universität wien

# DIPLOMARBEIT

Titel der Diplomarbeit

## Optimization of the lentivirus production for the efficient transduction of primary murine macrophages

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)


| | |
|---|---|
| Verfasserin / Verfasser: | Katrin Anna Christina Spiesberger |
| Matrikel-Nummer: | 0205777 |
| Studienrichtung /Studienzweig (lt. Studienblatt): | Genetik - Mikrobiologie |
| Betreuerin / Betreuer: | o. Univ. Prof. Dr. Thomas Decker |


Wien, am 02.10.2009

## List of abbreviations

| | |
|---|---|
| (c)PPT | (central) polypurine tract |
| (h)CMV | (human) cytomegalo virus |
| att sites | attachment sites |
| BIV | bovine immunodeficiency virus |
| BMM | bone marrow-derived macrophages |
| BMV | brome mosaic virus |
| CA | capsid protein |
| CAEV | caprine arthritis encephalitis virus |
| cDNA | complementary DNA |
| CTE | constitutive transport element |
| CTS | central termination signal |
| dNTP | deoxyribonucleotide triphosphate |
| EIAV | equine infectious anemia virus |
| Env | envelope glycoprotein |
| FIV | feline immunodeficiency virus |
| Gag | group specific antigen |
| GFP | green fluorescent protein |
| gp | glycoprotein |
| HIV | human immunodeficiency virus |
| hrs | hours |
| IN | integrase |
| kb | kilobases |
| kDa | Kilodalton |
| LTR | long terminal repeat |
| MA | matrix protein |
| Min | minutes |
| MOI | multiplicity of infection |
| MVV | maedi-visna virus |
| NC | nucleocapsid |
| Nef | negative factor |
| NLS | nuclear localization signal |
| nts | nucleotides |
| o/n | over night |
| Pbs | primer binding site |
| PCR | polymerase chain reaction |
| pDNA | plasmid DNA |
| PERT | product-enhanced reverse transcriptase assay |
| PIC | pre integration complex |
| PM | peritoneal macrophages |
| Pol | polymerase |
| PR | protease |
| rcf | relative centrifuge force |
| RCR | replication competent retrovirus |
| Rev | regulator of expression of viral proteins |
| rpm | round per minute |
| RRE | rev responding element |
| RT | reverse transcriptase |
| RT | room temperature |
| RTC | reverse transcription complex |
| SIN | self-inactivating |
| SIV | simian immunodeficiency virus |
| SU | surface glycoprotein |
| TAR | transactivation response RNA element |
| Tat | transactivator of transcription |
| TM | trans-membrane glycoprotein |
| TU/ml | transducing units per mililiter |
| U | unit |
| Vif | viral infectivity factor |
| Vpr | viral protein r |
| Vpu | viral protein u |
| VSV-G | vesicular stomatitis virus envelope G glycoprotein |
| w/o | without |
| WPRE | woodchuck hepatitis post-transcriptional response element |

## 1. Summary / Abstract

In contrast to other retroviruses, lentiviruses do not require mitosis for the efficient integration of viral DNA into the host genome, and thus, they are able to transduce non-dividing cells. To date, gene transfer to a wide variety of dividing and non-dividing primary cells has been successfully performed.

In this study we evaluated the ViraPower™ T-REx™ Lentiviral Expression System (Invitrogen) in terms of the efficient transduction of differentiated primary cells. This system consists of a transfer expression vector containing the gene of interest, and a three plasmid packaging system required for vector particle production. To assess the transduction efficiency of viral preparations, the green fluorescent protein (GFP) was used as a reporter gene.

The virus was produced according to the manufacturer's protocol and the viral titers were determined by selection of antibiotic-resistant cell clones, as recommended. By this means, we obtained titers in the range of 5 – 9.7 x $10^3$ transducing units/ml. The achieved multiplicity of infection (MOI) was too low for the successful transduction of primary cells.

Subsequently, we applied modifications to the original protocol in order to increase the viral titers. These approaches for optimization included the use of a two plasmid packaging system, the scale-up of the virus production by the increase of the total amount of transfected cells and the concentration of viral supernatants by ultracentrifugation. All approaches resulted in a clear improvement of the titer. Consequently, the transduction of primary macrophages yielded a moderate to strong expression of GFP.

In addition to the conventional titration, the reverse transcriptase (RT) activities of the viral supernatants were determined by a product-enhanced reverse transcriptase (PERT) assay analysis. The measured RT activity values were in accordance with the transduction results of the primary cells.

In summary, we report the successful improvement of lentivirus production leading to distinctly increased titers compared to the original protocol. As a result, primary macrophages were efficiently transduced. In addition, we show, that the PERT assay is a reliable method to predict the transduction capacity of lentiviral supernatants.

## 2. Zusammenfassung

Lentiviren können im Gegensatz zu allen anderen Retroviren ihre virale DNS unabhängig vom Zellteilungsstadium der Wirtszelle in das Wirtsgenom integrieren. Dadurch können sie mitotisch inaktive Zellen transduzieren. Mittlerweile ist der Gentransfer in eine Vielzahl von sowohl mitotisch aktiven als auch mitotisch inaktiven Zellen erfolgreich nachgewiesen worden.

In dieser Arbeit haben wir getestet, ob das "ViraPower™ T-REx™ Lentiviral Expression System" (Invitrogen) geeignet ist primäre Makrophagen effizient zu transduzieren. Dieses System besteht aus einem Expressionsvektor, der das zu transferierende Gen enthält, und einem Verpackungssystem bestehend aus drei Plasmiden, das für die Vektorpartikelproduktion benötigt wird. Um die Transduktionseffizienz bewerten zu können, verwendeten wir das grün fluoreszierende Protein (GFP) als Reportergen.

Die Virusproduktion und die Bestimmung der viralen Titer durch Selektion Antibiotika-resistenter Zellklone wurden entsprechend dem Originalprotokoll durchgeführt. Mit dieser Methode erhielten wir virale Titer von 5 bis 9.7 x $10^3$ transduzierte Units/ml. Aufgrund dieser niedrigen Titerwerte wurde kein ausreichendes Verhältnis von infektiösen Viruspartikeln zu Zellen erreicht, welches zu einer erfolgreichen Transduktion der Zellen geführt hätte.

In der Folge versuchten wir durch Modifikationen des Originalprotokolls die viralen Titer zu erhöhen. Diese umfassten die Virusproduktion unter der Verwendung eines Verpackungssystems bestehend aus zwei Plasmiden, eine Virusproduktion im größeren Maßstab durch die Erhöhung der Anzahl der Virus produzierenden Zellen und die Konzentration der viralen Überstände durch Ultrazentrifugation. All diese Ansätze führten zu einem deutlichen Titeranstieg, sodass wir in primären Zellen eine mittlere bis starke GFP Expression nachweisen konnten.

Zusätzlich zur konventionellen Titration wurde die Aktivität der viralen reversen Transkriptase (RT) durch eine „product-enhanced reverse transcriptase" (PERT) Analyse bestimmt. Die gemessenen RT Aktivitäten stimmten mit den Transduktionsergebnissen der primären Zellen überein.

Zusammenfassend berichten wir in dieser Arbeit von erfolgreichen Optimierungsschritten des Originalprotokolls zur Produktion von Lentiviren, die zu

deutlichen Titeranstiegen führten. Dadurch konnten wir primäre Makrophagen effizient transduzieren und zeigen, dass die PERT Analyse eine verlässliche Methode ist um die Transduktionskapazität von lentiviralen Überständen einzuschätzen.

# 3. Introduction

## 3.1. Classification of lentiviruses

Lentiviruses belong to the large family of retroviruses. Retroviruses are enveloped viruses carrying two copies of single-strand positive RNA. The virions are 80-100nm in diameter and the comprised virion RNA is 7-12kb in size. The replicative strategy of the *Retroviridae* includes the reverse transcription of the virion RNA into linear double-stranded DNA, which is integrated into the host cell's genome, hence the designation "retro" [1].

Retroviruses are broadly divided into the categories simple and complex, according to the organization of their genomes. Simple viruses carry only structural genes (*gag*, *pol,* and *env*), whereas complex viruses additionally code for regulatory proteins. Defined by evolutionary relatedness, retroviruses can further be divided into the genera Lentivirus, Spumavirus and a group of "oncogenic" viruses [2].

The name lentivirus (lentus, latin – slow) originates from the uniquely prolonged incubation period needed for the virus to induce a disease. The genus Lentivirus comprises a variety of primate (human immunodeficiency virus (HIV)-1 and 2, simian immunodeficiency virus (SIV)) and non-primate viruses (Maedi-Visna virus (MVV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), caprine arthritis encephalitis virus (CAEV) and bovine immunodeficiency virus (BIV)) [3].

## 3.2. Structure of the lentiviral genome

The lentiviral genome consists of *cis*-acting sequences, which do not encode proteins, and *trans*-acting viral elements encoding structural, regulatory, and accessory proteins. *Cis*-acting determinants include long terminal repeats (LTRs), the packaging signal psi ($\psi$), the rev-response element (RRE), the polypurine tract (PPT), and attachment (att) sites.

LTRs are homologous regions at both ends of the lentiviral provirus, which are required for virus replication, integration, and expression. LTRs can be divided into three regions, U3, R, and U5. U3 comprises basal-, enhancer-, and modulatory-promoting elements.

The R region is involved in the Tat-mediated transactivation (see 3.2.2). Furthermore, the first nucleotide of the R region corresponds to the transcription initiation. LTRs also contain signals for RNA capping and polyadenylation in the R region. U5 is sufficient for reverse transcription and thus infectivity of viral particles. Psi is required for the encapsidation of the genomic transfer RNA. The RRE interacts with the *rev* gene and is essential for processing and the transport of viral RNAs. The PPT is necessary for priming of the plus-strand synthesis and the att sites for viral DNA integration [2-4].
Besides the structural genes *gag, pol,* and *env* (see 3.2.1.), common to all retroviruses, HIV-1 additionally comprises regulatory (*tat, rev*) (see 3.2.2.) and accessory genes (*vif, vpr, vpu, nef*) (see 3.2.3.), involved in viral gene expression, viral particle assembly, and infectivity (Fig. 1). HIV-1 has become the best-studied and most frequently used lentiviral vector system (see 3.4.) [3].

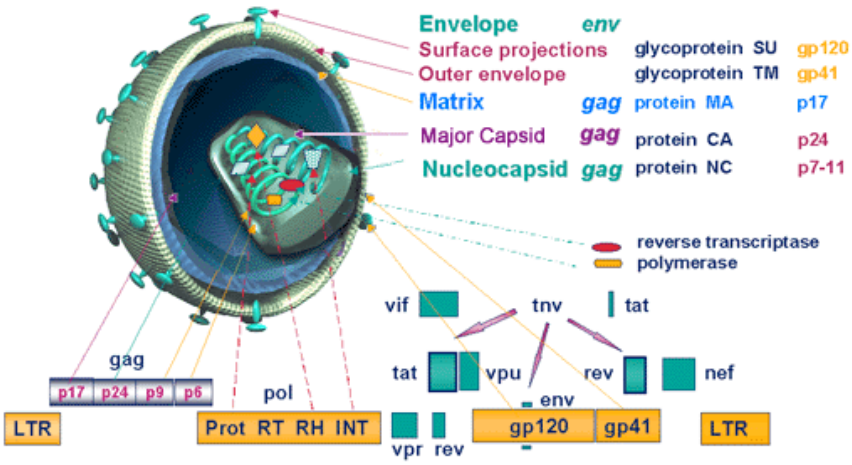



**Figure 1:** Genome map of a lentivirus. The organization of the lentiviral genome and the localization of the viral proteins are schematically depicted. Picture by C. Büchen-Osmond and J. Whitehead. (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.061.1.06.htm)

### 3.2.1. Structural genes of HIV-1

Common to all retroviruses the HIV-1 genome encodes the three structural proteins Gag (group-specific antigen), Pol (polymerase), and Env (envelope glycoprotein). Gag and Pol proteins are initially synthesized as polypeptide precursors p55 Gag and p160

Gag-Pol. During or after virus budding from the host cell the precursors are cleaved by the viral protease into mature products. The cleavage of the 55-kDa Gag precursor generates the structural proteins p17 matrix (MA), p24 capsid (CA), and p7 nucleocapsid (NC) (Fig. 1). The processing of the 160-kDa Gag-Pol precursor generates the viral enzymes p12 viral protease (PR), p51/66 reverse transcriptase (RT), and p31 integrase (IN) [2, 5].

Env, synthesized as polyprotein precursor gp160, is cleaved by a cellular protease into mature proteins gp120, the surface glycoprotein (SU), and gp41, the trans-membrane glycoprotein (TM) (Fig. 1) [2, 6].

### 3.2.2. Regulatory genes of HIV-1

The HIV-1 regulatory genes *tat* and *rev* encode transactivator proteins essential for replication. Tat (transactivator of transcription) is a 15-kDa transcription factor consisting of several domains including a RNA binding domain and a nuclear localization signal (NLS). The basal transcription activity from the HIV-1 LTR is very low. The interaction of Tat with its respective transactivation response RNA element (TAR) results in the phosphorylation of the C-terminal domain of RNA polymerase II and thus, in a dramatic enhancement of the transcriptional activity [7-9].

Rev (regulator of expression of viral proteins) is a 21-kDa phosphoprotein, which is involved in the translocation of transcripts from the nucleus to the cytoplasm. There are three classes of viral mRNAs [10]:

1, unspliced genomic RNA, which functions as the mRNA for the Gag and Gag-Pol polyprotein precursors;

2, partially spliced mRNAs, which encode the Env, Vif, Vpu, and Vpr proteins (see 3.2.3.);

3, fully spliced mRNAs, which are translated into Rev, Tat, and Nef (see 3.2.3.)

Binding of Rev to RRE, which is present in all unspliced and partially spliced HIV-1 RNAs, enables the transport of unspliced and partially spliced RNAs to the cytoplasm. Thus, Rev-RRE interaction is indispensable for the virus replication [11].

### 3.2.3. Accessory genes of HIV-1

Vif (viral infectivity factor) is a 23-kDa phosphorylated protein required for the productive infection *in vivo*. The protein is synthesized in the late phase of the viral life cycle during assembly and/or maturation of virions. The requirement of Vif for efficient HIV-1 replication depends on the cell type, suggesting that its functions are specific for host cellular factors rather than viral factors [12].

Vpr (viral protein r) is a 14-kDa protein present only in primate lentiviruses. After virus entry the reverse transcription of the viral RNA takes place in the cytoplasm of the target cell within the reverse transcription complex (RTC). As part of this RTC, Vpr has an effect on the accuracy of the reverse transcription process. Vpr is also an integral component of the pre-integration complex (PIC) and thus, participating in the nuclear translocation of the viral DNA into non-dividing cells [13].

Vpu (viral protein u), a 16-kDa membrane protein, is expressed in infected host cells during the late stage of infection. The two domains of Vpu are responsible for its functions. The N-terminal trans-membrane domain appears to form ion channels and plays a role in virion release enhancement [14]. The cytoplasmic domain is involved in the degradation of CD4 surface molecules [15].

Nef (negative factor) is a 27- to 34-kD protein essential for viral infectivity *in vivo*. It acts as a modulator of host cell pathways leading to the amplification of viral replication. Further functions of Nef are: The downregulation of CD4 and MHC class I cell surface molecules, the regulation of cellular activation through several kinases, and the enhancement of HIV-1 infectivity by protecting the viral core from post-fusion degradation [16].

## 3.3. Life cycle of lentiviruses

### 3.3.1. Attachment and entry

*In vivo* HIV-1 is mainly targeting T-cells, macrophages and dendritic cells according to the cell surface receptors required for HIV-1 infection. The gp120 interacts with specific

receptors and co-receptors, like CD4, CCR5, and CXCR4 on host cells. Gp41 anchors the gp120/gp41 complex in the membrane and is also responsible for catalyzing the membrane fusion reaction between the viral and host cell lipid bilayers during virus entry [6, 7].

HIV-1 can also attach to cells in a CD4-independent way, by interacting with sugars or lectin-like domains on cell surface receptors. The chemokine receptor CCR5 is predominantly used as co-receptor *in vivo*. Contrasting, additional co-receptors were identified to support HIV-1 infection *in vitro* [17, 18].

Beside a receptor binding function, the glycoproteins of enveloped viruses include a fusion protein function. The interactions of HIV-1 particles with cell surface receptors lead to a rearrangement of gp41 and the exposure of the fusion domain induces the fusion of the membranes to release the nucleocapsid [19]. The RTC is released following the removal of the lipid bilayer. Subsequent to the disassembly of the virion the nucleoprotein complex is delivered into the cell, where the reverse transcription starts [7].

