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Development of a novel reverse genetics system to assess the functional interplay of Lassa virus proteins

Bachelor Thesis

University of Veterinary Medicine Vienna

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1 Introduction

1.1 Lassavirus and the family of *Arenaviridae*

The family *Arenaviridae* is a viral family with three distinct genera: *Mammarenavirus*, *Reptarenavirus* and *Hartmanivirus*. Whereas the reptarenaviruses and hartmaniviruses have no clinical relevance as human pathogens, some mammarenaviruses are the cause of various infections in humans, including hemorrhagic fevers. The genus *Mammarenavirus* is further divided in viruses of the New World (e.g. Junín virus (JUNV), Machupo, Guanarito, Sabia, and Chapare virus) and of the Old World (e.g. Lassa and Lujo virus) (Hallam et al., 2018a). This distinction in New World and Old World is based on antigen features and the geographic location of these viruses, with New World viruses mostly occurring in south America, while Old World viruses are more common in Europe and Africa. The New World Arenaviruses are further divided into clades A, B, C, and A/Rec or D (Günther & Lenz, 2004; Hallam et al., 2018; Wulff et al., 1977).

Lassa virus (LASV) is the clinically most relevant of the Old World Arenaviruses. A high genetic diversity was shown within LASV strains, so that they are subdivided into lineages I-IV. This distinction correlates with the geographic origin of LASV isolates, with the lineages I-III mostly occurring in Nigeria, and lineage IV in Guinea, Sierra Leone, and Liberia (Bowen et al., 2000). Furthermore, two more lineages were proposed for new isolates from Mali and the Ivory Coast (lineage V), as well as from Togo (lineage VI) (Manning et al., 2015; Whitmer et al., 2018). LASV is found in these areas in its natural rodent host (*Mastomys natalensis*), causing chronic infection and lifelong virus excretion in urine and faeces. Therefore, human infection may occur through contact with infectious rodent excretions via contaminated food supplies or consumption of meat from diseased rodents. (Salazar-Bravo et al., 2002). Moreover, human-to-human transmission is possible via inhalation of aerosols or direct contact with infectious bodily fluids (Maiztegui et al., 1998). Furthermore, Lassa Fever manifests in about 30% of infections with symptoms varying from mild febrile symptoms to severe hemorrhagic fever with multiorgan failure, leading to case fatality rate (CRF) of ~1% (WHO fact sheet Lassa fever, 2017).

1.2 Morphology and genome structure

LASV is an enveloped negative strand RNA virus with a bisegmented genome, with both segments encoding two proteins in ambisense orientation, separated by a non-coding intergenic region. Viral particles appear as spherical or pleomorphic with an average diameter

of 50-300nm (Schlie et al., 2010). The S-segment (3.5 kb) encodes the glycoprotein precursor molecule preGPC in positive orientation and the nucleoprotein (NP) in negative orientation, whereas the L-segment (7.3 kb) encodes the viral polymerase L in negative and matrix protein Z in positive orientation (Auperin et al., 1986a; Ferron et al., 2017; Lukashevich et al., 1997; Clegg, 1985; Strecker, Eichler, Meulen, et al., 2003). The LASV particle contains the genomic RNA, which is encapsidated by NP, and together with NP and the associated viral Polymerase L forms the ribonucleoprotein complex (RNP). Moreover, the matrix protein Z and trimeric glycoprotein complexes (GPC) are found on the inside (Z) or mainly outside (GPC) of the viral membrane (Schlie et al., 2010; Strecker, Eichler, ter Meulen, et al., 2003). GPC consist of three subunits GP1, GP2 and the stable signal peptide (SSP), which are formed through several protein processing steps of precursor glycoprotein. Specifically, co-translational processing generates the stable signal peptide through cleavage, followed by post-translational processing to form GP1 and GP2 (Ferron et al., 2020).

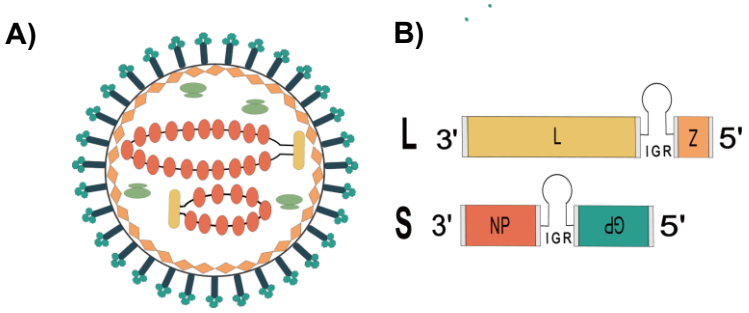

Figure 1: Schematic display of LASV Virion and Genome. (A) The LASV genome is associated with the nucleoprotein (NP) and the viral polymerase L, forming the RNP complex. The inner membrane is covered with matrix protein Z, whereas trimeric GPC consisting of GP1, GP2 and SSP is embedded in the lipid layer. (B) The LASV genome consists of 2 segments, each coding for two proteins in ambisense orientation, interrupted by non-coding intergenic regions.

1.3 Life cycle and replication

For cell entry through endocytosis the GPC subunit GP1 interacts with the cellular receptor alpha-dystroglycan (Acciani et al., 2017). GP2-mediated fusion of the viral membrane with cell membrane then results in RNP complexes being released into the cytoplasm (Eschli et al., 2006). After this, the transcription of proteins in negative orientation, L and NP, also called early phase proteins, is started. Subsequently, expressed proteins facilitate genome replication, resulting in the antigenome, which serves as template for transcription of late phase proteins Z and preGPC, as well as as template for replication of new genomes (Pinschewer et al., 2003). Synthesized RNA is co-transciptionally encapsidized by NP and forms, together with associated L, new RNP complexes, which are transported to the plasma membrane. After

transcription the matrix protein Z is transported to the plasma membrane. There, interactions between Z and NP cause the RNP complexes to adhere to the cell membrane. Furthermore, after processing and posttranslational modifications the trimeric GPC is transported to the plasma membrane through the secretory pathway. Finally, viral budding mediated by the matrix protein Z and supported by components of the endosomal sorting complexes required for transport (ESCRT) machinery takes place (Eichler et al., 2004, Perez, Craven, & Torre, 2003).

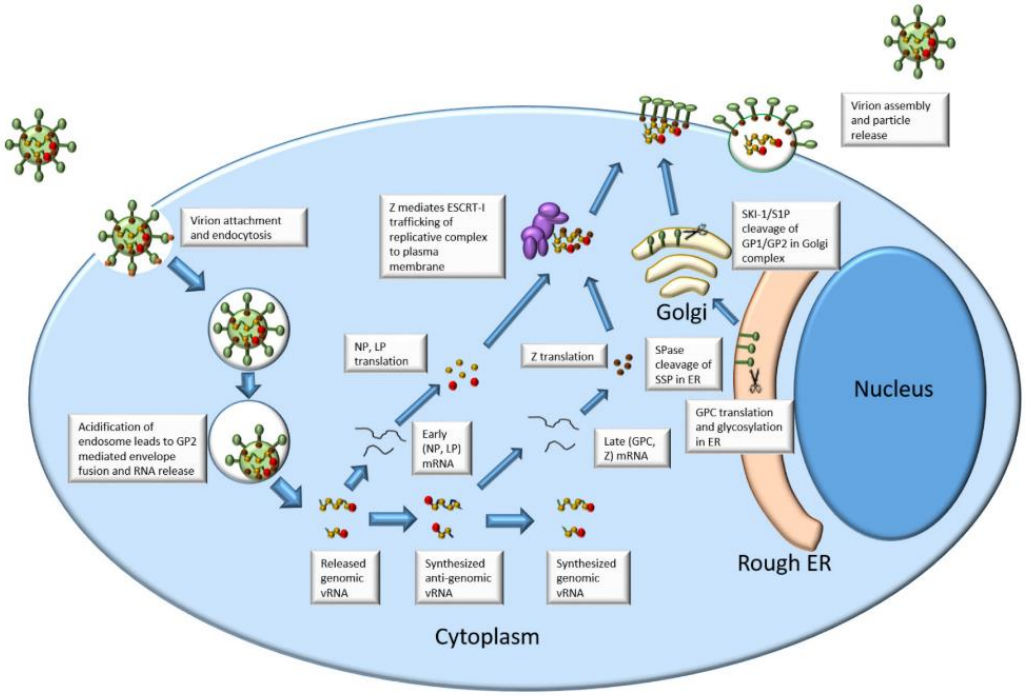

Figure 2: Schematic display of the LASV Life Cycle. The illustration depicts virion entry by fusion followed by genome replication and gene expression. After translation preGPC undergoes post-translational modifications in the endoplasmatic reticulum and golgi complex. Subsequently, all viral proteins are transported to cell membrane for viral budding and virion release. Figure reproduced from Hallam et al., 2018, under a CC-BY 4.0 license.

1.4 Lassa virus protein Z

The small RING Finger domain protein Z, which represents the arenavirus matrix protein, is 90-99 amino acids long (depending on the species of arenavirus) and its sequence is located on the L segment in positive orientation (Capul et al., 2011a; Hallam et al., 2018a). Therefore, it is expressed through transcription of the antigenome and classified as a late phase protein in the viral life cycle. There are several functional domains within Z. The late domain motifs PTAP (amino acids 81 to 84) and PPPY (amino acids 94 to 97) are highly conserved among arenavirus species and were shown to be required for the release of enveloped viral particles

(Strecker, Eichler, Meulen, et al., 2003). Moreover, the central RING domain was shown to be required for downregulation of RNA synthesis by Z (Cornu et al., 2001). Additionally, Z carries a myristoylation site at position 2, which is essential for its budding function and GP interactions (Capul et al., 2007; Perez et al., 2003; Perez, Greenwald, & de La Torre, 2004).

Figure 3: Schematic display of functional domains of LASV matrix protein Z. The RING finger domain is mandatory for downregulation of RNA synthesis and the proline rich late domain motifs as well as the myristoylation site are required for the formation and release of viral particles. Illustration was kindly provided by Dr. Lisa Wendt.

Nine conserved amino acid residues important for this thesis have been previously investigated by Capul (Capul et al., 2011) were analyzed in detail. These conserved amino acid residues included G27, L71 and P72, which are conserved in the whole Arenavirus family, as well as P21, D22, P28 and K68, which are conserved among Old World Arenaviruses. Finally, R16 and T73, which are conserved among Arenaviruses except New World clade A and B (figure 4) (Capul et al., 2011b), were investigated.

The findings of Capul et al., 2011 suggest that none of the altered amino acid residues has an effect on budding activity of Z as well as its plasma membrane localization and interaction with GP. However, the R16A, P72A and T73A mutants as well as the P21A+D22A double mutant lead to loss of both Z-mediated inhibition of viral RNA synthesis and infectivity of produced virus-like particles (VLPs). These VLPs are produced by expression of Z, and can incorporate GPC as well as nucleocapsids, resulting in VLPs that can infect target cells. Moreover, the G27A mutant displayed loss of the Z inhibitory effect on RNA synthesis, as well as a marginal increase in VLP infectivity. Finally, P28A retained and K68A lead to an increased inhibitory effect on RNA synthesis but were associated with loss of VLP infectivity.

