Generation of a packaging cell line for the production of gamma retroviral vectors.

Profile research project for GSLS master Drug Innovation
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0. Layman's Abstract

CAR T-cell therapy is a innovative cancer therapy that leverages the body's immune system to combat cancer. This therapy begins with the collection of patient's blood, after which a type of white blood cells, named T cells, are isolated. These cells are an essential part of the immune system, responsible for identifying and destroying harmful cells, such as those infected by viruses or cancerous cells. However, cancer cells are sometimes able to evade detection, making it difficult to target and eliminate them. To overcome this challenge the chimeric antigen receptor (CAR) was developed, to enhance the cancer fighting capabilities of T cells. For CAR T-cell therapy, the isolated T-cells are genetically modified to express the CAR. This receptor is designed specifically to recognize and bind to a protein found on the surface of cancer cells. After this modification the CAR T-cells are introduced back into the patient to combat the cancer.

Even though CAR T-cell therapy has revolutionized the treatment of cancer, the complex coordination between pharmaceutical companies and academic hospitals leads to long production times and high costs. Academic hospitals, therefore, could benefit from producing CAR-T cell therapy in-house. One way to genetically modify CAR-T cells is by using gamma retroviral vectors. Gamma retroviral vectors are viruses modified to safely "infect" T cells and genetically equip them with the CAR, without causing harm. In our study, we aimed to produce a packaging cell line, which are cells that act as small biological factories to produce these viruses. We compared our packaging cell line to a commercially available cell line. We could see that our packaging cell line's virus production and T-cell modification capabilities were similar to the commercial cell line. This indicates that our cell line holds potential for large-scale production of gamma retroviral vectors for CAR-T cell therapy.

1. Abstract

The usage of chimeric antigen receptor (CAR) T cell therapy has revolutionized the treatment of hematological malignancies, but its application still faces logistical challenges. These challenges, involve the complex coordination required between hospitals and pharmaceutical companies, resulting in longer production times and high costs. Academic hospitals would therefore benefit from the production of in-house CAR-T cell therapies. Gamma retroviral vectors are well-studied vectors for the transduction of T cells. The production of gamma retroviral vectors is preferred in a packaging cell line due to scalability and simplified production processes. In this study it was aimed to generate a packaging cell line for the production of gamma retroviral vectors at academic hospital sites. We transfected HEK293T cells with helper expression plasmids pRD114.EGFP.PuroR and pGagPol.mCherry.PuroR, followed by selection using Puromycin and sorting of eGFP and mCherry positive cells through fluorescent activated cell sorting (FACS). This process lead to the production of our HEK293T-PACK cell line. Our HEK293T-PACK cell line generated gamma retroviral vector particles that effectively transduced Jurkat cells and outperformed those produced by the commercially available 293Vec-RD114 packaging cell line. This indicates that our packaging cell line holds the potential for large-scale production of gamma retroviral vector for CAR-T cell therapy.

Keywords:

chimeric antigen receptor (CAR), CAR-T cell, gamma retroviral vector, murine leukemia virus (MLV), GagPol, RD114, 293Vec-RD114, antibiotic selection, FACS, stable cell line.

2. Abbreviation list

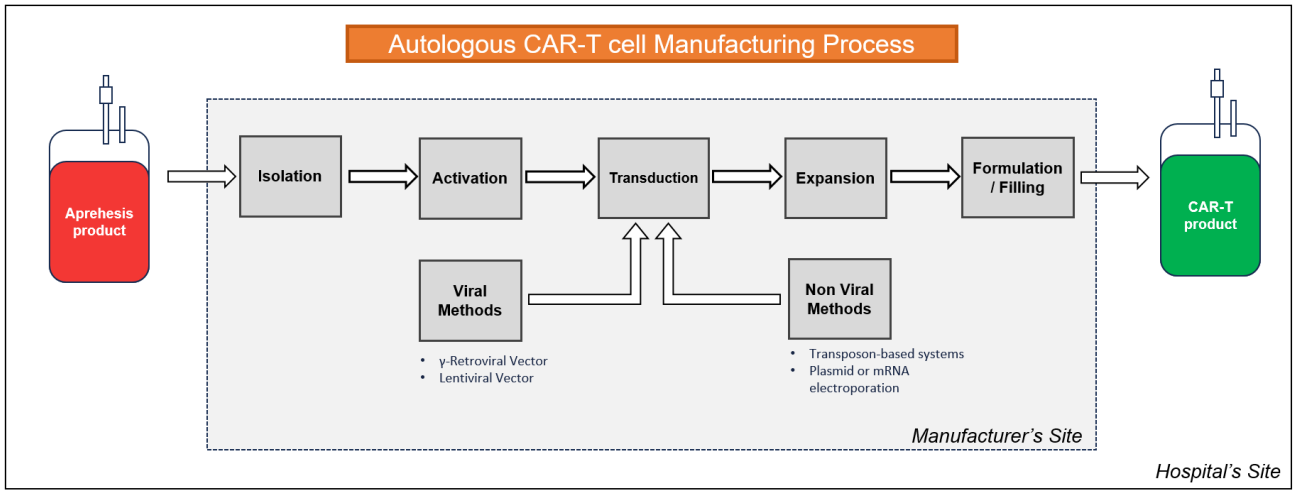
ALL	acute lymphocytic leukemia
BleoR	phleomycin/zeocin-binding protein
DMEM	Dulbecco's modified eagle medium
eGFP	enhanced green fluorescent protein
ENV	envelope
CAR	chimeric antigen receptor
CMV	Cytomegalovirus
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCS	frontward scatter
HSCT	hematopoietic stem cell transplantation
HygR	aminoglycoside phosphotransferase gene
LTR	long terminal repeats
MLV	murine leukemia virus
MM	multiple myeloma
NEB	New England Biolabs
NHL	non-Hodgkin lymphoma
PBMCs	peripheral blood mononuclear cells
PBS	primer binding site
PPT	polypurine tract
PS	penicillin and streptomycin
PuroR	puromycin N-acetyl-transferase gene
RCR	replication competent retroviruses
RT	room temperature
SSC	sideward scatter
SV40	simian-virus 40
WRPE	woodchuck hepatitis virus post-transcriptional regulatory element
ψ	packaging signal

3. Introduction

3.1. CAR-T Cell Therapy: Progress and Challenges

The treatment of hematological malignancies has advanced significantly over the years, yet these diseases remain a major cause of cancer-related deaths worldwide (Hemminki et al., 2023). Traditionally, treatment methods have included radiotherapy, hematopoietic stem cell transplantation (HSCT), and chemotherapy. However, with a deeper understanding of tumor immunology and the molecular mechanisms underlying immune cell and cancer cell interactions, new immunotherapy approaches have emerged. One particularly revolutionary approach is the transfer of cytotoxic T cells that are genetically modified to express a chimeric antigen receptors (CAR; Larson et al., 2021; Abbasi et al., 2022). A CAR is an artificial hybrid receptor, wherein T-cell signaling domains are fused to a antigen recognizing monoclonal antibody, allowing the CAR-T cell to recognize proteins or antigens found on cancer cells (Linder et al., 2020). This method has transformed the treatment landscape for conditions such as acute lymphocytic leukemia (ALL), non-Hodgkin lymphoma (NHL), and multiple myeloma (MM) (Zhang et al., 2022; Wang et al., 2023). Yet, despite the fast rise in approved therapies, autologous CAR-T cell treatment faces logistical challenges. These challenges are shown in the complex coordination required for CAR-T cell therapy between hospitals and pharmaceutical companies, leading to longer production times and higher costs (See figure 1; Zhang et al., 2022; Elsallab et al., 2023; Wang et al., 2023;). In short, for the production of autologous CAR-T cell therapy, patient peripheral blood mononuclear cells (PBMCs) are harvested in the hospital. The patient material is then sent to the manufacturers site, where the T-cells of the patient's PBMCs are isolated and activated. Following this, the T-cells undergo transduction, during which the genetic material of the CAR sequence is integrated into the cells. These cells are then expanded and assessed by quality control testing. Afterwards, the final CAR-T cell product is ready to be injected back into the patient (Vormittag et al., 2018).

Figure 1. Flow Scheme of the CAR-T Cell Manufacturing Process. *Adapted from Vormittag et al. (2018)*



3.2. Gamma Retroviral Vectors Transduction for CAR-T Cell Production

The transduction of the T cells can be achieved through either non-viral or viral methods (Lukjanov et al., 2021). The most common alternative to viral methods are transposon methods, with the Sleeping Beauty and PiggyBac transposon-based systems being the most promising in clinical trials (Feldman et al., 2014; Prommersberger et al., 2021, Singh et al., 2022; Yagu & Nakazawa, 2023). These non-viral methods are advantageous over viral methods due to their lower risk of insertional mutagenesis. As their integration is random, which reduces the risk of integration near gene-regulatory region (Lukjanov et al., 2021). However, lentiviral and retroviral vectors remain the preferred methods for T cell transduction in approved therapies and new clinical trials, due to their high transduction efficiency and well-established effectiveness (Wang et al., 2023).

