

UNIVERSITY OF TARTU
Faculty of Science and Technology
Institute of Bioengineering

Natavan Aliyeva

Engineering and Validation of Single-Cycle Alphavirus Replicon Particles

Bachelor's Thesis (12 ECTS)
Curriculum Science & Technology

Supervisor:
MSc Evelin Väljaots

Tartu 2026

Engineering and Validation of Single-Cycle Alphavirus Replicon Particles

MAYV, RRV, ONNV, and BFV are understudied alphaviruses, often investigated using tools developed for model viruses, such as SINV and CHIKV. This study aimed to develop and evaluate VRP systems for a panel of alphaviruses using ZsGreen and mCherry reporter replicons and a split-helper system in BHK-21 cells. The VRPs were analysed by second-round infection using fluorescence microscopy. The results showed that VRP production differed between alphavirus backbones. SINV and MAYV produced relatively high titers of VRPs, whereas RRV and BFV were less efficient. Removal of the capsid enhancer sequence improved ONNV VRP production but reduced RRV VRP titers and abolished detectable BFV VRP titers. Second-round infection confirmed that the generated VRPs were single-cycle. Overall, this thesis demonstrates that alphavirus VRP production is virus-specific and that regulatory elements optimised for model alphaviruses cannot be assumed to function universally across the genus.

Keywords: alphavirus, virus-like replicon particles, capsid enhancer, split-helper RNA system.

CERCS: B230

Institute of Bioengineering, Laboratory of Applied Virology

Alfaviiruste replikone sisaldavate partiklite konstrueerimine ja valideerimine

MAYV, RRV, ONNV ja BFV on olulised, kuid vähem uuritud alfaviirused, mida uuritakse sageli mudelviiruste, nagu SINV-i ja CHIKV-i jaoks välja töötatud tööriistadega. Käesoleva töö eesmärk oli luua ja hinnata viirus-replikon partikleid (VRP-d) mitmele alfaviirusele. ZsGreen ja mCherry reporter-replikone kandvad VRP-d toodeti "*split-helper*"-RNA süsteemiga ning analüüsiti teise ringi nakatamisega kasutades fluorestsentsmikroskoopiat. Tulemused näitasid, et VRP-de tootmise varieeruvus sõltuvalt alfaviiruse taustast. SINV ja MAYV partiklitel oli suhteliselt kõrge tiiter, aga RRV ja BFV olid madalamate partiklite tiitritega. Kapsiidi valgusünteesi võimendaja eemaldamine parandas ONNV partiklite tootmist, kuid vähendas RRV tiitrit ja BFV tiiter kadus täielikult. Teise ringi nakkus kinnitas, et moodustatud VRP-d olid ühetsüklilised. Kokkuvõttes näitab käesolev töö, et alfaviiruste VRP-de tootmine on viiruse spetsiifiline ning mudelalfaviiruste jaoks optimeeritud regulatoorsed elemendid ei toimi universaalselt kogu viirusperekonna ulatuses.

Märksõnad: alfaviirus, viirus-laadsed replikon-partiklid, kapsiidi valgusünteesi võimendaja, "*split-helper*" RNA süsteem.

CERCS: B230

Bioinseneeria instituut, rakendusviroloogia uurimisrühm

Table of Contents

List of abbreviations – in alphabetical order	5
Introduction	7
1 Literature review.....	8
1.1 Classification of alphaviruses	8
1.2 Genome organisation and structure	10
1.3 Functions of alphavirus non-structural proteins	11
1.3.1 nsP1.....	11
1.3.2 nsP2.....	11
1.3.3 nsP3.....	12
1.3.4 nsP4.....	12
1.4 Functions of alphavirus structural proteins.....	12
1.4.1 Capsid protein.....	13
1.4.2 Envelope associated proteins (E1, E2, E3, 6K and TF).....	13
1.5 Alphavirus replication cycle	13
1.6 Pathogenicity	15
1.7 Replicon systems	17
1.7.1 Definition and concept of alphavirus replicon systems.....	17
1.7.2 Molecular design of the alphavirus replicon	18
1.7.3 Advantages and limitations of alphavirus replicons	18
1.8 Viral replicon particles (VRPs).....	20
1.8.1 Definition and concept of the VRPs	20
1.8.2 Single helper systems	21
1.8.3 Split-helper system	23
1.8.4 VRP production (co-transfection, packaging, and lack of propagation).....	24
2 Aims of the thesis	25
3 Experimental part	26
3.1 Materials and methods	26
3.1.1 Cloning of plasmid constructs	26
3.1.3 <i>In vitro</i> transcription	29
3.1.4 Production of viral replicon particles (VRPs).....	30
3.1.5 Titration of VRPs using fluorescent microscopy.....	30
3.1.6 Second round of infection.....	31
3.2 Result and discussion.....	32
3.2.2 Generation of VRPs	34
3.2.3 First round infection (titration of VRPs).....	36

3.2.4 Second round of infection.....	42
Summary.....	44
References	46
Appendix	50
Non-exclusive license to reproduce thesis and make thesis public.....	57

List of abbreviations

BFV	Barmah Forest virus
BHK-21	Baby hamster kidney 21 cells
BSL	Biosafety level
C	Capsid protein
CHIKV	Chikungunya virus
CSE	Conserved sequence element
DLP	Downstream loop
dsRNA	Double-stranded RNA
E	Envelope glycoprotein
EEEV	Eastern equine encephalitis virus
eIF2 α	Eukaryotic initiation factor 2 alpha
G3BP	Ras-GAP-associated endoribonuclease
GMEM	Glasgow minimum essential medium
GOI	Gene of interest
icDNA	Infectious complementary DNA
iu	Infectious unit
IVT	In vitro transcription
kb	Kilobase
LB	Lysogeny broth
MAYV	Mayaro virus
ns	Non-structural
NTP	Nucleotide triphosphate
ONNV	O'nyong-nyong virus
ORF	Open reading frame
PS	Packaging signal
RCV	Replication-competent virus