### 3.3.2. Reverse transcription and nuclear import

After infection, HIV-1 converts its RNA genome into double-stranded DNA. In HIV-1, the priming occurs at a purine-rich sequence known as the central PPT (cPPT). RNAseH removes the tRNA bound to the primer-binding site and second-strand transfer takes place. The synthesis stops at the central termination signal (CTS). Since the CTS is 3' of the cPPT, about 100nts of the plus-strand DNA are displaced, resulting in the formation of a "DNA flap". It supports the nuclear import of the PIC to the nucleus [7]. The PIC consists of the viral DNA/DNA double-strand forms, MA, IN, Vpr viral proteins, and cellular factors. The nuclear import is triggered by the interaction of NLS with specific cell proteins [20].

### 3.3.3. Integration and synthesis of viral proteins

Subsequent to the nuclear import of the viral PIC, IN catalyzes the stable insertion of the viral DNA into the host cell genome. The integration is mainly directed by

interactions between the PIC and the chromatin. Recently it was shown, that the integration of HIV-1 does not occur completely randomly, but favors sites of active transcription and symmetric sequences [21]. Following the integration into the host genome, the provirus serves as a template for the synthesis of the viral RNAs (see 3.2.2.).

### 3.3.4. Virion assembly, release and maturation

The assembly of HIV-1 takes place at the plasma membrane of the infected cell. Gag is responsible for targeting and stably binding the plasma membrane. The encapsidation of HIV-1 RNAs into virus particles is mediated by the interactions of the packaging signal and the NC domain of Gag. The mature envelope glycoproteins are generated by the cleavage of gp160 (see 3.2.1.). After cleavage gp41 anchors the Env complex in the host cell membrane. The final step in the assembly process is the budding of the virus particle from the host cell plasma membrane. Following the virus particle release, the viral PR cleaves the Gag and Gag-Pol polyprotein precursors to generate mature proteins. This cleavage triggers structural rearrangements leading to the maturation of the virion [7].

## 3.4. Lentiviral vector systems

Due to the fact, that HIV-1 is the most extensively studied human pathogen to date, HIV-1 based vectors are most commonly used. Alternatively, HIV-2, EIAV, FIV, MVV, CAEV, and BIV (see 3.1.) are considered as lentiviral vectors. However, their usage in gene transfer is still restricted because of low titers and the limited transducibility of human tissues [22, 23].

For reasons of biosafety, currently used HIV-1 vector systems consist of a transfer construct including the transgene of interest, one or more packaging constructs, encoding all proteins required for particle production and transduction of target cells, and an envelope construct, that encodes the envelope glycoproteins [24].

The major safety issue is to avoid the generation of replication-competent retroviruses (RCR). RCR are produced by recombination events at sites of partial homology

between the vector's sequences, the packaging construct, and a retroviral element in the producer cells [25].

### 3.4.1. Modification of the packaging system

The packaging system includes all HIV-1 genes required for vector particle production and efficient transduction of target cells [24]. The initial packaging construct was developed by Naldini et al. (1996) and is referred as the first generation packaging cassette [26]. It consists of one plasmid that contains all HIV-1 accessory and regulatory genes except *vpu*. *Env* is provided on a separate plasmid. The human cytomegalovirus (hCMV) promoter drives the expression of all viral proteins required in *trans*. The packaging signal ψ was deleted from the 5' untranslated region and the 3' LTR was substituted with a poly(A) site from the insulin gene [26].
Further investigations led to the development of the second generation packaging system. In contrast to the packaging system of the first generation, the plasmid lacks the sequences encoding the accessory genes. Virus produced with the second generation packaging is able to transduce most target cells *in vitro* and *in vivo* [27].
Since a main objective concerning lentiviral vectors is the prevention of RCR, a third generation packaging system was developed. To reduce the possibility of recombination events between the helper vector and the transfer construct the *gag, pol*, and *rev* coding sequences were segregated onto separate plasmids. The requirement for *tat* was overcome by the replacement of the 5' LTR of the transfer construct with a strong constitutive promoter (e.g. deriving from CMV or Rous sarcoma virus (RSV)) [28].

### 3.4.2. Modification of the transfer construct

Self-inactivating (SIN) HIV-1 vectors were developed to improve biosafety by minimizing the risk of generating RCR. The viral transcriptional promoter and enhancer elements were eliminated and mutations were positioned in the U3 region of the 3' LTR of the vector DNA. By reverse transcription the modified 3´ LTR is duplicated in the 5' LTR. As

SIN vectors are devoid of their parental enhancer/promoter sequences, they lack the ability to transcribe full-length vector RNA [25, 29].
The incorporation of cPPT and CTS elements (see 3.3.2.) accelerate the transduction kinetics of HIV-1 vectors. Consequently, the DNA flap improves the nuclear importation of the PIC. In addition, the incorporation of cPPT and CTS increased the transduction efficiency by 3-10-fold in a variety of cells [24].
The RRE permits the transport of viral RNAs by interaction with the Rev protein, which is critical in producing high-titer virus. In a variety of vectors it has been replaced by homologous nuclear export signals such as the Mason-Pfizer monkey virus constitutive transport element (CTE) or the woodchuck hepatitis post-transcriptional response element (WPRE). The insertion of WPRE was shown to increase the expression and the stability of the transgenic mRNA in the target cell by 5-8 fold [22, 29].

### 3.4.3. Modification of the envelope construct

Originally, the envelope was coded by HIV-1 *env* sequences. HIV-1 virions are permissive for the incorporation of non-HIV membrane proteins, which allowed the development of pseudotyped vectors. HIV-1 particles can be pseudotyped by envelope glycoproteins from a variety of other viruses, including VSV. Due to the beneficial properties, VSV-G is almost exclusively used. VSV-G stabilizes vector particles and therefore allows vector concentration by ultracentrifugation. HIV-1/VSV-G pseudotypes are 20-130-fold more infectious than non-pseudotyped. VSV-G is broadening the vector tropism dramatically and it directs the vector entry to an endocytic pathway, which reduces the requirements for viral accessory proteins [29, 30]. An obvious drawback of VSV-G pseudotyped envelopes is the distinct cytotoxicity during its constitutive expression [31].

## 3.5. Transduction of primary cells by lentivirus

In contrast to other retroviruses, lentiviruses do not require mitosis for efficient integration. To date, a large diversity of dividing and non-dividing primary cells have

been successfully transduced including cells of the hematopoietic lineage, epithelial cells, or tumor cells (for review see [22, 24] and references therein).

The nuclear import of viral DNA as part of the PIC is the rate-limiting step in the lentiviral life cycle. Since HIV-1 can access the nucleus before mitosis, it has been assumed that the PIC contains proteins with NLS that are essential for the infection of non-dividing cells. These proteins include the viral structure protein MA, the viral IN, the viral accessory protein Vpr, and in addition the *cis*-acting DNA sequence cPPT [32].

Several reports prove the importance of the MA-NLS for the nuclear import of the PIC. Mutations in the MA-NLS abolished the nuclear import function and decreased the infection of macrophages [33, 34]. Contrasting, MA-NLS independent infection was demonstrated [35].

The deletion of *vpr* resulted in a diminished transport of the viral genome to the nucleus and a reduced infection rate of macrophages [36]. However, Vpr is only a component of primate lentiviruses and the lack of both, Vpr and MA-NLS, does not abolish the replication in non-dividing cells [32].

Viral IN governs the nuclear import of the PIC in a NLS-mediated way. However, mutation studies showed a critical role for HIV-IN in viral infection of both non-dividing and dividing cells [37, 38].

In addition, mutations in the HIV-1 genome, which prevent the formation of the DNA flap are replication defective. Though mutations in the cPPT affect the infection of dividing as well as non-dividing cells, it is not absolutely required for infection of non-dividing cells, because HIV-1 based vectors lacking the cPPT are still able to transduce non-diving cells [39, 40].

Hence, the essential factors for the successful infection of non-dividing cells are still unidentified, since even viruses lacking any of the identified NLS still remain infectious in non-dividing cells [41].

It was recently reported that the CA protein is a dominant determinant in the infection of non-dividing cells. CA regulates the uncoating process due to its interaction with cytoplasmatic factors. If incoming virions can enter the nucleus only after uncoating has proceeded, than uncoating rather than nuclear import might be the rate-limiting step in the infection of non-dividing cells [42].

## 4. Aim of the project

The lentiviral technology is one of the most promising tools for gene transfer to primary, non-dividing cells. So far, limiting factors for the efficient transduction are the generation of high-titer and non-toxic lentiviral stocks.

Therefore, the aim of this work was to evaluate if the ViraPower™ T-REx™ Lentiviral Expression System (Invitrogen) could be routinely used for the transduction of primary macrophages. We produced virus according to the manufacturer's protocol and determined the titers. In order to enhance viral titers and transduce primary macrophages more efficiently, we optimized the original protocol for lentiviral production in terms of using different packaging systems, scaling-up the production, and concentrating the viral supernatants. We compared the viral preparations and their transduction efficiencies and tested different titer determination methods for their applicability.

# 5. Materials and Methods

## 5.1. Plasmids

### 5.1.1. GFP

For this study the green fluorescent protein (GFP) was used as a reporter gene. In comparison to the wild type protein the photostability and fluorescence are improved, due to a single point mutation (S65T) [43]. The plasmid pKC/GFP (S65T) (5515bp) [44] was kindly provided by Dr. Thomas Czerny (FH Campus Vienna). Experiments were additionally performed with an enhanced GFP originally cloned in the vector pmaxGFP (Lonza).

### 5.1.2. Lentiviral vectors

For the generation of the lentiviral transfer vector the plasmid pLenti4/TO/V5-DEST, supplied in the ViraPower™ T-REx™ Lentiviral Expression System (Invitrogen), was used as a backbone. Cloning of the expression plasmid is based on the Gateway® Technology (Invitrogen) (Fig. 2). In brief, at first the gene of interest is cloned into an entry vector. Consequently, the transfer to the expression vector takes place by site-specific recombination.
In the following subchapters the features of the plasmids used for generating the entry and the expression clones are described.

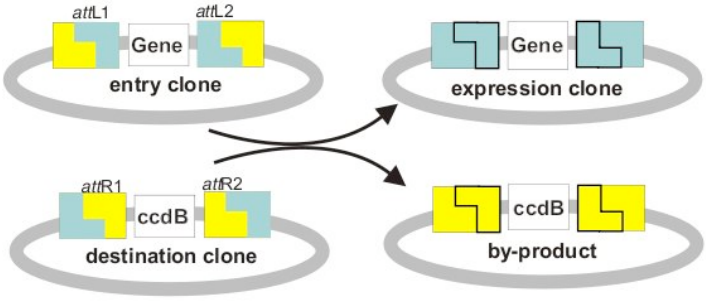



**Figure 2**: Schematic depiction of the Gateway® cloning system. The gene of interest is cloned into an entry vector, where the gene is flanked by attachment sites (*att*L). The recombination of the *att*L sites with the corresponding attachment sites of the destination vector (*att*R) (= LR recombination) leads to the generation of the expression clone.

#### 5.1.2.1. pENTR ™1A

Due to its suitable restriction and recombination sites pENTR™1A (Invitrogen) (for vector map see Appendix 9.1.) was chosen as an entry vector. pENTR™1A (2717bp) includes two site-specific recombination sites (*att*L1 and *att*L2) for recombination with the Gateway® destination vector pLenti4/TO/V5-DEST, the *ccdB* gene for negative selection in ccdB sensitive E.coli, and a kanamycin resistance gene.

#### 5.1.2.2. pLenti4/TO/V5-DEST

The pLenti4/TO/V5-DEST (Invitrogen) (8599bp) (for vector map see Appendix 9.2.) contains a tetracycline-regulatable hybrid promoter consisting of the CMV promoter and two tetracycline operator 2 sites (TetO2). It also includes elements that allow packaging of the construct into virions (e.g. 5' and 3' LTRs, and the packaging signal $\psi$)and reverse transcription of the viral mRNA. Furthermore it carries the ampicillin and Zeocin™ resistance marker genes for selection of stably transduced cell lines. The pLenti4/TO/V5-DEST is adapted for Gateway® cloning and includes two recombination sites, *att*R1 and *att*R2, for cloning of the gene of interest from an entry clone. Furthermore, a C-terminal tag, the V5 epitope, is provided for the detection of the recombinant protein.

### 5.1.3. Packaging plasmids

Due to biosafety, an optimized mixture of packaging plasmids is needed for the appropriate production of lentivirus. These plasmids supply the helper functions as well as structural and replication proteins in *trans* required to produce the lentivirus. In this study two different systems of packaging were used, which are described in detail in the following chapters.

#### 5.1.3.1. 3$^{rd}$ generation packaging

For this purpose the ViraPower™ Packaging mix (Invitrogen) was used. It consists of three different packaging plasmids in an optimized ratio. It contains pLP1 (8889bp),

pLP2 (4180bp), and pLP/VSVG (5821bp). pLP1 comprehends the HIV-1 *gag* gene, the HIV-1 *pol* gene as well as the HIV-1 Rev response element (RRE). pLP2 encodes the *rev* gene. pLP/VSVG encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow the production of a pseudotyped retrovirus with a broad host range (for vector maps see Appendix 9.3. – 9.5.).

#### 5.1.3.2. 2nd generation packaging

Plasmids for the 2nd generation packaging system were kindly provided by Prof. E. Muellner (MFPL, MUV). psPAX2 (10703bp) combines the functions of pLP1 and pLP2. It includes the *gag* and *pol* genes as well as the RRE. pMD2.G (5824bp) encodes the VSV glycoprotein (for vector maps see Appendix 9.6., 9.7.).

## 5.2. Cloning

### 5.2.1. Isolation of plasmid DNA

Plasmid DNA (pDNA) was isolated by alkaline lysis. 3ml of L-Broth (LB)-medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH7.5) containing the appropriate antibiotic were inoculated with a single bacteria colony and incubated o/n at 37°C. Cells were harvested by centrifugation at 10000rpm for 2 min (Eppendorf, Centrifuge 5415D), resuspended in 250µl P1 (50mM Tris-HCl pH7.5, 10mM EDTA pH8, 0.05mg/ml RNAse A), and lysed by addition of 250µl P2 (0.2M NaOH, 1% SDS). The reaction was neutralized by addition of 250µl P3 (2.55M KOAc pH 4.8) and 10µl $CHCl_3$ and centrifuged for 10min at 13000rpm (Eppendorf, Centrifuge 5415D). The supernatant was collected and mixed with 0.7vol isopropanol. After 10min of centrifugation at 13000rpm (Eppendorf, Centrifuge 5415D), the pellet was washed with 70% EtOH, dried, and resuspended in an adequate amount of 1 x TE (10mM Tris, 1mM EDTA) buffer.

### 5.2.2. Plasmid midiprep

Plasmid DNA isolation in the scale of midipreps was performed with the JETSTAR 2.0 Midiprep Kit (Genomed) according to the manufacturer's protocol.

### 5.2.3. Determination of DNA concentration

The concentration of isolated plasmid DNA was analyzed by measuring the extinction (OD, optical density) of a 1:100 dilution (in 1 x TE buffer) at λ=260nm with a photometer (Gene Quant II, Pharmacia, RNA/DNA Calculator). The DNA concentration was calculated as follows:

DNA concentration (µg/ml)=$OD_{260}$ x dilution factor x multiplication factor (50 for dsDNA)

### 5.2.4. Digestion of DNA with restriction enzymes

Usually about 500ng of DNA were restricted with restriction endonucleases provided by MBI Fermentas or Invitrogen, applying 1U enzyme/1µg DNA and the according buffer. The reaction with a final volume of 20µl was incubated at the appropriate temperature for at least 4hrs.

### 5.2.5. Ligation and transformation

A ligation reaction of 15µl contained equimolar ratios of vector and insert DNA, 1µl T4 DNA ligase and 1.5µl 10x buffer for T4 DNA ligase. The reaction was incubated for 4 hours at 15-16°C and the ligase was inactivated for 15 min at 65°C.
For the transformation chemically competent cells (protocol by M. Scott, USF, California, USA), available as a stock at the institute, were used.
50µl competent E.coli Top10F' (Invitrogen) and 5µl ligation reaction were incubated on ice for 30min, followed by a heat shock at 42°C for 30sec, and then kept on ice for 5min. 450ml LB-medium were added and the reactions were incubated for 90min at 37°C on a rotary shaker (Innova 4080, New Brunswick Scientific) before plating onto LB-plates with appropriate antibiotics.

### 5.2.6. LR recombination reaction

For the LR recombination reaction 150ng of the expression vector pLenti4/TO/V5-DEST were mixed with 150ng of the entry clone containing the GFP cDNA. After the addition of 2µl Gateway® LR Clonase™II enzyme mix the reaction was incubated for 18hrs at 25°C in a final volume of 10µl. Subsequently, 1µl proteinase K was added, the reaction was incubated for 10min in a water bath at 37°C and transformed into commercial competent OneShot® StBl3™ E.coli (Invitrogen) according to the manufacturer's protocol.