Retroviral vectors are derived from the *Retroviridae* family. They are single stranded RNA viruses that reverse transcribe their genome into double stranded DNA, which then can be stably integrated into the host cell genome. Gamma retroviral vectors are specifically derived from the Murine Leukemia Virus (MLV). The reverse transcribed and integrated proviral DNA of MLV is flanked by long terminal repeats (LTRs) structured into the U3, R, and U5 regions (See figure 2). Transcription starts at the 5′ U3 region, producing RNA that includes the primer binding site (PBS), major splice donor (SD), and packaging signal (ψ) upstream of the Gag/Pol coding region, encoding for structural and enzymatic proteins. Downstream of the Gag/Pol coding region, the envelope (ENV) gene is expressed via a splice acceptor in Pol, and the 3′untranslated region, including the polypurine tract (PPT) and polyadenylation signal. To develop a safe viral vector and avoid the creation of replication-competent retroviruses (RCR), the Gag/Pol gene and ENV gene must be separated from the retroviral genome. This is achieved through a split packaging design (See Figure 2). The transfer plasmid retains the ψ, PBS, and LTRs but carries the gene of interest instead of the structural and enzymatic genes. The Gag/Pol and ENV genes are supplied by separate helper expression plasmids, which lack the ψ, reducing the risk of a recombination event (Miller et al., 1990; Maetzig et al., 2011).

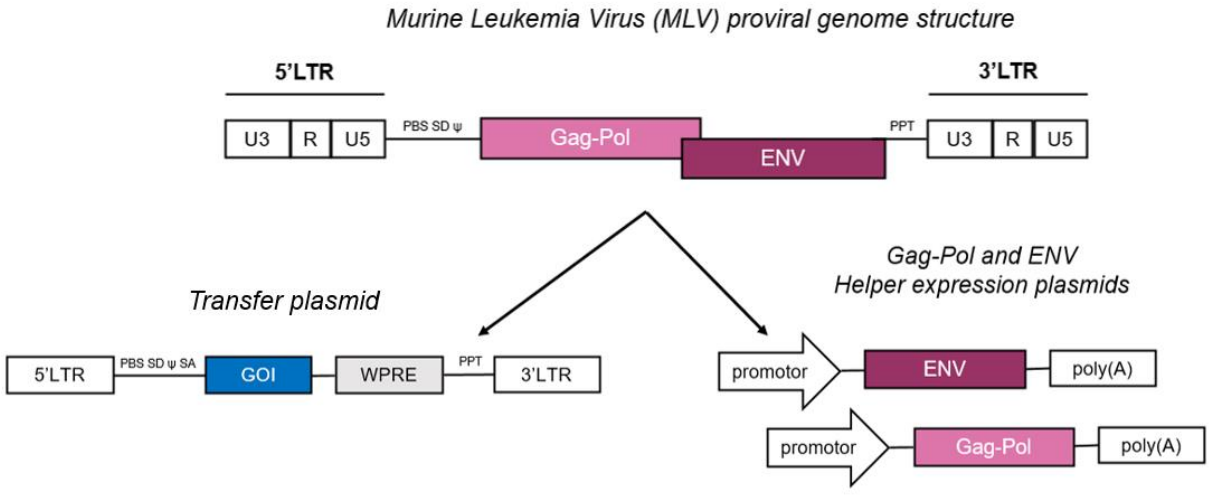


Figure 2. Split packaging design for gamma retroviral vector production. *Adapted from Maetzig et al. (2011)*

3.3. Developing Gamma Retroviral Vector Packaging Cell Line for CAR-T Cell Production

The production of gamma retroviral vectors can be achieved through two methods. The first method is transient triple expression, where the transfer plasmid and helper expression plasmids are introduced in a cell line through triple transfection. The second method utilizes packaging cell lines that already stably express the Gag/Pol and ENV genes. As the Gag/Pol and ENV lack the ψ, only after transfection with the transfer plasmid these packaging cells will produce gamma retroviral vectors (Park et al., 2018). The production of gamma retroviral vectors is preferred to be performed in a packaging cell line over transient triple transfection, as this method can be easily upscaled, simplifies the production, and increases production yields (Miller et al., 1990; Watanabe & McKenna, 2022).

The usage of packaging cell lines can also facilitate easy in-house manufacturing of CAR-T cells by academic hospitals. Manufacturing of CAR-T cells in-house by academic hospitals would circumvent the complex coordination with pharmaceutical companies, leading to shorter production times and less costs (Jacoby et al., 2018; Straetemans et al., 2018; Castella et al, 2019; Elsallab et al., 2023). The presented study is part of the Life Science Made Better project (Amsterdam UMC) in which a new CAR-T cell therapy will be developed and tested in a phase I clinical trial in-house. The produced CAR-T cells will be transduced by gamma retroviral vectors. Therefore, the aim of this study is to generate a packaging cell line that would enable the large-scale production of gamma retroviral vectors for CAR-T cell therapy clinical trials in an academic setting.

A packaging cell line is aimed to be produced by transfection of the helper expression plasmids, expressing Gag/Pol and ENV. Each of these helper expression plasmids is designed as a bicistronic expression cassette, meaning that they not only contain a cassette for expression of the Gag/Pol and ENV, but also a cassette for expression of an unique fluorescent protein and antibiotic resistance gene. This design allows for the selection of a pool of packaging cells that stably express Gag/Pol and ENV. The selection is carried out through antibiotic selection and FACS (Kaufman et al., 2008; Lanza et al., 2013; Guo et al., 2021). After selection, the established pool of cells stably expressing Gag/Pol and ENV will be transfected with a transfer plasmid. The viral titer production and transduction efficiency of the produced gamma retroviral vectors will then be compared with those of a commercial packaging cell line to determine the upscale potential of the produced packaging cell line (Ghani et al., 2007; Van Der Loo et al., 2011; Ghani et al., 2019).

4. Material and Methods

4.1. Plasmids

For the generation of the packaging cell line two bicistronic expression vectors were designed. The first generation bicistronic vectors pRD114.EGFP.BleoR and pGagPol.mCherry.HygR were based on pLTR-RD114A (Addgene plasmid #17576; http://n2t.net/addgene:17576; RRID:Addgene_17576) and pUMVC (Addgene plasmid #8449; http://n2t.net/addgene:8449; RRID:Addgene_8449) (Stewart et al., 2003; Zhang et al., 2004). The expression vectors were custom designed and synthesized by GenScript (See figure 3).

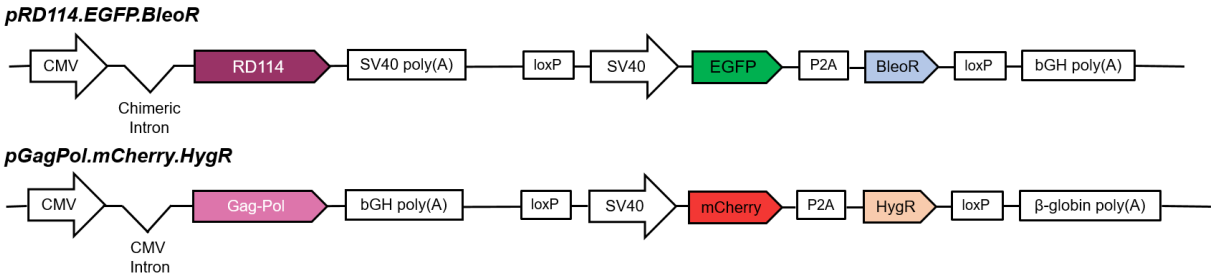


Figure 3. Schematic diagram of the first generation of bicistronic expression cassettes designed for generation of gamma-retroviral vector packaging cell lines. **pRD114.EGFP.BleoR** drives expression of the RD114 envelope glycoprotein under the cytomegalovirus (CMV) promotor, and the co-expression of enhanced green fluorescent protein (eGFP) and phleomycin/zeocin-binding protein (BleoR) under the Simian-virus 40 (SV40) promoter. **pGagPol.mCherry.HygR** drives expression of MLV gag-pol under the CMV promotor, and the co-expression of mCherry and the aminoglycoside phosphotransferase gene (HygR) is under the SV40 promoter. In cassettes, co-expression of the corresponding fluorescent protein and the antibiotic resistance gene is enabled by the P2A self-cleaving peptide. In addition, both cassettes include loxP excision sites located before the SV40 promoter and after the antibiotic resistance gene to facilitate knocking out of the fluorescent protein and the antibiotic resistance gene.