RdRp	RNA-dependent RNA polymerase
RRV	Ross River virus
SFV	Semliki Forest virus
SG	Subgenomic
SINV	Sindbis virus
ssRNA	Single-stranded RNA
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis virus
VGM	Viral growth medium
VRP	Viral replicon particle
WEEV	Western equine encephalitis virus

Introduction

The genus Alphavirus, within the family Togaviridae, comprises a group of small, enveloped, positive-sense, single-stranded RNA viruses primarily transmitted by mosquito vectors (1). These viruses represent a significant global public health threat, generally categorised into Old World alphaviruses, which cause debilitating musculoskeletal disease characterised by fever, rash, and chronic polyarthritis (e.g., Chikungunya (CHIKV), Mayaro (MAYV), and Ross River (RRV) viruses), and New World alphaviruses, which are associated with life-threatening encephalitis (e.g., Venezuelan equine encephalitis virus (VEEV)) (2). Despite the severe socioeconomic burden imposed by these pathogens, many species, such as O'nyong-nyong (ONNV) and Barmah Forest (BFV), remain under-researched, and there are currently no widely licensed human vaccines or targeted antiviral therapies for most species (3).

The study of highly pathogenic alphaviruses requires high-containment biosafety facilities (BSL-3) to handle infectious wild-type strains (4). To mitigate these risks and facilitate research in more accessible BSL-2 settings, molecular tools such as Virus-Like Replicon Particles (VRPs) have been developed (4). VRPs are engineered by replacing the viral structural genes with a gene of interest (GOI), such as a fluorescent reporter, while retaining the non-structural proteins (nsP1–4) essential for RNA replication (5). These "suicide vectors" can undergo only a single cycle of infection; they can efficiently enter and replicate within a target cell to express high levels of the GOI, but they lack the genetic information necessary to produce new progeny virions (6).

A major challenge in alphavirus engineering is that foundational molecular knowledge is largely derived from model organisms like Sindbis virus (SINV) (1). It is often assumed that regulatory tools developed for these models function universally across the genus, yet studies have revealed substantial species-specific differences in virus-host interactions (5,7).

This study aims to develop and evaluate VRP production systems for a panel of arthritogenic alphaviruses (SINV, MAYV, CHIKV, BFV, RRV, and ONNV). This research uses a three-part helper system in which the capsid and envelope genes are supplied separately on distinct RNA molecules. This approach improves biosafety and reduces the risk of creating replication-competent viruses (RCVs). The study then investigates how efficiently different alphavirus backbones integrate. A central objective is to assess the species-specific role of the capsid enhancer and determine if its removal can optimise VRP yields for understudied pathogens. Finally, the study employs second-round infection assays to validate the particles' propagation-defective nature, ensuring they remain strictly single-cycle and safe for broader laboratory use.

1 Literature review

1.1 Classification of alphaviruses

The genus Alphavirus belongs to the family *Togaviridae* and, as of 2021, includes 32 recognised species. They are small, enveloped viruses with a single-stranded, positive-sense RNA genome that is roughly 11.5-12 kilobases (kb) in length (1).

Alphaviruses are classified into Old World and New World viruses. Old-world alphaviruses include (among others) Semliki Forest virus (SFV), SINV, ONNV, BFV, and RRV. They are usually found in Africa, Asia, and Australia, whereas the CHIKV is spread worldwide. Typically, most of them cause fever, rash, and severe joint pain. New World alphaviruses, such as Eastern Equine Encephalitis Virus (EEEV) and VEEV, are found in the Americas and can cause life-threatening encephalitis in humans and equines (8).

Alphaviruses are classified into antigenic complexes based on the structural and immunogenic properties of their envelope surface glycoproteins 1 and 2 (E1 and E2). The E2 is the primary target for neutralising antibodies, and viruses that share it exhibit strong neutralising cross-reactivity, while the E1 glycoprotein is more highly conserved across the genus and serves as a tool for broader classification, as evidenced by antibodies that inhibit virus-induced haemagglutination (8).

Based on phylogenetic data, the viruses that this thesis will be discussing further belong to the following clades or antigenic complexes: Semliki Forest (SF) complex (includes CHIKV, ONNV, RRV, and MAYV), Sindbis complex (includes SINV), Barmah Forest complex (includes BFV, which is a sole member of its own clade) (Figure 1) (8,9).

alphavirus species: release of the genomic RNA to the infected cell cytoplasm, processing of the non-structural protein precursor (expression of non-structural proteins/formation of replication complexes), synthesis of genomic RNA through the formation of negative-strand in the replication complexes, and transcription of 26S subgenomic mRNA for expression of structural proteins (1). More about viral replication will be mentioned in the next sections.

1.2 Genome organisation and structure

The alphavirus genome contains a 5' cap and a 3' poly(A) tail. The genome contains two open reading frames (ORFs). 2/3 from the 5' side encodes for non-structural (ns) proteins, nsP1-4, that are necessary for replication, while the remaining 1/3 from the 3' side encodes the structural proteins that make up the capsid and glycoproteins needed for virion packaging. The non-coding regions flank the viral genome – the 5' untranslated region (UTR) and the 3' UTR, and the non-coding junction sequence between the two ORFs – the subgenomic (SG) promoter. UTRs are regulatory regions that initiate translation, regulate RNA replication, and facilitate virion packaging (Figure 2) (1).

In different parts of the genome, there are four conserved sequence elements (CSEs) that the replication process depends on. CSE 1 (5' UTR) is located at the 5' end of the genome and has a complementary structure at the 3' end. CSE 2 is 51 nucleotides long and is located within the nsP1 coding region and is required for the minus-strand RNA synthesis. Both CSEs function in the synthesis of the plus-strand RNA and as a part of the genomic promoter for the minus-strand RNA synthesis. CSE 3 (subgenomic (SG) promoter), located in the junction region between the non-structural and structural coding ORFs, promotes the transcription of the SG RNA. CSE 4, located at the 3' end right before the poly(A) tail, serves as the promoter for initiating minus-strand RNA synthesis on the plus-strand template (Fig. 2) (1,10).