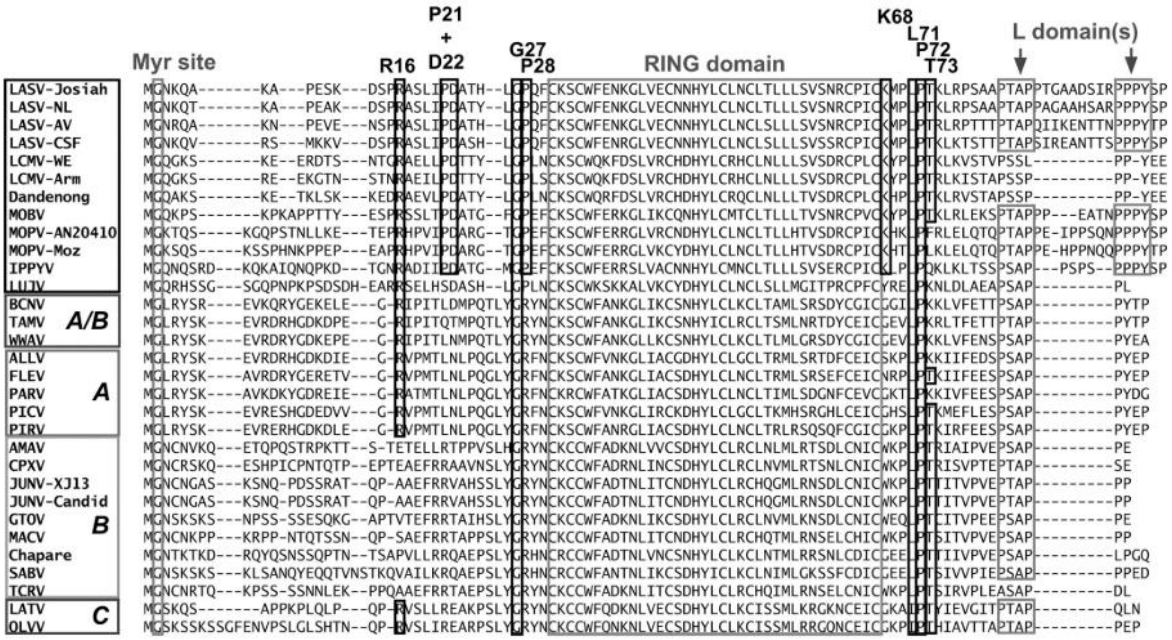

Figure 4 ClustalW alignments of arenavirus Z proteins. The Z proteins were arranged by clade as seen on the left side. Already well-known conserved regions like myristoylation site (G2), RING domain and Late domains are highlighted in grey boxes, whereas conserved residues outside of these boxes and targeted for mutagenesis are outlined in black. The position of each residue in LASV Z is indicated above the alignment, and the Z amino acid sequences from the following species (indicated by GenBank accession no. or NCBI reference sequence no.) were used: NP_694871.1 (LASV Josiah), AAO59510.1 (LASV NL), AAO59508.1 (LASV AV), AAO59514.1 (LASV CSF), AAD03395.1 (LCMV WE), ABC96003 (LCMV Armstrong), ABY20731 (Dandenong virus), ABC71138.1 (Mobala virus [MOBV] Acar), AAV54106.1 (Mopeia virus [MOPV] AN20410), ABC71136.1 (MOPV Mozambique), ABC71142.1 (Ippy virus [IPPYV] Da- kAnB), YP_002929492 (Lujo virus [LUJV]), YP_001649224 (Bear Canyon virus [BCNV]), YP_001911117 (Tamiami virus [TAMV]), YP_001911119 (Whitewater Arroyo virus [WWAV]), YP_001649213 (Allpahuayo virus [ALLV]), YP_001936023 (Flexal virus [FLEV]), YP_001936027 (Parana virus [PARV]), YP_138535 (Pichinde virus [PICV]), YP_025092 (Pirital virus [PIRV]), YP_001649217 (Amapari virus [AMAV]), YP_001649219 (Cupixi virus [CPXV]), NP_899216 (JUNV XJ13), AAV68494 (JUNV Candid), NP_899220 (Guanarito virus [GTOV]), NP_899214 (Machupo virus [MACV]), YP_001816784 (Chapare virus), ABY59837 (Sabia virus [SABV]), Q88470 (Tacaribe virus [TCRV]), YP_001936025 (Latino virus [LATV]), and YP_001649215 (Oliveros virus [OLVV]) (Capul et al., 2011). Figure used with permission of American Society for Microbiology - Journals, from Journal of Virology, American Society for Microbiology, volume 85, issue 7, 2011.

1.5 Life cycle modelling systems

1.5.1 Minigenome system

Minigenome systems are the simplest life cycle modelling system (i.e. reverse-genetics based systems that allow to model the life cycle of viruses) and can be used for investigation of viral

replication, transcription, and protein expression in isolation. The core of this system is the usage of a miniature version of the viral genome (hence the name “minigenome”), in which all viral open reading frames (ORFs) have been removed and replaced by a reporter gene. These systems can encode either one (monocistronic) or multiple (multicistronic), but not all, viral proteins (Ebihara et al., 2005).

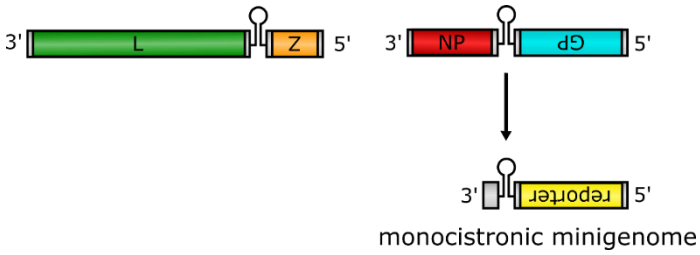

Figure 5: Schematic display of LASV genome segments L and S as well as the minigenome coding for a reporter instead of GP. The LASV genome consists of 2 segments, each coding for two proteins in ambisense orientation, interrupted by non-coding intergenic regions. To create a monocistronic minigenome both ORFs of the small genome segment are deleted and substituted with a single reporter gene. Illustration was kindly provided by Dr. Lisa Wendt.

Moreover, addition of the viral proteins necessary for replication and transcription of the viral genome is essential. In case of LASV these proteins are NP and L and are provided by expression plasmids in *trans*. For initial transcription of the minigenome the T7-polymerase of T7 bacteriophage is used. Therefore, the minigenome plasmids includes the T7-promotor, upstream of the 3’ untranslated region (UTR), and the T7-terminator, downstream of the 5’-UTR. Moreover, downstream of the reporter gene the hepatitis delta virus (HDV) ribozyme is included in the construct. Its self-catalytic function is necessary to provide a precise 3’-end, as the T7 polymerase attach further non-encoded nucleotides to 3’ ends of RNAs. The T7 polymerase is provided in *trans* as Pol-II driven plasmid. In this thesis a monocistronic minigenome of LASV was developed with nanoluciferase (nluc) incorporated at the GP locus. After transfection of minigenome plasmids and corresponding plasmids encoding RNP proteins in the respective amount, the T7-driven initial transcription of minigenomes takes place. RNP proteins then drive minigenome replication and secondary transcription. Ultimately, this leads to reporter expression which can be measured with a luminometer, and the results correlate directly with viral replication and secondary transcription. Moreover, firefly luciferase (FF) was co-transfected as a tool for normalization between samples.

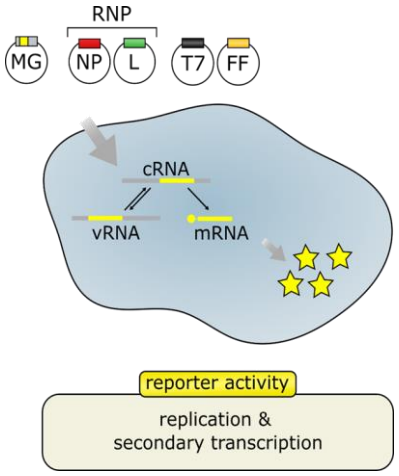

Figure 6: schematic overview of minigenome assay performed in eukaryotic cells. Crucial components of a minigenome assay are transfected as plasmids in eukaryotic cells, including the minigenome construct, RNP proteins, T7 polymerase and FF luciferase. Within the cell transcription and replication takes place, followed by expression of the reporter gene. The expression levels can then be measured and correlate with minigenome replication and secondary transcription. Illustration was kindly provided by Dr. Lisa Wendt.

1.5.2 Transcription and replication-competent virus-like particle system

The transcription and replication-competent virus-like particle (trVLP) system is another life cycle modelling system, which can not only be used to investigate replication and gene expression, but also production of viral particles and cell entry. These systems use a further advanced minigenome encoding proteins necessary for viral budding and entry, in the case of arenaviruses GP and Z are the minimum requirement for these steps (Lee et al., 2002). For LASV such system exists so far only as a chimeric approach utilizing a Lymphocytic Choriomeningitis Virus (LCMV) minigenome system, which is often used as prototype for Old World Arenaviruses, in combination with LASV Z (Perez et al., 2003, Capul et al., 2011). In this thesis a bicistronic minigenome system suitable for a trVLP assay and solely based on LASV components was developed. Therefore, the GP-ORF was replaced by Z and the NP-ORF was replaced by GP linked with nluc. Moreover, for this linkage the T2A peptide was used, which leads to self-cleavage during translation (Liu et al., 2017), resulting in the production of GP and nluc as separate proteins from one ORF (Liu et al., 2017). This minigenome with its corresponding RNP proteins and T7-polymerase could be used to transfect producer cells (p0), where transcription and replication competent virus-like particles are formed and released into the supernatant. The cleared supernatant could then be used to infect the next cell passage (p1). Therefore, reporter activity from p0 correlates with replication and secondary transcription in p0, whereas reporter activity from p1 correlates with replication

(p0 & p1), secondary transcription (p0 & p1), assembly and budding (p0) and lastly with viral entry (p1). Furthermore, to take differences in gene expression between samples into account, firefly luciferase (FF) was co-transfected and used as internal normalization control.

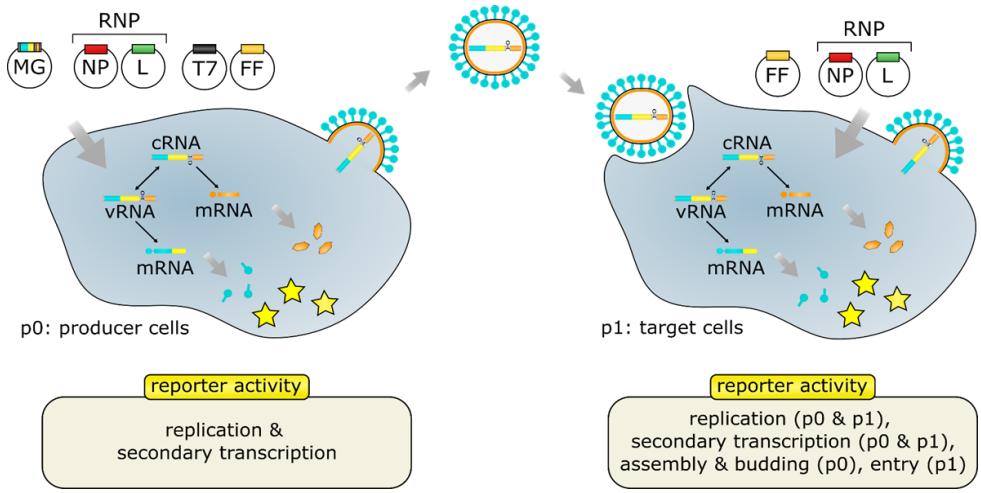

Figure 7: Schematic display of transcription and replication virus-like particle assay performed in eukaryotic cells. The components of a trVLP assay, the minigenome construct including sequences for GP and Z, the RNP proteins, T7 polymerase, and FF luciferase, are provided as expression plasmids and transfected in producer cells (p0). Within the cells minigenome expression takes place leads to formation of virus-like particles enveloping minigenome constructs. Moreover, reporter expression can be measured and correlate with replication and secondary transcription. The virus-like particles are then used to infect target cells (p1). The reporter activity in p1 cells correlates with replication and secondary transcription of both p0 and p1 as well as with assembly and budding in p0 and entry in p1. and Illustration was kindly provided by Dr. Lisa Wendt.