The second generation of bicistronic expression vectors were designed based on the first generation bicistronic expression cassettes (See figure 4). In this second generation both the antibiotic resistance genes have been replaced with the puromycin N-acetyl-transferase gene (*PuroR*). For the generation of pRD114.EGFP.PuroR the *BleoR* gene was replaced by *PuroR* using restriction-enzyme based cloning. A plasmid containing the *PuroR* and pRD114.EGFP.BleoR were enzymatically digested using restriction enzymes BmtI, PflMI, and HindIII from New England Biolab (NEB). Agarose gel electrophoresis was used to separate the backbone and the insert from each other, and the appropriate DNA bands were isolated and purified following gel purification kit protocol (NucleoSpin). A 1:3 ligation, using purified backbone and insert, respectively, was performed using T4 DNA ligase (NEB). The ligation product was transformed into XL-10 gold ultracompetent cells (Agilent). Single colonies were picked and success of the cloning was confirmed with whole plasmid sequencing by Macrogen Europe. For the generation of pGagPol.mCherry.PuroR the *HygR* gene was replaced by *PuroR* using Gibson Assembly cloning. The pGagPol.mCherry.HygR was used as backbone and the *PuroR* insert was derived from the same *PuroR* containing plasmid. Overlapping constructs were PCR generated using the appropriate primers (Biolegio, See supplementary table 1) and following manufactures guidelines for Q5 High-Fidelity 2X Master Mix (NEB). Following PCR, agarose gel electrophoresis was used to separate the backbone and the insert from each other, and the appropriate DNA bands were isolated and purified following gel purification

kit protocol (NucleoSpin). A 1:3 ligation, using purified overlapping backbone and insert, respectively, was performed using Gibson Assembly Master Mix (NEB). After ligation the same steps for the generation of pRD114.EGFP.PuroR were followed to confirm successful cloning.

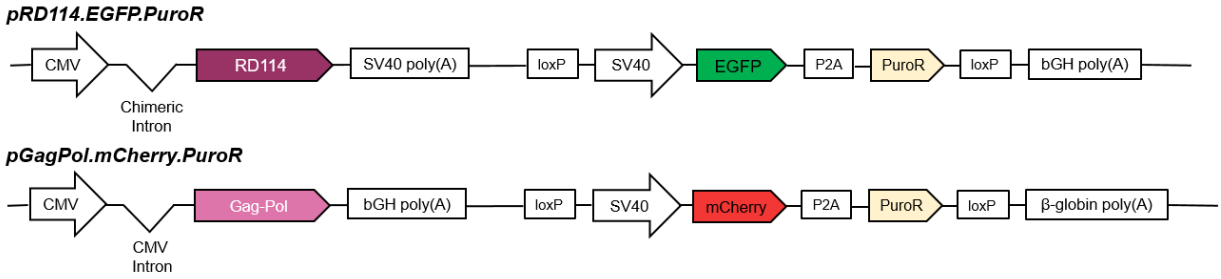


Figure 4. Schematic diagram of the second generation of bicistronic expression cassettes designed for generation of gamma-retroviral vector packaging cell lines. **pRD114.EGFP.PuroR** and **pGagPol.mCherry.PuroR** are designed based on the first generation of bicistronic expression cassettes. In this second generation, the antibiotic resistance genes phleomycin/zeocin-binding protein (BleoR) and aminoglycoside phosphotransferase (HygR) have been replaced with the puromycin N-acetyl-transferase gene (PuroR).

For the production of gamma retroviral vector the transfer plasmid pCAR.GFP was used (See figure 5). This plasmid contains a CAR insert and an eGFP fluorescent marker separated by a P2A self-cleaving peptide. The eGFP is added to indicate the percentage of cells transduced by the gamma retroviral vectors (See section 4.8 Transduction of Jurkat Cell Line).

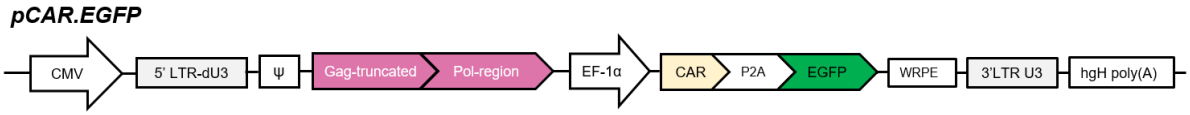


Figure 5. Schematic diagram of the transfer plasmid designed for the production of gamma retroviral vector. CAR.P2A.EGFP is designed as a self-inactivated replicating viral vector for the transduction of a CAR and EGFP. The CMV promotor drives the MLV 5′ LTR. The EF-1α promotor drives the co-expression of a CAR construct and eGFP separated by self-cleaving peptide P2A. The construct end is characterized by woodchuck hepatitis virus post-transcriptional regulatory element (WRPE) region, MLV 3′ LTR and a hgH poly(A) signal.

4.2. Cell lines

HEK293T and 293Vec-RD114 (Biovec Pharma; Ghani et al., 2019) cells were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (PS; ThermoFisher Scientific). The cells were cultured in a t75 flask passaging and were passaged twice a week. Jurkat cells were grown in RPMI 1640 (Gibco) medium supplemented with 10% FBS and PS (ThermoFisher Scientific). The cells were cultured in a t25 flask, and were monitored three times per week to keep the number of cells in culture between 1e6 and 3e6 per mL (Jahan et al., 2023).

4.3. Antibiotic Selection Kill Curves on Transfected and Non-Transfected HEK293T Cells

In a 6-well plate 1.5 x 10^5 HEK293T cells were seeded in 2 mL DMEM + 10% FBS + PS. Cells were incubated at 37 °C with 5% CO2 overnight. The next day transfection was performed using GeneJammer (Agilent). In short, 3 µL GeneJammer was mixed with 96 µL OptiMEM and incubated for five minutes at room temperature (RT). Subsequently, 1 µL (1 µg/µL) DNA was added to the GeneJammer OptiMEM mix. After mixing and incubating for 45 minutes at RT, it was added to the appropriate well. Cells were then again incubated overnight at 37 °C with 5% CO2. The day after transfection, the transfected and non-transfected cells were subjected to antibiotic pressure. The growth medium was removed from each well, followed by washing with phosphate buffered saline (PBS). New growth medium supplemented with either Zeocin (Invivogen), Hygromycin B Gold (Invivogen), or Puromycin (Invivogen) in a dilution series was added to the cells. After 48 hours of incubation at 37 °C with 5% CO2 and exposure to antibiotic-supplemented growth medium, the cells' medium was refreshed with new antibiotic supplemented growth medium. After 72 hours post medium refreshment and incubation at 37 °C with 5% CO2, the cells were imaged at 10x zoom using an EVOS-FL system (Invitrogen).

4.4. Selection of Packaging Cell Line through FACS and Antibiotic Selection

In a 6-well plate 2e5 HEK293T cells were seeded in 2 mL DMEM + 10% FBS + PS. Cells were incubated at 37 °C with 5% CO2 overnight. The next day transfection was performed using GeneJammer (Agilent), 3 µL GeneJammer was mixed with 96 µL OptiMEM and incubated for five minutes at RT. Subsequently, either 1 µL (1 µg/µL) pGagPol.mCherry.PuroR, 1 µL (1 µg/µL) pRD114.EGFP.PuroR, or 0.5 µL of both was added to the GeneJammer OptiMEM mix. After mixing and incubating for 45 minutes at RT, it was added to the appropriate well. Cells were subsequent incubated at 37 °C with 5% CO2. After 48 hours cells were prepared for sorting. The growth medium was removed from each well, followed by washing with PBS. Afterward 200 µL Trypsin-EDTA (Gibco) was added to the wells and set for five minutes at 37 °C. The cells were subsequent resuspended in 2 mL of PBS + 2% FCS and filtered through a 30 µm strainer. Cell sorting was performed on a BD FACSmelody cell sorter (BD Bioscience). The sorter was equipped with a 488 nm and 561 nm laser to detect eGFP and mCherry fluorescence, respectively. Forward scatter (FSC) and side scatter (SSC) parameters were used to exclude debris, and doublets (See supplementary figure 1). Gates for sorting were set to identify double positive eGFP and mCherry cells based on fluorescence intensity measured in a non-transfected control and single transfected controls. Cells were sorted directly into DMEM + 10% FCS + PS medium and cultured in a 6-well format. The day after sorting the sorted cells were exposed to antibiotic pressure by refreshing the growth medium with DMEM + 10% FBS + PS and 0.2 µg/mL Puromycin (Pur; Invivogen). Over the course of 74 days the growth medium supplemented with Pur was refreshed every 2 to 3 days and when appropriate cells were expanded to a t75 flask. In total sorting of the double transfected HEK293T cell population occurred four times. Three times the double transfected HEK293T cell population percentages were assessed through flow cytometry (See figure 6).

