV-A-env	5'-CCACCTGGATCCATGCATCC-3'	64.34
Reverse-backbone-PERV-B	5'-GGATGCATGGATCCAGGTGG-3'	64.34
Reverse-PERV-A-env	5'-TAGTTCTAATCTTAGAACTG-3'	39.71

Table A.5.: Sequencing Primers

Name	Sequence	T_m
env-start-Reverse	5'-GTCCACAAGGCGTTTACCATTTAACTTGAGGAGTT-3'	71.57
Forward-env-1	5'-TTGACCACACCAACGGCTGT-3'	69
Forward-env-2	5'-TTGGCAACAGCGGGTACAAA-3'	68.5
Forward-env-3	5'-TTTAATCGAACCTCTGAGAG -3'	56.1
Forward-env-4	5'-GGGTGGTTTGAAGGATGGTT -3'	64.8
Reverse-env-1	5'-ATCCTTTCATTCCCCACTTC -3'	61.7
Reverse-env-2	5'-TTACAATTCGATGTAGGTTA-3'	52.2
Reverse-env-3	5'-CAACAAGAAGAGGTAGCCTC-3'	58.1
Reverse-env-4	5'-CCAATTCCCATCATTAGAAG-3'	58.5

A.10. Vectors



Comments for pCR®2.1-TOPO®
3931 nucleotides

*Lac*Zα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Invitrogen™
life technologies

Figure A.2.: pCR®2.1-TOPO® 3.9 kb map.