1.6 Objectives of the study

Work with infectious Lassa virus (LASV) particles is restricted to a few high containment laboratories worldwide. To overcome this bottle neck, reverse genetics systems are a powerful tool to investigate highly pathogenic viruses under BSL1/2 conditions, making research more accessible. In the past such systems for LASV sometimes comprised both LASV and LCMV components in form of chimeric sytems (Capul et al., 2011). Moreover, arenaviruses and functional properties of their proteins are often investigated with systems solely based on the prototypic arenavirus LCMV (Iwasaki & de la Torre, 2018; Perez et al., 2003; Pinschewer et al., 2003). In this thesis two reverse genetics systems solely based on LASV components should be developed as well as used to investigate effects of conserved amino acid residues within LASV Z on viral RNA synthesis and egress. The individual tasks were specified as followed:

- A novel minigenome system for LASV, solely based on LASV components should be developed. To this end, the LASV genomic S-segment should be used as a basis and nanoluciferase should be introduced as reporter gene. Moreover, optimal assay conditions should be identified through titration of RNP proteins and comparing assay performance in different cell lines.

- Secondly, a novel transcription and replication competent virus like particle assay solely based on LASV components should be developed. To this end, the genomic LASV S-segment was once again to be used as a starting point and modified to encode LASV Z as well as GPC linked with nanoluciferase.

- Lastly, both developed systems should be used to investigate the role of nine conserved amino acid residues within LASV Z.

2 Material and Methods

2.1 Molecular biology methods

2.1.1 Reverse transcription

Reverse transcription is a method to generate cDNA from RNA *in vitro* using a reverse transcriptase, e.g. Superscript IV (Thermo Fisher Scientific, USA). Components for an RT-reaction using this enzyme are deoxynucleotide triphosphates (dNTPs), oligo $d(T)_{20}$ primers, template RNA, Dithiotreitol (DTT) (Carls Roth, Germany), ribonuclease inhibitor and the reverse transcriptase.

In the first step RNA, primers and dNTPs were mixed and incubated shortly at 65°C to remove secondary structures of RNA and enable annealing of the random primer. During incubation, the reaction mix was prepared containing reaction buffer, DTT, ribonuclease inhibitor and the transcriptase. The reaction mix was stored on ice until further use. After primer annealing followed a cooling step on ice to prevent reforming of secondary structures of the RNA. Afterwards the reaction mix was added, briefly mixed (without vortexing) and incubated at 50°C for 10 min followed by 80°C for 10 min.

2.1.2 Polymerase chain reaction

The polymerase chain reaction (PCR) is a well-established and commonly used method to amplify DNA fragments *in vitro* (Mullis et al., 1986). Necessary components of the reaction are heat resistant DNA-dependent-DNA-Polymerase, template DNA containing the desired sequence, forward and reverse primer, dNTPs and Mg^{2+}.

The following reaction took place in a C1000 Thermal Cycler (Bio-Rad). At first the reaction started with an incubation step at 95°C leading to dissociation of the double stranded DNA to single strand DNA making it accessible for primers. Each primer has its individual optimal annealing temperature depending on length and GC-content. The annealing is followed by an elongation step at the optimal working temperature of the polymerase in which the polymerase uses the dNTPs to elongate the desired DNA sequence starting at the primers. The amplified DNA of the preceding cycles can be used as template for the following ones. Therefore, multiple cycles consisting of denaturation, primer annealing and elongation lead to a theoretically exponential growing DNA yield. However, the yield is not exponentially growing due to decreasing primer to amplicon ratios. The experimental setup and cycling conditions used are displayed in Table 1 and

Table **2**. In this thesis the KAPA HiFi HotStart Readymix (Roche, Switzerland) was used. Moreover, details on all used primers are listed in the appendix 8.1.6.

Table 1 experimental set up for PCR

LASV-cDNA (Josiah) or Plasmid	1 µL LASV-cDNA (Josiah)	50 ng Plasmid
Primer fwd	1.5 µL	
Primer rev	1.5 µL	
KAPA HiFi HotStart Readymix	25 µL	
Nuclease-free water	Fill up to 50 µL	
∑	50 µL	

Table 2 PCR cycling conditions

Step		Temperature	Time
initial denaturation		95 °C	3 min
35x	Denaturation	98 °C	20 s
	primer annealing	primer specific	15 s
	Elongation	72 °C	60 s/kb
final elongation		72 °C	60 s/kb

2.1.3 Analytical and preparative restriction digest

The method of restriction digest is used as a tool to cut DNA with bacterial restriction enzymes, which recognize specific DNA sequences, called recognition sites, and cut both DNA strands. The digest could result in overhanging ends called “sticky ends” or without an overhang called “blunt ends”. To perform the reaction, DNA was mixed with restriction enzyme(s) and their corresponding buffer. The setup of reaction depends on the intend of the method, for an analytical restriction digest 5 µL of purified plasmid was incubated with 0.5 µL of each used enzyme. Whereas for a preparative restriction digest 3 µg of plasmid were used and 1 µL per used enzyme. The reaction was then incubated at 37 °C for either one hour (analytical restriction digest) or over-night to ensure complete digestion of DNA for preparative restriction digest.

2.1.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a useful tool to image DNA after PCR or restriction digest. At first agarose was mixed with 1x TAE buffer for a final concentration of 1-2 % and boiled until

the agarose was fully dissolved and cooled down to about 55 °C before pouring the gel into the mold. After the gel is polymerized, it was moved into a horizontal electrophoresis chamber filled with 1x TAE buffer, and DNA samples mixed with 6x Purple Loading Dye could be loaded into the gel pockets, additionally a DNA Ladder (1kb) was loaded into a separate gel pocket, and the gel was run at 100-120 V.

After electrophoresis the gel was either stained with ethidiumbromide (for analytical gels) or Fast Blast (for preparative gels). If the gel was stained with ethdiumbromide (15min in a 0.5 µg/ml ethidiumbromide solution), it is necessary to illuminate the gel with UV light to make DNA with intercalated ethidiumbromide visible. If the gel was dyed with Fast Blast (three min in 100x Fast Blast solution), it was washed with warm water, with about three to five water changes, until the DNA bands become visible.

2.1.5 Purification of DNA from agarose gel

After preparative restriction digest or PCR, DNA fragments of different lengths could be separated by agarose gel electrophoresis, after which the desired DNA fragment band was cut out with a scalpel. For the purification the Kit NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) was used and according to protocol the gel was weighted and double the amount of NTI buffer was added. Afterwards the mixture was incubated at 55 °C for five to ten minutes until the gel had fully dissolved. Now the mixture could be loaded onto the provided spin column, which binds the DNA to its membrane during centrifugation. The resulting flow-through could then be discarded. Afterwards washing steps were performed, followed by the elution process, where the column was incubated with elution buffer at 70 °C for five minutes, after which the DNA was eluted by centrifugation.

2.1.6 Purification of PCR products

For the Purification of PCR products, the kit NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) was used. At first double the amount of NTI-buffer was added to 50 µL PCR product, the mixture was loaded onto the spin column and a bind wash protocol was performed according to manufacturer's instructions. For the elution step an incubation step at 70 °C for 5 min was performed and lastly the DNA was eluted by centrifugation.

2.1.7 Dephosphorylation

The method of Dephosphorylation was used to avoid religation of linear vector DNA. Since the ligase needs a 5'-phosphate to ligate DNA strands, an alkaline shrimp phosphatase (rSAP) is used to eliminate the 5'-phosphate of cut vector DNA. Therefore, dephosphorylated vector DNA without insert should not be capable of religation, whereas vector with ligated insert contains a 5'-phosphate and therefore should ligate. To perform this method, 2 µL rSAP were

added to digest product and incubated at 37 °C for 60 min. If the digest product was purified prior to dephosphorylation, an appropriate amount of 10x CutSmart buffer is required to add to the reaction.

2.1.8 Ligation

Ligation is a method to combine 2 (or more) DNA fragments. Usually, vector DNA is ligated with an insert to form a plasmid which can then be transformed into bacterial cells. The enzyme catalyzing this reaction is called ligase. In this thesis the T4 ligase (New England Biolabs, Ipswich, USA) was used. During the reaction a phosphodiester bond is formed between the 5' end and the 3' end. Normally, an excess of insert is used either as 2 (vector) : 9 (insert) volume ratio or as 1 (vector) : 2 (insert), 1 (vector) : 3 (insert), 1 (vector) : 5 (insert) molar ratio. It is important to perform a religation control where the amount of insert is substituted with dH_2O, as this allows to draw conclusions regarding the amount of background colonies after transformation of the ligation product. If the vector was not cut or dephosphorylation took not place properly, there will be colonies on the control plate which incorporated circular vector DNA without the gene of interest. Therefore, also the colonies on the ligation product plate have a high chance of not containing the gene of interest, and thus a high number of colonies should be picked for screening. On the other hand, if there are few to no colonies on the control plate, there is a high possibility that the colonies on the sample plates incorporated vector containing the gene of interest, so that only few colonies needed to be screened for a successful insertion of the gene of interest in the next step.

2.1.9 Preparation of chemically competent bacteria

In order to prepare chemically competent bacterial cells the mix and go *E.coli* Transformation Kit (Zymo Research) was used. First, an aliquot of competent XL1-blue *E.coli* was thawed on ice and added to 5 mL of LB medium without antibiotics followed by incubation overnight at 37 °C. Then, 1 mL of the overnight culture was used to inoculate 100 mL SOC medium, which was incubated at 26 °C until an OD_{600nm} between 0.4 – 0.6 was reached. Afterwards, the culture was placed on ice for ten minutes followed by pelleting the cells by centrifugation at 1600-2500 x g for ten minutes at 4 °C. The resulting cell pellet was resuspended in 5 mL ice cold 1x wash buffer and re-pelleted through centrifugation at 1600-2500 x g for ten minutes at 4 °C. The supernatant was removed, and the pellet resuspended in 5 mL ice cold 1x competent buffer. Then the competent cells were distributed into 50 µL aliquots and shock frosted in liquid nitrogen before long term storage at -80 °C.

2.1.10 Transformation of chemically competent bacteria

Two different chemically competent bacteria strains were used in this thesis. For constructs smaller than 6 kb the Escherichia coli XL1-blue strain was used, whereas for constructs longer than 6 kb or problematic constructs the Escherichia coli NEB 10-beta strain was used. Both strains are stored as 50 µL aliquots at -80 °C. In order to perform transformation, cells were thawed on ice and 1-10 µL of DNA was added. After 30-60 min incubation on ice the XL1-blue cells could be plated on a selection plate, whereas the NEB 10-beta were incubated 30 min on ice followed by a 30 s heat shock at 42°C and once again incubation on ice for five minutes. Then 950 µL SOC (outgrowth medium) were added to the reaction tube and incubated at 37 °C with shaking for an hour before spreading on a selection plate. Whereas in most cases plates were incubated at 37 °C overnight, problematic constructs were incubated at 30 °C or room temperature for 24 to 48 hours.