Flow cytometry was performed on the BD LSRFortessa (BD Bioscience). Growth medium was removed from the cells, followed by washing with PBS. Afterward the cells were trypsinized and incubated for five minutes at 37 °C. The cells were subsequent resuspended in 2 mL of growth medium and transferred to 5 mL FACS tubes. The cells were spun down for five minutes at 1500 rpm. The supernatant was removed before adding 1 mL of PBS supplemented with 4% paraformaldehyde (PFA). After 10 minutes of incubation the PBS + 4% PFA solution was diluted by adding 3 mL PBS supplemented with 0.1 % HSA + 0.05% Azide. Cells were spun down for five minutes at 1500 rpm. The supernatant was removed and 3 mL PBS + 0.1% HSA + 0.05 % Azide was added before the cells were quantitively measured on the BD LSRFortessa. Flow cytometry and sorting data analysis was performed with FCS express 6 flow cytometry software.

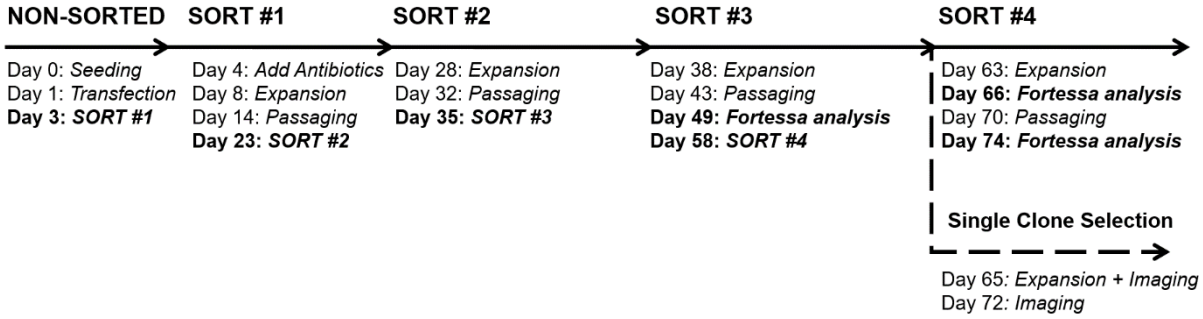


Figure 6. Timeline showing the sorting events for the production of the HEK293T packaging cell line. The time line shows the days of the sorting events indicated by (*SORT*), analysis time points indicated by (*Fortessa analysis)*, the expansion from a 6-well format to a t75 flask (*Expansion*) and the first time passaging from a t75 flaks to another t75 flask (*Passaging*). Furthermore the timeline of the single clone selection is indicated in the figure, showing the day of *expansion and imaging*.

4.5. Single Clone Selection

After the fourth sort the eGFP and mCherry positive cells were diluted to an average concentration of 0.8 cells per 200 µL DMEM + 10% FCS + PS and cultured in a 96-well flat plate. The cells were incubated at 37 °C with 5% CO2. After 7 days of incubation, upon the appearance of a cell mass the expression of eGFP and mCherry was determined by imaging at 10x zoom using an EVOS-FL system. Cells were selected to be expanded based on expression and consistency of eGFP and mCherry throughout the cell mass. Growth medium was removed and subsequent wells were washed with 100 µL PBS. 200 µL of Trypsin-EDTA was added to the wells and incubated for 5 minutes at 37 °C. Afterward the wells were resuspended in 1 mL growth medium and transferred to a 24-well format. The cells were incubated at 37 °C with 5% CO2. Again after 7 days of incubation expression and consistency of eGFP and mCherry throughout the cell mass was assessed by imaging.

4.6. Gamma Retroviral Vector Production

In a 10-cm dish 5e6 HEK293T cells, 293Vec-RD114 (Biovec Pharma), or the produced HEK293T-PACK were seeded in 10 mL DMEM + 10% FBS + PS. Cells were incubated at 37 °C with 5% CO2 O/N. The day after transfection was performed using GeneJammer (Agilent), 18 µL GeneJammer was mixed with 576 µL OptiMEM and incubated for five minutes at RT. To each experimental condition, also a condition without transfer plasmid was added. After mixing and incubating for 45 minutes at RT, it was added to the appropriate dish. Cells were incubated at 37 °C with 5% CO2 O/N. Two days in a row cell medium was harvested and stored at 4 °C while cells were supplemented with new growth medium. Afterward the harvested cell medium was concentrated using Retro-X™ Concentrator (Clontech) following manufacturer guidelines. The subsequent day, the medium + Retro-X™ Concentrator was spun down at 1500 rpm for 45 minutes. The supernatant was carefully removed and the pellet was resuspended in 1 mL PBS, aliquoted and stored at -80 °C until future usage.

4.7. qPCR

Concentrated supernatant collected after production (See section 4.6) was purified using NucleoSpin Viral RNA isolation kit (MACHERY-NAGEL). Afterward, the sample was treated with DNase following Retro-X qRT-PCR Titration Kit User Manual (Takarabio). In short, the RNA sample, DNAse, and DNase buffer were mixed and incubated at 37 °C for 30 minutes followed by 70 °C for 5 minutes. The number of retroviral particles in each sample was quantified based on RT-qPCR analysis by counting the amount of minus-strand strong-stop DNA molecules synthesized in the viral particle. The thermal cycling conditions were performed following protocol. The supplemented standard curve was generated and concentrations of the viral samples were determined using linear regression.

4.8. Transduction of Jurkat Cell line

In a 24-well plate 3e5 Jurkat cells were plated in 2 mL RPMI + 10% FBS + PS supplemented with 4 µg/mL polybrene. To the appropriate well either diluted or undiluted aliquoted gamma retroviral vector was added. The plate is spinoculated for one hour at 3000 rpm at RT. Afterward the cells were incubated at 37 °C with 5% CO2 overnight. Six days after incubation the cells are assessed for GFP expression by flow cytometry performed on BD LSRFortessa (BD Bioscience). Samples were fixated and prepared before being measured (See section 4.4). Forward scatter (FSC) and side scatter (SSC) parameters were used to exclude debris, and doublets (See supplementary figure 2). Flow cytometry data analysis was performed with FCS express 6 flow cytometry software.

5. Results

5.1. Puromycin Demonstrates Decreased Antibiotic Pressure on Transfected HEK293T Cells in Contrast to Hygromycin and Zeocin

The HEK293T packaging cell line was aimed to be produced through sorting and antibiotic selection. Before antibiotic selection is performed for the generation of the packaging cell line, the impact of the antibiotics Zeocin and Hygromycin is qualitatively assessed using imaging of a kill curve (See figure 7A). Non-transfected cells exposed to antibiotic pressure of Hygromycin showed signs of cell death at a concentration of 125 μg/mL, while transfected cells showed clear signs of cell death at 62.5 μg/mL (See figure 7B). For the Zeocin exposed cells a similar trend can be seen where the non-transfected cells their fitness showed to be affected at a concentration of 100 μg/mL, while transfected cells showed signs of cell death at 50 μg/mL (See figure 7B). In general, the Zeocin and Hygromycin exposed non-transfected cells showed to have better fitness in comparison to the cells transfected with either first generation bicistronic expression vector. As the Zeocin and Hygromycin exposed non-transfected cells showed to have better fitness in comparison to the transfected cells, the antibiotic resistance genes in the first generation of bicistronic expression vectors were switched for *PuroR*. In a similar fashion as kill curves for Zeocin and Hygromycin, the impact of the antibiotic Puromycin is qualitatively assessed using imaging. It can be seen for the non-transfected cells that they showed signs of cell death at a concentration of 0.2 μg/mL. At a concentration of 0.4 μg/mL the transfected cells showed limited signs of cell death and slight signs of compromised cellular fitness, while the non-transfected cells showed no signs of viable cells (See figure 7B). This indicates that Puromycin can be utilized for the generation of a packaging cell line.