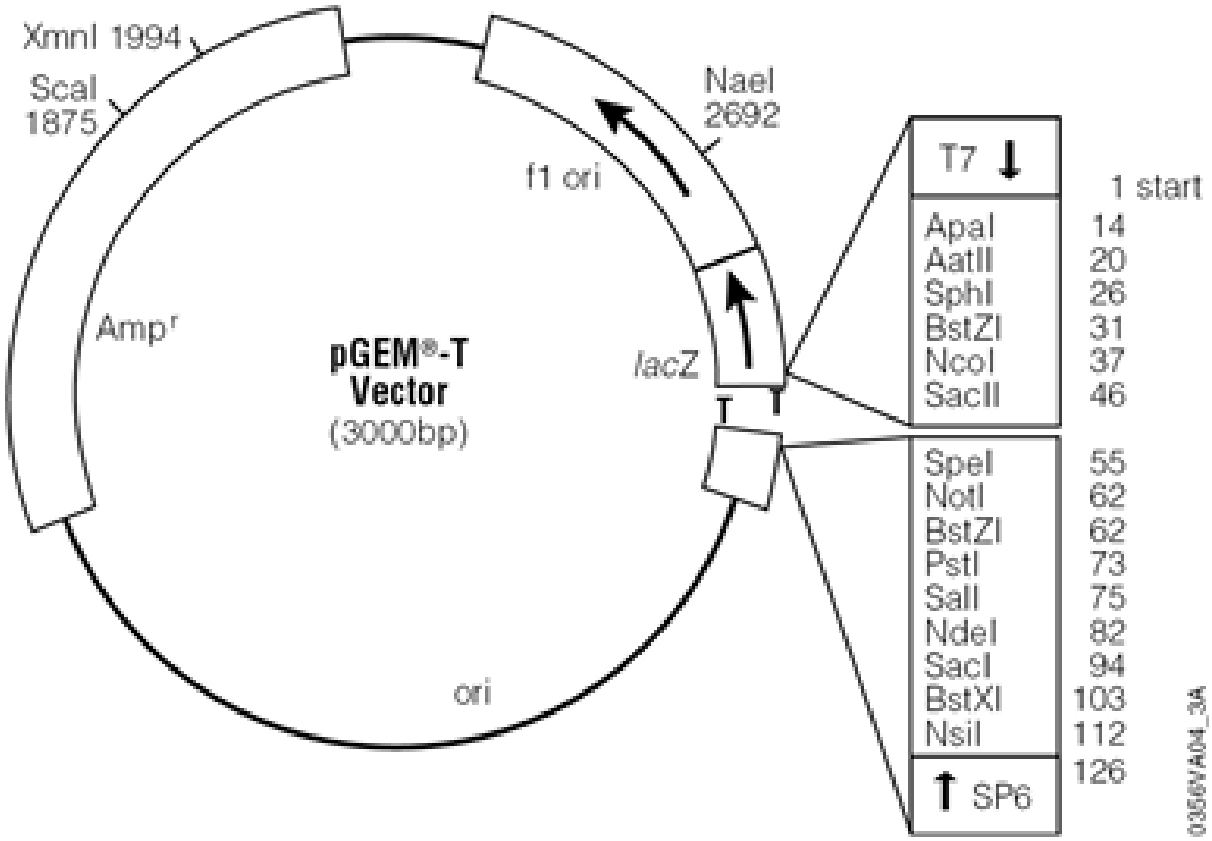


Figure A.3.: 3015bp pGEM®-T Easy Vector used in cloning applications.

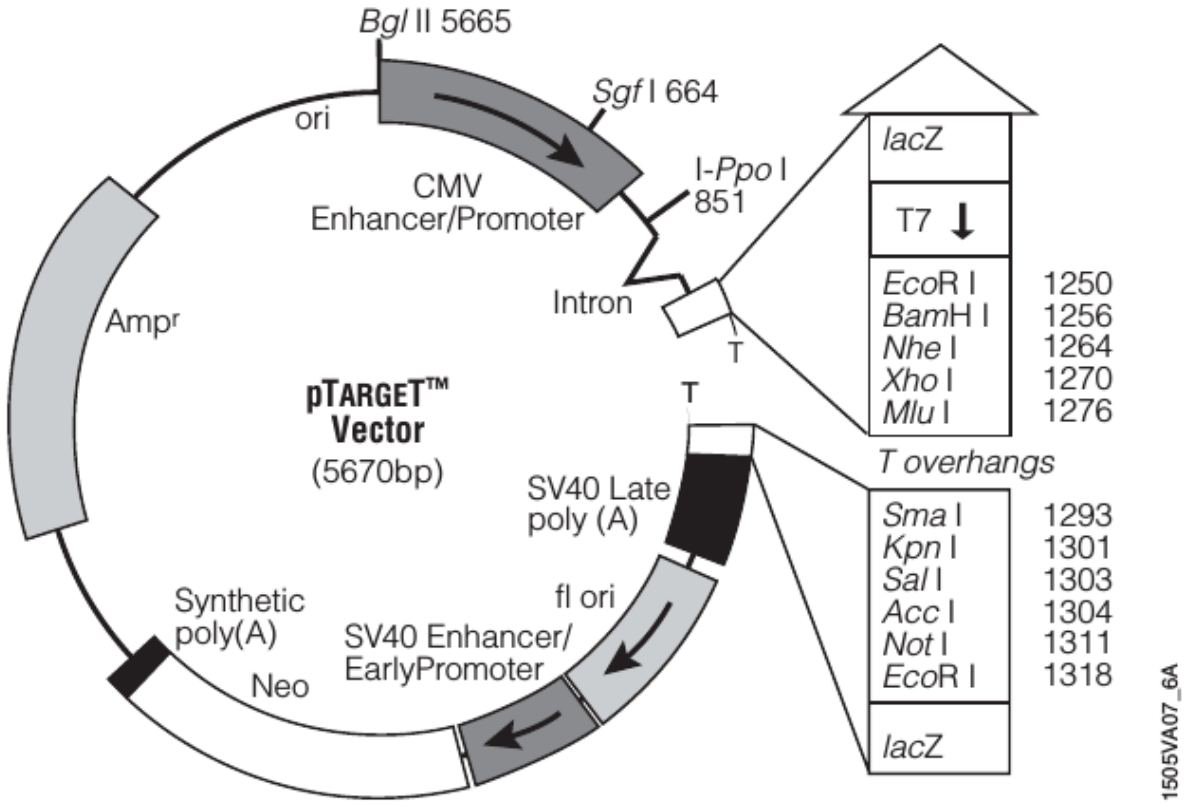


Figure A.4.: 5670bp ptarget®-Mammalian Expression Vector System for protein secretion.