2.1.11 Preparation of plasmid DNA from bacterial cultures

For the preparation of plasmid DNA from bacterial cultures two different kits were used, depending on culture scale 5 mL or 100-200 mL (Mini: NucleoSpin Plasmid EasyPure/ Midi: NucleoBond Xtra Midi). Both kits use the principle of alkaline lysis of bacterial cells, but differ in their purification methods. The mini kit utilizes a silica membrane for DNA binding , whereas the midi kit uses an anion exchange column. Therefore, both methods differ in their salt conditions, and ethanol precipitation after performing a midi kit preparation is necessary. Both protocols were started with the resuspension of pelleted bacteria, followed by alkaline lysis and neutralization. Then, with a centrifugation step cell debris, precipitated protein and genomic DNA were pelleted and the clear supernatant was transferred onto a column, which captures plasmid DNA. Afterwards washing steps and elution followed.

For minipreparation a volume of 5 mL LB-medium containing the Ampicillin (1 mg/mL) was inoculated with transformed *E.coli* and incubated at 37 °C overnight, whereas for midi preparation 100 mL (high copy plasmid) or 200 mL (low copy plasmid) LB-medium with antibiotic was inoculated with 1 mL of miniculture and incubated overnight under the same conditions. After conducting the plasmid preparation according to the manufactorer's manual the DNA concentration was measured by adding 1 µL of solution into the NanoPhotometer P300 (Implen, Germany)

2.1.12 Sequencing

In order to control the created constructs, Sanger-Sequencing was performed by Seqlab (Microsynth) or GATC (Eurofins genomics). Afterwards the data was aligned with reference sequences of the LASV strain “Josiah”, or reference sequences created using Clone Manager (Sci Ed Software). For details on used primer see appendix 8.1.6.

2.1.13 Site directed mutagenesis

Site directed mutagenesis (SDM) is a method used to replace amino acid residues within a gene in a controlled manner. To this end, primers encoding the desired point mutations are designed and PCR is performed with said mutagenesis primers and a plasmid, containing the gene of interest as template. In our case conserved amino acid residues within LASV matrix protein Z were substitutated by alanin using the bicistronic mingenome as template. Since this template plasmid was purified from bacterial culture, it was methylated. In contrast, the PCR product was unmethylated. Therefore, a restriction digest with DpnI (NEB, Ipswich, USA) which only digests methylated DNA was performed and only PCR product should remain intact for the following transformation in *E.coli.*

Since the original plasmid and the mutagenesis product are almost identical, three colonies of each SDM attempt were picked and analyzed by sanger sequencing. All used primes are listed in the appendix 8.1.6.

2.2 Tissue culture methods

Almost all created constructs were used in cell-based assays using the mammalian cell lines human hepatocarcinoma cells (Huh7, provided by Stephan Becker, Philipps University Marburg, Germany), baby hamster kidney (BHK-21) cells which constantly express T7 and T5 (BsrT7/5, CCLV-RIE0583 provided by Stefan Finke, Friedrich-Loeffler-Institut, Germany) or human embryonic kidney (HEK-293T) cells (Collection of Cell Lines Veterinary Medicine CCLV-RIE1018).

2.2.1 Cultivation of mammalian cells

The cell lines HEK-293T and Huh7 were grown in cell culture fiasks (T75) using Dulbecco’s modified Eagles minimum essential medium (DMEM, FLI internal distribution) supplemented with 10 % fetal bovine serum (FBS) and 1x GlutaMAX (Thermo Fisher Scientific) as well as 100U/mL penicillin and 100µg/mL streptomycin (P/S; ThermoFisher scientific). BsrT7/5 cells were maintained in Glasgows modified Eagles minimum essential medium (GMEM, FLI Internal distribution) supplemented with 10 % newborn calf serum (NCS) and 100 U/mL penicillin and 100µg/mL streptomycin (P/S; ThermoFisher scientific, USA). To every second

passage of BsrT7/5 cells 1 mg/mL Geneticin (Thermo Fisher Scientific, USA) was added to secure T7 and T5 expression. All cells were incubated at 37 °C with 5 % carbon dioxide. Every 3 to 4 days cells were splitted in order to maintain healthy growth. For splitting of the cells, the supernatant was carefully removed, and the appropriate amount of Alsever's trypsin versene (ATV) solution (3 mL for T75) was added to detach the adhering cells from the flask surface. After a few minutes (depending on cell line) at room temperature all cells were detached, and culture medium (7 mL for T75 flask) could be added to stop the trypsin reaction. Afterwards an aliquot of the cell mixture was transferred into a sterile falcon tube and diluted with culture medium to maintain healthy growth in a new culture flask.

2.2.2 Minigenome assay

The minigenome assay is a life cycle modelling system and can be used to analyze the expression of a protein of interest. To this end, the minigenome construct includes the non-coding regions of the small LASV genome segment with the NP ORF removed, and the ORF of GPC substituted with the reporter gene nluc, Therefore, luminescence signal of nluc correlates with gene expression.

The minigenome assay was performed in three different cell lines, Huh7, BsrT7/5 and HEK-293T, starting with the seeding of cells in a 12-well plate. This was followed by transfection of plasmids encoding NP (0-1000 ng), L (0-1000 ng), minigenome (125 ng), T7 (125 ng) and firefly-luciferase (FF, 12.5 ng) 24 h after seeding at approximately 50% confluency.

The transfection of cells was performed with TransIT LT-1 (Mirus Bio LLC) as transfection reagent, which was mixed with OptiMEM (Thermo Fisher Scientific, USA) and the plasmids listed above. After 15 minutes at room temperature DMEM supplemented with 5% FBS was added, and the mixture was carefully added to the cells followed by a centrifugation step at 1000xg for 10 minutes and room temperature. Subsequently, the cells were incubated for 24 hours at 37 °C and 5% carbon dioxide. Then, the growth medium was exchanged, followed by incubation for 24 h at the same conditions than before. Finally, luciferase assays were performed to acquire data on minigenome reporter activity as well as activity of firefly luciferase, which is used as an internal process and standardization control. In general, luciferases are enzymes which catalyze a light-emitting chemical reaction, and the intensity of the emitted light correlates with the expression of the luciferase, and thus in case of the LASV minigenome with viral RNA synthesis.

To measure luciferase activity, the medium was removed from the cells and 200 µL of lysis juice (Promega) was added. After 10 min incubation at room temperature, the cell suspension was transferred to a microcentrifuge tube and centrifuged for 3 min at 10000 x g. Subsequently,

40 µL of the cleared supernatant was mixed with 40 µL of the corresponding luciferase substrate solution. This was a mixture of Nano-Glo Luciferase Assay Buffer (Promega) and Nano-Glo substrate (Promega) for the nanoluciferase assay, and a mixture of Beetle juice (PJK GmbH) with D-Luciferin (PJK GmbH) for firefly assay. Finally, the luminescence was measured in a Tecan Infinite F200 Pro.

2.2.3 Transcription and replication virus like particle assay

The transcription and replication virus like particle (trVLP) system is an extension of the minigenome system and can be used to investigate the whole viral life cycle. Here, two cell passages are involved: The producer cells p0 are initially transfected and release virus-like particles into the supernatant, which can be used to infect the next cell passage p1. The reporter signal of both allow drawing different conclusions for either gene expression, viral egress or cell entry.

First, cells were split (1:6 for Huh7, 1:4 for BsrT7/5 and HEK-293T), seeded in a 12-well plate and grown in medium overnight (p0). This was followed by transfection of expression plasmids for T7 (125 ng), NP (125 ng), a bicistronic minigenome (125 ng), L (500 ng) and firefly luciferase (FF, 12,5 ng) on the next day. For the negative control it was important to keep the overall quantity of transfected plasmid constant; therefore, 500 ng of empty vector or GFP were used instead of L. Then, the medium was changed after 24h post transfection (to medium containing 5 % FKS, 2 mL per well). The next day reverse transfection of p1 cells was performed. To this end, plasmids encoding NP (125 ng), L (500 ng) and FF (12,5 ng) were mixed with 50 µL OptiMEM and TransIT. The cells were diluted, added to the TransIT mix, and incubated for 15 min at room temperature. The whole 1000 µL containing plasmids and cells were then seeded in one well of a 12-well plate. On day 3 post transfection of p0, the supernatant of p0 was removed and centrifuged (10 min at 1000 x g), whereas the cells were used for a luciferase-reporter-assay. Simultaneously, 24 hours post transfection of p1, the supernatant of p1 was removed and replaced by the cleared supernatant of p0. After centrifugation at 1000xg and room temperature for 10 min the cells were incubated at 37 °C and 5 % carbon dioxide for one hour. Afterwards, the supernatant of p1 (inoculated with trVLPs of p0) was replaced by fresh medium. The p1-cells were harvested 72 hours after infection and used for a luciferase-reporter-assay, performed as described in section minigenome assay.

2.3 Statistical methods

Statistics were performed using two-way ANOVA (analysis of variance) for data analysis of minigenome performance in different cell lines, and ordinary one-way ANOVA for data analysis

of minigenome systems with and without decoy GFP ORFs and analysis of Z mutants in minigenome and trVLP assay. All statistical methods were conducted in GraphPad – Prism (version 9.4.1).

3 Results

Since work with infectious LASV is restricted to high containment laboratories, novel approaches to investigate its life cycle under BSL-1/2 conditions are needed. The presented thesis includes the development of two life cycle modelling systems for LASV able to achieve this goal. Moreover, nine conserved amino acid residues within LASV Z were modified and their effects were studied using the developed reverse genetic systems.

3.1 Cloning of expression plasmids containing LASV proteins

For performing a minigenome assay the viral RNP proteins L and NP must be added as expression plasmids in *trans*. Moreover, for the later development of a transcription and replication competent virus-like particle assay an expression plasmid coding LASV GP was needed as well. Therefore, the first step was to either obtain or generate these necessary plasmids.

In the cases of LASV NP and GP, the construct pCAGGS-LASV-NP was already available at the facility and a plasmid containing LASV GP was kindly provided by PD Dr. Allison Groseth (Head of the Laboratory for Arenavirus Biology at the Friedrich-Loeffler-Institut), and GP could be successfully cloned from this template into pCAGGS.

However, the gene sequence of LASV L exceeds 6.5 kb and, therefore, two strategies were attempted to clone this gene into the plasmid. Specifically, LASV L was either amplified in 3 fragments per PCR and restriction digests with XhoI, BbvCL and PspXI were performed before ligation with pCAGGS. Alternatively, amplification of LASV L as one fragment by PCR with LASV cDNA as template was performed and followed by restriction digest with XhoI and ligation with pCAGGS.

After each of the resulting clones from the two different cloning strategies showed at least one mutation in the L sequence, two constructs were combined through restriction digest and ligation. This resulted in successfully generating the pCAGGS-LASV-L construct.

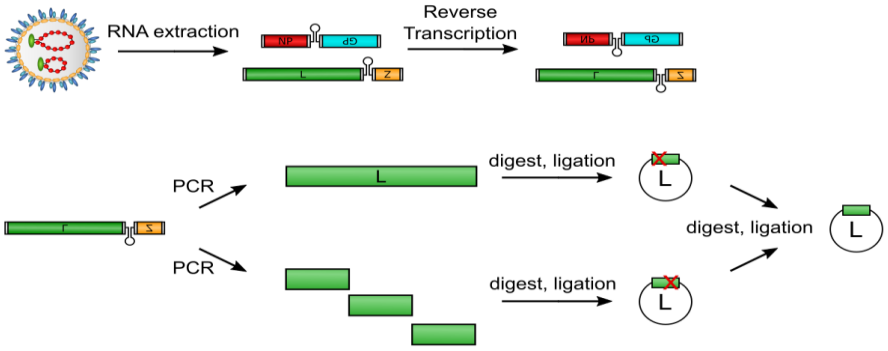

Figure 8: Schematic display of cloning process for LASV L. Cloning started with reverse transcription of the viral genome, followed by using the resulting cDNA as template for PCR. This was followed by restriction digest of the PCR product(s) and vector as well as ligation of these. After none of the clones resulted in intact constructs, two clones each containing one point mutation at different ends of the protein (indicated with red X) were combined through restriction digest and ligation, ultimately resulting in the generation of a successful clone. Illustration was kindly provided by Dr. Lisa Wendt.