Another important element is the packaging signal (PS), which ensures that the viral genomic RNA, rather than the more abundant host cell mRNA, is encapsulated into the viral capsid. The location of this signal is not conserved across the genus and can vary. For most alphaviruses, it is located within the nsP coding region (nsP1 – for SINV, VEEV, EEEV, WEEV), nsP2 – for (CHIKV, ONNV, SFV, RRV) (Fig. 2) (10). Because the PS is typically located in the non-structural region, removing the structural ORF does not affect packaging of genomic RNA into viral particles. This allows replacing the structural genes with, for example, reporter protein-coding sequences, which can then be used to generate virus-like replicon particles (VRPs) (11,12).

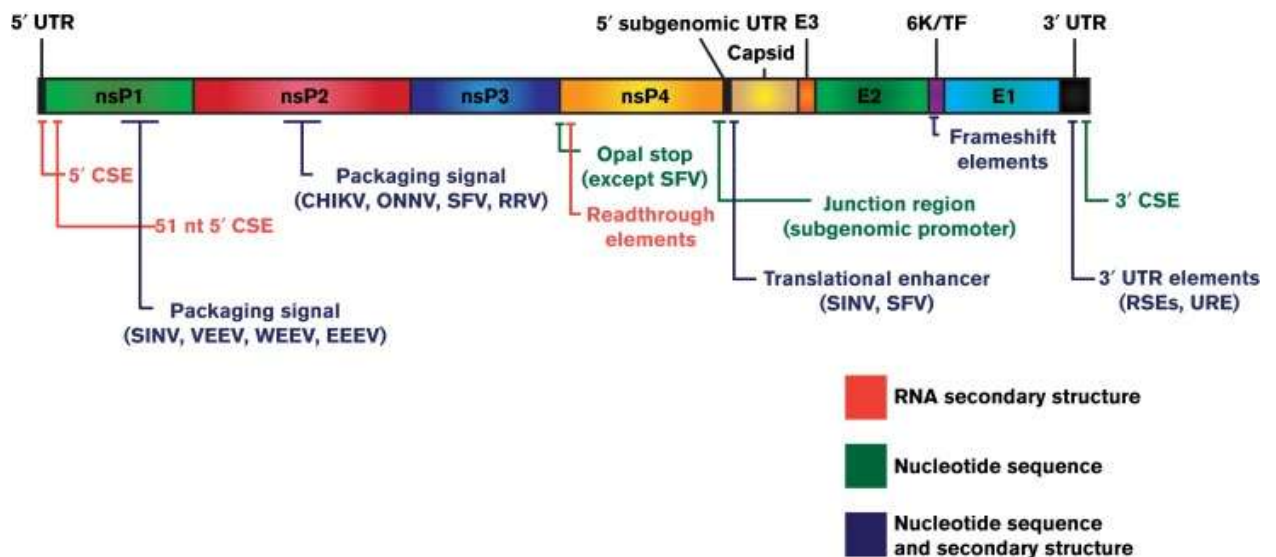


Figure 2. Schematic organisation of the alphavirus RNA genome and conserved cis-acting elements. The genome contains the 5' UTR, non-structural protein-coding sequences nsP1–nsP4, the subgenomic promoter region, structural protein-coding sequences capsid–E3–E2–6K/TF–E1, and the 3' UTR. Major regulatory elements involved in RNA replication, translation, subgenomic RNA synthesis, and genome packaging are indicated, including conserved sequence elements, packaging signals, the opal stop/readthrough element, translational enhancer, frameshift element, and 3' UTR elements. Colour coding distinguishes elements defined by RNA secondary structure, nucleotide sequence, or both (13).

1.3 Functions of alphavirus non-structural proteins

1.3.1 nsP1

Non-structural proteins (nsPs) are essential components of a membrane-bound replicase complex to drive alphavirus RNA synthesis (1). nsP1 acts as the RNA capping enzyme and anchors the replication complex to the host cell membrane. The nsP1 proteins form a dodecameric (12 copies of nsP1) ring interacting with negatively charged phospholipids in the cell membrane. This dodecamer ring forms the basis of the alphavirus replication complex, which protects the viral genome from host immune responses, allowing the exit of newly synthesised positive-strand RNAs into the infected cell cytoplasm (1).

1.3.2 nsP2

nsP2 is the largest non-structural protein of alphaviruses and serves as the primary regulator of viral-host interactions and polyprotein processing. (1) The N-terminus of this protein contains a superfamily 1 RNA helicase and a nucleoside-triphosphatase domain that, together, can unwind intermediate double-stranded RNA. The C-terminus of the protein is a cysteine protease that cleaves the viral nsP1234 polyprotein into separate units, and this is essential for the conversion

of the early replicase to the late replicase to control the synthesis of minus-strand and plus-strand RNA (1). nsP2 is also a key factor in inhibiting host cell functions in Old World alphaviruses, including the degradation of cellular RNA polymerase II, thereby shutting down host cell transcription (1).

1.3.3 nsP3

nsP3 of alphaviruses has three domains and serves as a bridge between the virus and the host cell machinery. The macrodomain at the N-terminus is highly conserved and essential for disassembling host stress granules – clumps of mRNAs that the cell forms to defend against any stress. This is achieved by removing adenosine diphosphate-ribose from the host factor G3BP, which acts as a central switch in stress granule formation (1). The hypervariable domain is a highly disordered C-terminus of nsP3 that serves as a centre for recruiting host G3BP factors to alphavirus replication complexes. This domain forms a protective layer around the replication compartments, shielding newly synthesised RNA from degradation by cellular enzymes (14).

1.3.4 nsP4

nsP4 is the most conserved non-structural alphavirus protein that serves as the catalytic subunit of the alphavirus replication complex – RNA-dependent RNA polymerase (RdRp) (1). It is primarily responsible for synthesising the genomic and subgenomic plus-strand RNAs, as well as the minus-strand full-length intermediate (1). nsP4 can recognise and bind to sequence motifs on minus-strand RNA to synthesise new plus-strand RNAs. nsP4 is produced in smaller quantities due to an opal stop codon upstream of its coding sequence. Cellular ribosomes stop the translation at this codon, producing nsP123, but sometimes read through to produce the full-length P1234, which is then cleaved to release nsP4 (15). The alphavirus nsP4 protein harbours a destabilising N-terminal tyrosine residue, which targets it for degradation via the N-end rule pathway (1). When nsP4 is incorporated into a functional replication complex, the tyrosine becomes less accessible to the cell machinery (10).