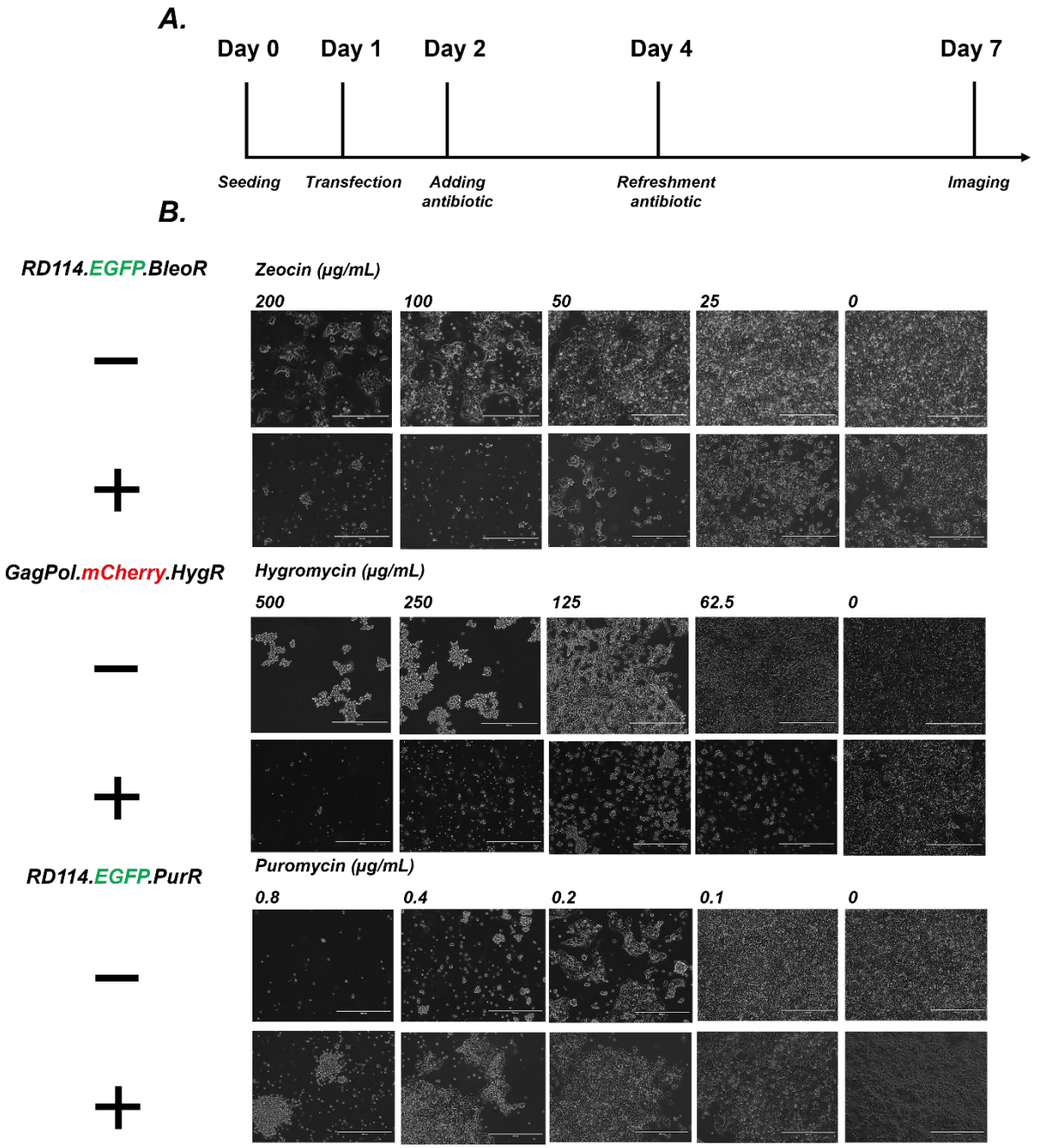


Figure 7. Assessment of antibiotic selection efficiency for first and second generation bicistronic expression cassettes in transfected HEK293T cells. A. Timeline of the experimental procedure. Cells were seeded on day 0, transfected on day 1, and antibiotics were added on day 2. The medium was refreshed with antibiotics on day 4, and imaging was performed on day 7. **B.** Representable microscopy images show the condition of HEK293T cells transfected with pGagPol.mCherry.HygR, pRD114.EGFP.BleoR, or pRD114.EGFP.PuroR (+) or non-transfected cells (-) under different antibiotic selection pressures. HEK293T cells transfected with pGagPol.mCherry.HygR were subjected to a decreasing concentration series of Hygromycin (500, 250, 125, 61.3, and 0 µg/mL), cells transfected with pRD114.EGFP.BleoR to a decreasing concentration series of Zeocin (200, 100, 50, 25, and 0 µg/mL), and cells transfected with pRD114.EGFP.PuroR to decreasing concentration series of Puromycin (0.8, 0.4, 0.2, 0.1, and 0 µg/mL). Imaging was performed on an EVOS FL system at 10x magnification.

5.2. FACS and Antibiotic Selection Generates a Semi-Stable Packaging Cell Line

The generation of a stable HEK293T packaging cell line was aimed to be achieved through FACS and antibiotic selection. For this HEK293T cells were transfected with helper expression plasmids pRD114.EGFP.PuroR and pGagPol.mCherry.PuroR. These helper expression plasmids were designed to express the antibiotic resistance gene *PuroR* and either fluorescent protein eGFP or mCherry, allowing to sort for HEK293T cells expressing both helper expression plasmids. Cells were continuously cultured in growth medium supplemented with Pur (0.2 μg/mL) and were sorted a total of four times. The percentage of eGFP, mCherry and double positives was measured at every sort, between sort #3 and #4, and two timepoints after sort #4 (See figure 8). It can be seen that over the sorts the percentage of double positives increases from 38% to 79%, while the percentage of non-fluorescent cells decreases from 56% to 3%. It can also be noticed that the percentage of single eGFP and mCherry increases over time without the initial sort having a high percentage of eGFP positive (3%) and mCherry positive cells (2%). After sort #4 the cell population distribution was analyzed two more times without sorting. It can be seen that the distribution of the cell population seemed to stabilize over these measurements.

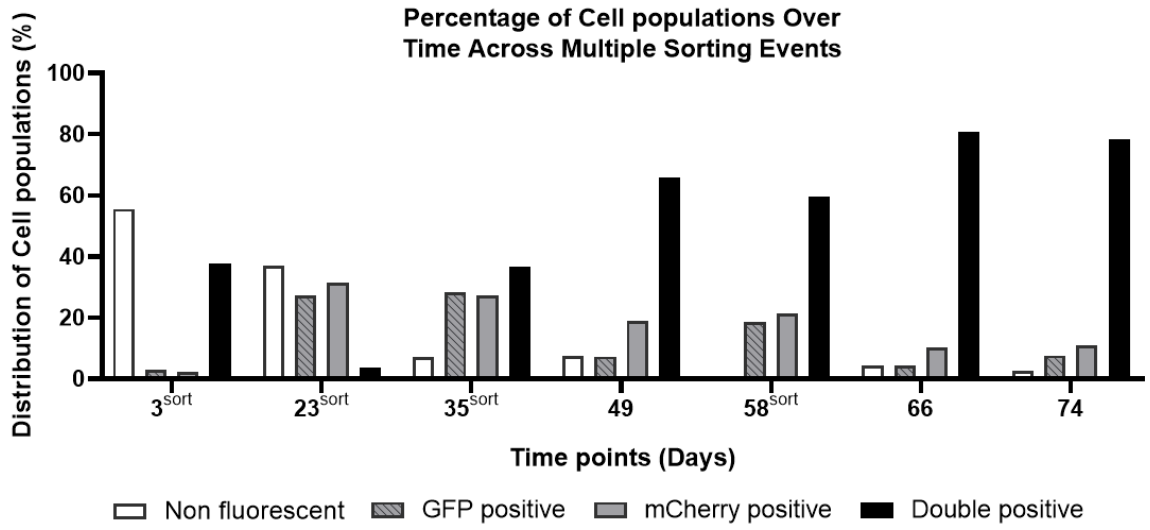


Figure 8. Stacked histogram displaying the percentages of cell population over time across multiple sorting events. The cell populations are categorized in non-fluorescent (white), GFP positive (grey striped), mCherry positive (grey), and double positive (black). Sorting events are indicated on the x-axis by a superscript sort (^{sort}), and were performed on day 3, 23, 35, and 58. Each bar represents the normalized percentage of cell populations per timepoint.

5.3. Single Clone Dilution Lead to the Generation of Stably Expressing Single Clones

After sort #4 a part of sorted population was diluted to retrieve a single clone population, that stably expresses fluorescent eGFP and mCherry without antibiotic selection pressure. The criteria for the expansion of the single clones was kept to the clones expressing both eGFP and mCherry in a consistent matter throughout their cell mass. It can be seen on day 7 after seeding that there was island formation from single clones with cells stably and consistently over the island expressed EGFP and mCherry (See figure 9). However it was also observed that some single cell colonies showed either inconsistent expression of eGFP and mCherry (7F2) or only stable expression of one fluorescent protein (8B8), these colonies were not taken along for expansion. Singe cell colonies that were expanded showed to either keep their stable and consistent expression of both fluorescent proteins (8E5 and 2G5), or lose the expression of one of the fluorescent proteins (2H8).