### 5.2.7. Polymerase chain reaction (PCR)

#### 5.2.7.1. Primer sequences

| PRIMER | SEQUENCE |
|---|---|
| GFP forward | 5´- ATG GTG AGC AAG GGC GAG G - 3´ |
| GFP reverse | 5´- ACT TGT ACA GCT CGT CCA TG - 3´ |
| GFP stop-reverse | 5´- TTA CTT GTA CAG CTC GTC CAT G - 3´ |
| pENTR-sequ forward | 5´- TGA CTG ATA GTG ACC TGT TCG - 3´ |
| pENTR-sequ reverse | 5´- GTA ACA TCA GAG ATT TTG AGA CAC - 3´ |
| CMV forward | 5´- CGC AAA TGG GCG GTA GGC GTG - 3´ |
| V5 reverse | 5´- ACC GAG GAG AGG GTT AGG GAT- 3´ |

#### 5.2.7.2. Standard PCR reaction

A PCR standard reaction in a final volume of 25µl contained:
10pm primer each (Invitrogen), 1 x PCR buffer (200mM TrisHCl pH8.8, 500mM KCl), 0.2mM dNTPs, 1.5mM $MgCl_2$ and 1unit Biotaq.

Cycling conditions were as follows:
5min at 95°C for initial denaturation, followed by 30-35 cycles of 30sec denaturation, 30-40sec annealing at a temperature according to primer sequences, and polymerization at 72°C for 1min/1000bp.

PCR fragments for sequence analysis were amplified with a proof-reading polymerase (Pfu DNA polymerase, Fermentas) according to the manufacturer's protocol.

#### 5.2.7.3. Precipitation of PCR products by polyethyleneglycol (PEG)

The PCR reaction was replenished with water to a volume of 100µl. Then it was mixed with 1 vol $PEG^{6000}$ (24% $PEG^{6000}$, 3M NaCl) and incubated at 37°C for 10min. After centrifugation at 13000rcf at 4°C for 30min (Sigma, 1-15K) the pellet was washed twice with 70% EtOH, dried in a vacuum concentrator (Concentrator 5301, Eppendorf) and resuspended in an adequate amount of 1 x TE buffer.

#### 5.2.7.4. Sequencing of PCR products

100ng of the precipitated PCR products were analyzed by dye-terminator-sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. The BigDye® reaction products were precipitated with 50µl 100% EtOH and 2µl 3M NaOAc, centrifuged for 30min at RT at 18000rcf (Sigma, 1-15K), washed with 70% EtOH, dried in the vacuum concentrator (Concentrator 5301, Eppendorf), and resuspended in 20µl $H_2O$. Sequencing was done with the Megabace™1000 (Molecular Dynamics), a capillary dye-terminator-sequencer. Sequences were analyzed and aligned with the Sequence Navigator-ABI PRISM software (Applied Biosystems).

### 5.2.8. DNA gel electrophoresis

PCR products and DNA fragments were analyzed by agarose gel electrophoresis. Samples were mixed with 1/10 volume 10x loading buffer (30% glycerol, 5mM EDTA pH8.0, 0.25% bromphenolblue) and separated on 0.8% to 1.5% agarose gels (containing 0.05µg/ml ethidium bromide) with 0.5x TBE buffer (5.4g Tris, 2.75g boric acid, 0.4g EDTA in 1l $H_2O$). The 1kb+ ladder was used as a standard. DNA fragments were visualized with a UV-transilluminator (EAGLE EYE II, Stratagene).

## 5.3. Cell culture

### 5.3.1. Cell culture reagents

| Reagent | abbreviation | Art. No. |
|---|---|---|
| Dulbecco's Modified Eagle Medium, High Glucose | DMEM | Invitrogen #41966-029 |
| Fetal Calf Serum*) | FCS | Invitrogen #10270-106 |
| L-Glutamine 200mM (100x) | L-Glu | Invitrogen #25030-024 |
| Penicillin-Streptomycin, 10000U/ml Penicillin and 10000µg/ml Streptomycin (100x) | P/S | Invitrogen #15140-122 |
| 2-Mercaptoethanol 55mM (1000x) | β-ME | Invitrogen #31350-010 |
| Phosphate-Buffered Saline (1x), pH 7.4 | PBS | Invitrogen #10010-015 |
| Dimethyl sulfoxide | DMSO | Sigma #D2650 |
| G418 (Geneticin) | | Biochrom #A 2912 |
| Non-Essential Amino Acids solution 10mM (100x) | NEAA | Invitrogen #11140-050 |
| MEM Sodium Pyruvate Solution 100mM (100x) | SP | Invitrogen #11360-070 |
| Opti-MEM I Reduced Serum Medium | Opti-MEM | Invitrogen #31985-062 |
| Trypsin-EDTA 0.05% | | Invitrogen #25300-054 |
| Zeocin™ | | Invitrogen #R250-05 |

*) Before use FCS has been inactivated at 56°C for 30min (except FCS for 293FT cells).

Cells were incubated at 37°C and 5% $CO_2$ in standard medium (DMEM + 10%FCS, 2mM L-Glu, 100µg/ml P and 100U/ml S) except where otherwise indicated.

### 5.3.2. Cell lines

#### 5.3.2.1. 293FT cells

293FT cells (Invitrogen) are derived from the 293 cell line (ATCC #CRL-1573) descending from primary embryonic human kidney. The *E1A* adenovirus gene participates in transactivation of some viral promoters, allowing the cells to produce high levels of protein. Furthermore the 293FT cell line stably expresses the SV40 large T antigen controlled by the CMV promoter.
293FT cells were grown in standard medium supplemented with 10% not heat inactivated FCS, 1x NEAA, 1xSP and 500μg/ml G418.

#### 5.3.2.2. 2fTGH cells

The 2fTGH cell line is a mutant HT1080 human sarcoma cell line transfected with a vector encoding a selectable marker regulated by interferon [45]. 2fTGH cells were used for titration experiments due to their beneficial characteristics. They grow in an adherent non-migratory manner and exhibit a doubling time in the range of 18-25hrs. 2fTGH cells were grown in standard medium.

#### 5.3.2.3. HeLa cells

The HeLa cell line (ATTC #CCl-2) is an immortal cell line derived from cervical cancer cells.
HeLa cells were grown in standard medium.

### 5.3.3. Freezing of cells

The cells were washed with PBS, trypsinized, and centrifuged for 5min at 1000rpm (Hereaus Multifuge 1S, ThermoScientific). The supernatant was removed and the cells were thoroughly resuspended in pre-cooled freezing-medium (90% FCS + 10% DMSO). 1ml aliquots were transferred into pre-cooled cryotubes and incubated for further 30min on ice. Vials were stored at –80°C o/n and transferred into liquid nitrogen (bone

marrow-derived macrophages (BMM)) or to a freezer at -152°C (cell lines) for long-term storage.

### 5.3.4. Thawing of cells

Cells were thawed carefully in a water bath at 37°C, transferred into 10ml DMEM and centrifuged for 5min at 1000rpm (Hereaus Multifuge 1S, ThermoScientific). After discarding the supernatant, the cell pellet was resuspended in a suitable volume of appropriate medium and seeded onto cell culture dishes.

### 5.3.5. Preparation of L929 conditioned medium

L929 cells (ATCC #CCL-1) grown to full confluency on cell culture dishes (Ø10cm) were split onto 30 culture dishes (Ø 15cm). Cells were grown in standard medium until approximately 70% confluency. The medium was removed and the cells were incubated additional 10 days with starving medium (standard medium without FCS) under standard conditions. Afterwards the medium was collected and filter-sterilized (Vacuum filter, 0.22µm). Aliquots were stored at –20°C and thawed at 4°C o/n prior to use.

### 5.3.6. Isolation of bone marrow-derived macrophages (BMM)

BMM were isolated and grown in standard medium for BMM (supplemented with 50µM β-ME and 15% conditioned medium from L929 cells) [46]. Mice were sacrificed by cervical dislocation. Tibia and femur were isolated and transferred into PBS. Bones were cut at both ends and flushed with 1ml standard medium for BMM per bone. The cells were pooled, resuspended and plated onto three non-tissue culture dishes (Ø 10cm). At day 3 after isolation the medium was changed. The cells were split (1:2 or 1:3) upon confluency and seeded for experiments on tissue culture dishes at day 8 after isolation.

### 5.3.7. Isolation of thioglycollate-elicited peritoneal macrophages (PM)

Mice were injected intra-peritoneally with 2ml of one-week-old 4% Thioglycollate-medium and sacrificed 4 days later by cervical dislocation. The peritoneum was flushed with 8ml PBS and the obtained cells were plated onto cell culture dishes. After 2hrs incubation period the cells were washed with PBS and fresh medium was added. PM were grown in standard medium with 5% FCS and supplemented with 50µM β-ME.

## 5.4. Production of lentivirus

### 5.4.1. Transfection with Lipofectamine™ 2000

9µg ViraPower™ Packaging mix (Invitrogen) and 3µg pLenti4/TO/V5-GFP were resuspended in 1.5ml Opti-MEM. Lipofectamine™ 2000 (3µl/µg DNA) was solubilized in 1.5ml Opti-MEM. After an incubation of 5min at RT the solutions were mixed and incubated at RT for 20min. In the meantime 293FT cells were washed with PBS, trypsinisized, pooled, and counted. The DNA-Lipofectamine complexes (3ml) were plated onto 10cm cell culture dishes together with 2ml 293FT medium w/o G418. 6 x $10^6$ 293FT cells in 5ml medium were seeded onto the complexes and incubated o/n at standard conditions. On the following day the medium containing the DNA-Lipofectamine™ 2000-complexes was removed and replaced by standard complete medium w/o G418. 48-72hrs after transfection the viral supernatant was harvested, centrifuged for 5min at 1000 rpm (Hereaus Multifuge 1S, ThermoScientific), and the supernatant was stored at –80°C.

### 5.4.2. Transfection with *Trans*IT®-LT1 Transfection Reagent

On the day before transfection 2 x $10^6$ 293FT cells were plated on 6cm cell culture dishes. 20µl *Trans*IT®-LT1 transfection reagent (4µl/µg DNA) were dissolved in 500µl Opti-MEM and incubated 10-15min at RT. 5µg DNA (packaging plasmids and respective expression clones) were added and incubated 20-25min at RT. After the removal of the medium the *Trans*IT®-LT1 reagent / DNA complexes were dropped onto

the cells. 12-14 hrs post transfection the medium was removed and 293FT medium supplemented with 30% FCS was added. On the following 3 days the medium was collected, centrifuged (5min at 1000 rpm, Hereaus Multifuge 1S, ThermoScientific), and stored at –80°C while fresh medium (+ 30% FCS) was added.

### 5.4.3. Concentration of the lentivirus

During virus production the medium was collected on consecutive days and stored at –80°C. The collected supernatants were thawed, pooled, and ultracentrifuged at 4°C, 24000 rpm for 1:45hrs (Sorvall Ultra Pro 80, Rotor Sorvall AH-828, DuPont Instruments). The pellets were incubated in 200µl Opti-MEM on ice o/n to dissolve. On the next day the pellets were resuspended and 20µl aliquots were stored at –80°C.

### 5.4.4. Killing curve experiment

Due to the Zeocin™ -resistance of the pLenti4/TO/V5-DEST constructs the sensitivity of 2fTGH and HeLa cells had to be determined. Therefore 25% confluent cells plated on 10cm dishes were grown with different concentrations of antibiotics (50 - 1000µg/ml). Cells grown with the appropriate antibiotic concentration died at day 10-14 after antibiotic addition.

### 5.4.5. Titration of the lentiviral stock

2 x $10^5$ 2fTGH and HeLa cells were plated in each well of a 6-well dish (day 1). On the next day the lentiviral stock was thawed and 10-fold dilutions were prepared ($10^{-2}$ - $10^{-6}$) in 1ml medium. After removing the old medium, the lentiviral dilutions and 6µg/ml hexadimethrine bromide (Polybrene®) were added. Dishes were incubated at 37°C and 5% $CO_2$ o/n. On day 3 the medium was changed. Antibiotic selection started at day 4. The medium was removed, the cells were washed with PBS, trypsinizied, transferred onto cell culture dishes (Ø 10cm), and the appropriate amount of antibiotic, corresponding to the resistance of the plasmids, was added.
Every 2-3 days the medium containing the antibiotic was changed. On day 14-16 (post plating cells) the medium was removed, the cells were washed twice with PBS and

stained for 10min with Crystal violet solution. The Crystal violet solution was removed and the cells were washed twice with PBS. Blue-stained colonies were counted and the transducing units per ml (TU/ml) were determined according to the manufacturer's protocol.

### 5.5. Product-enhanced reverse transcriptase (PERT) assay

The PERT assay is a very sensitive method to detect reverse transcriptase (RT) activity within the virions. It is a quantitative real-time-PCR assay, based on the enzymatic capacity of the viral RT to synthesize DNA from RNA templates [47].
The viral supernatants were mixed 1:2 with disruption buffer (40mM Tris-HCl pH8.1, 50mM KCl, 20mM Dithiothreitol (DTT), 0.2% NP-40, 0.2% Triton X-100), incubated for two minutes at room temperature, and stored on ice. The samples were diluted 1:1000 with the dilution buffer (20mM Tris-HCl pH7.5, 50mM KCl, 0.25mM EDTA pH8, 0.025% Triton X-100, 50% Glycerol, 0.2mM DTT). The real-time RT-qPCR was performed at VetOMICs (Core Facility for Research, VetMedUni Vienna). Dilutions from Moloney murine leukemia virus (MMLV) reverse transcriptase molecules (Promega) served as standard concentrations ($10^{-12}$ – $10^{-5}$ pU/10µl). Brome mosaic virus (BMV) RNA was used as a RNA template. The PCR reaction included:

| | | |
|---|---|---|
| $H_20$ | 4.26 | µl |
| 10 mM Tris-HCl, pH 8.3 | 0.25 | µl |
| 25 mM KCl | 0.63 | µl |
| 0.175 % Triton X-100 | 0.24 | µl |
| 200 µM each dNTPs | 2.00 | µl |
| 2.5 mM $MgCl_2$ | 2.50 | µl |
| 1 mM DTT | 0.25 | µl |
| 0.5µM primer BMV-forward | 1.25 | µl |
| 1µM primer BMV-reverse | 2.50 | µl |
| 0.15 µM BMV probe | 0.38 | µl |
| 25 nM ROX Ref. Dye | 0.50 | µl |
| 1.25 U AmpliTaq Gold | 0.25 | µl |
| 20 ng BMV RNA | 5.00 | µl |
| Sample | 5.00 | µl |
| | 25.00 | µl |

| | |
|---|---|
| primer BMV-forward | 5' - GCT CGC TGG TGA TTT GAT CTT - 3' |
| primer BMV-reverse | 5' - CAC AAC GTT CCT ACC TGG AAC A - 3' |
| BMV probe | 5' - FAM-CTC TGT GTG AGA CCT CTG CTC GAG GAG A - TAMRA - 3' |

Cycling conditions were as follows:
45min at 37°C, 10min at 95°C, followed by 45 cycles of 15sec at 95°C and 1min at 60°C.

The activity of transcribing the BMV RNA was specified as pU/10µl, measured by a 7500 Real-time PCR System (Applied Biosystems) and analyzed by SDS Software Version 1.3.1. (Sequences Detection Software, Applied Biosystems).

### 5.6. Transduction of primary cells

2 x $10^4$ to 2 x $10^5$ primary cells (BMM and PM) were plated in 24-, 48-, and 96-well dishes (for more details see Table 3) and transduced with an arbitrary volume of viral supernatant by adding 6µg/ml Polybrene®. 24 and 48hrs post transduction the expression of GFP was checked with an ultraviolet (UV)-microscope (Diaphot 300, Nikon). Pictures were taken with the AxioCam Hrc by Zeiss and analyzed with the software AxioVision Release 4.6.3. (4-2007).