1.4 Functions of alphavirus structural proteins

The structural proteins of alphaviruses are translated from a 26S SG RNA as a single polyprotein precursor (3). This polyprotein is processed by both viral and host proteases into individual components of the virion: the capsid (C) protein and the envelope glycoproteins E3, E2, 6K/TF, and E1 (1). These proteins are essential for viral assembly, maturation, and entry into the host cell (5).

1.4.1 Capsid protein

The capsid protein is multifunctional, consisting of two distinct domains (10). The N-terminal domain binds viral RNA via electrostatic interactions (10). The C-terminal domain is highly conserved and possesses a serine protease activity that allows the protein to cleave itself from the nascent structural polyprotein (16).

The capsid protein recognises a unique cis-acting packaging signal (PS) on the genomic RNA, ensuring that only the viral genome is incorporated into the final virion (10). Also, the C-terminal domain interacts with the cytoplasmic tail of the E2 glycoprotein to provide the necessary energy for the nucleocapsid budding (17).

1.4.2 Envelope associated proteins (E1, E2, E3, 6K and TF)

The viral envelope has 80 trimeric spikes, each composed of E1-E2 heterodimers (10). E2 glycoprotein functions as the primary attachment protein. It interacts with cellular receptors, such as the laminin receptor, to facilitate entry (10). It is also the principal target for neutralising antibodies (8,16). E1 glycoprotein is a class II membrane fusion protein which contains a highly conserved fusion loop that is hidden in the heterodimer until the pH of the endosome drops. Then, E1 undergoes conformational changes that expose the fusion loop, leading to fusion between the viral and endosomal membranes (10).

E3 primarily acts as a scaffold to ensure the proper folding of E2 (17).

The region encoding the 6K protein contains a slippery sequence that induces a ribosomal frameshift in approximately 10-15% of translational events, leading to the production of the TF protein (1). The palmitoylated TF protein, produced via a programmed ribosomal frameshift, is essential for proper alphavirus assembly at the plasma membrane and its preferential incorporation into mature virions, where it simultaneously acts as a key antagonist to the host's innate immune response (17). 6K protein forms ion channels in host membranes that facilitate the transport of glycoproteins and the efficient budding of new virions (17).

1.5 Alphavirus replication cycle

Alphavirus replication is a coordinated, efficient process that occurs entirely within the host cell cytoplasm (Figure 3). The first step of the process is entry and uncoating of the virion. The virion binds to a receptor on the host cell surface via the E2 glycoprotein. Because alphaviruses have a

wide range of hosts, there is also no universal receptor (10). The virus is internalised via clathrin-dependent endocytosis, and viral virulence directly depends on the efficiency of this entry pathway.

After endocytosis, the endosome acidifies (18). The low pH triggers conformational rearrangements in the E1-E2 heterodimer, exposing the fusion loop and driving the fusion of the viral envelope with the endosomal membrane (10). This fusion releases the nucleocapsid into the cytoplasm, where it is disassembled by the host ribosomal large subunit, which interacts with the capsid proteins. After this dissociation, translation of the ns proteins begins (17). Immediately after translation, the nsP2 protease cleaves P1234, releasing the mature nsP4 polymerase and the P123 precursor. The early replicase machinery, consisting of P123 and nsP4, is responsible for synthesising minus-strand RNA from the genomic plus-strand.

As the concentration of nsP2 increases, it begins to cleave P123 into nsP1, nsP2, and nsP3, which together with nsP4, form the late replicase. The late replicase is responsible for synthesising the plus-strand genomic RNA from the minus-strand and for producing the SG RNA from the SG promoter. The SG RNA encodes a polyprotein containing the structural proteins, which is produced from the SG promoter on the negative RNA strand (1). The capsid protein cleaves itself almost immediately after synthesis from the structural polyprotein chain by utilising its own serine protease activity. The remaining polyprotein chain complex is directed to the endoplasmic reticulum (ER) (10). Within the ER, cleavage produces the 6K and E1 proteins, and E1 and precursor E2 form heterodimers required for transport to the Golgi (1,10,17). Final maturation occurs in the trans-Golgi network, where furin cleaves precursor E2 into E2 and E3, preparing the glycoproteins for membrane fusion during the next infection cycle. In the cytoplasm, the capsid protein recognises a packaging signal within the non-structural coding region of the genomic RNA, ensuring that only the genomic RNA is packaged (13) (Figure 3).

During glycoprotein processing, the nucleocapsid is assembled in the cytoplasm. The capsid protein binds to the packaging signal located within the non-structural part of the genomic RNA, either in nsP1 (for SINV and VEEV) or in nsP2 (for the SF complex) (10) (13). The SG RNA lacks this signal, and only full-length genomic RNA is packaged into the virion (Figure 3) (1).

The pre-assembled nucleocapsids interact with the C-terminal cytoplasmic tails of the E2 glycoproteins in the plasma membrane. This interaction provides the energy to drive the budding of the virion through the host membrane, which becomes the virus envelope (Figure 3) (10).