3.1.1 Cloning of expression plasmids containing LASV GP linked with nanoluciferase

pCAGGS-LASV-GP was amplified by PCR with modified primers to enable insertion via restriction digest and ligation into either pCAGGS-oligo-T2A-nluc or pCAGGS-oligo-T2A-GFP, resulting in a vector containing LASV GP linked to either nanoluciferase or GFP. These constructs were the basis for developing a bicistronic minigenome suitable for the trVLP assay.

3.2 Cloning of expression plasmids containing LASV minigenomes

The core of a minigenome system is a simplified version of the viral genome lacking viral ORFs and instead encoding a reporter. To generate such a system, the non-coding regions of the S-segment of LASV were synthesized and cloned into the vector pAmp via the restriction enzyme sites NotI and RsrII. Afterwards, this construct was used as a basis for minigenome variations. Therefore, the next step was to cut the sequence for nanoluciferase out of another vector and insert it into the empty minigenome casette, resulting in pAmp-LASV-S-dNP-dGP_nluc. Further, for imaging a minigenome with GFP instead of nanoluciferase was additionally cloned.

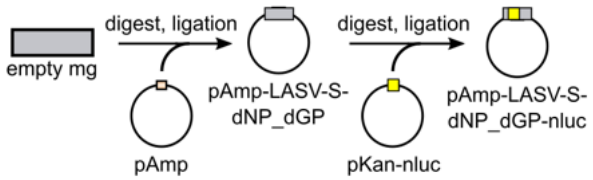

Figure 9: Schematic display of generation of LASV minigenome with nanoluciferase as reporter. This started with the empty minigenome cassette, which was cloned into the vector pAmp, followed by insertion of nluc at the GP locus. Illustration was kindly provided by Dr. Lisa Wendt.

Since unspecific translation of reporters can be a source of background signals, decoy ORFs were introduced. These serve as decoys instead of the actual reporter for translation from cryptic promoters, while not interfering with signal output of the assay and, therefore, minimizing background noise. From a practical point of view the nanoluciferase minigenome was cloned via NotI and RsrII restriction sites into a vector already encoding eGFP decoy ORFs. These were present in reverse orientation (3'-5'; left, L) before and after the T7 terminator (1L2L), since this orientation was shown to be the most efficient in lowering background signals for a Junín virus minigenome (Dunham et al., 2018).

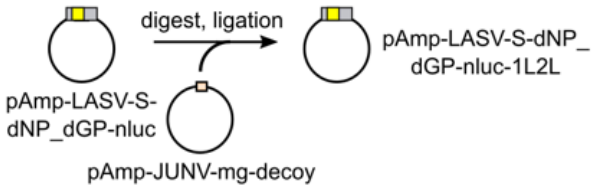

Figure 10: schematic display of generation of LASV minigenome with nanoluciferase as reporter and additional eGFP decoy ORFs. The minigenome construct was cut out of the original vector and instead got inserted into a vector already containing eGFP decoy ORFs. Illustration was kindly provided by Dr. Lisa Wendt.

3.3 Establishment of optimal conditions for a LASV minigenome assay

The performance of minigenome assays is dependent on various factors, including the type of cell line in which the assay is performed, and in which ratios RNP proteins are provided. Therefore, possible cell lines were screened and RNP proteins were titrated to determine optimal assay conditions. Furthermore, decoy eGFP ORFs were implemented to minimize background noise.

3.3.1 Determination of optimal cell lines for a LASV minigenome assay

The performance of a minigenome assay is dependent on the cell line it is performed in. Thus, the LASV minigenome assay underwent evaluation in Huh7, BsrT7/5, and HEK293T cell lines, all previously validated for compatibility with the JUNV minigenome assay and hence considered viable candidates for implementing the LASV minigenome system. Theoretically, in a minigenome assay lacking viral polymerase L no fluorescence signal should occur. However, due to cryptic promotor activity, a baseline background signal persists. Therefore, the assay performance is assessed by comparing the signal intensity difference between negative (-L) and positive (+L) samples.

The results of these preliminary tests are displayed in Figure 11, where the y-axis represents reporter activity in logarithmic levels of relative light units (RLU). Moreover, results for the already well-established JUNV minigenome assay are seen on the left side, acting as benchmark to identify optimal assay conditions for the LASV minigenome assay. The positive LASV samples exceed the background by approximately 2 log levels, while not showing significant differences between cell lines. However, considering the established compatibility of Huh7 cells with the JUNV minigenome system, subsequent experiments with the LASV minigenome system were conducted exclusively with Huh7 cells.

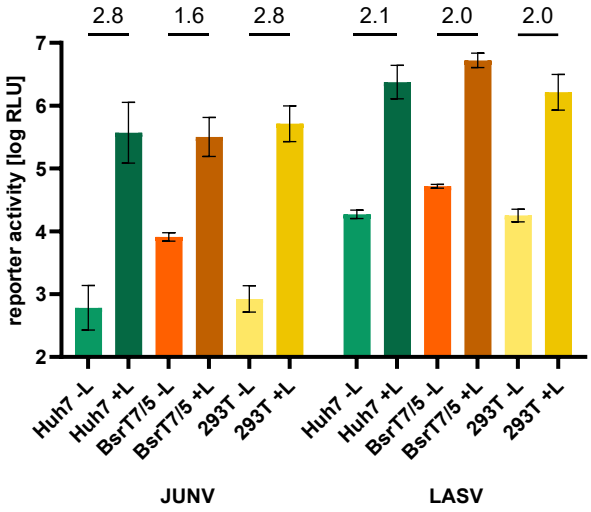

Figure 11: Comparison of JUNV and LASV minigenome systems in the cell lines Huh7, BSrT7/5 and Hek293T. The right side represents LASV minigenome assay performance in each cell line, left side displays the JUNV minigenome as reference. Means and standard deviations of 3 independent experiments are shown. The numbers above the bars indicate the dynamic range (positive control (+L) signal minus corresponding negative control (-L) signal) for each investigated cell line.

3.3.2 Influence of eGFP decoy ORFs

The next step for optimization of assay performance was further reduction of background signal. One potential option is adding decoy eGFP ORFs, which would be expressed by cryptic promotor activity, instead of the minigenome-encoded reporter.

Figure 12 illustrates the comparison between the established JUNV minigenome system with (1L2L) and without (w/o) eGFP decoy ORFs on the left, and the newly developed LASV minigenome system with (1L2L) and without (w/o) eGFP decoy ORFs on the right. Both systems display reduced background noise due to the implementation of eGFP decoy ORFs.

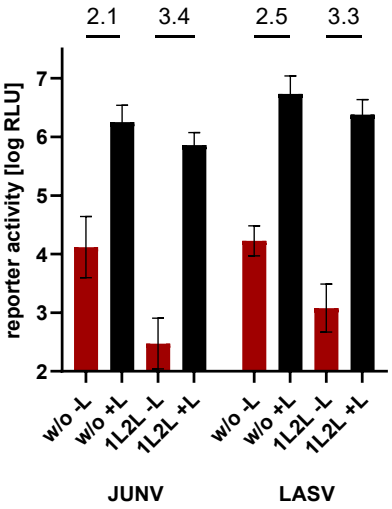

Figure 12: Comparison of minigenome assays with and without eGFP decoy ORFs. Minigenome assays for JUNV (left side) and LASV (right side) with (1L2L) and without (w/o) eGFP decoy ORFs were run. Means and standard deviations of 3 independent experiments are shown. The numbers above the bars indicate the dynamic range (positive control (+L) signal minus corresponding negative control (-L) signal) for each minigenome construct.

3.3.3 Titration of RNP proteins

Necessary for the functionality of a minigenome assay is the supply of RNP proteins, which are not encoded by the minigenome itself. The respective amount of RNP proteins needed for optimal assay performance can be determined through titration of NP or L. As depicted in Figure 13, the minigenome system efficiency initially increases in correlation with the amount of transfected NP. However, it reaches a small plateau between 62.5 and 125 ng of transfected pCAGGS-NP after which the signal intensity decreases. Therefore, further experiments were conducted with 125 ng of transfected pCAGGS-NP.

On the other hand, the titration series of L displays no clear maximum, but rather a steady increase of reporter activity with increasing amount of L plasmid transfected. However, to ensure comparability to a previously established minigenome system for JUNV, and for practical reasons, 500 ng was chosen as amount of pCAGGS-L to be transfected in future experiments.

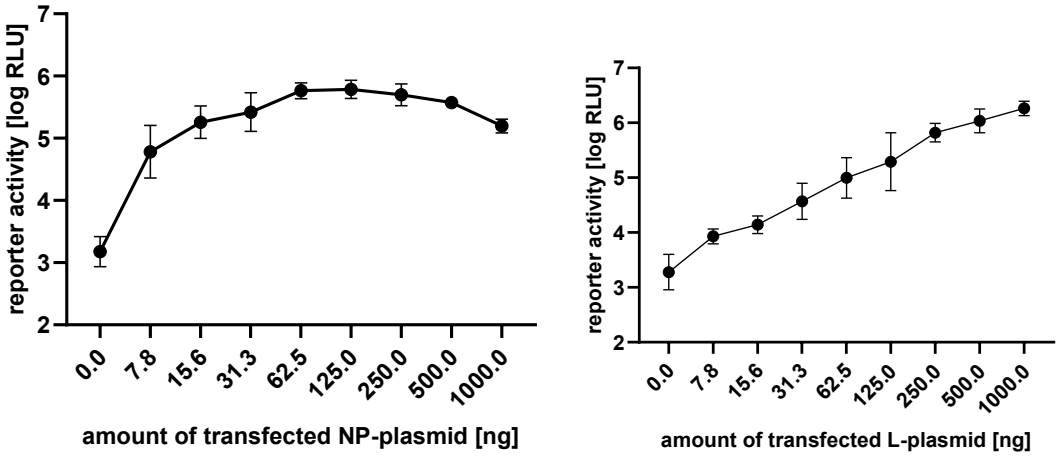

Figure 13: Titration series of transfected RNP proteins. The titration series of NP plasmid with the range of 0.0 ng up to 1000 ng is seen on the left side, and the titration series of L-plasmid with a range of 0.0 ng up to 1000 ng is seen on the right side. n=3

3.4 Cloning of a bicistronic LASV minigenome

For the transcription and replication-competent virus-like particle (trVLP) assay, creation of a bicistronic minigenome that encodes both LASV GP and Z was necessary. Thus, the expression plasmid containing GP, linked with nanoluciferase via the self-cleaving T2A peptide, was incorporated into the NP locus of the empty minigenome using BspMI restriction sites. This was followed by the insertion of Z into the GP locus through BsmBI restriction sites.