A.11. Sequences

A.11.1. PERV-A(42): Accession number: AJ133817

>gi|6688947|emb|AJ133817.1| Porcine endogenous retrovirus type C roviral gag, pol and env genesand LTR (class A, clone 42)

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CCAATGTACATGGGGATCCCAAAATAAGCTTACCCTTACTGAGGTTTCTGGAAAAGGCACCTGCATAGGG

ATGGTTCCCCCATCCCACCAACACCTTTGTAACCACACTGAAGCCTTTAATCGAACCTCTGAGAGTCAAT

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GCAGTCCTTGATAAATATGACTATAGATATAATCGGCCAAAAAGAGAGCCCATATCCCTGACACTAGCTG

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GAAAATGCAACCTAACCCTCCCAGAACCCAGGAAGTTAATAAAAAGCTCTAAATGCCCCCGAATTCCAGA

CCCTGCTGGCTGCCAGTAAATAGGTAGAAGGTCACACTTCCTATTGTTCCAGGGCCTGCTATCCTGGCCT

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AATGATTGGTCCACGGAGCGCGGGCTCTCGATATTTTAAAATGATTGGTCCATGGAGCGCGGGCTCTCGA

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CCTCTTGCTGTTTGCATCAAGACCGCTTCTTGTGAGTGATTTGGGGTGTCGCCTCTTCCGAGCCCGGACG
AGGGGGATTGTTCTTTTACTGGCCTTTCA

A.11.2. PERV-B(33): Accession number: AJ133816.1

>gi|6688945|emb|AJ133816.1| Porcine endogenous retrovirus type C
proviral gag, pol and env genes and LTR (class B, clone 33)

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ATGATTGGTCCACGGAGCGCGGGCTCTCGATATTTTAAAATGATTGGTCCACGGAGCGCGGGCTCTCGAT

ATTTTAAAATGATTGGTTTGTGACGCACAGGCTTTGTTGTGAACCCCATAAAAGCTGTCCCGATTCCGCA

CTCGGGGCCGCAGTCCTCTACCCCTGCGTGGTGTACGACTGTGGGCCCCAGCGCGCTTGGAATAAAAATC
CTCTTGCTGTTTGCATCAAGACCGCTTCTCGTGAGTGATTTGGGGTGTCGCCTCTTCCGAGCCCGGACGA
GGGGGATTGTTCTTTTACTGGCCTTTCA

A.11.3. Multiple sequence alignment with PERV-A(42)-env

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
1	M	M	.	.
2	H	H	.	.
3	P	P	.	.
4	T	T	.	.
5	L	L	.	.
6	S	S	.	.
7	R	R	.	.
8	R	R	.	.
9	H	H	.	.
10	L	L	.	.
11	P	P	.	.
12	I	I	.	.
13	R	R	.	.
14	G	G	.	.
15	G	G	.	.
16	K	K	.	.
17	P	P	.	.
18	K	K	.	.
19	R	R	.	.
20	L	L	.	.
21	K	K	.	.
22	I	I	.	.
23	P	P	.	.
24	L	L	.	.
25	S	S	.	.
26	F	F	.	.
27	A	A	.	.
28	S	S	.	.
29	I	I	.	.
30	A	A	.	.
31	W	W	.	.
32	F	F	.	.
33	L	L	.	.
34	T	T	.	.
35	L	L	.	.
36	S	S	.	.
37	I	I	.	.
38	T	T	.	.
39	P	P	.	.
40	Q	Q	.	.
41	V	V	.	.
42	N	N	.	.
43	G	G	.	.
44	K	K	.	.
45	R	R	.	.
46	L	L	.	.
47	V	V	.	.
48	D	D	.	.
49	S	S	.	.
50	P	P	.	.
51	N	N	.	.
52	S	S	.	.
53	H	H	.	.
54	K	K	.	.
55	P	P	.	.
56	L	L	.	.
57	S	S	.	.
58	L	L	.	.
59	T	T	.	.
60	W	W	.	.
61	L	L	.	.
62	L	L	.	.