## 5.7. List of materials and reagents

Agrobiogen (Hilgertshausen, Germany)
Biotaq DNA - polymerase

BD Bioscience Austria (Schwechat, Austria)
Falcon™ cell culture dishes

Beckmann (Krefeld, Germany)
centrifuge adaptors, centrifuge tubes

Fermentas (St. Leon-Rot, Germany)
2´-desoxyribonukleosid-5´-triphosphate (dNTPs), Pfu-DNA Polymerase, restriction enzymes, T4 DNA Ligase

Greiner (Kremsmünster, Austria)
cryotubes, non-tissue culture dishes

Invitrogen (Lofer, Austria)
1kb+ DNA ladder, UltraPure Agarose, Gateway® LR Clonase™II enzyme mix, Lipofectamine™ 2000, Virapower™ Packaging Mix, chemically competent OneShot® StBl3™ E.coli, Proteinase K, restriction enzymes, RNAse A

Merck (Vienna, Austria)
brome phenol blue, magnesium chloride, sodium chloride, sodium hydroxide, ampicilline sodium salt, kanamycine disulfate

Mirus (Wisconsin, USA)
*Trans*IT®-LT1 Transfection Reagent

Österreichische Alkoholhandels GmbH (Spillern, Austria)
ethanol

Roth (Karlsruhe, Germany)

boric acid, chloroform, dithiothreitol (DTT), ethidium bromide, ethylenediaminetetraacetic acid (EDTA), hydrochloric acid, isopropanol, polyethylene glycol (PEG6000), potassium acetate, potassium chloride, sodium acetate, Tris(hydroxymethyl)-aminomethane (Tris), tryptone, yeast extract

Sarstedt (Wr. Neudorf, Austria)

cell culture dishes, vacuum filter 0.22µm, laboratory consumables (pipette tips, tubes)

Sigma-Aldrich (Vienna, Austria)

Crystal violet solution, glycerol, nonyl phenoxylpolyethoxylethanol (NP-40), Polybrene®, sodium dodecyl sulfat (SDS), thioglycollate, Triton X-100

# 6. Results

## 6.1. Cloning strategy of the lentiviral vector

The commercial Virapower™ T-REx™ lentiviral expression system (Invitrogen) includes the destination plasmid pLenti4/TO/V5-DEST (for details see 5.1.2., 5.1.2.2. and Appendix 9.2.) as a backbone for the generation of the lentiviral transfer vector.
The cloning system is based on the Gateway® Technology (Invitrogen). For this purpose firstly an entry vector is cloned, where the gene of interest is flanked by *att*L recombination sites. Accordingly, this gene is inserted into the destination plasmid containing *att*R sites by a LR recombination reaction, i.e. by the recombination of the L and R attachment sites of the respective clones, catalyzed by LR Clonase™ II enzyme (Fig. 2 and 5.2.6.).
The destination vector includes the option to append a C-terminal fusion protein (V5-epitope), which allows the detection of the recombinant protein by anti-V5 antibodies. Therefore, two different expression clones (pLenti4-GFP/V5 and pLenti4-GFP/ΔV5) were created to check, if this fusion tag exerts influence on the expression of the GFP cDNA. In the case of the clone pLenti4-GFP/V5 the GFP cDNA is tagged with the V5 epitope. The clone pLenti4-GFP/ΔV5 lacks the expression of the fusion tag by using the stop codon of the GFP cDNA.
The plasmid pmaxGFP (for details see Appendix 9.8.), encoding the GFP from copepod *Pontellina sp*., was introduced by a company as a novel bright fluorescent reporter protein. Based on the experience in our laboratory, pmaxGFP exceeds the signal strength of other routinely used GFP proteins. Therefore, a third expression clone, referred to as pLenti4-maxGFP, harboring the cDNA sequence of pmaxGFP, was generated. The aim was to assess the impact of the signal strength of the GFP expression on the transduction results.

### 6.1.1. Generation of the entry clones

The cloning of the expression vector is based on site-specific recombination (Gateway® Technology, Invitrogen). Therefore, the generation of entry clones was required. The

Gateway® pENTR™ Vector pENTR™1A (Invitrogen) (for details see 5.1.2.1. and Appendix 9.1.) was used as a backbone for generating the entry clones.
In case of pENTR-GFP/V5 and pENTR-GFP/ΔV5, the entry clones for the expression vectors pLenti4-GFP/V5 and pLenti4-GFP/ΔV5, respectively, the GFP cDNA (720bp) was amplified by Pfu-polymerase using the pKC/GFP (S65T) DNA as a template and the primers GFP forward, reverse and GFP stop-reverse, respectively (Fig. 3).

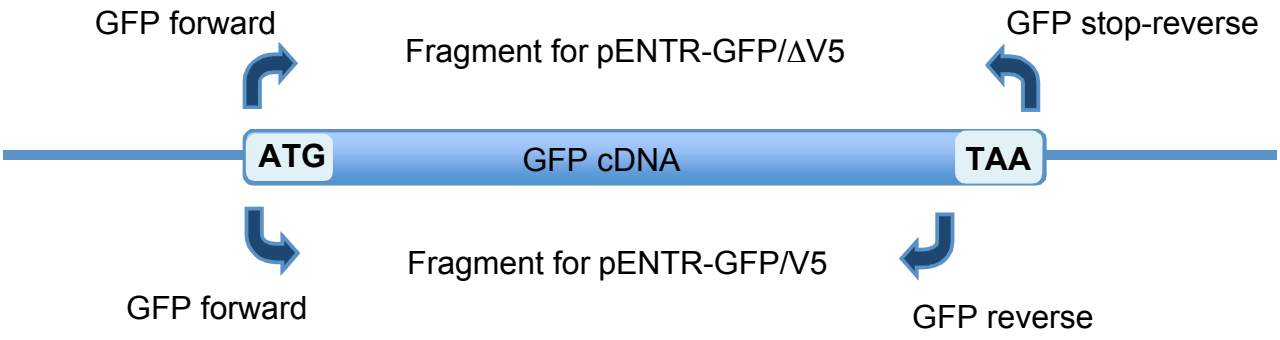



**Figure 3:** Primer binding sites for the amplification of the GFP cDNA. Amplification with the primers GFP forward / GFP stop-reverse (indicated by arrows) produced fragments for cloning the entry clone resulting in the final expression clone without the V5 epitope tag. Amplification with the primers GFP forward / GFP reverse produced fragments for cloning the entry clone resulting in the final expression clone including the V5 epitope tag.

In terms of pENTR-maxGFP, the entry clone for the expression vector pLenti4-maxGFP, the GFP cDNA was isolated by enzymatic restriction of the original plasmid with *Eco47*III and *Xho*I (see Appendix 9.8.).
The precipitated amplification products for generating pENTR-GFP/V5 and pENTR-GFP/ΔV5 were ligated into the blunt ended entry vector pENTR™1A (*Xmn*I/*Eco*RV). cDNA fragments derived from pmaxGFP were ligated into pENTR™1A (*Xmn*I/*Xho*I).
The ligated plasmids were transformed into E.coli Top10F' and the bacteria were grown on LB plates by kanamycin selection. A PCR screen, using the primers GFP forward, reverse and stop-reverse, respectively (Fig. 3), identified positive pENTR-GFP/V5 and pENTR-GFP/ΔV5 clones. A restriction analysis (*Ava*II/*Pvu*I) revealed two fragments (2249 and 715 bp) for positive clones (Fig. 4). Positive pENTR-maxGFP clones were identified by enzymatic restriction (*Ban*II) of the pDNA, giving rise to four fragments (1050, 770, 700, and 430 bp) (Fig. 5).

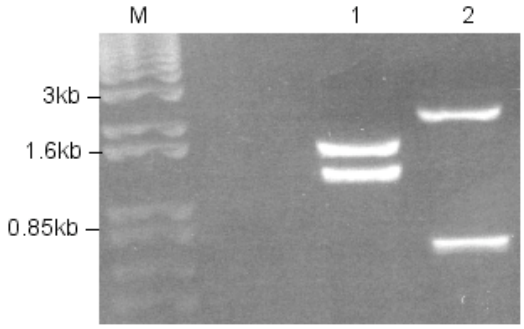



**Figure 4:** Restriction analysis of pENTR-GFP/V5. Samples were digested with *Ava*II and *Pvu*I. Lane 1: Pattern of a negative clone where the GFP cDNA fragment was not integrated; Lane 2: Pattern of a positive clone (#9) giving rise to two fragments (2249 and 715bp); M: 1kb+ ladder

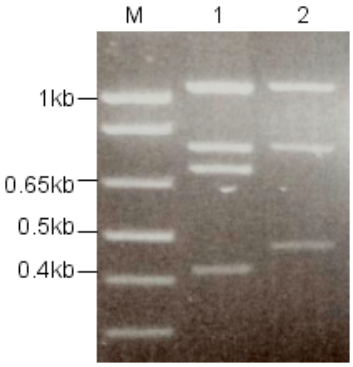



**Figure 5:** Restriction analysis of pENTR-maxGFP. Samples were digested with *Ban*II. Lane 1: Pattern of a positive clone giving rise to four fragments (1050, 770, 700, and 430bp); Lane 2: Pattern of a negative clone where the GFP cDNA fragment was not inserted; M: 1kb+ ladder

Subsequently, the integrity of the sequences of the positive clones was confirmed by sequence analysis using the primers pENTR-sequ forward and reverse, which hybridize sequences of pENTR™1A next to the insertion site of the cloned fragments. Sequences were aligned to the published vector sequence (http://tools.invitrogen.com/content/sfs/vectors/pentr1a_seq.txt) and to the GFP mRNA sequence (GenBank Access. No. DR631190.1). The sequence of maxGFP cDNA is unpublished but the sequence taken from the original vector showed a high homology to the GFP mRNA of *Pontellina plumata* (GenBank Access. No. AY268072.1).

Following clones were chosen as entry vectors for the final expression clones: pENTR-GFP/V5 – clone #9, pENTR-GFP/ΔV5 – clone #13, and pENTR-maxGFP – clone #1.

### 6.1.2. Generation of the expression clones

The expression clones pLenti4-GFP/V5, pLenti4-GFP/ΔV5, and pLenti4-maxGFP were created by a recombination reaction between the *att*R sites of the pLenti4/TO/V5-DEST destination vector and the *att*L sites of the respective entry clones (Fig. 6), followed by the transformation into OneShot® StBl3™E.coli.

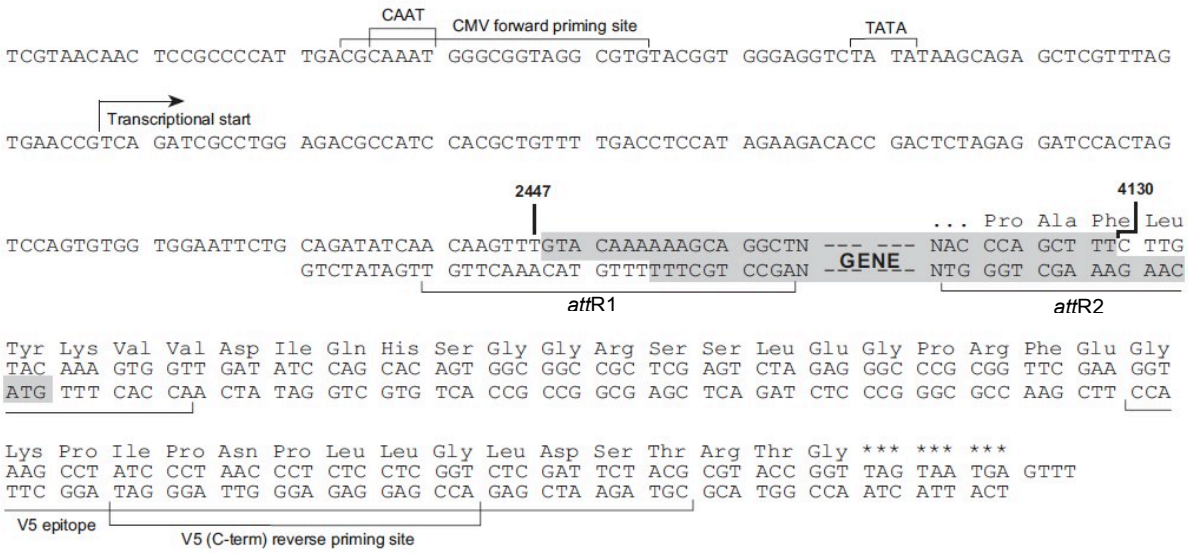



**Figure 6:** Recombination region of pLENTI4/TO/V5-DEST. The shaded region corresponds to the sequences that are replaced by the sequences of the entry clone by the recombination event. The binding sites for the primers used for the sequence analyses (CMV forward / V5 reverse) are depicted. The V5 epitope is only expressed in the expression clone pLenti4-GFP/V5.

A PCR screen, using either primers binding the GFP cDNA sequence (GFP forward, reverse and stop-reverse, respectively) (Fig. 3) or primers binding vector sequences adjacent to the integration site (CMV forward and V5 reverse) (Fig. 6) was performed to identify positive clones. The plasmid DNA of putative positive clones was isolated and restricted with *Ava*I or *Eco*RV. Positive clones gave rise to four fragments (5557, 1515, 596, and 20bp) or two fragments (6885 and 795bp), respectively (Fig. 7). In case of pLenti4-maxGFP, positive clones restricted with *Pvu*II showed three fragments of 4991, 2513, and 795bp (Fig. 8). Subsequently, the correctness of the sequences of the positive expression clones was confirmed by sequence analysis using the primers CMV forward and V5 reverse.

Sequences were aligned to the published vector sequence (https://www.lablife.org/ct?f=c&a=viewvecseq&vectorid=91). The GFP cDNA sequence was aligned as described before (6.1.1.)

Following clones were chosen as final expression clones: pLenti4-GFP/V5 – clone #16, pLenti4-GFP/ΔV5 – clone #29, and pLenti4-maxGFP – clone #20.

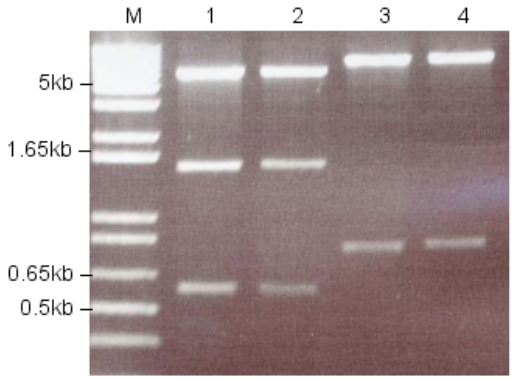



**Figure 7:** Restriction analysis of pLenti4-GFP/V5 and ΔV5. Lane 1 and 2: Pattern of positive clones (#16 and #32) digested with *Ava*I giving rise to four fragments (5557, 1515, 596, and 20bp); Lane 3 and 4: Pattern of positive clones (#16 and #32) digested with *EcoR*V giving rise to two fragment (6885 and 795bp); M: 1kb+ ladder

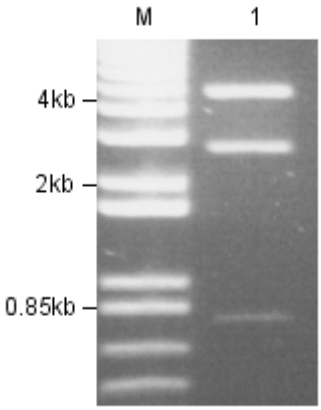



**Figure 8:** Restriction analysis of pLenti4-maxGFP. Samples were digested with *Pvu*II. Lane 1: Pattern of a positive clone giving rise to three fragments (4991, 2513, and 776bp); M: 1kb+ ladder

To test the expression of GFP, 6 x $10^6$ 293FT cells were transfected with the expression vectors pLenti4-GFP/V5, pLenti4-GFP/ΔV5, and pLenti4-maxGFP, respectively, using Lipofectamine™ 2000. 24-36hrs after transfection the expression of GFP was visualized using UV microscopy. At least 90% of the cells were successfully transfected independent of the transfected expression vector. Nevertheless, the intensity of the GFP expression was stronger in cells transfected with pLenti4-maxGFP, compared to the other constructs (Fig. 9, page 47). These observations were reproducible in at least three independent experiments. The use of HeLa cells for transfection instead of 293FT cells gave comparable results. Thus, the superior signal strength of the reporter protein encoded by maxGFP cDNA can be beneficial, if a low or moderate transduction efficiency is expected. In terms of pLenti4-GFP/V5 and pLenti4-GFP/ΔV5 no reproducible significantly different expression levels were observed. Hence, the V5 epitope does not seem to affect the expression of the gene of interest.

## 6.2. Separation of the plasmids included in the ViraPower™ Packaging Mix

The production of infectious virus particles is based on the co-transfection of the expression clone and packaging plasmids encoding proteins responsible for virus assembly and encapsidation. The Virapower™ T-REx™ lentiviral expression system (Invitrogen) includes the plasmids for the 3rd generation packaging, namely pLP1, pLP2, and pLP/VSV-G (for details see 5.1.3.1. and Appendix 9.3. – 9.5.)

These plasmids are originally provided by the company as a mixture with a not indicated ratio of the respective plasmids. To transfect cells with a defined ratio of packaging and envelope plasmid DNA, the plasmid-mix was split. Therefore, the pDNA of the packaging mix was transformed into E.coli Top10F'. The plasmids were discriminated by the differing restriction pattern obtained by the enzymatic digestion with *Eco*RI (Fig. 10).

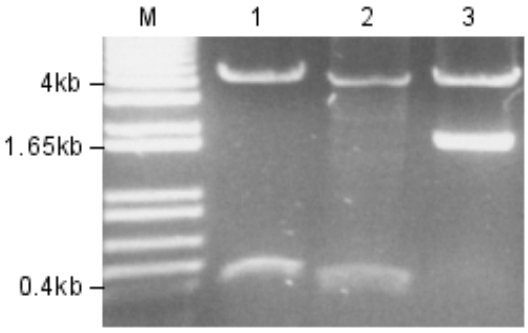



**Figure 10:** Restriction analysis of pLP1, pLP2, and pLP/VSVG. Samples were digested with *EcoR*I. Lane 1: Pattern of a positive pLP1 clone giving rise to three fragments (4335, 4153, and 402bp); Lane 2: Pattern of a positive pLP2 clone giving rise to two fragments (3869 and 311bp); Lane 3: Pattern of a positive pLP/VSVG clone giving rise to two fragments (4153 and 1668bp); M: 1kb+ ladder

## 6.3. Production of lentivirus using the 3rd generation packaging

For the Virapower™ T-REx™ Lentiviral Expression System (Invitrogen) the use of the 3rd generation packaging is recommended. Virus was produced according to the manufacturer's protocol. 6 x $10^6$ 293FT cells were transfected by lipofection with Lipofectamine™ 2000 (5.4.1.) with the 3rd generation packaging mix and the expression vectors pLenti4-GFP/V5 and pLenti4-GFP/ΔV5, respectively. 10ml viral supernatant were harvested, aliquoted, and stored at –80°C. The determination of the lentiviral titer was performed by the selection of stably transduced cells using Zeocin™ as a selection agent. A preliminary killing curve experiment revealed the minimum concentration of

Zeocin™ needed for the efficient killing of untransduced cells. Consequently, 100µg/ml Zeocin™ were used for the titering experiments. The titer of the lentiviral stock was calculated as transforming units (TU)/ml.