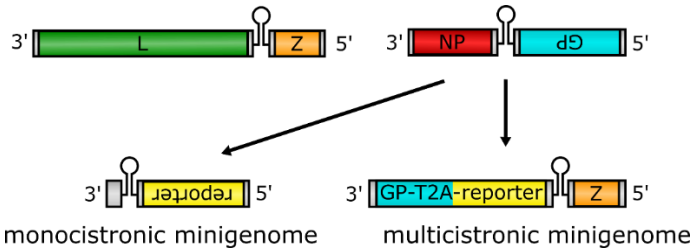

Figure 14: Schematic display of generation of both monocistronic and multicistronic minigenomes for LASV. For creation of a monocistronic minigenome a reporter gene was inserted into the GP locus of the small genome segment. On the other hand the bicistronic minigenome was obtained through inserting Z into the GP locus and the reporter linked with GP through a T2A linker was inserted in the NP locus. Illustration was kindly provided by Dr. Lisa Wendt.

3.5 Establishment of optimal conditions for a LASV trVLP assay

The transcription and replication virus like particle (trVLP) assay is a more advanced reverse genetics technique and was used to investigate the functionality of Z regarding its effect on

producing virus like particles able to infect target cells. Compared to overexpression analysis of Z in context of a minigenome system, the trVLP assay mimics natural expression of Z since it is provided by the bicistronic minigenome and not externally from an expression plasmid. The parameters determined by RNP titration series with the monocistronic minigenome system could be translated to the trVLP assay. However, determination of cell lines most suitable for both producer cells (p0) and target cells (p1) had to be carried out, since in comparison to the minigenome assay additional steps of the viral life cycle are modelled in this assay.

3.5.1 Determination of best fitting cell lines for a LASV trVLP assay

First, the trVLP assay was evaluated regarding the most suitable cell lines, both for p0 and p1 cells. To this end, the cell lines 293T and Huh7 were compared based on their signal output of the reporter assay in both p0 and p1, as well as background levels. Ideally, signal intensities of positive samples (+L) should exceed background noise (-L) by several log levels. Considering this, Huh7 cells resulted in better assay performance, by exceeding the background by 1.3 log levels in producer cells and 3.6 log levels in p1. These results are depicted in Figure 15, displaying reporter activity in logarithmic levels of relative light units (RLU) at the y-axis. On the other hand, no significant difference between positive and negative samples could be seen with HEK293T cells, indicating no compatibility with this LASV trVLP system.

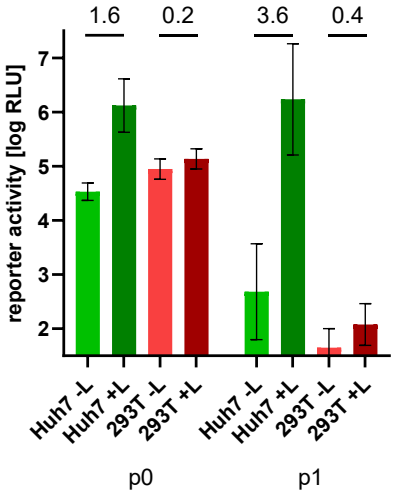

Figure 15: Results of transcription and replication competent virus like particle (trVLP) assay conducted with different cell lines for p0 and p1. Reporter activity in p0 (left side) and p1 (right side) of trVLP assays in Huh7 and Hek-293T are displayed. Means and standard deviations of 5 independent experiments are shown. The numbers above the bars indicate the dynamic range (positive control (+L) signal minus corresponding negative control (-L) signal) for each tested cell line.

3.6 Overexpression of LASV Z protein in a minigenome assay

The late phase protein LASV Z plays a crucial role not only in viral budding but also in its interaction with the viral polymerase L, leading to inhibition of viral RNA synthesis (Cornu et al., 2001). We confirmed these inhibiting effects through overexpression of LASV Z, showing reporter activity equal to negative control if 25 ng or more of pCAGGS-Z is transfected. Moreover, we proved that Z functionality is not influenced by attaching a myc-tag on the C-terminus of the protein, as seen in Figure 16 comparing titration series with pCAGGS- Z-myc (green) and pCAGGS-Z (black). The control without pCAGGS-L is marked in red as cutoff indicator.

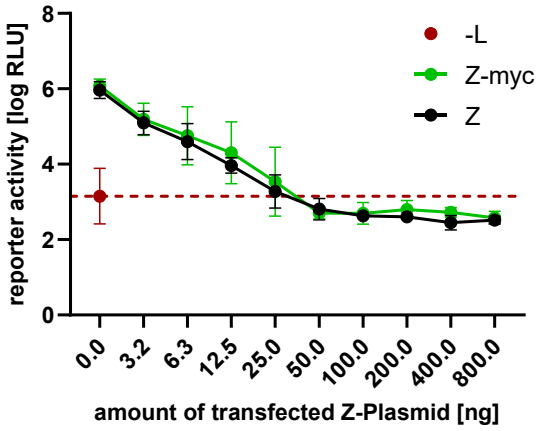

Figure 16: Comparison of Z-Plasmid titration with and without C-terminal myc tag. The titration series of Z with the range of 0.0 ng up to 800 ng is displayed. The control lacking L is highlighted in red and marks the cutoff of minigenome assay performance. n=3

3.7 Site directed mutagenesis of LASV Z

There are several conserved amino acid residues outside of functional domains within LASV Z proposed by (Capul et al., 2011) including R16, P21, D22, G72, P28, K68, L71, P72 and T73. These were investigated on their effects on Z functionality with both reverse genetic systems developed as part of this thesis. Moreover, the well-described myristoylation site at G2, which leads to loss of Z's budding functionality, was used as control (Perez et al., 2004). To this end, these amino acid residues of interest were altered by site directed mutagenesis to result in alanin. On one hand site directed mutagenesis was performed on pCAGGS-Z, creating nine different variations which could be utilized for overexpression analysis in minigenome assay. On the other hand, the bicistronic minigenome was used as template for

site directed mutagenesis as well, generating constructs suitable for investigation in the trVLP assay.

3.7.1 Overexpression analysis of altered amino acid residues within LASV Z

Using the established LASV minigenome system the functionality of Z regarding its inhibiting properties through interaction with the polymerase L could be investigated in detail. To this end, the generated expression plasmids of LASV Z were studied regarding their effects on viral RNA transcription using this system. The experimental setup included a set of the following controls, a negative control lacking viral Z and L, a positive control including addition of L but lacking Z, as well as a reference sample of wild type (WT) Z. As seen in Figure 17 the addition of wild type Z leads to loss of any reporter activity above background levels. The same applies to the altered amino acid residues G2A, R16A, P21A, D22A, G27A, P28A, K68A, T73A. These results suggest that none of these amino acid residues are affecting interactions between polymerase L and Z. However, samples L71A and T73A display a significant increase in minigenome reporter activity, suggesting an importance for the inhibitory activity of Z in viral RNA synthesis.

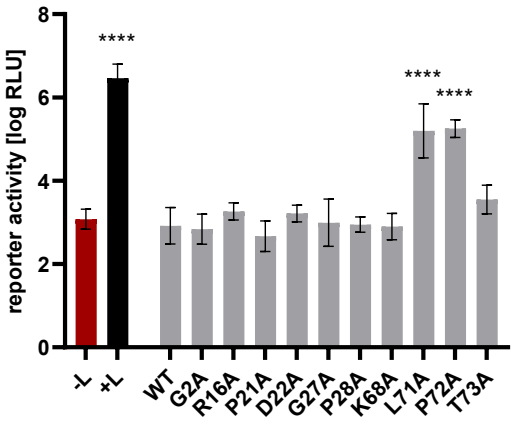

Figure 17: **Results of overexpression analysis in minigenome assay of WT as well as altered amino acid residues within Z**. Negative control lacking L and Z is highlighted in red, whereas positive control including L but lacking Z is seen in black. Ten different Z variants where investigated. Means and standard deviations of 4 independent experiments are shown. Stars above the bars marking differences in significancy compared to WT Z reporter activity. (**** $p < 0{,}0001$; *** $p<0{,}001$; ** $p < 0{,}01$; * $p < 0{,}05$).

3.7.2 Analysis of altered conserved amino acid residues within LASV Z in a trVLP assay

The transcription and replication competent virus like particle (VLP) assay can be used to investigate replication and secondary transcription as well as assembly, budding and viral entry. Since Z is well described as essential budding factor (Capul et al., 2007) and is required for the formation of infectious viral particles (Casabona et al., 2009), this assay is well suited to assess these functions. Specifically, reporter activity in p1 cells in the assay should correlate with the formation of infectious virus-like particles.

The trVLP system was used to investigate the LASV Z variants G2A, R16A, P21A, D22A, G27A, P28A, K68A, L71A, P72A and T73A on their effects on Z functionality in more detail. As expected, none of the Z variants performed significantly different from wild type in producer cells (p0). However, differences in reporter activity of p1 cells seen in figure 18 suggest an effect of the altered amino acid residues G2A, D22A and L71A on the formation of infectious virus-like particles mediated by Z. These results were to be expected for the already well described G2A mutation.

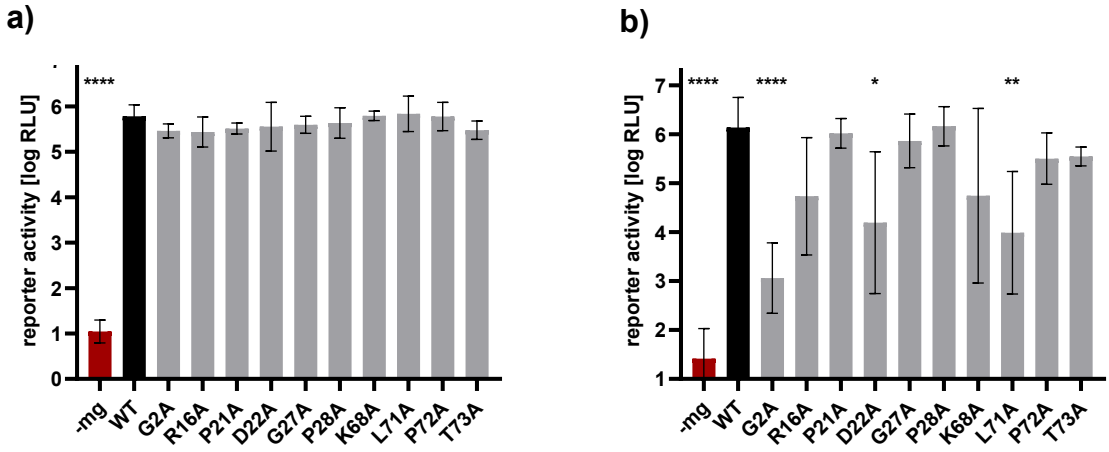

Figure 18: Altered amino acid residues within Z investigated in trVLP assay with a) displaying reporter activities in p0 and b) reporter activities in p1. As negative control served trVLP assay lacking minigenome was run. Ten different Z variants and WT Z were investigated. Means and standard deviations of 4 independent experiments are shown. Stars above the bars marking differences in significancy compared to WT Z reporter activity. (**** p < 0,0001; *** p<0,001; ** p < 0,01; * p < 0,05).

4 Discussion

LASV is classified as biosafety level 4 pathogen, which restricts work with infectious particles to a few high containment laboratories worldwide. Consequently, research progress is constrained and methods for investigating the LASV life cycle under BSL1/2 conditions are required. Today reverse genetics systems are a powerful tool to overcome these limitations, however such systems are currently limited for LASV. Therefore, the aim of this project was to develop two reverse genetic systems solely based on LASV components. Moreover, these systems should be used to investigate conserved amino acid residues within LASV Z on their effects on Z functionality.