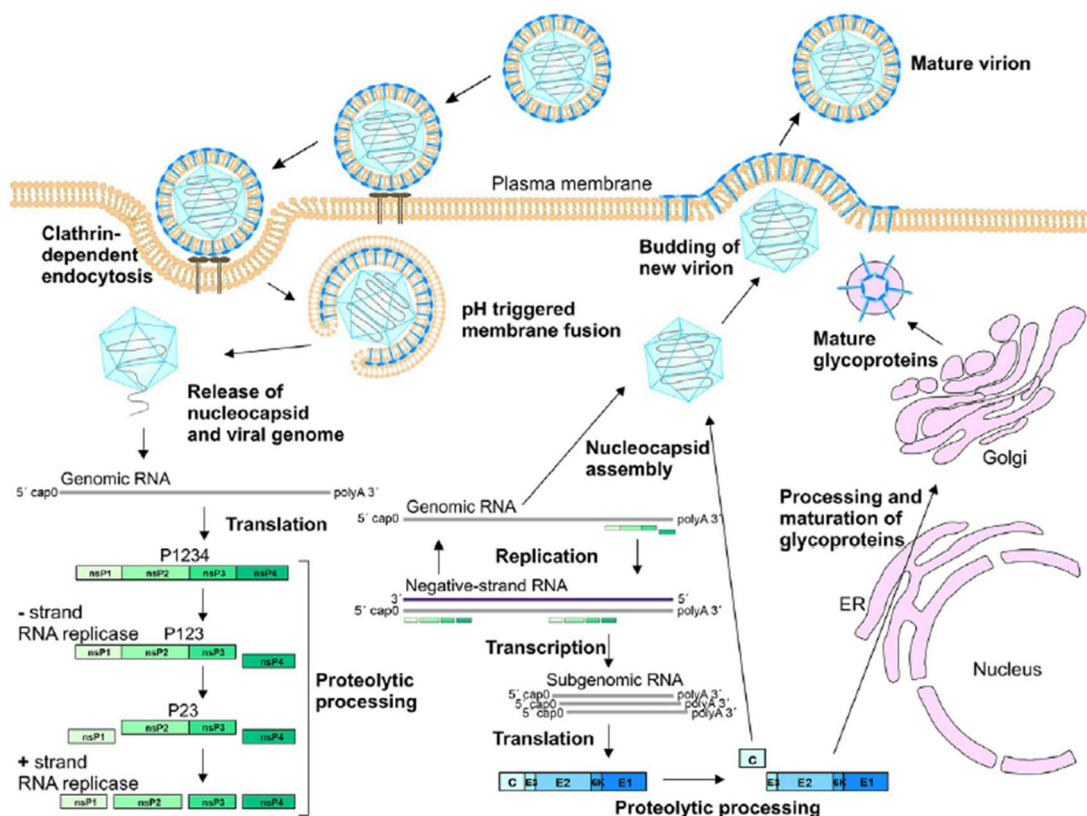


Figure 3. Overview of the alphavirus replication cycle. The schematic summarises the main stages of alphavirus infection, including receptor-mediated entry by clathrin-dependent endocytosis, low-pH-triggered membrane fusion, release of the viral genomic RNA, translation and processing of nonstructural and structural polyproteins, RNA replication and subgenomic RNA transcription, nucleocapsid assembly, glycoprotein maturation through the ER and Golgi, and budding of mature virions from the plasma membrane (1).

1.6 Pathogenicity

The pathogenicity of the viruses in this study and, more generally, of viruses in the genus *Alphavirus* depends on replication efficiency, host immune response, and specific genetic determinants that affect these traits in both host and virus.

CHIKV causes acute febrile illness and debilitating polyarthrititis by infecting joint tissues, which, along with immune-mediated inflammation, leads to arthritis (19,20). Since its discovery in 1953, CHIKV has established a permanent epidemic and endemic infections in Africa, Asia, the Indian Ocean, and the Americas (21). There are no specific antiviral therapies, so medical care focuses on supportive treatment to manage fever and joint inflammation (22) As of 2026, two CHIKV vaccines have received regulatory approval. In late 2023, the Food and Drug Administration licensed IXCHIQ (VLA1553), the first live-attenuated vaccine for CHIKV (23). More recently, in

early 2025, the Food and Drug Administration and European Commission approved Vimkunya, a virus-like particle (VLP) vaccine developed by Bavarian Nordic (23).

ONNV is closely related to CHIKV and is responsible for the sporadic outbreaks (with milder symptoms) of infection with fever and joint pain in Africa (16). Unlike CHIKV, which has spread worldwide, ONNV has been confined to Africa (10). While the first outbreak in 1959 involved over 2 million cases, subsequent outbreaks have been described as sporadic (16).

MAYV causes symptoms similar to those of CHIKV infection, which typically include sudden high fever, rash, severe joint pain, headache, muscle pain, nausea, vomiting, and eye pain (24,25). This virus is primarily endemic to the tropical forests of Central and South America, with a heavy concentration in the Amazon basin (25). The true epidemiological burden of MAYV is grossly underreported, as there is a symptomatic overlap with other tropical diseases that also cause fever and serological cross-reactivity, making it difficult to distinguish past MAYV infection using standard antibody tests (24,25). Currently, no specific antiviral therapies or licensed vaccines are approved for MAYV infection, and medical management is strictly supportive (25).

SINV is the most widespread alphavirus, with a geographic distribution spanning Europe, Africa, and Australia (10). The highest reported human burden is in Northern Europe, where the virus is endemic and causes cyclical outbreaks every few years. Sindbis fever is characterised by fever, rash, and joint pain that can last for several months or even years. The epidemiological burden is difficult to assess due to underreporting from subclinical cases and misdiagnosis, given the relatively mild nature of the disease. No specific treatment or licensed vaccines are available (17).

RRV is the most common arbovirus in Australia, causing epidemic polyarthritis (26). In 1979-1980, the virus spread significantly to the South Pacific, affecting Fiji, American Samoa and New Caledonia. Australia reports an average of 4745 cases annually, and the socioeconomic burden is substantial due to long-term morbidity and lost productivity. No specific treatment or vaccines are available; however, an inactivated whole-virus vaccine candidate was developed and showed promise in preclinical trials, though clinical trials were difficult to fund because the disease caused by RRV is not fatal and is mainly restricted to Australia (27).

Similarly, BFV causes symptoms like those of RRV and is considered the second most prevalent virus in Australia. BFV differs from RRV by a more prominent and intense rash, but a lesser joint pain and swelling. Australia reports approximately 1000 confirmed cases annually. Because BFV and RRV share the same endemic areas and produce overlapping symptoms, patients in these

regions are typically tested for both viruses (9,27). No specific treatment or licensed vaccines are available.