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
63	T	T	.	.
64	D	D	.	.
65	S	S	.	.
66	G	G	.	.
67	T	T	.	.
68	G	G	.	.
69	I	I	.	.
70	N	N	.	.
71	I	I	.	.
72	N	N	.	.
73	S	S	.	.
74	T	T	.	.
75	Q	Q	.	.
76	G	G	.	.
77	E	E	.	.
78	A	A	.	.
79	P	P	.	.
80	L	L	.	.
81	G	G	.	.
82	T	T	.	.
83	W	W	.	.
84	W	W	.	.
85	P	P	.	.
86	E	E	.	.
87	L	L	.	.
88	Y	Y	.	.
89	V	V	.	.
90	C	C	.	.
91	L	L	.	.
92	R	R	.	.
93	S	S	.	.
94	V	V	.	.
95	I	I	.	.
96	P	P	.	.
97	G	G	.	.
98	L	L	.	.
99	N	N	.	.
100	D	D	.	.
101	Q	Q	.	.
102	A	A	.	.
103	T	T	.	.
104	P	P	.	.
105	P	P	.	.
106	D	D	.	.
107	V	V	.	.
108	L	L	.	.
109	R	R	.	.
110	A	A	.	.
111	Y	Y	.	.
112	G	G	.	.
113	F	F	.	.
114	Y	Y	.	.
115	V	V	.	.
116	C	C	.	.
117	P	P	.	.
118	G	G	.	.
119	P	P	.	.
120	P	P	.	.
121	N	N	.	.
122	N	N	.	.
123	E	E	.	.
124	E	E	.	.
125	Y	Y	.	.
126	C	C	.	.

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
127	G	G	.	.
128	N	N	.	.
129	P	P	.	.
130	Q	Q	.	.
131	D	D	.	.
132	F	F	.	.
133	F	F	.	.
134	C	C	.	.
135	K	K	.	.
136	Q	Q	.	.
137	W	W	.	.
138	S	S	.	.
139	C	C	.	.
140	I	I	.	.
141	T	T	.	.
142	S	S	.	.
143	N	N	.	.
144	D	D	.	.
145	G	G	.	.
146	N	N	.	.
147	W	W	.	.
148	K	K	.	.
149	W	W	.	.
150	P	P	.	.
151	V	V	.	.
152	S	S	.	.
153	Q	Q	.	.
154	Q	Q	.	.
155	D	D	.	.
156	R	R	.	.
157	V	V	.	.
158	S	S	.	.
159	Y	Y	.	.
160	S	S	.	.
161	F	F	.	.
162	V	V	.	.
163	N	N	.	.
164	N	N	.	.
165	P	P	.	.
166	T	T	.	.
167	S	S	.	.
168	Y	Y	.	.
169	N	N	.	.
170	Q	Q	.	.
171	F	F	.	.
172	N	N	.	.
173	Y	Y	.	.
174	G	G	.	.
175	H	H	.	.
176	G	G	.	.
177	R	R	.	.
178	W	W	.	.
179	K	K	.	.
180	D	D	.	.
181	W	W	.	.
182	Q	Q	.	.
183	Q	Q	.	.
184	R	R	.	.
185	V	V	.	.
186	Q	Q	.	.
187	K	K	.	.
188	D	D	.	.
189	V	V	.	.
190	R	R	.	.