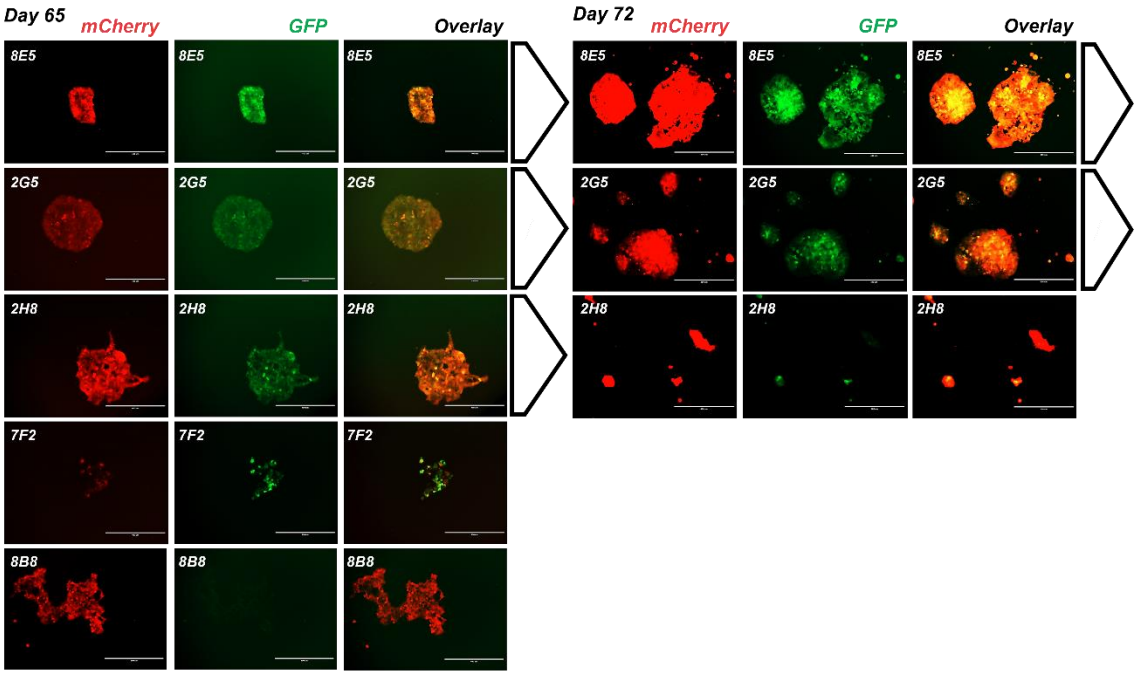


Figure 9. Single cell colonies derived after sort #4. Representative images of single cell colonies after sorting of day 58. Single cell colonies were imaged for mCherry positivity (red) and GFP positivity (green) and overlay (yellow). Cell colonies that showed a consistent signal for both fluorescent proteins (as seen in 8E5, 2G5, and 2H8) were expanded, while colonies that showed consistent signal but for one fluorescent protein (8B8) or non-consistent expression of both fluorescent protein (7F2) were not expanded.

5.4. Gamma Retroviral Vector Produced with 293Vec-RD114 or HEK293T-PACK leads to Comparable Viral Titer and Transduction Efficiency on Jurkat Cells.

After sorting, the established pool of HEK293T-PACK (HEK293T packaging cell line) is transfected with a transfer plasmid for gamma retroviral vector production. The vector production of our established cell line is compared with commercially available cell line 293Vec-RD114. The genomic copy number per condition was determined through RT qPCR. It could be seen that the transfection of the HEK293T-PACK (6.944e6 CN/mL) and 293Vec-RD114 (9.944e6 CN/mL) with the transfer plasmid lead to comparable titer (See figure 10). Also the mock conditions showed comparable background signal with the HEK293T-PACK mock (4.817e1 CN/mL) and 293ec-RD114 mock (2.164e2 CN/mL) having similar genomic copy numbers per mL.

After production the transduction efficiency of the gamma retroviral vector batches was tested on Jurkat cells. Jurkat cells were transduced by spinoculation and transduction efficiency was measured based on eGFP expression by flow cytometry (See figure 11). It could be seen that HEK293T-PACK mock (0.13%), and 293Vec-RD114 mock (0.15%) showed a similar percentage of cells positive for GFP as the negative control (0.15%; data not shown). It could be seen that the gamma retroviral vectors produced by packaging cell lines produced showed a higher percentage GFP positive cells in comparison to their mock. With the HEK293T-PACK (35.30%) outperforming the 293Vec-RD114 produced gamma retroviral vectors (28.55%).

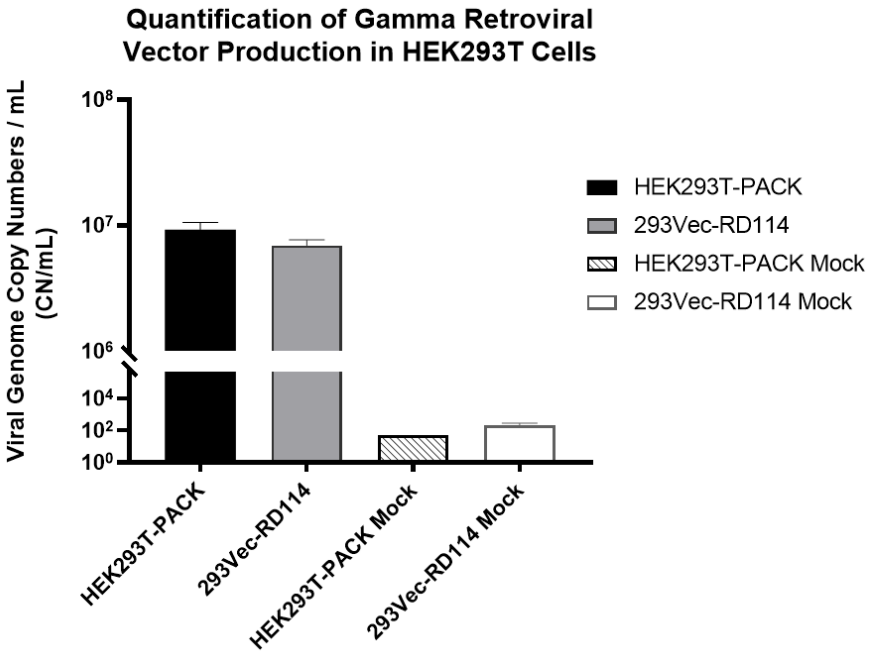


Figure 10. Quantification of gamma retroviral vector production in HEK293T Cells. Viral genome copy numbers per mL (CN/mL) was determined through RT qPCR transfer plasmid transfected HEK293T-PACK (black), transfer plasmid transfected 293Vec-RD114 (gray), HEK293T-PACK Mock (striped), and 293Vec-RD114 Mock (white). Titer data analysis is performed in triplicate from one gamma retroviral vector production.

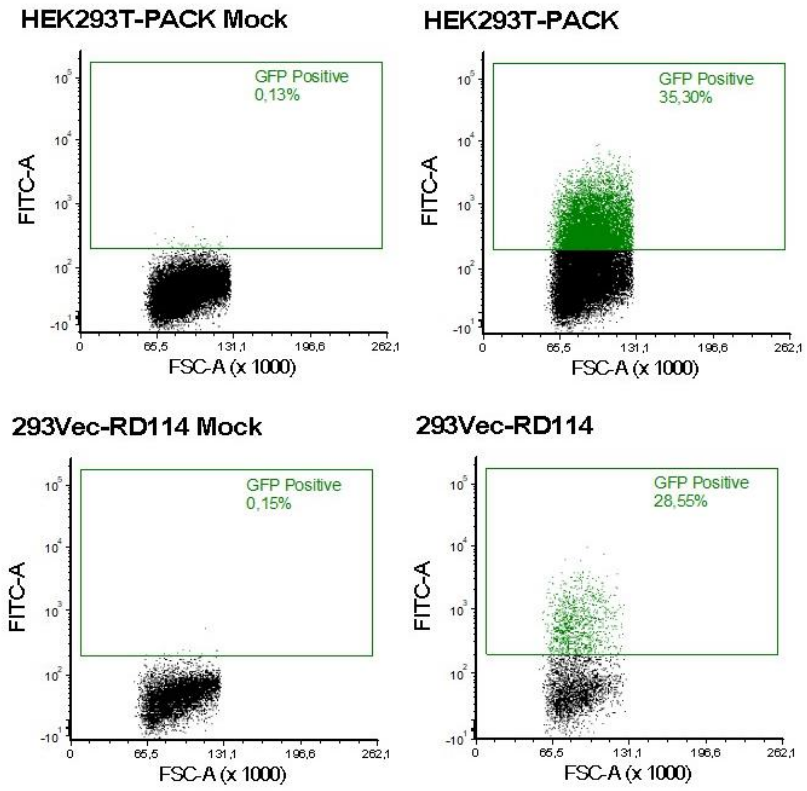


Figure 11. Transduction efficiency of produced gamma retroviral vectors on Jurkat Cells. Transduction efficiency on Jurkat cells is measured based on GFP fluorescence intensity. X-axis showing forward scatter. Y-axis showing GFP fluorescence on FITC-A

6. Discussion

The aim of this study was to generate a packaging cell line for large-scale production of retroviral vectors for CAR-T cell therapy in an academic setting. We transfected HEK293T cells with helper expression plasmids pRD114.EGFP.PuroR and pGagPol.mCherry.PuroR. Following this, we selected the double transfected cells using Puromycin and sorted the eGFP and mCherry positive cells. This process generated our HEK293T-PACK cell line. Our HEK293T-PACK cell line produced viral vector particles capable of transducing Jurkat cells. The production of these particles and transduction efficiency was similar to those produced by the 293Vec-RD114 cell line, indicating that our packaging cell line has the potential for large-scale production of gamma retroviral vectors.