Their significant global public health impact drives the need to research alphavirus replication mechanisms and life cycles. Despite the severe socioeconomic burden these viruses impose, there are currently no approved targeted antiviral therapies or widely licensed human vaccines for most viral species (13). The main issue with researching these viruses is the need for BSL-3 conditions. So, moving alphavirus research into lower-level conditions using replicons and VRPs is beneficial for identifying new drug targets and advancing self-amplifying RNA technologies for future vaccines and therapeutics (28).

1.7 Replicon systems

1.7.1 Definition and concept of alphavirus replicon systems

Replicons are engineered by replacing the viral structural protein genes (approximately 4.1 kb) with a gene of interest under the control of an alphavirus SG promoter. The principle of viral replicons is that replication, SG RNA transcription, and protein expression occur without the formation of an infectious virus. So, this system allows the creation of self-amplifying RNAs that mimic the virus's replication cycle but are propagation-incompetent. Thus, alphavirus-based replicon systems cannot produce infectious progeny (3).

In a typical alphavirus replicon, all structural genes are removed. In previous studies on CHIKV, the entire region corresponding to the structural ORF was deleted. This has also been done with SINV, SFV and VEEV (3,12). Because the essential packaging signals are located in the non-structural ORF (Figure 2), these deletion-containing genomes can still be packaged to form virus-like replicon particles (VRPs) if the structural proteins are provided separately (*in-trans*) within the “helper” RNAs (5).

The heterologous gene of interest (GOI), such as a reporter or a vaccine antigen, is typically inserted downstream of the native SG promoter (3). This maximises heterologous protein expression level and ensures the stability of the replicon system. Stability here refers to the genetic integrity and functional persistence of the gene of interest across consecutive cell passages (Figure 4) (29).

Replicon-transfected cells produce two species of positive-strand RNAs – the full-length genomic replicon and shorter SG RNAs, which encode for the reporter or GOI – because the viral replication machinery can recognise two different promoter sites on the minus-strand RNA template – the

genomic and SG promoters. The genomic promoter is located at the 3' end of the minus-strand, and the SG promoter is in the junction region (Figure 4) (1). Because the proportion of these RNAs is naturally asymmetric, favouring the production of SG RNAs, the heterologous protein yield is very high, and its expression can be clearly monitored in transfected cells (1,3). An alphavirus replicon system functions as a self-amplifying RNA (saRNA). Once inside the cytoplasm, the RNA is translated to produce the viral replication machinery, which then generates many additional RNA copies from the original template. Unlike conventional mRNA, which is a static template for translation – meaning it is a single-use, non-replicating molecule that can only be translated into protein until it is naturally degraded – a replicon carries the genetic instructions for its own RNA-dependent RNA polymerase (RdRp).



Figure 4. Schematic structure of an alphavirus replicon carrying a gene of interest. The replicon contains the 5' untranslated region (5' UTR), the nonstructural protein coding region nsP1–nsP4, a subgenomic promoter (SG prom) driving expression of the inserted gene of interest (GOI), followed by the 3' untranslated region (3' UTR) and poly(A) tail. The structural genes are replaced by the GOI, allowing RNA replication and reporter/transgene expression without production of infectious virus particles in the absence of helper structural proteins.

1.7.2 Molecular design of the alphavirus replicon

To conclude the previous part, if we were to design an alphavirus replicon, it would be based on the alphavirus genome's genetic separation into structural and non-structural units. This allows us to replace the structural genes with a gene of interest without affecting replication (5).

To retain the ability to replicate, the replicon genome must include certain minimal sequences – the CSEs and the entire non-structural ORF. The CSEs are CSE 1, CSE 2, CSE 3 (SG promoter), CSE 4, and the poly(A) tail (at least 11-12 residues) (13). The important parts are the UTRs, CSE1, both the 5' and 3' ends, as they are necessary for the formation of functional replication complexes.

1.7.3 Advantages and limitations of alphavirus replicons

The use of alphavirus replicons offers distinct advantages for high-level heterologous gene expression and vaccine development, though they are subject to specific biological and safety limitations.

One of the main advantages of the replicon system is its self-amplifying nature. Alphavirus replicons retain the viral replicase complex (nsP1-4), where a single RNA molecule can produce up to 200,000 copies within the host cytoplasm (30). This allows for a 1,000-fold reduction in the required RNA dose compared to conventional mRNA vaccines (31). Additionally, because this system exploits the SG promoter, which naturally directs the production of structural proteins, the expression of the gene of interest can be very high, constituting as much as 25% of total cellular proteins (6,10).

Furthermore, alphavirus replicons replicate exclusively in the cytoplasm, preventing chromosomal integration and increasing safety while avoiding the need for nuclear splicing (6,10). The replication process also involves double-stranded RNA intermediates, which are strong triggers for the host's immune system and provide a natural self-adjuvant effect, enhancing the subsequent adaptive immune response (5,32).

Disadvantages, on the other hand, include the following. Firstly, replicon expression is transient, meaning that it is short-lived. This is due to RNA's short half-life, the absence of chromosomal integration and strong cytotoxic effects that eventually lead to death of the host cell (6). In SFV-based systems, protein expression usually lasts 75 hours before declining (10). That is why the engineering of non-cytopathic replicons is important, enabling persistent replication for an unlimited number of passages, especially under selective pressure (6).

The previously mentioned dsRNA intermediates are also a disadvantage of the system, as during replication, the cell detects viral dsRNA (12,33). This activates the interferon type I pathway, which shuts down global protein production (33). As a result, the synthesis of the protein of interest is reduced, potentially preventing the successful assembly and packaging of viral particles (33). During replication, the cell detects viral dsRNA and triggers an immune response (12,33). This activates the kinase PKR, which halts global protein synthesis (33). As a result, the synthesis of the protein of interest is reduced, potentially preventing the successful assembly and packaging of viral particles (33).

Also, there is a documented risk of RNA recombination between the replicon and helper RNAs, which could potentially restore the structural genes and produce a replication-competent virus (5). To minimise this risk, a split-helper system is used, in which the capsid and envelope structural genes are located on separate RNAs. This significantly lowers the risk of recombination (3). Further, using promoterless helper RNAs that lack the subgenomic promoter can also lower the risks (11).