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
191	N	N	.	.
192	K	K	.	.
193	Q	Q	.	.
194	I	I	.	.
195	S	S	.	.
196	C	C	.	.
197	H	H	.	.
198	S	S	.	.
199	L	L	.	.
200	D	D	.	.
201	L	L	.	.
202	D	D	.	.
203	Y	Y	.	.
204	L	L	.	.
205	K	K	.	.
206	I	I	.	.
207	S	S	.	.
208	F	F	.	.
209	T	T	.	.
210	E	E	.	.
211	K	K	.	.
212	G	G	.	.
213	K	K	.	.
214	Q	Q	.	.
215	E	E	.	.
216	N	N	.	.
217	I	I	.	.
218	Q	Q	.	.
219	K	K	.	.
220	W	W	.	.
221	V	V	.	.
222	N	N	.	.
223	G	G	.	.
224	I	I	.	.
225	S	S	.	.
226	W	W	.	.
227	G	G	.	.
228	I	I	.	.
229	V	V	.	.
230	Y	Y	.	.
231	Y	Y	.	.
232	G	G	.	.
233	G	G	.	.
234	S	S	.	.
235	G	G	.	.
236	R	R	.	.
237	K	K	.	.
238	K	K	.	.
239	G	G	.	.
240	S	S	.	.
241	V	V	.	.
242	L	L	.	.
243	T	T	.	.
244	I	I	.	.
245	R	R	.	.
246	L	L	L	.
247	R	R	R	.
248	I	I	I	.
249	E	E	E	.
250	T	T	T	.
251	Q	Q	Q	.
252	M	M	M	.
253	E	E	E	.
254	P	P	P	.

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
255	P	P	P	.
256	V	V	V	.
257	A	A	A	.
258	I	I	I	.
259	G	G	G	.
260	P	P	P	.
261	N	N	N	.
262	K	K	K	.
263	G	G	G	.
264	L	L	L	.
265	A	A	A	.
266	E	E	E	.
267	Q	Q	Q	.
268	G	G	G	.
269	P	P	P	.
270	P	P	P	.
271	I	I	I	.
272	Q	Q	Q	.
273	E	E	E	.
274	Q	Q	Q	.
275	R	R	R	.
276	P	P	P	.
277	S	S	S	.
278	P	P	P	.
279	N	N	N	.
280	P	P	P	.
281	S	S	S	.
282	D	D	D	.
283	Y	Y	Y	.
284	N	N	N	.
285	T	T	T	.
286	T	T	T	.
287	S	S	S	.
288	G	G	G	.
289	S	S	S	.
290	V	V	V	.
291	P	P	P	.
292	T	.	T	.
293	E	.	E	.
294	P	.	P	.
295	N	.	N	.
296	I	.	I	.
297	T	.	T	.
298	I	.	I	.
299	K	.	K	.
300	T	.	T	.
301	G	.	G	.
302	A	.	A	.
303	K	.	K	.
304	L	.	L	.
305	F	.	F	.
306	S	.	S	.
307	L	.	L	.
308	I	.	I	.
309	Q	.	Q	.
310	G	.	G	.
311	A	.	A	.
312	F	.	F	.
313	Q	.	Q	.
314	A	.	A	.
315	L	.	L	.
316	N	.	N	.
317	S	.	S	.
318	T	.	T	.

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
319	T	.	T	.
320	P	.	P	.
321	E	.	E	.
322	A	.	A	.
323	T	.	T	.
324	S	.	S	.
325	S	.	S	.
326	C	.	C	.
327	W	.	W	.
328	L	.	L	.
329	C	.	C	.
330	L	.	L	.
331	A	.	A	.
332	S	.	S	.
333	G	.	G	.
334	P	.	P	.
335	P	.	P	.
336	Y	.	Y	.
337	Y	.	Y	.
338	E	.	E	.
339	G	.	G	.
340	M	.	M	.
341	A	.	A	.
342	R	.	R	.
343	G	.	G	.
344	G	.	G	.
345	K	.	K	.
346	F	.	F	.
347	N	.	N	.
348	V	.	V	.
349	T	.	T	.
350	K	.	K	.
351	E	.	E	.
352	H	.	H	.
353	R	.	R	.
354	D	.	D	.
355	Q	.	Q	.
356	C	.	C	.
357	T	.	T	.
358	W	.	W	.
359	G	.	G	.
360	S	.	S	.
361	Q	.	Q	.
362	N	.	N	.
363	K	.	K	.
364	L	.	L	.
365	T	.	T	.
366	L	.	L	.
367	T	.	T	.
368	E	.	E	.
369	V	.	V	.
370	S	.	S	.
371	G	.	G	.
372	K	.	K	.
373	G	.	G	.
374	T	.	T	.
375	C	.	C	.
376	I	.	I	.
377	G	.	G	.
378	M	.	M	.
379	V	.	V	.
380	P	.	P	.
381	P	.	P	.
382	S	.	S	.