For the determination of the titer of preparation (Prep) A (transfected with pLenti4-GFP/V5) and Prep B (transfected with pLenti4-GFP/ΔV5) the cell line 2fTHG was used. Prep A showed a titer of 5 x $10^3$ TU/ml and Prep B showed a titer of 9.7 x $10^3$ TU/ml (Table 1, page 40). A repetition of the titering experiment demonstrated the reproducibility of the results.

According to the protocol a titer of at least 5 x$10^5$ TU/ml should be achievable. Since our titers were about one to two logs beneath the expected values, we decided to modify the original protocol by introducing optimization steps to the virus production procedure.

| No. | Prep name | Packaging | Transfected cells | UC | Titer-2fTGH | Titer-HeLa | Recovery | PERT (pU/10µl) |
|---|---|---|---|---|---|---|---|---|
| A | pLenti4-GFP/V5 | 3rd | 6 x 10e6 293FT | - | 5 x 10e3 TU/ml | n.d. | n.d. | 1.48 x 10e12 |
| B | pLenti4-GFP/dV5 | 3rd | 6 x 10e6 293FT | - | 9.7 x 10e3 TU/ml | n.d. | n.d. | 1.18 x 10e12 |
| C | pLenti4-GFP/V5 | 2nd | 3.6 x 10e8 293FT | + | 4.9 x 10e5 TU/ml | n.d. | n.d. | 2.22 x 10e13 |
| D | pLenti4-mGFP | 2nd | 1.2 x 10e8 293FT | + | 1.7 x 10e5 TU/ml | 4.4 x 10e6 TU/ml | n.d. | 1.81 x 10e13 |
| E | pLenti4-mGFP | 2nd | 7.2 x 10e7 293FT | + | 2 x 10e5 TU/ml | 4.2 x 10e5 TU/ml | n.d. | 8.23 x 10e12 |
| F | pLenti4-GFP/V5 | 2nd | 4 x 10e6 293FT | - | 9.5 x 10e2 TU/ml | n.d. | n.d. | 1.71 x 10e11 |
| G | pLenti4-GFP/dV5 | 2nd | 4 x 10e6 293FT | - | 1.1 x 10e3 TU/ml | n.d. | n.d. | 1.57 x 10e11 |
| H | pLenti4-mGFP | 2nd | 4 x 10e6 293FT | - | 6 x 10e2 TU/ml | n.d. | n.d. | 2.85 x 10e11 |
| I | pLenti4-GFP/V5 | 3rd | 4 x 10e6 293FT | - | 1 x 10e2 TU/ml | n.d. | n.d. | 7.71 x 10e10 |
| J | pLenti4-GFP/dV5 | 3rd | 4 x 10e6 293FT | - | < 1 x 10e2 TU/ml | n.d. | n.d. | 2.97 x 10e10 |
| K | pLenti4-mGFP | 3rd | 4 x 10e6 293FT | - | < 1 x 10e2 TU/ml | n.d. | n.d. | 7.64 x 10e10 |
| L | pLenti4-GFP/V5 | 2nd | 4 x 10e6 293FT | + | 7.2 x 10e3 TU/ml | 5.6 x 10e4 TU/ml | 13% | 2.27 x 10e12 |
| M | pLenti4-GFP/dV5 | 2nd | 4 x 10e6 293FT | + | 4.6 x 10e3 TU/ml | 3.2 x 10e4 TU/ml | 7% | 6.79 x 10e11 |
| N | pLenti4-mGFP | 2nd | 4 x 10e6 293FT | + | 3.3 x 10e4 TU/ml | 7.8 x 10e4 TU/ml | 92% | 3.13 x 10e12 |
| O | pLenti4-GFP/V5 | 3rd | 4 x 10e6 293FT | + | 1.3 x 10e3 TU/ml | 1 x 10e4 TU/ml | 22% | 3.09 x 10e11 |
| P | pLenti4-GFP/dV5 | 3rd | 4 x 10e6 293FT | + | 2 x 10e2 TU/ml | 1.6 x 10e4 TU/ml | n.d. | 3.34 x 10e11 |
| Q | pLenti4-mGFP | 3rd | 4 x 10e6 293FT | + | 1 x 10e3 TU/ml | 1.3 x 10e4 TU/ml | n.d. | 2.23 x 10e12 |

**Table 1:** Overview of the lentiviral preparations.
(UC) ultracentrifugation; (n.d.) not determined

## 6.4. Optimization of the lentivirus production

To improve the titer levels several approaches for optimizing the virus production were performed. These approaches considered parameters like the packaging system, the transfection method, the cell density and the collection of the viral supernatant. Since the 2nd generation packaging is still the most widely and successfully used system in lentivirus production, we co-transfected 293FT cells with the expression clone pLenti4-GFP/V5 and the packaging plasmids psPAX2 and pMD2.G (for details see 5.1.3.2. and Appendix 9.6., 9.7.). A modified protocol of the *Trans*IT®-LT1 Transfection Reagent (see 5.4.2.) was used for lipofection according to an established protocol from the laboratory of Prof. E. Muellner (MFPL, MUV). A total of 18ml viral supernatant per virus preparation were collected on three consecutive days and were frozen at -80°C. The pooled supernatants of each preparation were concentrated by ultracentrifugation. All titers were determined using 2fTGH cells. In table 2 the different approaches (Prep) 1–5 are listed displaying the variations with respect to the standard procedure (Prep 1):

| Procedure | Prep 1 (standard procedure) | Prep 2 | Prep 3 | Prep 4 | Prep 5 |
|---|---|---|---|---|---|
| **number of transfected cells** | 2 x 10e6 cells plated on day before transfection | s.p. | s.p. | 1 x 10e6 cells plated on day before transfection | 4 x 10e6 cells plated on day of transfection |
| **packaging system** | 2nd generation | 3rd generation | s.p. | s.p. | s.p. |
| **transfection procedure** | DNA-complexes dropped onto cells | s.p. | s.p. | s.p. | cells seeded onto DNA-complexes |
| **storage of viral supernatants** | stored at -80°C | s.p. | stored at 4°C | s.p. | s.p. |
| **viral titer (TU/ml)** | 4.5 x 10e3 | 1.4 x 10e3 | 3.2. x 10e4 | 3.75 x 10e4 | 1.8 x 10e5 |

**Table 2:** Approaches for the optimization of the virus production. The standard procedure is given for Prep 1. For Prep 2 – Prep 5 the variations of the standard procedure are indicated.
(s.p.) according to standard procedure

The repetition of the virus production according to Prep 5 gave reproducible results. As a consequence, the following virus preparations were produced according to the last approach (Prep 5).

## 6.5. Optimization of the virus production by the scale-up and concentration of the virus preparations

For the efficient transduction of primary non-dividing cells a high number of infectious virus particles is needed. Thus, a considerable amount of viral supernatant is required to perform several transduction experiments, especially if the needed multiplicity of infection (MOI) is only achievable by increasing the inoculum volume.

To achieve sufficient amounts of concentrated viral supernatants the scale of the virus preparation was increased. A total amount of $3.6 \times 10^8$ and $1.2 \times 10^8$ 293FT cells were transfected in 15cm cell culture dishes with the expression clones pLenti4-GFP/V5 and pLenti4-maxGFP, respectively. According to Prep 5 (see 6.4.) the 2nd generation packaging was used for the transfection reaction. The amounts of pDNA used for the transfection were adapted to the cell culture dish surface area. The viral supernatants were harvested, ultracentrifuged, and resuspended in an appropriate amount of Opti-MEM. The titering of Prep C (pLenti4-GFP/V5) and D (pLenti4-maxGFP) using 2fTGH cells revealed $4.9 \times 10^5$ TU/ml and $1.65 \times 10^5$ TU/ml, respectively.

In case of Prep D the determination of the titer was also performed with HeLa cells. A preliminary killing curve experiment resulted in an optimal concentration of 50µg/ml Zeocin™. Titration with HeLa showed a titer of $4.4 \times 10^6$ TU/ml.

Prep E (pLenti4-maxGFP) was produced as described for Prep D with a total amount of $7.2 \times 10^7$ 293FT cells using 10 cm cell culture dishes. The titer was determined using 2fTGH and HeLa cells and revealed $2 \times 10^5$ TU/ml and $4.2 \times 10^5$ TU/ml, respectively. Results are summarized in Table 1.

## 6.6. Influence of the concentration by ultracentrifugation on the viral titer

Concentrated viral preparations facilitate the transduction of cells with an optimal titer by using a minimal inoculum volume. Thus, we assessed the effective impact on the titer and the recovery rate of infectious virus particles, if the viral preparations were concentrated by ultracentrifugation. Furthermore, a second parameter, i.e. the use of the 2nd versus the 3rd generation packaging system was included in the experimental design. The virus was produced in a small scale according to the protocol of Prep 5 (see 6.4.). Thus, $4 \times 10^6$ 293FT cells per 6cm dish were transfected with the expression

clones pLenti4-GFP/V5 (Prep F, I, L, O), pLenti4-GFP/ΔV5 (Prep G, J, M, P), and pLenti4-maxGFP (Prep H, K, N, Q). For the preparations F, G, H, L, M, N the 2nd generation packaging and for the preparations I, J, K, O, P, Q the 3rd generation packaging was applied. Two identical charges of transfections were done for each expression clone and the respective packaging system. Viral supernatants of the first charge (Prep F – K) were collected, aliquoted and stored at –80°C, whereas supernatants of the second charge (Prep L - Q) were concentrated by ultracentrifugation before storing the aliquots at -80°C. Titer determination was performed using 2fTGH and HeLa cells.

The results of these experiments are summarized in Table 1. In brief, in all cases the concentration by ultracentrifugation led to increased titer values, although to a variable extent (4 – 55-fold). The recovery rate also showed a high variability (7 – 91 %). Titer values for preparations performed with plasmids of the 2nd generation packaging always exceeded those achieved with the 3rd generation packaging system.

### 6.7. Evaluation of the quality of the virus preparations

A certain amount of infectious virus particles is needed to achieve an efficient transduction of cells. Hence, it is valuable to determine the quality of a virus preparation in order to predict the transduction efficiency with respect to reproducible results using different preparations.

A common method is the determination of the viral titer by selecting for stably transduced antibiotic-resistant cells. For several preparations (Prep D, E, L - Q) we performed these experiments using two different cell lines, i.e. 2fTGH and HeLa. Titering using HeLa cells constantly revealed higher titer values than those obtained when using 2fTGH cells (Fig. 11) and notably influenced the calculation of the MOI (Table 3, page 46).

Ideally, the titer determination of a virus preparation is done on the same cell line, which is used in the experimental setup. When working with primary cells the titering has to be performed with other cell lines owing a completely different transduction capacity. Thus, we tried to assess the quality of the viral preparations directly by circumventing the procedure of the titer determination. For this purpose we measured the reverse transcriptase (RT) activity, hence the amount of functional virus particles, in the lentiviral

supernatants (Prep A – Q) by a PERT assay analysis. Values for the RT activity (pU/10μl) of the respective viral supernatants are shown in Table 1. The effective RT activity per transduction reaction is shown in Table 3. The values obtained for the RT activity were in accordance with the results of the titering experiments (Fig. 11). Therefore, the PERT assay analysis is a cost- and time-effective alternative to estimate the quality of a virus preparation.

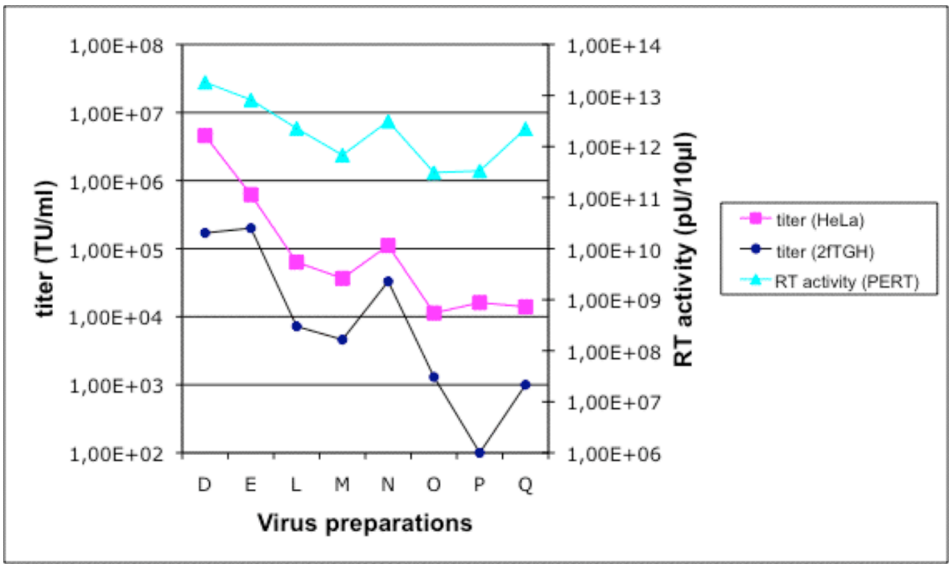



**Figure 11:** Comparison of the viral titers obtained by selecting stably transduced cells with the RT activity of the respective viral supernatants. Values obtained by titering using 2fTGH and HeLa cells are scaled to the left y-axis. Values of the RT activity obtained by the PERT assay analysis are scaled to the right y-axis.

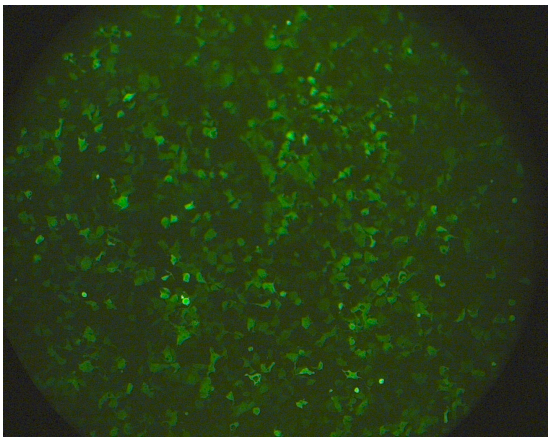
## 6.8. Lentiviral transduction of primary cells

BMM and PM were isolated according to their respective protocols and an appropriate amount of cells was seeded onto 96-, 48-, and 24-well cell culture dishes (Table 3). On the following day the media was replaced by variable amounts of the supernatants derived from the different virus preparations (Table 3). Polybrene® (6µg/ml), a polymer, which increases the infectivity of retroviruses [48] was added. 24hrs later the viral supernatants were discarded and complete medium was added. 36 and 48hrs post infection the cells were checked for the expression of GFP and cell viability. The results of the transduction experiments are summarized in Table 3. Representative examples of transduced primary macrophages are shown in Fig. 12.