Alphavirus replicons are generally considered genetically stable during short-term use, with divergence rates estimated at 1×10^{-4} and 6×10^{-4} per nucleotide per year. However, because the alphavirus RNA-dependent RNA polymerase lacks proofreading activity, suppressor or compensatory mutations can arise very rapidly if the replicon is functionally impaired (8).

In summary, alphavirus replicon systems are a powerful tool due to their self-amplifying RNA nature and strong transgene production. Their design ensures replication competence even in the absence of structural genes. However, there are limitations, including cytotoxicity driven largely by nsP2-mediated host transcription shutoff, strong innate immune activation due to double-stranded RNA intermediates, transient expression, and the theoretical risk of recombination with helper RNAs. The successful application of alphavirus replicons requires careful genetic design to balance replication efficiency, expression strength, safety, and genetic stability.

1.8 Viral replicon particles (VRPs)

1.8.1 Definition and concept of the VRPs

A virus replicon particle (VRP) is a single-round infectious unit (IU) that packages a self-amplifying alphavirus replicon RNA within a viral protein shell (3). The key difference between a replicon and a VRP is that a replicon is naked genetic material that requires artificial introduction into the cell via transfection, whereas a VRP is a fully evolved piece of machinery that can infect cells independently, allowing for effective infection (5).

Structurally, VRPs are antigenically equivalent to a wild-type virion because they utilise the same protein/membrane shell, which consists of a viral capsid, cell membrane, and envelope glycoproteins. Because the surfaces of the VRPs are identical to those of the authentic virus, they interact with cellular receptors in the same way (4).

Just like wild-type alphaviruses, VRPs have spherical particles 70 nm in diameter (4). They consist of an icosahedral nucleocapsid surrounded by a host-derived lipid bilayer. The envelope is studded with 80 trimeric spikes composed of the heterodimers of E1 and E2 glycoproteins (13). These spikes are the primary target of the host organism's neutralising antibodies, and the VRPs are antigenically identical to the virus from which they are derived. The only difference is that, instead of an 11-12 kb viral genome, VRPs carry a replicon RNA lacking structural genes, making them single-cycle vectors (4). They are single-cycle (also called “suicide particles”) because they cannot undergo multiple rounds of infection. They can efficiently enter a target cell and replicate the

incorporated RNA to high levels, but the infected cell cannot assemble new VRPs because the delivered replicon RNA lacks the structural genes (12).

The practical significance of all this equivalence is that it makes VRPs ideal safe particles for research. As VRPs are identical in their early stages of infection but do not produce viral progeny, it is possible to safely study dangerous pathogens under biosafety level 2 (BSL-2) conditions (4). Furthermore, their ability to mimic the authentic virus and trigger an immune reaction makes them highly potent for potential vaccine development (12).

1.8.2 Single helper systems

To produce VRPs, helper RNAs are required in addition to replicon RNA. In a single-helper system, the production of the virus replicon relies on a single RNA molecule providing all the necessary components for virion assembly *in trans*. The single-helper RNA encodes the entire alphavirus structural polyprotein: the capsid (C) protein, the small peptides E3 and 6K, and the E1 and E2 glycoproteins (3). These sequences are placed under the control of a subgenomic promoter (Figure 5) (34).

The Helper RNA is engineered to contain only the CSEs required for the recognition by the viral replication machinery, while the genes for the replication proteins (nsP1-4) are entirely deleted. These CSEs include 5' and 3' UTRs and the 51-nucleotide element from the nsP1 coding sequence. These are required for the viral polymerase to bind and initiate RNA synthesis (Figure 5)(3).

The alphavirus structural proteins are not translated directly from the genomic RNA; they are expressed only after the viral replicase recognises the SG promoter on the negative-strand template and transcribes the SG RNA, which then serves as the specific mRNA for their translation (1). The production of structural proteins occurs only when replicon RNA (encoding for the functional replicase) is present. After co-transfection of the replicon RNA and helper RNA, the replicon RNA is immediately translated by the host cell to produce the nsP1-4 proteins, which assemble into the early replicase complex. The early replication complex recognises the 5' and 3' signals of the helper RNA as if they were its own and produces complementary minus-strand intermediates (4). The late replicase complex then uses the minus-strand RNA molecules as templates to produce positive-strand RNAs. These positive-strand RNAs are then translated by the cellular translational machinery into structural proteins (12) (11).

To improve safety, single-helper RNAs lack packaging signals. This ensures that only the replicon RNA, which does contain the packaging signal in the nsP1-2 coding sequences, is incorporated into the resulting VRPs (12).