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
383	H	.	H	H
384	Q	.	Q	Q
385	H	.	H	H
386	L	.	L	L
387	C	.	C	C
388	N	.	N	N
389	H	.	H	H
390	T	.	T	T
391	E	.	E	E
392	A	.	A	A
393	F	.	F	F
394	N	.	N	N
395	R	.	R	R
396	T	.	T	T
397	S	.	S	S
398	E	.	E	E
399	S	.	S	S
400	Q	.	Q	Q
401	Y	.	Y	Y
402	L	.	L	L
403	V	.	V	V
404	P	.	P	P
405	G	.	G	G
406	Y	.	Y	Y
407	D	.	D	D
408	R	.	R	R
409	W	.	W	W
410	W	.	W	W
411	A	.	A	A
412	C	.	C	C
413	N	.	N	N
414	T	.	T	T
415	G	.	G	G
416	L	.	L	L
417	T	.	T	T
418	P	.	P	P
419	C	.	C	C
420	V	.	V	V
421	S	.	S	S
422	T	.	T	T
423	L	.	L	L
424	V	.	V	V
425	F	.	F	F
426	N	.	N	N
427	Q	.	Q	Q
428	T	.	T	T
429	K	.	K	K
430	D	.	D	D
431	F	.	F	F
432	C	.	C	C
433	V	.	V	V
434	M	.	M	M
435	V	.	V	V
436	Q	.	Q	Q
437	I	.	I	I
438	V	.	V	V
439	P	.	P	P
440	R	.	R	R
441	V	.	V	V
442	Y	.	Y	Y
443	Y	.	Y	Y
444	Y	.	Y	Y
445	P	.	P	P
446	E	.	E	E

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
447	K	.	K	K
448	A	.	A	A
449	V	.	V	V
450	L	.	L	L
451	D	.	D	D
452	K	.	K	K
453	Y	.	Y	Y
454	D	.	D	D
455	Y	.	Y	Y
456	R	.	R	R
457	Y	.	Y	Y
458	N	.	N	N
459	R	.	R	R
460	P	.	P	P
461	K	.	K	K
462	R	.	R	R
463	E	.	E	E
464	P	.	P	P
465	I	.	I	I
466	S	.	S	S
467	L	.	L	L
468	T	.	T	T
469	L	.	L	L
470	A	.	A	A
471	V	.	V	V
472	M	.	M	M
473	L	.	L	L
474	G	.	G	G
475	L	.	L	L
476	G	.	G	G
477	V	.	V	V
478	A	.	A	A
479	A	.	A	A
480	G	.	G	G
481	V	.	V	V
482	G	.	G	G
483	T	.	T	T
484	G	.	G	G
485	T	.	T	T
486	A	.	A	A
487	A	.	A	A
488	L	.	L	L
489	I	.	.	I
490	T	.	.	T
491	G	.	.	G
492	P	.	.	P
493	Q	.	.	Q
494	Q	.	.	Q
495	L	.	.	L
496	E	.	.	E
497	K	.	.	K
498	G	.	.	G
499	L	.	.	L
500	S	.	.	S
501	N	.	.	N
502	L	.	.	L
503	H	.	.	H
504	R	.	.	R
505	I	.	.	I
506	V	.	.	V
507	T	.	.	T
508	E	.	.	E
509	D	.	.	D
510	L	.	.	L