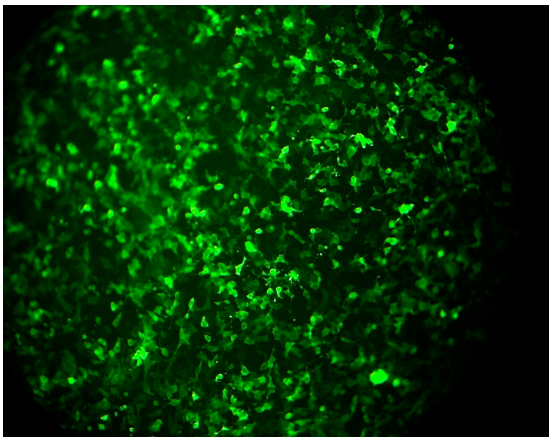
**Table 3 (next page):** Overview of transduced primary cells.
GFP expression: (-) missing, (+) weak, (++) moderate, (+++) strong; cell viability: (1) not impaired, (2) impaired, (3) strongly impaired; (n.d.) not determined; (vol) inoculum volume

**Table 3**

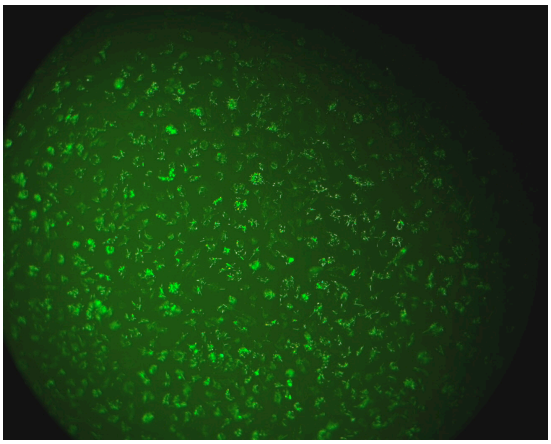
| prep | celltype | well | cell density | vol. | expression | cell health | MOI (2fTGH) | MOI (HeLa) | RT activity (pU) |
|---|---|---|---|---|---|---|---|---|---|
| A | BMM | 96 | 2 x 10e4 | 50µl | - | 1 | 0.01 | n.d. | 7.4 x 10e12 |
| | | | | 100µl | - | 1 | 0.03 | n.d. | 1.5 x 10e13 |
| B | BMM | 96 | 2 x 10e4 | 50µl | - | 1 | 0.02 | n.d. | 5.9 x 10e12 |
| | | | | 100µl | - | 1 | 0.05 | n.d. | 1.2 x 10e13 |
| C | BMM | 24 | 2 x 10e5 | 20µl | - | 1 | 0.05 | n.d. | 4.4 x 10e13 |
| | | | | 40µl | - | 2 | 0.1 | n.d. | 8.9 x 10e13 |
| | | | | 100µl | + | 3 | 0.25 | n.d. | 2.2 x 10e14 |
| | | 96 | 2 x 10e4 | 50µl | + | 3 | 1.23 | n.d. | 1.1 x 10e14 |
| | | | | 100µl | +/++ | 3 | 2.45 | n.d. | 2.2 x 10e14 |
| | PM | 48 | 2 x 10e5 | 20µl | + | 2 | 0.05 | n.d. | 4.4 x 10e13 |
| | | | | 50µl | + | 2 | 0.12 | n.d. | 1.1 x 10e14 |
| | | | | 100µl | + | 2 | 0.25 | n.d. | 2.2 x 10e14 |
| | | | | 200µl | + | 3 | 0.49 | n.d. | 4.4 x 10e14 |
| D | BMM | 24 | 2 x 10e5 | 20µl | + | 1 | 0.02 | 0.44 | 3.6 x 10e13 |
| | | | | 40µl | ++ | 1 | 0.03 | 0.88 | 7.2 x 10e13 |
| | | | | 80µl | +++ | 2 | 0.07 | 1.76 | 1.5 x 10e14 |
| | | | | 120µl | +++ | 3 | 0.1 | 2.64 | 2.2 x 10e14 |
| | | 96 | 2 x 10e4 | 50µl | ++/+++ | 2 | 0.41 | 11 | 9.1 x 10e13 |
| | | | | 100µl | ++/+++ | 3 | 0.83 | 22 | 1.8 x 10e14 |
| | PM | 48 | 2 x 10e5 | 20µl | + | 2 | 0.02 | 0.44 | 3.6 x 10e13 |
| | | | | 40µl | +/++ | 2 | 0.03 | 0.88 | 7.2 x 10e13 |
| | | | | 80µl | ++ | 2 | 0.07 | 1.76 | 1.5 x 10e14 |
| | | | | 120µl | ++ | 3 | 0.1 | 2.64 | 2.2 x 10e14 |
| E | BMM | 24 | 2 x 10e5 | 20µl | -/+ | 1 | 0.02 | 0.04 | 1.7 x 10e13 |
| | | | | 80µl | + | 2 | 0.08 | 0.17 | 6.6 x 10e13 |
| | | | | 120µl | + | 2 | 0.12 | 0.25 | 9.9 x 10e13 |
| | | 96 | 2 x 10e4 | 50µl | +/++ | 2 | 0.5 | 1.05 | 4.1 x 10e13 |
| | | | | 100µl | +/++ | 3 | 1 | 2.1 | 8.2 x 10e13 |
| | PM | 96 | 4 x 10e4 | 5µl | - | 2 | 0.03 | 0.05 | 4.1 x 10e12 |
| | | | | 10µl | + | 2 | 0.05 | 0.11 | 8.2 x 10e12 |
| | | | 1 x 10e5 | 5µl | -/+ | 2 | 0.01 | 0.02 | 4.1 x 10e12 |
| | | | | 10µl | -/+ | 2 | 0.02 | 0.04 | 8.2 x 10e12 |
| F | BMM | 96 | 2 x 10e4 | 100µl | - | 1 | <0.01 | n.d. | 1.7 x 10e12 |
| G | BMM | 96 | 2 x 10e4 | 100µl | - | 1 | <0.01 | n.d. | 1.6 x 10e12 |
| H | BMM | 96 | 2 x 10e4 | 100µl | - | 1 | <0.01 | n.d. | 2.9 x 10e12 |
| I | BMM | 96 | 2 x 10e4 | 100µl | - | 1 | <0.01 | n.d. | 7.7 x 10e11 |
| J | BMM | 96 | 2 x 10e4 | 100µl | - | 1 | <0.01 | n.d. | 3.0 x 10e11 |
| K | BMM | 96 | 2 x 10e4 | 100µl | - | 1 | <0.01 | n.d. | 7.7 x 10e11 |
| L | BMM | 96 | 2 x 10e4 | 5µl | - | 1 | <0.01 | 0.01 | 1.1 x 10e12 |
| | | | | 10µl | - | 1 | <0.01 | 0.03 | 2.3 x 10e12 |
| | | | | 20µl | - | 1 | <0.01 | 0.06 | 4.5 x 10e12 |
| | | | | 50µl | + | 1 - 2 | 0.02 | 0.14 | 1.1 x 10e13 |
| M | BMM | 96 | 2 x 10e4 | 5µl | - | 1 | <0.01 | <0.01 | 3.4 x 10e11 |
| | | | | 10µl | - | 1 | <0.01 | 0.02 | 6.8 x 10e11 |
| | | | | 20µl | - | 1 | <0.01 | 0.03 | 1.4 x 10e12 |
| | | | | 50µl | -/+ | 1 - 2 | 0.01 | 0.08 | 3.4 x 10e12 |
| N | BMM | 96 | 2 x 10e4 | 5µl | - | 1 | <0.01 | 0.02 | 1.6 x 10e12 |
| | | | | 10µl | - | 1 | 0.02 | 0.04 | 3.1 x 10e12 |
| | | | | 20µl | + | 2 | 0.03 | 0.08 | 6.3 x 10e12 |
| | | | | 50µl | - | 2 - 3 | 0.08 | 0.2 | 1.6 x 10e13 |
| O | BMM | 96 | 2 x 10e4 | 5µl | - | 1 | <0.01 | <0.01 | 1.5 x 10e11 |
| | | | | 10µl | - | 1 | <0.01 | <0.01 | 3.1 x 10e11 |
| | | | | 20µl | - | 1 | <0.01 | 0.01 | 6.2 x 10e11 |
| | | | | 50µl | + | 2 - 3 | <0.01 | 0.03 | 1.5 x 10e12 |
| P | BMM | 96 | 2 x 10e4 | 5µl | - | 1 | <0.01 | <0.01 | 1.7 x 10e11 |
| | | | | 10µl | - | 1 | <0.01 | <0.01 | 3.3 x 10e11 |
| | | | | 20µl | - | 1 | <0.01 | 0.02 | 6.7 x 10e11 |
| | | | | 50µl | + | 2 - 3 | <0.01 | 0.04 | 1.7 x 10e12 |
| Q | BMM | 96 | 2 x 10e4 | 5µl | - | 1 | <0.01 | <0.01 | 1.1 x 10e12 |
| | | | | 10µl | - | 1 | <0.01 | <0.01 | 2.2 x 10e12 |
| | | | | 20µl | - | 1 | <0.01 | 0.01 | 4.5 x 10e12 |
| | | | | 50µl | - | 3 | <0.01 | 0.03 | 1.1 x 10e13 |



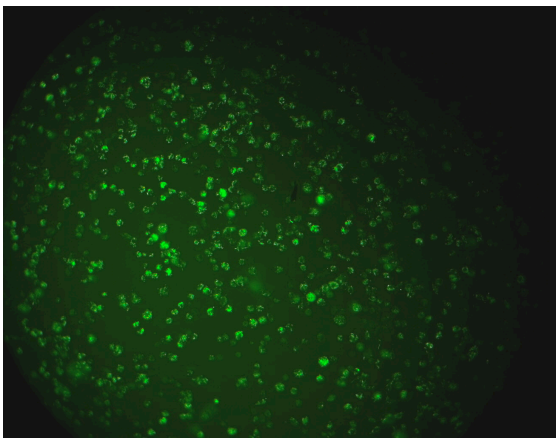
9A



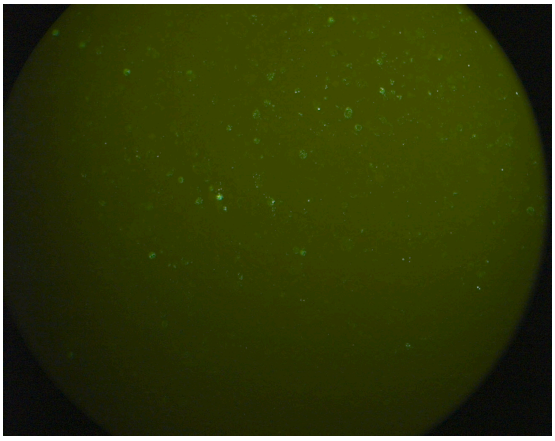
9B



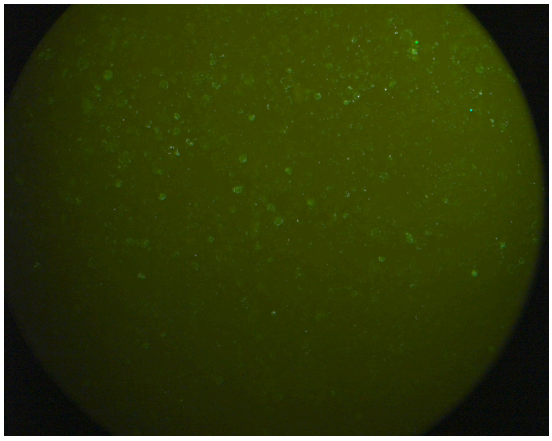
12A



12B



12C



12D

**Figure 9 and Figure 12**

**Figure 9:** Transfection of 293FT cells with the expression vector.
6 x $10^6$ cells were transfected with 3µg of (A) pLenti4-GFP/ΔV5 and (B) pLenti4-maxGFP, respectively. 36hrs after transfection, more than 90% of the cells were successfully transfected. The intensity of the GFP expression was considerably higher for cells transfected with pLenti4-maxGFP. Results for cells transfected with pLenti4-GFP/V5 were comparable to (A).

**Figure 12:** Transduction of primary macrophages.
2 x $10^5$ BMM were transduced with 40µl (A) and 120 µl (B) of Prep D, respectively. More than 90% of the cells were successfully transduced in both cases. However, cells inoculated with a higher volume showed a strongly impaired viability (B).
2 x $10^5$ PM were transduced with 40µl (C) and 80 µl (D) of Prep D, respectively. Approximately 40% (C) and 80% (D) cells showed a weak to moderate expression of GFP. In both cases an impairment of the cell viability was observed.

## 7. Discussion

The lentivirus technology is one of the most promising tools for gene delivery to primary cells. The generation of high-titer and non-toxic lentiviral stocks is crucial for the successful infection especially of differentiated, non-dividing cells. In this work we wanted to evaluate if a new lentiviral vector system – the ViraPower™ T-REx™ Lentiviral Expression System (Invitrogen) – meets demands for the routinely use in transduction of non-dividing cells.

The production of virus according to the manufacturer's protocol resulted in low titers, i.e. 5 - 9.7 x $10^3$ TU/ml. As a consequence, independent of the inoculum volume the calculated multiplicity of infection (MOI) was low (0.013-0.49) and the infected macrophages failed to express readily detectable GFP.

Due to the stability of VSV-G pseudotypes, the concentration by ultracentrifugation of HIV-1 based lentiviral vectors is widely used to augment titers [30]. We compared equally produced concentrated and unconcentrated preparations and observed both varying increase of the titer (4 - 55-fold) and recovery rates (7 - 91%). Our results are in accordance with published data, wherein the variability is attributed to the conditions of the concentration procedure and the size of the transfer vector [49-51].

The efficient production of lentivirus by means of the 3$^{rd}$ generation packaging was proven previously [28]. However, in some reports the direct comparison of the packaging systems showed clear advantages in favor of the 2$^{nd}$ generation packaging [50, 52]. To assess the influence of the packaging in our system we analyzed titer levels and the transduction capacity of virus preparations differing only in the applied packaging. We showed, that viruses transfected with the 2$^{nd}$ generation packaging achieved explicitly higher titers with an increase in the range of 9.5 – 33x compared to the 3$^{rd}$ generation group. The refinement in terms of biosafety by introducing an additional packaging plasmid might negatively influence virus particle production.

We determined the titer of our virus preparations by the selection of antibiotic-resistant, thus virus-DNA containing cell colonies. Selection for stable integration of virus-DNA is based and therefore dependent on transduction of the cells used for titering. The conditions of the transduction process (e.g. inoculum volume, sensitivity of target cells, cell density, incubation period) are crucial for titer levels and the subsequent calculation

of the MOI. More than 50-fold difference in titer level or MOI was reported for the same vector stock using different cell numbers or incoculum volumes [53].
Since HT1080 cells were recommended by the original protocol for optimal titration results we used 2fTGH cells, descending from HT1080, as the standard cell line for the titration experiments. However, the titration of the same virus preparations with HeLa cells revealed reproducibly higher titer levels (18.3–fold, median value) leading to strong discrepancies between calculated MOI values dependent on the cell line used for titration. Thus, with this titering method we could not specify standard MOI values, which guarantee reproducible transduction results.
Alternatively, p24 antigen ELISA or RT-PCR-analysis of virus-DNA and –RNA in transduced cells or supernatants, respectively, are routinely used for titer determination. Titers assessed by DNA-analysis provide a reliable estimate, whereas RNA analysis as well as p24 titers are rather poor in predicting transduction efficiency, due to overestimation by non-functional vector particles [54, 55].
As an alternative method to assess the quality of our virus preparations we applied the PERT assay analysis for quantitative detection of the RT activity [56, 57]. The RT activity was measured directly on the harvested viral supernatant, thus avoiding influences by cell transduction. By transducing primary cells (BMM and PM) we revealed moderate to strong GFP expression, if cells were inoculated with at least 1 x $10^{14}$ pU of RT activity. Furthermore, beneath an activity of $10^{13}$ pU the cell viability was not essentially impaired. With increasing activity the cell health dropped drastically, which referred rather to the increase of the inoculum volume than to RT activity levels. In our study the PERT assay turned out to be a valuable tool to assess, if a virus preparation fulfills the requirements for the efficient transduction of macrophages, i.e. transduction with a minimum of 1 x $10^{14}$ pU of RT activity in a preferably small inoculum volume.

In summary, we showed the successful transduction of primary macrophages, provided that the transduction occurs with a minimum RT activity. For the routinely use of this lentiviral expression system the optimization of the original protocol is indispensible. Virus preparations must be prepared in a large scale to assure sufficient amounts following the concentration of the virus. A repeated concentration of the virus might be required in order to transduce sensitive cells with small volumes of highly concentrated

virus. Furthermore, we showed, that the measurement of the RT activity in the viral supernatants by the PERT analysis proved to be a time and cost saving tool for the reliable prediction of the transduction efficiency.