4.1 Development of a LASV minigenome and trVLP assay

For the development of a monocistronic minigenome assay determination of the optimal cell line for assay performance was necessary and the results were in accordance with the previous experiences with the already well-established JUNV minigenome assay available on site. In short, Huh7 proved to be most suitable for optimal assay performance. Moreover, further optimization by implementing eGFP decoy ORFs before and after the T7 terminator led to an expected decrease in background signal of the minigenome system, since cryptic promotor activity would now lead to expression of eGFP instead of nanoluciferase (Dunham et al., 2018). However, these eGFP decoy ORFs could not be implemented in the trVLP system within the given time of the project and will be the subject of future advancement of the assay. Compared to other T7-driven reverse genetics systems for arenaviruses, such as those for LCMV, the quantity of transfected RNP proteins and minigenomes varied significantly (Lee et al., 2000). Possible species-specific differences in RNP protein quantities necessary for proper assay performance might underlie these differences.

In the past, T7-driven reverse genetics systems based solely on LASV components have been developed, primarily in form of a runoff minigenome system (Hass et al., 2004). In this system, the minigenome is not supplied from an expression plasmid, but rather from a PCR product that is either transfected directly in form of a DNA that is then T7-transcribed inside cells, or after *in vitro* transcription.. This method has drawbacks, including high nonspecific luciferase activity. The systems presented in this thesis, which provides minigenome sequence in form of expression plasmids, demonstrate significantly more robust reporter activities with less background noise.

In conclusion, two novel reverse genetic systems solely based on LASV components could be developed and optimal assay conditions were determined.

4.2 Overexpression analysis of LASV Z variants

The RING finger protein Z serves as the driving budding factor and is essential for inhibiting RNA transcription during the late phase of viral life cycle. The effects of altered conserved amino acid residues within LASV Z were investigated utilizing the developed minigenome assay. These experiments concluded most of the tested amino acid residues (G2A, R16A, P21A, D22A, G27A, P28A, K68A, T73A) not having significant effects on the interaction of Z and L. However, the variants L71A and P72A displayed a significant increase in reporter activity and therefore suggest a loss of proper inhibition of viral polymerase by LASV Z. The results for P72A are in accordance with previous descriptions in the literature (Capul et al., 2011). Nonetheless, according to the literature most of the other conserved amino acid residues (G2A, R16A, P21A, D22A, G27A, P28A) were described to result in increased minigenome expression as well. On the other hand, K68A was associated with decreased reporter activity, suggesting greater inhibition of viral polymerase than wild type Z (Capul et al., 2011). However, experiments conducted in literature were utilizing a chimeric reverse genetic system comprising LCMV minigenome system and RNP proteins and LASV Z. Consequently, variations in minigenome expression between the two systems could be caused by effects related to the compatibility of LASV and LCMV

4.3 Analysis of altered conserved amino acid residues within LASV Z in trVLP Assay

The transcription and replication-competent virus-like particle (trVLP) system is another life cycle modelling system, which enables not only examination of replication and gene expression, but also viral egress and cell entry. Consequently, this system was used to investigate the conserved amino acid residues described above (G2, R16, P21, D22, G72, P28, K68, L71, P72, T73) within LASV Z. As expected, mutation of myristoylation site G2 lead to loss of Z budding functionality (Perez et al., 2004). Moreover the findings of these experiments suggest that the modified amino acid residues D22A and L71A may impact formation of viral particles or cell entry. Moreover, this observation regarding L71 corresponds with previous literature, as this position in LASV Z corresponds to L79 in JUNV, which is known to be critical for the formation of infectious particles (Casabona et al., 2009). However, these effects were not described by literature for D22A. These discrepancies could be a result of the different minigenome systems used, or of a species-specific function of this amino acid. While this study utilized a minigenome system solely based on LASV components, the literature used a chimeric system comprised of LASV and LCMV components. As a result, differences in

minigenome expression of the two systems may be cause by underlying effects of compatibility between LASV and LCMV.

4.4 Conclusion

In conclusion, in this work two novel reverse genetic systems based solely on LASV components were successfully developed. Specifically, on one hand a monocistronic minigenome system was established, and on the other hand a multicistronic transcription and replication competent virus-like particle system. For both systems different assay conditions were investigated, indicating Huh7 as most suitable cell line among those tested for both assays. Moreover, RNP titration resulted in determination of optimal transfection amounts for NP (125 ng) and L (500 ng). Furthermore, the implementation of eGFP decoy ORFs resulted in minimal background levels for the minigenome system.

Both systems were then used to investigate the impact of conserved amino acid residues within LASV Z (G2, R16, P21, D22, G72, P28, K68, L71, P72, T73). Results indicate an importance of L71 for both proper inhibition of viral RNA synthesis by Z, and formation of infectious trVLPs, since alteration affected both reverse genetic systems used for investigation. Further, D22 appears to be important for the formation of infectious trVLPs, whereas P72A was associated with impaired inhibition of minigenome reporter activity, indicating an importance of P72 for the polymerase inhibiting properties of Z.

Moving forward, the developed reverse genetic systems and insights gained from this study will serve as valuable tools for further understanding of LASV biology.

Abstract

The Lassa virus (LASV) is an Old World arenavirus endemic to West Africa and causes hemorrhagic fever in humans. Since LASV is classified as biosafety level 4 pathogen, work with infectious LASV is restricted to high containment laboratories. Fortunately, life cycle modelling systems are a powerful tool to investigate highly pathogenic BSL4 viruses such as LASV under biosafety level 1 conditions. The simplest life cycle modelling system is a minigenome system, which uses a miniature version of the viral genome (called “minigenome”). This system can be used to investigate the processes of viral replication and transcription in eukaryotic cells. To examine the whole viral life cycle including budding and entry, minigenome systems can be further advanced into transcription and replication-competent virus-like particle (trVLP) assays. This type of system includes proteins needed for viral budding and entry, either supplied by expression plasmids or from the minigenome itself (“multicistronic trVLP assay”).

In this thesis, both a minigenome and a trVLP system solely based on LASV components were successfully developed. Moreover, these life cycle modelling systems were then used to investigate conserved amino acid residues within the LASV matrix protein Z, which is essential for viral budding and inhibition of RNA synthesis. The mutation of the conserved amino acid residues leucine (L) at position 71, and prolin (P) at position 72 were associated with loss of proper RNA synthesis inhibition by Z. Furthermore, mutation of L71 and aspartic acid (D) at position 22 displayed abnormalities in the formation of infectious viral particles.

In conclusion, two reverse genetics systems capable of overcoming biosafety level 4 restrictions were developed and used to investigate conserved amino acid residues within LASV Z in detail.

Zusammenfassung

Das Lassa-Virus (LASV) ist ein Altweltarenavirus, das in Westafrika endemisch ist und beim Menschen hämorrhagisches Fieber verursacht. Da LASV als Pathogen der Risikostufe 4 klassifiziert ist, ist die Arbeit mit infektiösem Viren nur in Laboren der höchster Biosicherheitsstufe 4 zulässig. Glücklicherweise sind Lebenszyklus-Modellierungssysteme eine zuverlässige Methode, um hochpathogene Viren wie LASV unter Bedingungen der Biosicherheitsstufe 1 zu untersuchen. Das einfachste Lebenszyklus-Modellierungssystem ist ein Minigenom-System, welches eine Miniaturversion des viralen Genoms (genannt "Minigenom") verwendet. Mit diesem System kann der Prozess der viralen Replikation und Transkription in eukaryotischen Zellen untersucht werden. Um den gesamten viralen Lebenszyklus einschließlich Knospung und Zelleintritt zu untersuchen, können Minigenomsysteme zu Transkriptions- und Replikations-kompetenten virusähnlichen Partikeln (trVLP)-Assays weiterentwickelt werden. Für diese Systeme werden Proteine, die für die Knospung und den Zelleintritt benötigt werden, entweder durch Expressionsplasmide oder aus dem Minigenom selbst ("multicistronischer trVLP-Assay") zur Verfügung gestellt.

In dieser Arbeit wurde sowohl ein Minigenom als auch ein trVLP-System, welche ausschließlich auf LASV-Komponenten basieren, erfolgreich entwickelt. Darüber hinaus wurden diese Lebenszyklus-Modellierungssysteme dann verwendet, um konservierte Aminosäurereste innerhalb des LASV-Matrixproteins Z zu untersuchen, welches für die virale Knospung und Hemmung der RNA-Synthese essenziell ist. Die Mutation der konservierten Aminosäurereste Leucin (L) an Position 71 und Prolin (P) an Position 72 war mit dem Verlust der RNA-Synthesehemmung durch Z assoziiert. Darüber hinaus zeigte die Mutation von L71 und Asparaginsäure (D) an Position 22 Anomalien in der Bildung infektiöser Viruspartikel.

Zusammenfassend lässt sich sagen, dass zwei Reverse-Genetik Systeme entwickelt wurden, welche Untersuchungen des LASV in Laboren der Sicherheitsstufe 1 oder 2 zulässt. Außerdem wurden diese Systeme verwendet, um konservierte Aminosäurereste innerhalb von LASV Z im Detail zu untersuchen.

List of abbreviations

ANOVA	Analysis of variance
ATV	Alsever's trypsin versine
BSL	Biosafety level
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's minimum essential medium
DTT	Dithiothreitol
ESCRT	Endosomal Sorting Complexes Required for Transport
FBS	Fetal bovine serum
FF	Firefly Luciferase
GFP	Green fluorescent protein
GPC	Glycoprotein Complex
GP1	Glycoprotein 1
GP2	Glycoprotein 2
HDV	Hepatitis Delta Virus
HEK293T	Human Embryonic Kidney 293 cells, transformed
JUNV	Junín Virus
kb	Kilobase pairs
L	viral RNA dependent polymerase
LB	lysogeny broth
LASV	Lassa virus
LCMV	Lymphocytic Choriomeningitis Virus
NCS	Newborn calf serum
NLuc	Nanoluciferase
NP	Nucleoprotein
OD	Optical density
ORF	Open Reading Frame
PCR	Polymerase chain reaction
P/S	Penicillin and streptomycin
RLU	Relative Light Units
RNP	Ribonucleoprotein complex
rSAP	Alkaline Shrimp Phosphatase
SOC	Super optimal broth with catabolite repression
SSP	Stable Signal Peptide

SDM	Site directed mutagenesis
T7	T7 Bacteriophage
trVLP	Transcription and Replication Competent Virus Like Particle
WT	Wild type

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6 Figures

7 Tables

8 Appendix

8.1 Material

8.1.1 Chemicals and Reagents

Buffer/Solution/Reagent	Company	Article/CAS number
NEBuffer r3.1	New England Biolabs	B9200
2 log Ladder	New England Biolabs	N3200L
10x T4 DNA ligase buffer	New England Biolabs	B0202S
5x T4 ligase buffer	Invitrogen	46300018
6x Gel loading Dye Purple	New England Biolabs	B7024S
Agar bacteriological grade	MP Biomedicals	9002-18-0
Ampicillin	Carl Roth	K029.4
2 x Lysis-Juice	PJK	102517
2-Log DNA Ladder (0.1-10.0 kb)	New England Biolabs	N3200L
Acetic acid 100%, p.a.	Carl Roth	3738.2
Ammonium persulfate (APS)	Carl Roth	9592.3
Ampicillin sodium salt; ≥97%	Carl Roth	K029.4
Beetle-Juice Luciferase Assay Firefly	PJK	102511-1
D(+)-Glucose	Carl Roth	X997.2
Deoxynucleotide (dNTP) Solution Mix, 0.8mol of each	New England Biolabs	N0447S
Dithiothreitol (DTT)	Carl Roth	6908.2
Ethanol ≥96%	Carl Roth	64-17-5
Ethidium bromide solution 1%	Carl Roth	2218.1
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth	8040.1
Fast Blast DNA Stain 500x	Biorad	1660420EDU
Glycine	Carl Roth	3908.2
Isopropanol ≥99,8%, p.a.	Carl Roth	6752.3
LE Agarose (Lonza)	Biozym	#840004
Magnesium chloride-Hexahydrate ≥99%, p.a.	Carl Roth	2189.2
Monopotassium phosphate ≥99%	Carl Roth	3904.1