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
511	Q	.	.	Q
512	A	.	.	A
513	L	.	.	L
514	E	.	.	E
515	K	.	.	K
516	S	.	.	S
517	V	.	.	V
518	S	.	.	S
519	N	.	.	N
520	L	.	.	L
521	E	.	.	E
522	E	.	.	E
523	S	.	.	S
524	L	.	.	L
525	T	.	.	T
526	S	.	.	S
527	L	.	.	L
528	S	.	.	S
529	E	.	.	E
530	V	.	.	V
531	V	.	.	V
532	L	.	.	L
533	Q	.	.	Q
534	N	.	.	N
535	R	.	.	R
536	R	.	.	R
537	G	.	.	G
538	L	.	.	L
539	D	.	.	D
540	L	.	.	L
541	L	.	.	L
542	F	.	.	F
543	L	.	.	L
544	K	.	.	K
545	E	.	.	E
546	G	.	.	G
547	G	.	.	G
548	L	.	.	L
549	C	.	.	C
550	V	.	.	V
551	A	.	.	A
552	L	.	.	L
553	K	.	.	K
554	E	.	.	E
555	E	.	.	E
556	C	.	.	C
557	C	.	.	C
558	F	.	.	F
559	Y	.	.	Y
560	V	.	.	V
561	D	.	.	D
562	H	.	.	H
563	S	.	.	S
564	G	.	.	G
565	A	.	.	A
566	I	.	.	I
567	R	.	.	R
568	D	.	.	D
569	S	.	.	S
570	M	.	.	M
571	S	.	.	S
572	K	.	.	K
573	L	.	.	L
574	R	.	.	R

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
575	E	.	.	E
576	R	.	.	R
577	L	.	.	L
578	E	.	.	E
579	R	.	.	R
580	R	.	.	R
581	R	.	.	R
582	R	.	.	R
583	E	.	.	E
584	R	.	.	R
585	E	.	.	E
586	A	.	.	A
587	D	.	.	D
588	Q	.	.	Q
589	G	.	.	G
590	W	.	.	W
591	F	.	.	F
592	E	.	.	E
593	G	.	.	G
594	W	.	.	W
595	F	.	.	F
596	N	.	.	N
597	R	.	.	R
598	S	.	.	S
599	P	.	.	P
600	W	.	.	W
601	M	.	.	M
602	T	.	.	T
603	T	.	.	T
604	L	.	.	L
605	L	.	.	L
606	S	.	.	S
607	A	.	.	A
608	L	.	.	L
609	T	.	.	T
610	G	.	.	G
611	P	.	.	P
612	L	.	.	L
613	V	.	.	V
614	V	.	.	V
615	L	.	.	L
616	L	.	.	L
617	L	.	.	L
618	L	.	.	L
619	L	.	.	L
620	T	.	.	T
621	V	.	.	V
622	G	.	.	G
623	P	.	.	P
624	C	.	.	C
625	L	.	.	L
626	I	.	.	I
627	N	.	.	N
628	R	.	.	R
629	F	.	.	F
630	V	.	.	V
631	A	.	.	A
632	F	.	.	F
633	V	.	.	V
634	R	.	.	R
635	E	.	.	E
636	R	.	.	R
637	V	.	.	V
638	S	.	.	S

Table A.6

Table A.6

Amino Acid Position	PERV-A(42)-env Wild Type	1^{st} Section	2^{nd} Section	3^{rd} Section
639	A	.	.	A
640	V	.	.	V
641	Q	.	.	Q
642	I	.	.	I
643	M	.	.	M
644	V	.	.	V
645	L	.	.	L
646	R	.	.	R
647	Q	.	.	Q
648	Q	.	.	Q
649	Y	.	.	Y
650	Q	.	.	Q

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Statement of independent work

Herewith I confirm that I wrote this Master's Thesis in its entirety and that no additional assistance was provided, other than from the sources listed.

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Date Signature