## 8. References

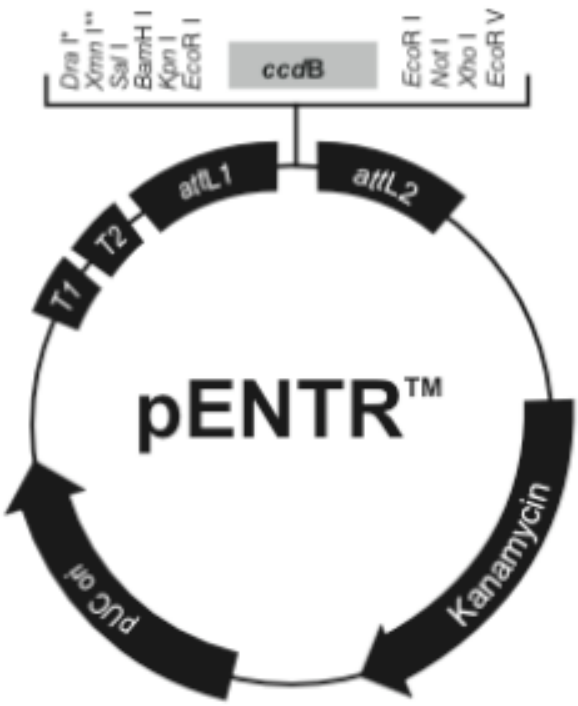

1. Coffin, J.M., S.H. Hughes, and H.E. Varmus, *Retroviruses*, ed. J.M. Coffin, S.H. Hughes, and H.E. Varmus. 1997, New York: Cold Spring Harbor Laboratory Press.
2. Federico, M., *From Lentiviruses to Lentivirus Vectors*, in *Lentivirus Gene Engineering Protocols*, M. Federico, Editor. 2003, Humana Press: Clifton, N.J. p. 3-15.
3. Klimatcheva, E., J.D. Rosenblatt, and V. Planelles, *Lentiviral vectors and gene therapy.* Front Biosci, 1999. **4**: p. D481-96.
4. Masuda, T., M.J. Kuroda, and S. Harada, *Specific and independent recognition of U3 and U5 att sites by human immunodeficiency virus type 1 integrase in vivo.* J Virol, 1998. **72**(10): p. 8396-402.
5. Freed, E.O., *HIV-1 gag proteins: diverse functions in the virus life cycle.* Virology, 1998. **251**(1): p. 1-15.
6. Wyatt, R. and J. Sodroski, *The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens.* Science, 1998. **280**(5371): p. 1884-8.
7. Freed, E.O., *HIV-1 replication.* Somat Cell Mol Genet, 2001. **26**(1-6): p. 13-33.
8. Jones, K.A., *Taking a new TAK on tat transactivation.* Genes Dev, 1997. **11**(20): p. 2593-9.
9. Brady, J. and F. Kashanchi, *Tat gets the "green" light on transcription initiation.* Retrovirology, 2005. **2**: p. 69.
10. Purcell, D.F. and M.A. Martin, *Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity.* J Virol, 1993. **67**(11): p. 6365-78.
11. Cullen, B.R., *Regulation of HIV-1 gene expression.* FASEB J, 1991. **5**(10): p. 2361-8.
12. Lake, J.A., et al., *The role of Vif during HIV-1 infection: interaction with novel host cellular factors.* J Clin Virol, 2003. **26**(2): p. 143-52.
13. Le Rouzic, E. and S. Benichou, *The Vpr protein from HIV-1: distinct roles along the viral life cycle.* Retrovirology, 2005. **2**: p. 11.
14. Nomaguchi, M., M. Fujita, and A. Adachi, *Role of HIV-1 Vpu protein for virus spread and pathogenesis.* Microbes Infect, 2008. **10**(9): p. 960-7.
15. Margottin, F., et al., *A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif.* Mol Cell, 1998. **1**(4): p. 565-74.
16. Foster, J.L. and J.V. Garcia, *HIV-1 Nef: at the crossroads.* Retrovirology, 2008. **5**: p. 84.
17. Clapham, P.R. and A. McKnight, *HIV-1 receptors and cell tropism.* Br Med Bull, 2001. **58**: p. 43-59.
18. Clapham, P.R. and A. McKnight, *Cell surface receptors, virus entry and tropism of primate lentiviruses.* J Gen Virol, 2002. **83**(Pt 8): p. 1809-29.
19. Weissenhorn, W., A. Hinz, and Y. Gaudin, *Virus membrane fusion.* FEBS Lett, 2007. **581**(11): p. 2150-5.
20. Sherman, M.P. and W.C. Greene, *Slipping through the door: HIV entry into the nucleus.* Microbes Infect, 2002. **4**(1): p. 67-73.
21. Delelis, O., et al., *Integrase and integration: biochemical activities of HIV-1 integrase.* Retrovirology, 2008. **5**: p. 114.

22. Quinonez, R. and R.E. Sutton, *Lentiviral vectors for gene delivery into cells.* DNA Cell Biol, 2002. **21**(12): p. 937-51.
23. Lever, A.M., P.M. Strappe, and J. Zhao, *Lentiviral vectors.* J Biomed Sci, 2004. **11**(4): p. 439-49.
24. Kafri, T., *Gene delivery by lentivirus vectors an overview.* Methods Mol Biol, 2004. **246**: p. 367-90.
25. Kappes, J.C. and X. Wu, *Safety considerations in vector development.* Somat Cell Mol Genet, 2001. **26**(1-6): p. 147-58.
26. Naldini, L., et al., *In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector.* Science, 1996. **272**(5259): p. 263-7.
27. Zufferey, R., et al., *Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo.* Nat Biotechnol, 1997. **15**(9): p. 871-5.
28. Dull, T., et al., *A third-generation lentivirus vector with a conditional packaging system.* J Virol, 1998. **72**(11): p. 8463-71.
29. Srinivasakumar, N., *HIV-1 vector systems.* Somat Cell Mol Genet, 2001. **26**(1-6): p. 51-81.
30. Burns, J.C., et al., *Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 8033-7.
31. Cronin, J., X.Y. Zhang, and J. Reiser, *Altering the tropism of lentiviral vectors through pseudotyping.* Curr Gene Ther, 2005. **5**(4): p. 387-98.
32. Vodicka, M.A., *Determinants for lentiviral infection of non-dividing cells.* Somat Cell Mol Genet, 2001. **26**(1-6): p. 35-49.
33. Bukrinsky, M.I., et al., *A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells.* Nature, 1993. **365**(6447): p. 666-9.
34. von Schwedler, U., R.S. Kornbluth, and D. Trono, *The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes.* Proc Natl Acad Sci U S A, 1994. **91**(15): p. 6992-6.
35. Haffar, O.K., et al., *Two nuclear localization signals in the HIV-1 matrix protein regulate nuclear import of the HIV-1 pre-integration complex.* J Mol Biol, 2000. **299**(2): p. 359-68.
36. Connor, R.I., et al., *Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes.* Virology, 1995. **206**(2): p. 935-44.
37. Gallay, P., et al., *HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9825-30.
38. Bouyac-Bertoia, M., et al., *HIV-1 infection requires a functional integrase NLS.* Mol Cell, 2001. **7**(5): p. 1025-35.
39. Zennou, V., et al., *HIV-1 genome nuclear import is mediated by a central DNA flap.* Cell, 2000. **101**(2): p. 173-85.
40. Dvorin, J.D., et al., *Reassessment of the roles of integrase and the central DNA flap in human immunodeficiency virus type 1 nuclear import.* J Virol, 2002. **76**(23): p. 12087-96.
41. Yamashita, M. and M. Emerman, *The cell cycle independence of HIV infections is not determined by known karyophilic viral elements.* PLoS Pathog, 2005. **1**(3): p. e18.

42. Yamashita, M. and M. Emerman, *Retroviral infection of non-dividing cells: old and new perspectives.* Virology, 2006. **344**(1): p. 88-93.
43. Heim, R., A.B. Cubitt, and R.Y. Tsien, *Improved green fluorescence.* Nature, 1995. **373**(6516): p. 663-4.
44. Heimbucher, T., et al., *Gbx2 and Otx2 interact with the WD40 domain of Groucho/Tle corepressors.* Mol Cell Biol, 2007. **27**(1): p. 340-51.
45. Pellegrini, S., et al., *Use of a selectable marker regulated by alpha interferon to obtain mutations in the signaling pathway.* Mol Cell Biol, 1989. **9**(11): p. 4605-12.
46. Baccarini, M., F. Bistoni, and M.L. Lohmann-Matthes, *In vitro natural cell-mediated cytotoxicity against Candida albicans: macrophage precursors as effector cells.* J Immunol, 1985. **134**(4): p. 2658-65.
47. Pyra, H., J. Boni, and J. Schupbach, *Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement.* Proc Natl Acad Sci U S A, 1994. **91**(4): p. 1544-8.
48. Davis, H.E., J.R. Morgan, and M.L. Yarmush, *Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes.* Biophys Chem, 2002. **97**(2-3): p. 159-72.
49. Geraerts, M., et al., *Upscaling of lentiviral vector production by tangential flow filtration.* J Gene Med, 2005. **7**(10): p. 1299-310.
50. al Yacoub, N., et al., *Optimized production and concentration of lentiviral vectors containing large inserts.* J Gene Med, 2007. **9**(7): p. 579-84.
51. Zhang, B., et al., *A highly efficient and consistent method for harvesting large volumes of high-titre lentiviral vectors.* Gene Ther, 2001. **8**(22): p. 1745-51.
52. Siapati, E.K., et al., *Comparison of HIV- and EIAV-based vectors on their efficiency in transducing murine and human hematopoietic repopulating cells.* Mol Ther, 2005. **12**(3): p. 537-46.
53. Zhang, B., et al., *The significance of controlled conditions in lentiviral vector titration and in the use of multiplicity of infection (MOI) for predicting gene transfer events.* Genet Vaccines Ther, 2004. **2**(1): p. 6.
54. Geraerts, M., et al., *Comparison of lentiviral vector titration methods.* BMC Biotechnol, 2006. **6**: p. 34.
55. Sastry, L., et al., *Titering lentiviral vectors: comparison of DNA, RNA and marker expression methods.* Gene Ther, 2002. **9**(17): p. 1155-62.
56. Sastry, L., et al., *Product-enhanced reverse transcriptase assay for replication-competent retrovirus and lentivirus detection.* Hum Gene Ther, 2005. **16**(10): p. 1227-36.
57. Andre, M., S. Morgeaux, and F. Fuchs, *Quantitative detection of RT activity by PERT assay: feasibility and limits to a standardized screening assay for human vaccines.* Biologicals, 2000. **28**(2): p. 67-80.

# 9. Appendix

## 9.1. Vector map of pENTR ™

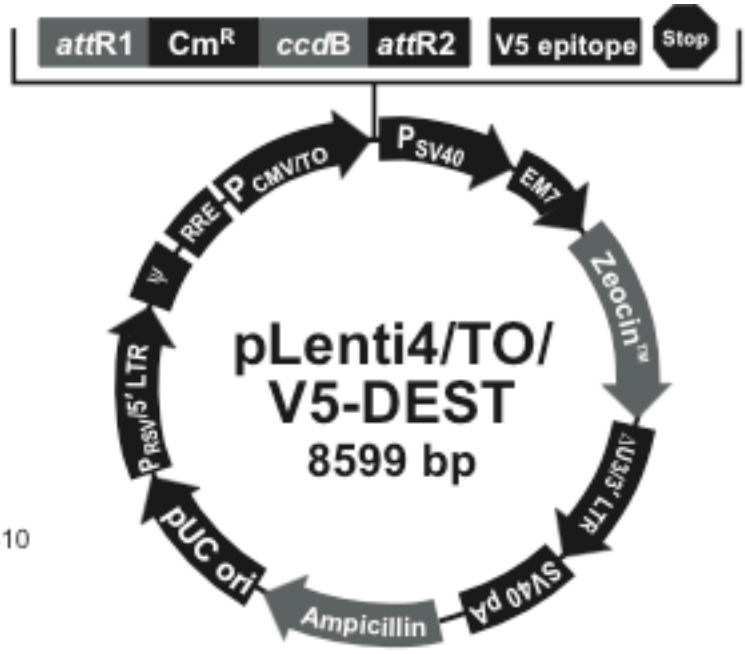



**Comments for pENTR™1A**
**2717 nucleotides**

*rrn*B T1 transcription termination sequence: bases 106-149
*rrn*B T2 transcription termination sequence: bases 281-308
*attL*1: bases 358-457 (complementary strand)
*ccd*B gene: bases 612-917
*attL*2: bases 946-1045
Kanamycin resistance gene: bases 1168-1977
pUC origin: bases 2041-2714

Vector map provided by Invitrogen (www.invitrogen.com)

## 9.2. Vector map of pLenti4/TO/V5-DEST




**Comments for pLenti4/TO/V5-DEST**
**8599 nucleotides**

RSV/5′ LTR hybrid promoter: bases 1-410
- RSV promoter: bases 1-229
- HIV-1 5′ LTR: bases 230-410

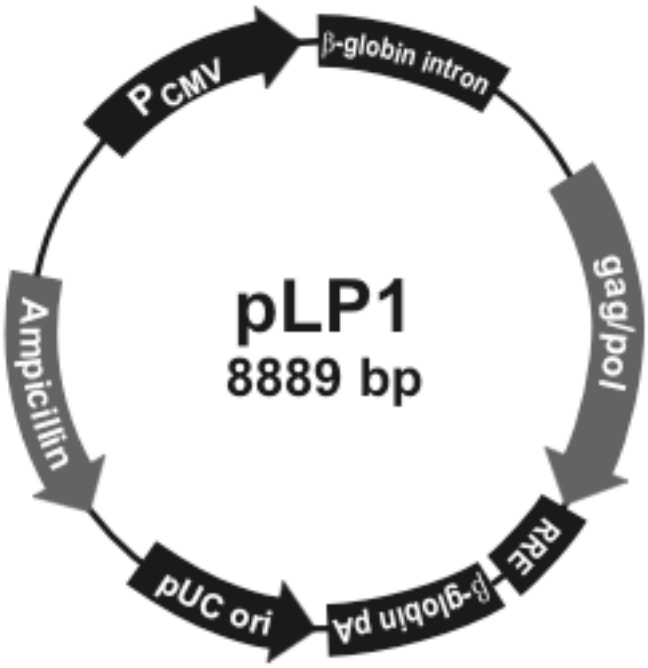
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
CMV/TO promoter: bases 1809-2364
- CMV promoter: bases 1809-2309
- TATA box: bases 2309-2315
- Tetracycline operator (2X $TetO_2$) sequences: bases 2325-2364

*att*R1 site: bases 2405-2529
Chloramphenicol resistance gene ($Cm^R$): bases 2638-3297
*ccd*B gene: bases 3639-3944
*att*R2 site: bases 3985-4109
V5 epitope: bases 4162-4203
SV40 early promoter and origin: bases 4258-4567
EM7 promoter: bases 4586-4652
Zeocin™ resistance gene: bases 4653-5027
ΔU3/3′ LTR: bases 5119-5353
- ΔU3: bases 5119-5172
- 3′ LTR: bases 5173-5353

SV40 polyadenylation signal: bases 5425-5559
*bla* promoter: bases 6415-6513
Ampicillin (*bla*) resistance gene: bases 6514-7374
pUC origin: bases 7519-8192

Vector map provided by Invitrogen (www.invitrogen.com)

## 9.3. Vector map of pLP1




**Comments for pLP1**
**8889 nucleotides**

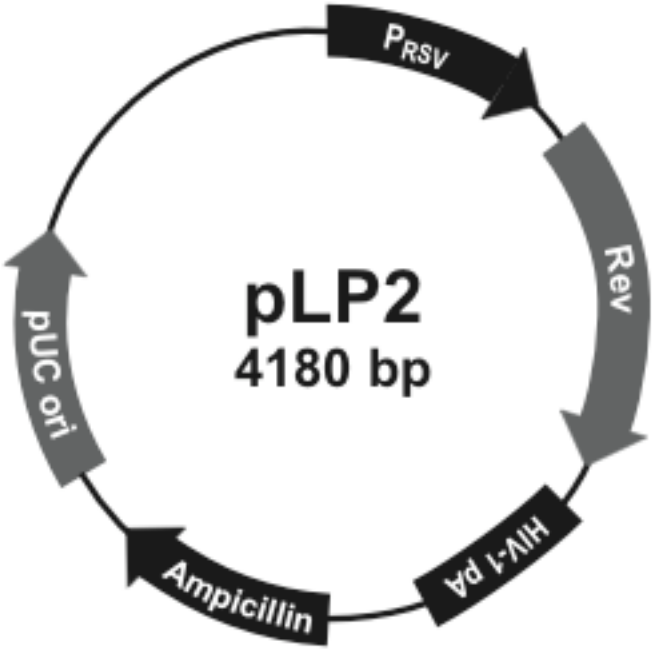
CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
HIV-1 gag/pol sequences: bases 1355-5661
gag coding sequence: bases 1355-2857
gag/pol frameshift: base 2650
pol coding sequence: bases 2650-5661
HIV-1 Rev response element (RRE): bases 5686-5919
Human β-globin polyadenylation signal: bases 6072-6837
pUC origin: bases 6995-7668 (C)
Ampicillin (*bla*) resistance gene: bases 7813-8673 (C)
*bla* promoter: bases 8674-8772 (C)

C=complementary strand

EcoRI cuts at bases 1333, 5668, and 6069

Vector map provided by Invitrogen (www.invitrogen.com)

## 9.4. Vector map of pLP2



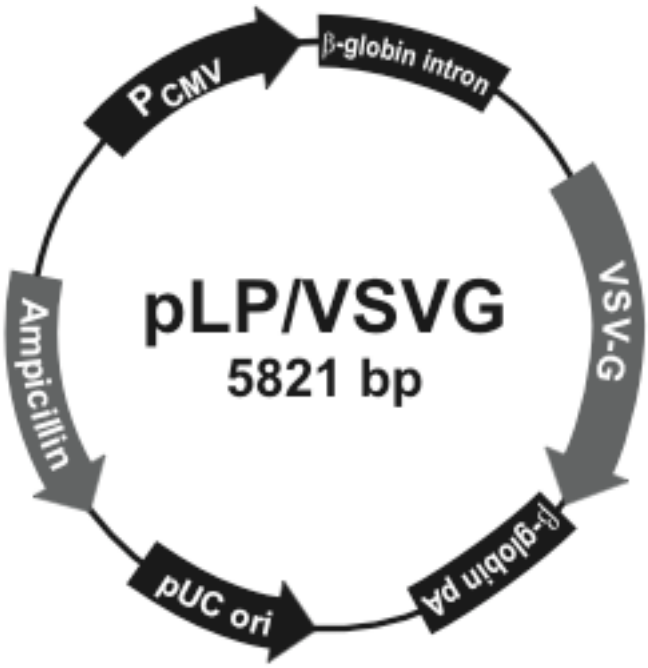

**Comments for pLP2**
**4180 nucleotides**

RSV enhancer/promoter: bases 1-271
TATA box: bases 200-207
Transcription initiation site: base 229
RSV UTR: bases 230-271
HIV-1 Rev ORF: bases 391-741
HIV-1 LTR polyadenylation signal: bases 850-971
*bla* promoter: bases 1916-2014
Ampicillin (*bla*) resistance gene: bases 2015-2875
pUC origin: bases 3020-3693