The generation of a stable HEK293T packaging cell line was initially based on Zeocin and Hygromycin selection. However, it was observed that non-transfected cells had higher fitness compared to transfected cells, both in the presence and absence of Zeocin and Hygromycin (See figure 7). This reduced fitness could be due to the effects of the transfection protocol or due to the lack of antibiotic resistance seen from the *BleoR* and *HygR*. Since other antibiotic resistance genes like *PuroR* are known for more robust expression, the antibiotic resistance genes *BleoR* and *HygR* were replaced with *PuroR*. This change resulted in the transfected cells exhibiting better fitness compared to the non-transfected cells. It was, therefore, chosen to continue with the generation of the packaging cell line using solely Puromycin as antibiotic selection marker. The generated HEK293T-PACK cell line was found to be stable, but with a small tendency of the double positive population depleting into either eGFP or mCherry single positives.

Using two different antibiotic selection markers might reduce this depletion. Since the antibiotic resistance from robust *PuroR* worked for antibiotic selection, it should be considered to use another antibiotic resistance gene that also exhibits robust expression like Blasticidin resistance gene (*BlstR)* with Blasticidin (Ghani et al., 2007; Kaufman et al., 2008). Preliminary results testing the selection with Blasticidin showed to be effective in our hands on day 9, but still showed reduced fitness of the transfected cells on day 7 (See supplementary figure 3). The reduced fitness could be attributed to the toxic effects of the transfection reagent, possible toxicity of the plasmid, or by insufficient expression of the antibiotic resistance gene. Preliminary results using a different transfection methodology and different promotor driving *HygR* gene expression showed to be effective in our hands (data not shown). This indicates that previously the expression of *HygR* could possibly have been too low to reach antibiotic resistance in the transfected cells or that the cells were affected too much with the combination of transfection and antibiotic selection. For the possible usage of *BleoR* and *HygR*, future experiments have to focus on determining ideal transfection conditions and determine stronger promotors to drive antibiotic resistance gene expression.

Furthermore, for the selection of a stable cell line the FACS selection method could be improved upon. In this study, sorting timepoints were partially based on cell number before sorting. Expanding the sorted cells after the first selection was time-consuming, and a big drop in double-positive cells was observed during this period (See figure 8). Increasing the starting material could improve yield after post-sorting, reducing the interval between sorts, and therefore potentially decrease the drop in double-positive cells after each sort. Gamma retroviral vectors production from our HEK293T-PACK cell line and the 293Vec-RD114 cell line lead to a similar viral titer and also the transduction efficiency on the Jurkat cell line showed to be similar for both experimental conditions.

To move towards the large scale production of gamma retroviral vector, future experiments have to focus on the production of gamma retroviral vectors under good manufacturers practices (GMP) conditions (Guidelines Good Manufacturing Practice for Advanced Therapy Medicinal Product, 2017; Straetemans et al., 2018; European Pharmacopeia, 2020). Furthermore, future experiments will have to focus on upscaling of the virus production by either producing virus in suspension, a fixed bed bioreactor or using HYPERStack® Cell Culture Vessels (Ghani et al., 2009; Strathearn & Rothenberg, 2013). Our selection of a single clone shows that it is possible to select for clones that stably express both helper expression plasmids (See figure 9). The expansion and testing of gamma retroviral vector production from single clones therefore has to be pursued in future experiments for the generation of a single clone packaging cell line.

Future generation of a packaging cell line would benefit from antibiotic selection based on two antibiotic resistance genes. Therefore, a third generation of bicistronic vectors would preferably have the integration of *PuroR* and *BlstR* or *HygR* (Kaufman et al., 2008; Lanza et al., 2013; Guo et al., 2021). Recombination events in our generated packaging cell line could possibly lead to the generation of RCR. To reduce the possibility of recombination events the helper expression plasmids homologous sequences should be reduced. The plasmids would therefore benefit from separate promotors that drive the RD114 and GagPol expression, like the usage of the EF-1α promotor next to the CMV promotor.

Overall, the selection method based on Puromycin resistance and sorting of cells expressing both fluorescent protein markers proved to be an effective strategy for generating a stable HEK293T packaging cell line. This study presents initial results demonstrating the feasibility of creating HEK293T packaging cell lines within an academic setting, yielding promising titers and transduction efficiencies comparable to those of commercial packaging cell lines. To fully implement this packaging cell line in an academic setting for gamma retroviral vector production for CAR T-cell clinical trial purposes, future research must focus on the characterization of the developed cell line following, the production of gamma retroviral vectors under GMP compliance, and the upscaling of the production processes. This study represents a foundational step towards achieving these goals.