Monosodium phosphate ≥98%	Carl Roth	K300.1
NEBuffer Pack for T4 DNA Ligase	NEB	B0202S
Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher Scientific	15140122
Potassium chloride ≥99,5%, p.a., ACS, ISO	Carl Roth	6781.1
Sodium chloride ≥99,5%, p.a., ACS, ISO	Carl Roth	3957.1
TEMED	Carl Roth	2367.3
TransIT-LT1 Transfection Reagent	Mobitec	MIR2306
Tryptone/Peptone ex-casein	Carl Roth	8952.2
Tween 20 Molecular biology grade	AppliChem	A4974
Yeast Extract Powder MP	Biomedicals	210330391

8.1.2 Restriction enzymes

Enzyme	Company	Article number
BamHI	Thermo Fisher Scientific	ER0051
BbvCI	Thermo Fisher Scientific	ER1561
BglII	Thermo Fisher Scientific	ER0052
BsmBI	Thermo Fisher Scientific	ER0451
BspMI	New England Biolabs	R0540S
DpnI	Thermo Fisher Scientific	ER1701
EcoRI	Thermo Fisher Scientific	ER0271
NdeI	Thermo Fisher Scientific	ER0541
NheI	Thermo Fisher Scientific	ER0581
NotI	Thermo Fisher Scientific	ER0582
PspXI	New England Biolabs	R0544S
RsrII	New England Biolabs	R0107S
SalI	hermo Fisher Scientific	ER0431
XhoI	Thermo Fisher Scientific	ER0681
XmaI	New England Biolabs	R0182S

8.1.3 Commercial kits

Kit	Company	Article number
NucleoSpin Plasmid EasyPure	Machery Nagel	740727250
NucleoSpin Gel and PCR Clean-up	Machery Nagel	740609250
NucleoBond Xtra Midi Plus; incl. Finalizers	Machery Nagel	740412.10
HiSpeed Plasmid Midi Kit	Qiagen	12643
Mix & Go E. coli Transformation Kit & Buffer Set	Zymo Research	T3002

8.1.4 Buffer Recipes

Buffer	Recipe
1 x Lysis juice	20 ml 2 x Lysis juice 20 ml dH_2O
10 x PBS	80 g/l NaCl 2 g/l KCl 11,5 g/l Na2HPO4 2 g/l KH2PO4 adjust pH to 7,3 auf 1 l mit A. dest auffüllen, autoklavieren
50x TAE-Puffer	242,28 g Tris Base 57,1 ml acetic acid 100 ml EDTA [0,5 mol/l] adjust pH to 8 ad 1 l dH2O Autoclave
10x TBS 88 g NaCl	24 g Tris base (121.1 g/mol) ad 1 l dH2O adjust pH to 7.6
ATV	8,5 g NaCl (Carl Roth) 0,4 g KCl (Carl Roth) 1,0 g Dextrose (Sigma 0,58 g NaHCO3 (Carl Roth 0,5 g Trypsin 1:250 (Invitrogen) 0,2 g EDTA (Serva) ad 1 l dH2O adjust pH to 7.2

8.1.5 Bacterial and cell culture media

Broth	Recipe
SOC	20 g/l Trypton 5 g/l Yeast Extract 0,5 g/l NaCl 2,5 ml/l KCl [1 mol/l] adjust pH to 7.0 Autoclave 20 ml/l Glucose [1 mol/l]
LB-Miller	10 g/l Trypton 5 g/l Yeast Extract 10 g/l NaCl adjust pH to 7.5 ad 1 l dH2O, Autoclave
LB-Agar	Prepare LB medium (LB-Miller) add 15 g/l agar Autoclaving 20-25 ml of LB agar per 10 cm petri dish
DMEM + 0% FBS	9,9 g DMEM high glucose 3,7 g NaHCO3 0,12 g Sodium pyruvate adjust pH to 7,1 330 mOsmol
DMEM + 5% FBS	500 ml DMEM + 0% FBS 25 ml Fetal Bovine Serum
DMEM + 10% FBS	500 ml DMEM + 0% FBS 50 ml Fetal Bovine Serum

8.1.6 Oligonucleotides

8.1.6.1 Primer for cloning of LASV RNP Proteins

#	Name	Sequence (5'→3')
5283	LASV-L-NotI_fwd	GATCGCGGCCGCATGGAGGAAGACATAGCCTGTGTC
5284	LASV-L-NheI_rev	GATCGCTAGCCTACTCAATGTCTTCGATGCATTCATC
5285	LASV-L-EcoRV_fwd	GATCATGATATCTTACCAGGTAAATTTTATGAAG
5292	LASV-GP_fwd	GATCCCCGGGCGTCTCtCATGGGACAAATAGTGACATTCTTCCAG
5293	LASV-GP_rev	GATCGCTAGCCGTCTCATCTATCTCTTCCATTTCACAGGCACAC
5298	LASV-dNP-GP_fwd	GATCGTCGACACCTGCagatGATGGGACAAATAGTGACATTCTTCCAG
5299	LASV-GP-T2A_rev	GATCGGATCCTCTCTTCCATTTCACAGGCACACC
5300	LASV-L-EcoRV_NheI_rev	GATCGCTAGCGTAAGATATCATGATCAAAGTCCTCAACC
5336	LASV-L-1x_fwd	gatcCCCGGGATGGAGGAAGACATAGCCTGTGTC
5337	LASV-L-1x_rev	GATCGCTAGCTTCTCGAGATCCCTCTTTGTTGACTCC
5338	LASV-L-2x_fwd	gatcCCCGGGtaCTCGAGAAATGGGTTGAGCGGAGGC
5339	LASV-L-2x_rev	GATCGCTAGCGCACCTCGAGTGAAAGTGGAAGACAAC
5340	LASV-L-3x_fwd	gatcCCCGGGCACTCGAGGTGCGCAAAAATTGC
5341	LASV-L-1bp_rev	GATCGCTAGCAACCTCAGCTGAAAATATTGCTGGAA
5342	LASV-L-2bp_fwd	gatcCCCGGGCAGCTGAGGTTTTACAAACAACAATAAGC
5298	LASV-dNP-GP_fwd	gatcGTCGACACCTGCagatGATGGGACAAATAGTGACATTCTTCCAG
5299	LASV-GP-T2A_rev	GATCGGATCCTCTCTTCCATTTCACAGGCACACC

8.1.6.2 Primer vor LASV Z site directed mutagenesis

#	Name	Sequence (5'→3')
5344	LASV-Z-G2A-fwd	gataCTCGAGCGTCTCtATGGCAAACAAGCAAGCCAAAGCC
5345	LASV-Z-R16A-fwd	GACAGTCCGGCAGCCAGCCTGATCCCAGATG
5346	LASV-Z-R16A-rev	GGCTGGCTGCCGGACTGTCTTTTGATTCTGGG
5347	LASV-Z-P21A-fwd	GCCTGATCGCAGATGCCACACATCTAGGGCCAC
5348	LASV-Z-P21A-rev	GATGTGTGGCATCTGCGATCAGGCTGGCTCTCGGAC
5349	LASV-Z-D22A-fwd	CCTGATCCCAGCTGCCACACATCTAGGGCCACAG
5350	LASV-Z-D22A-rev	GATGTGTGGCAGCTGGGATCAGGCTGGCTCTC
5351	LASV-Z-G27A-fwd	CACACATCTAGCGCCACAGTTCTGTAAGAGCTGCTG
5352	LASV-Z-G27A-rev	CAGAACTGTGGCGCTAGATGTGTGGCATCTGGGATCAG
5353	LASV-Z-P28A-fwd	CACATCTAGGGGCACAGTTCTGTAAGAGCTGCTGGTTC
5354	LASV-Z-P28A-rev	CTTACAGAACTGTGCCCCTAGATGTGTGGCATCTGGGATC
5355	LASV-Z-K68A-fwd	CATTTGCGCGATGCCTCTCCCCACAAAACTGAGACC
5356	LASV-Z-K68A-rev	GGGAGAGGCATCGCGCAAATGGGACACCTGTTGCTGAC
5357	LASV-Z-L71A-fwd	CAAGATGCCTGCACCCACAAAACTGAGACCATCAGCCG
5358	LASV-Z-L71A-rev	CAGTTTTGTGGGTGCAGGCATCTTGCAAATGGGACACCTG
5359	LASV-Z-P72A-fwd	GATGCCTCTCGCCACAAAACTGAGACCATCAGCCG
5360	LASV-Z-P72A-rev	CTCAGTTTTGTGGCGAGAGGCATCTTGCAAATGGGACACC
5361	LASV-Z-T73A-fwd	ATGCCTCTCCCCGCAAAACTGAGACCATCAGCCGCTC
5362	LASV-Z-T73A-rev	CTCAGTTTTGCGGGGAGAGGCATCTTGCAAATGGG

8.1.6.3 Sequencing Primer

#	Name	Sequence (5'→3')
1233	1233_pCAGGS_fw	CCTTCTTCTTTTTCCTACAG
1234	1234_pCAGGS_rev	CCTTTATTAGCCAGAAGTCAG
3610	pCA-GFP-NXF1-seq-fwd-2331	CGACAACCACTACCTGAGCAC
3629	seq_GFP_mCherry_19-fwd	ATGGTGAGCAAGGGCGAGG
4001	nluc_seq_fwd1	CCAGAATGCGTTCGCACAGC
4002	nluc_seq_rev1	GCTGTGCGAACGCATTCTGG
4477	4cis_Leader_fwd	GTTATGCTAGTTATTGCTCAGC
5313	LASV-L-813_fwd	GAGCCAGTTACTTAATGAACTTAAC
5314	LASV-L-1599_fwd	CAAGGTATCAAGTGGTCAGATGC
5315	LASV-L-2407_fwd	TGGTTCACTATTTAAGGAGAGGG
5316	LASV-L-3162_fwd	AGGTACACTCATTATAAGTCAAGG
5317	LASV-L-4004_fwd	GCTTATACCTCTAGTGATGATCAG
5318	LASV-L-4821_fwd	TTGCTGTCTGAAGCTATCAAC
5319	LASV-L-5617_fwd	GGTCCTTCACGTGGTTTCCAC
5320	LASV-L-6223_fwd	TACGGAGTATGACTTTGTATTGG

8.1.7 Devices

Device	Company
Dual 48/48 Fast Reaction Module	Bio-Rad
S1000 Thermal Cycler Chassis	Bio-Rad
Centrifuge 5430 R	Eppendorf
Centrifuge 5810 R	Eppendorf
NanoPhotometer P-Class	IMPLEN

PerfectSpin Mini Zentrifuge	Peqlab
CO2 Incubator	Sanyo
Luminometer	Promega
C1000 Thermal Cycler	Bio-Rad
Thermomixer 5430R	Eppendorf
The Butterfly	Carl Roth
Heidolph Duomax 1030	Heidolph Instruments
Mikrowelle	Siemens
Power-pac basic	Bio-rad
Molecular imager Gel-doc	Bio-Rad

8.1.8 Computer Software

Software	Company
Clone manager	Sci-Ed Software
Geneious	Biomatters Limited
Microsoft Office	Microsoft Corporation
Image Lab	Bio-Rad
ImageJ	NIH
Graphpad Prism	Graphpad Software, Inc
Inkscape	Open source vector graphic tool