EcoRI cuts at bases 176 and 4045

Vector map provided by Invitrogen (www.invitrogen.com)

## 9.5. Vector map of pLP/VSVG




**Comments for pLP/VSVG**
**5821 nucleotides**

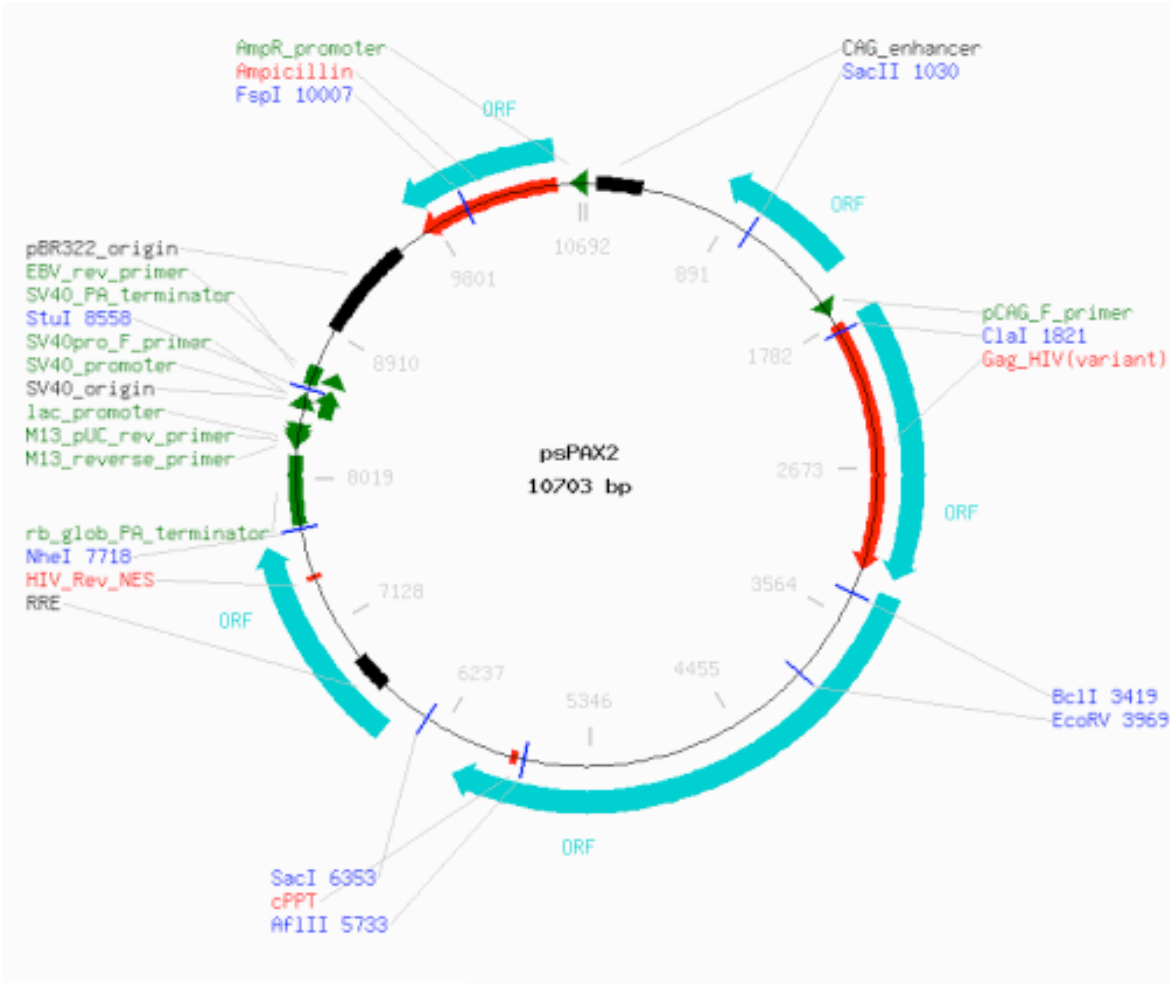
CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
VSV G glycoprotein (VSV-G): bases 1346-2881
Human β-globin polyadenylation signal: bases 3004-3769
pUC origin: bases 3927-4600 (C)
Ampicillin (*bla*) resistance gene: bases 4745-5605 (C)
*bla* promoter: bases 5606-5704 (C)

C=complementary strand

EcoRI cuts at bases 1333 and 3001

Vector map provided by Invitrogen (www.invitrogen.com)

## 9.6. Vector map of psPAX2

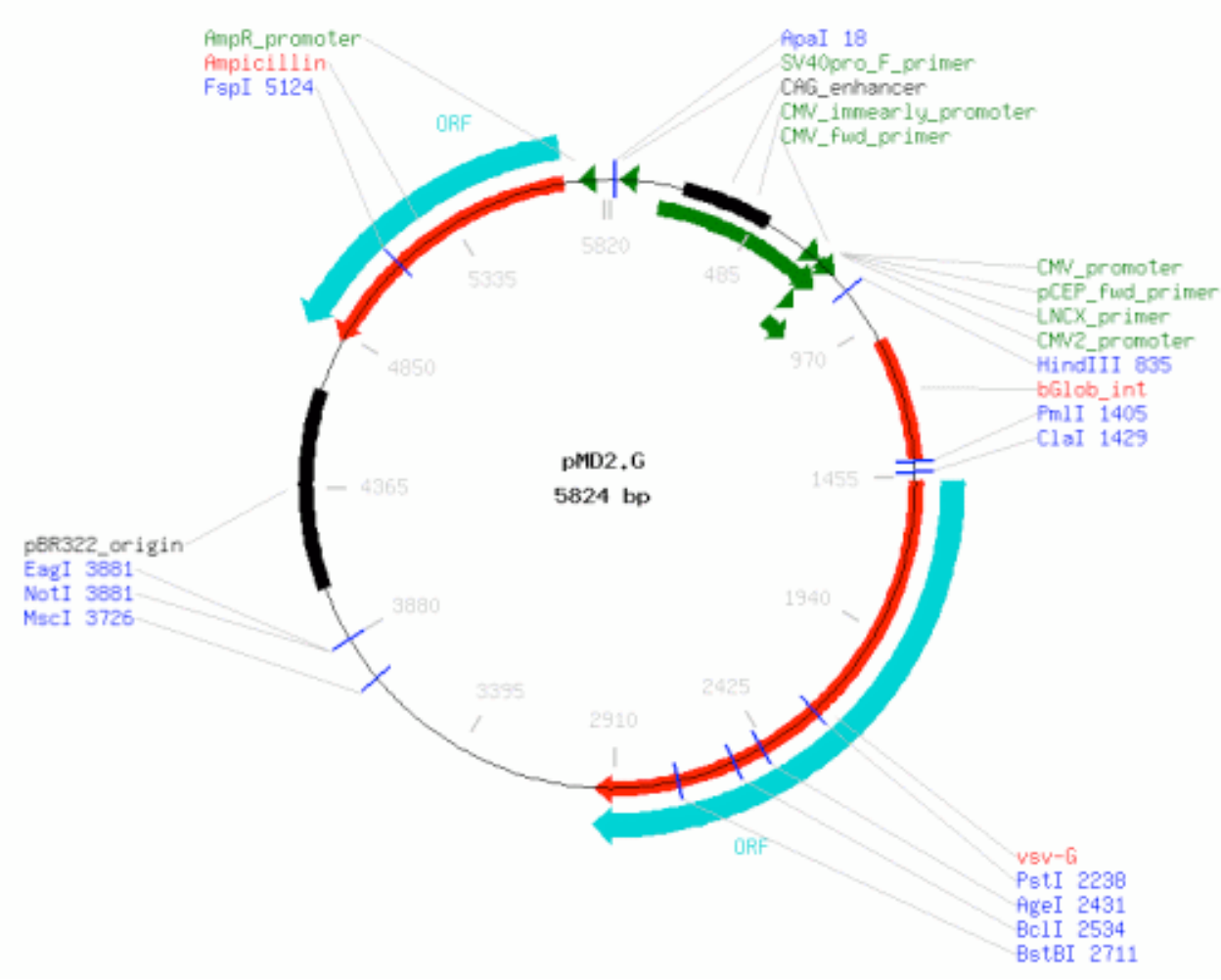



Selected features

| | |
|---|---|
| CAG_enhancer | 84 - 371 |
| ORF frame 1 | 1533 - 802 |
| pCAG_F_primer | 1678 - 1697 |
| ORF frame 1 | 1780 - 3282 |
| Gag_HIV(variant) | 1780 - 3282 |
| ORF frame 3 | 3348 - 6086 |
| cPPT | 5775 - 5790 |
| RRE | 6660 - 6893 |
| ORF frame 1 | 6538 - 7686 |
| HIV_Rev_NES | 7416 - 7445 |
| rb_glob_PA_terminator | 7731 - 8160 |
| M13_reverse_primer | 8220 - 8202 |
| M13_pUC_rev_primer | 8241 - 8219 |
| lac_promoter | 8284 - 8255 |
| SV40_origin | 8425 - 8502 |
| SV40_promoter | 8390 - 8569 |
| SV40pro_F_primer | 8487 - 8506 |
| SV40_PA_terminator | 8583 - 8714 |
| EBV_rev_primer | 8671 - 8690 |
| pBR322_origin | 9557 - 8938 |
| ORF frame 1 | 10572 - 9712 |
| Ampicillin | 10572 - 9712 |
| AmpR_promoter | 10642 - 10614 |

Vector map provided by Addgene Vector Database (http://www.addgene.org/12260)

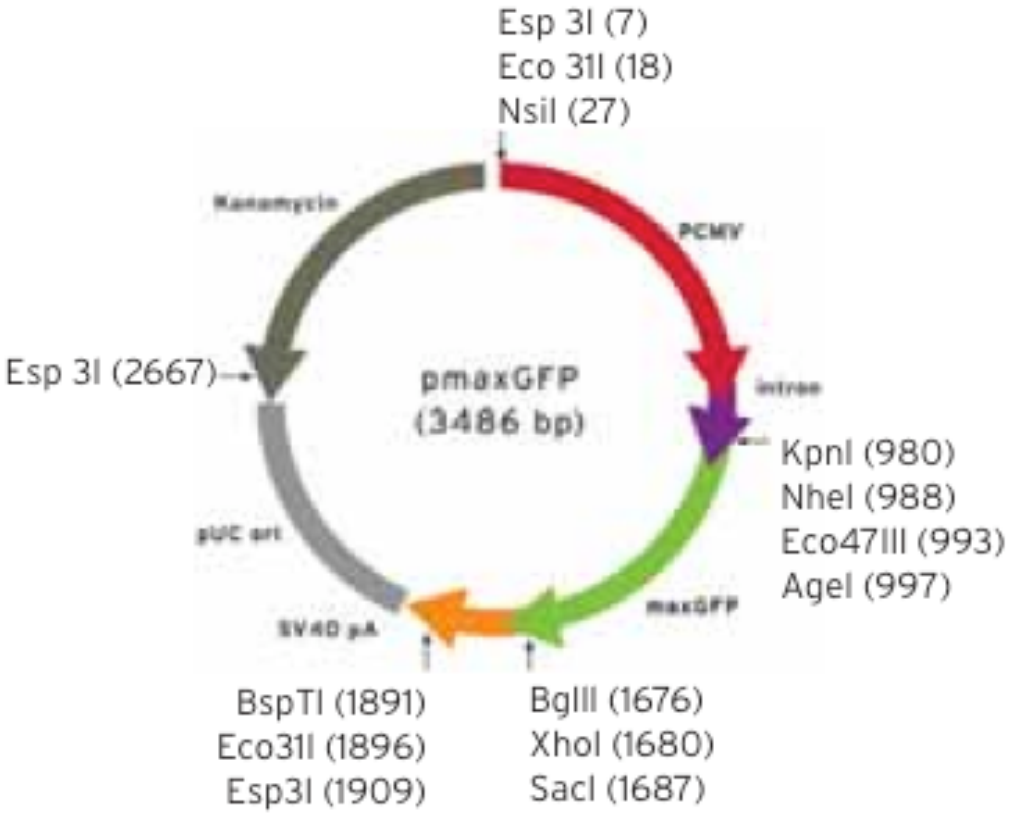
## 9.7. Vector map of pMD2.G




| Selected features | |
|---|---|
| SV40pro_F_primer | 59 - 40 |
| CAG_enhancer | 241 - 528 |
| CMV_immearly_promoter | 162 - 738 |
| CMV_fwd_primer | 695 - 715 |
| CMV_promoter | 696 - 765 |
| pCEP_fwd_primer | 739 - 758 |
| LNCX_primer | 741 - 765 |
| CMV2_promoter | 708 - 827 |
| bGlob_int | 1015 - 1398 |
| vsv-G | 1450 - 2973 |
| ORF frame 1 | 1450 - 2985 |
| pBR322_origin | 4674 - 4055 |
| ORF frame 2 | 5689 - 4829 |
| Ampicillin | 5689 - 4829 |
| AmpR_promoter | 5759 - 5731 |

Vector map provided by Addgene Vector Database (http://www.addgene.org/12259)

## 9.8. Vector map of pmaxGFP




Vector map provided by Lonza (www.lonzabio.com)

# 10. Danksagung

Herrn o. Univ. Prof. Dr. Thomas Decker danke ich für die Betreuung und Begutachtung dieser Diplomarbeit.
Herrn o. Univ. Prof. Dr. Mathias Müller möchte ich für die Bereitstellung des Themas, für die Ermöglichung meiner Diplomarbeit am Institut für Tierzucht und Genetik und die Korrektur dieser Arbeit herzlich danken.
Mein besonderer Dank gilt Frau Dr. Ursula Reichart, die mich intensiv betreut hat. Vor allem hat sie mein Interesse an der Arbeit mit Labortieren geweckt und mir alle dafür notwendigen Techniken beigebracht. Sehr dankbar bin ich auch für die gewissenhafte und umgehende Korrektur dieser Arbeit.
Vielen Dank auch an Mag. Rita Stiefvater, Dr. Nicole Leitner und Dr. Birgit Strobl, die mir bei Problemen aller Art zur Seite gestanden sind und mich von ihren Erfahrungen profitieren ließen.
Weiters danke ich Ass. Prof. Dr. Simone Müller, ohne deren Bemühungen ich vermutlich nie an dieses Institut gekommen ware.
Bei allen Kollegen der Tierzucht bedanke ich mich für ihre Hilfsbereitschaft, das ausgesprochen gute Arbeitsklima und die netten gemeinsam verbrachten Stunden. Auch der Arbeitsgruppe von Dr. Thomas Czerny bin ich zu Dank verpflichtet. Sie haben mich von Anfang an herzlich aufgenommen, mir bei verschiedensten Dingen geholfen und mit selbstgemachten Köstlichkeiten verpflegt.
Herzlichen Dank auch an o. Univ. Prof. Dr. Walter Baumgartner, der mir gestattete mich mit “seiner” Schafherde zu befassen und damit meine Leidenschaft für die Wissenschaften der kleinen Wiederkäuer entfacht hat. Die Arbeit mit den Schafen war und ist für mich ein wunderbarer und erfüllender Ausgleich zum Laboralltag.

Danke an meine Familie und Sebastian Schnell und Familie für die vielfältige Unterstützung und das Verständnis.

Diese Diplomarbeit möchte ich meiner Mutter Ingid Spiesberger widmen, die als einzige immer für mich da war, mich motiviert hat und mir alles ermöglicht hat. Danke, eine bessere Mutter hätte ich mir nicht wünschen können.

# 11. Lebenslauf

| | | |
|---|---|---|
| Name: | | Katrin Anna Christina Spiesberger |
| Geboren: | | 08.09.1984 in Wien 9 |
| Schule: | 1990-94 | Volksschule “Schubertschule”, Wien 9 |
| | 1994-2002 | Humanistisches Gymnasium, Wasagasse, Wien 9 |
| Universität: | 09/2002 | Beginn des Diplomstudiums der Biologie an der Universität Wien |
| | 04/2004 | 1. Diplomprüfung und Spezialisierung auf “Genetik und Mikrobiologie” |
| | 04/2008 | Beginn der Diplomarbeit am Institut für Tierzucht und Genetik an der Veterinärmedizinischen Universität Wien |