7. Literature

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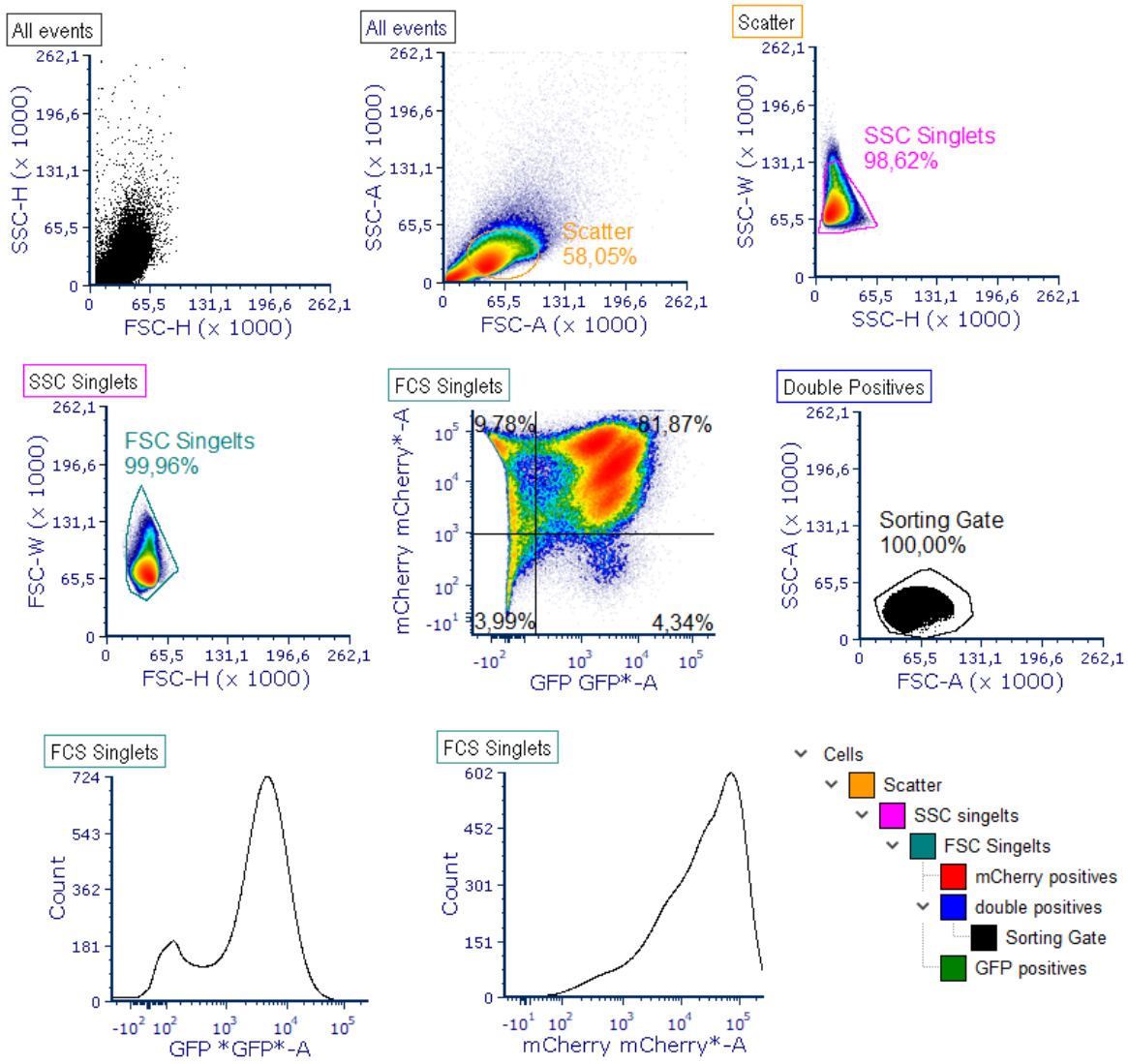
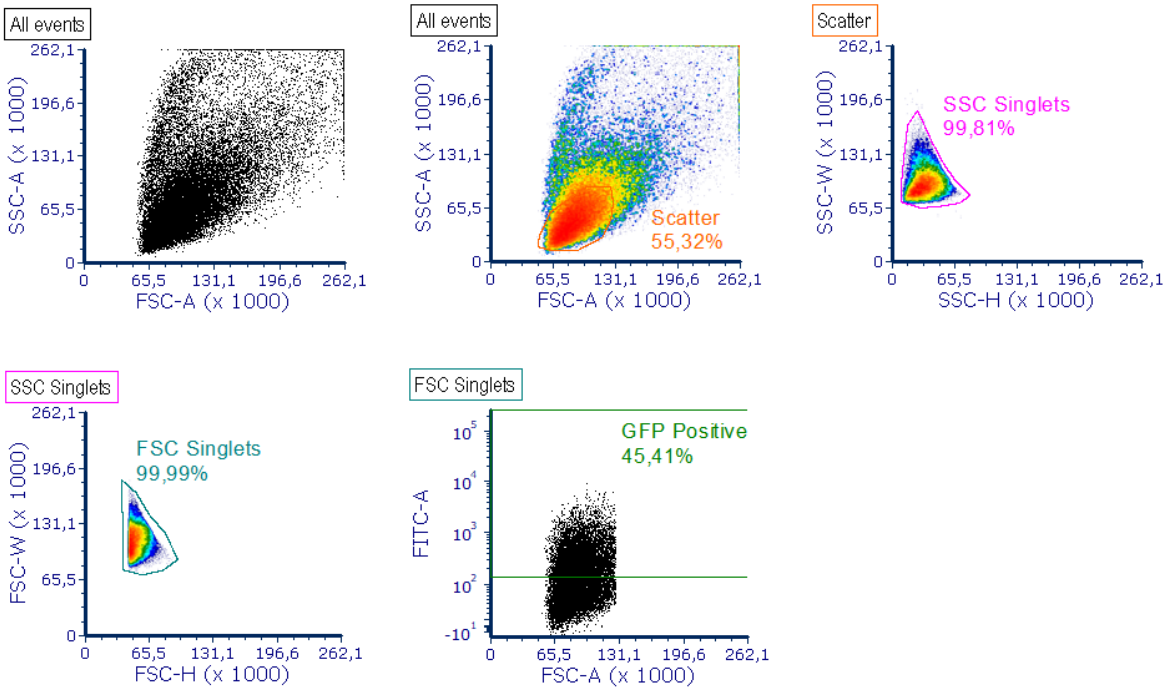
8. Supplementary

Supplementary Table 1. Primers used for the second-generation helper expression plasmid GagPol.mCherry.PuroR.

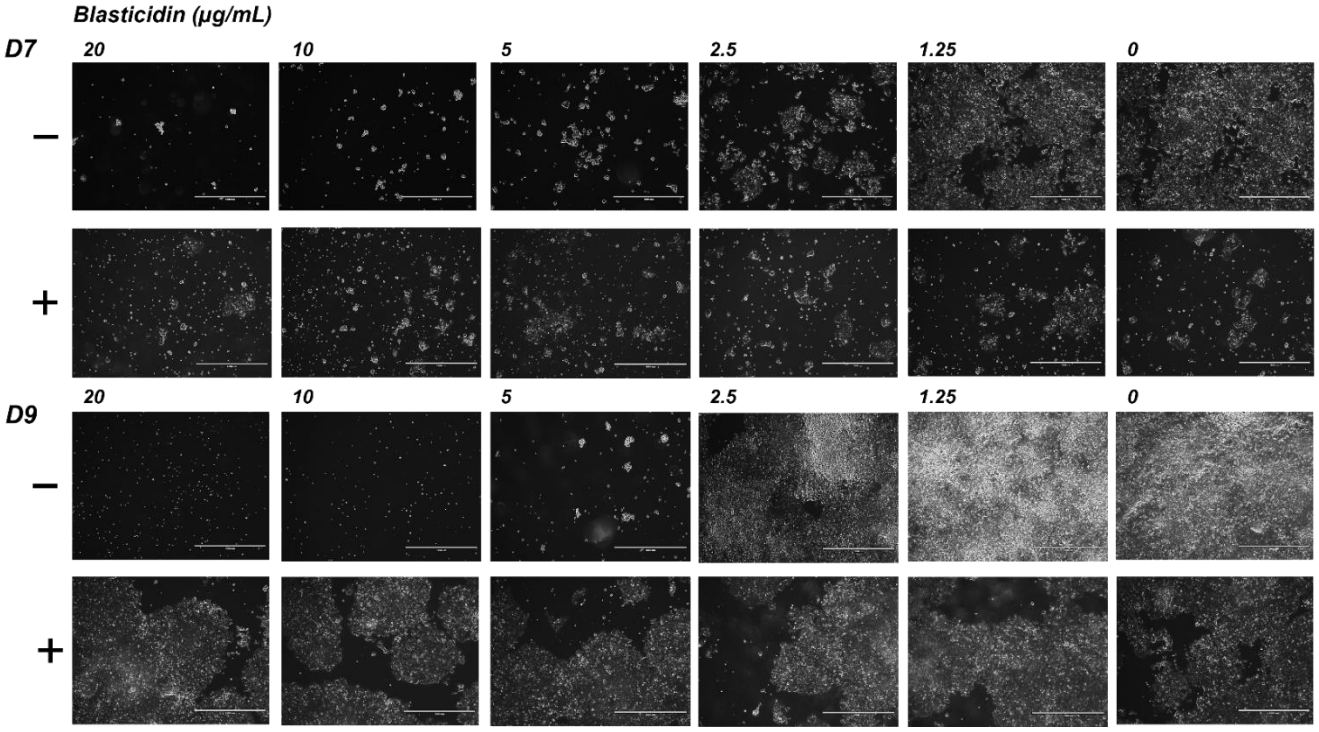
Vector Forward	TTGCATCCAAAGGCGGTAATACGG
Vector Reverse	TTTGTTGCTCCGGATCCCTTGT
Insert Forward	GATCCGGAGCAACAAACTTCTCAC
Insert Reverse	CGCCTTTGGATGCAATTTCCTCATTTTATTAGGAAAGGACAG



Supplementary Figure 1. Representative gating strategy for analyzing eGFP and mCherry positive HEK293T cells. Representative gating strategy from LSRFortessa data analysis day 66 is shown. Cell debris and doublets were excluded based on SSC and FSC parameters. The percentage of eGFP and mCherry positive cells has either been done on the BD FACSmelody based on parameters mCherry mCherry*-A (10^3) and GFP GFP*-A (20^2) or on the LSRFortessa based on parameters PE-CF594A (10^3) and FITC-A (20^2). Gating for double positive cells was performed by always including a non-fluorescent control, an eGFP fluorescent control, and a mCherry fluorescent control.



Supplementary Figure 2. Representative gating strategy for analyzing eGFP positive Jurkat cells. Representative gating strategy from LSRFortessa data analysis is shown. Cell debris and doublets were excluded based on SSC and FSC parameters. The percentage of eGFP positive cells has been determined on the LSRFortessa based on FITC-A. Gating for positive cells was performed by including a negative control.



Supplementary Figure 3. Assessment of Blasticidin antibiotic selection efficiency on transfected HEK293T cells. Representable microscopy images show the condition of HEK293T cells transfected with Blasticidin resistance gene (+) or non-transfected cells(-) under different antibiotic selection pressures of Blasticidin. Images were taken at day 7 (D7). After imaging the cells the medium was refreshed at D7 and new images were taken at day 9 (D9).HEK293T cells were subjected to decreasing concentrations of Blasticidin (20, 10, 5, 2.5, and 1.25 µg/mL). Imaging was performed on an EVOS FL system at 4x magnification.