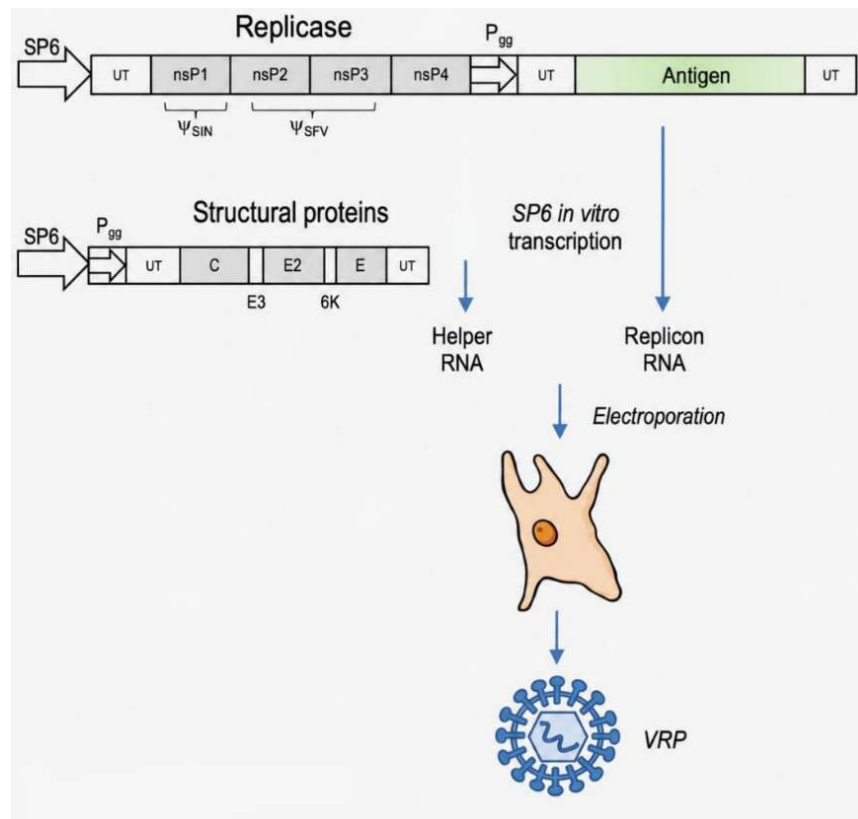


Figure 5. Production of alphavirus virus replicon particles using a single-helper RNA system. The replicon and helper constructs are first transcribed *in vitro* from SP6 promoters to generate replicon RNA and helper RNA. The replicon RNA encodes the viral replicase proteins nsP1–nsP4 and the gene of interest/antigen under the control of the subgenomic promoter, while the helper RNA provides the alphavirus structural proteins in trans. Both the replicon and the helper RNA include untranslated regions (UTRs) that are responsible for RNA replication, viral replicase recognition, and RNA stability. After co-electroporation into producer cells, the replicon RNA is replicated and packaged by helper-derived structural proteins, leading to the release of VRPs containing replicon RNA. (12, with modifications)

A major concern with single-helper RNA systems is the possibility that the helper RNA sequences could be incorporated into the replicon RNA by recombination, forming a single infectious RNA molecule. This leads to the formation of a replication-competent virus (RCV) (4,34). That's why the RCV formation is relatively high in the single-helper replicon system for VRP production (11).

Nonetheless, the single-helper systems are highly efficient at producing VRPs. Because all structural proteins are encoded by a single transcript, they can yield high titers on infectious particles, often reaching 10^8 - 10^9 iu per ml (34).

1.8.3 Split-helper system

The split-helper system is designed to increase biosafety while maintaining high levels of gene expression. In this system, the sequences for the viral capsid and envelope proteins are separated into two independent RNAs, which are co-transfected alongside the replicon RNA into the host cell (3).

The primary reason for separating the capsid and envelope coding RNAs is to prevent the generation of RCVs, as this system significantly reduces the likelihood of recombination. In a single-helper system, only a single recombination event is enough to restore a wild-type, full-length genome. In a split system, however, two independent and nearly perfect recombination events must occur to achieve an RCV (11). Just as in the single-helper system, the capsid and envelope RNAs both lack packaging signals but contain replication signals in their CSEs. The infection and replication cycles are identical to those of a single-helper system, and the final VRPs only contain the replicon RNA (12).

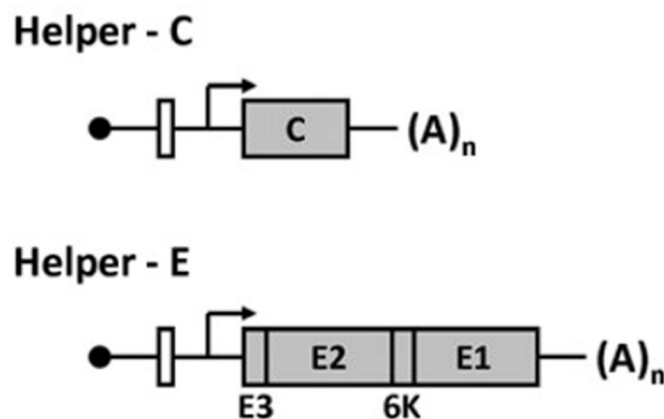


Figure 6. Schematic structure of split helper RNAs used for alphavirus VLP production. The structural genes are divided between two separate helper RNAs: Helper-C, which encodes the capsid protein, and Helper-E, which encodes the envelope glycoprotein cassette E3–E2–6K–E1. Each helper RNA contains the required untranslated and promoter elements for expression, followed by a poly(A) tail. This split-helper design provides structural proteins in trans while reducing the likelihood of recombination that could lead to a replication-competent virus. electroporation occurs as shown in Figure 5 ((3), with modifications)

Several modifications have been developed to further enhance the safety of split-helper systems. For example, the 5' ends of the helper RNAs can be cut down to the absolute minimum required for replication, reducing sequence overlap with the replicon and minimising the opportunity for homologous recombination (11).

The absence of RCV is confirmed through serial blind passages and plaque assays. This means that the researchers verify safety by performing multiple consecutive infections of cells with VRP stocks. If no viral spread is detected after two to five passages, the system is confirmed to be RCV-free (35).

1.8.4 VRP production (co-transfection, packaging, and lack of propagation)

In our case, we use a split-helper system to ensure safety. The first step in generating VRPs in this case is to produce 3 distinct RNA molecules via *in vitro* transcription of three linearised DNA molecules: a replicon with a reporter gene, helper C for the capsid protein, and helper E for the envelope glycoproteins (3).

Some of the frequently used cell lines for VRP generation and testing include BHK-21 (Baby Hamster Kidney) cells. These cells are fibroblasts that are highly permissive to alphavirus replication. The permissiveness of the cell line is characterised by a deficient interferon response (3,5).

The essential part of efficient VRP packaging is the replicon. The non-structural proteins (the replicase) translated from the replicon RNA act *in trans* to amplify the helper RNAs. This amplification then triggers the high-level transcription of subgenomic structural RNAs (11,12).

The selection of the encapsulated RNA is highly specific. The structural proteins, specifically the capsid, recognise the unique cis-acting packaging signal located within the non-structural coding region of the replicon RNA. Cis-acting means that they regulate major processes like replication, transcription, and translation only on the same nucleic acid molecule (10). Because the helper RNAs lack this packaging signal, the capsid protein selectively targets and packages only the replicon RNA (11)

Multiple factors determine the yield and quality of the VRP stock. The replicon and helper RNAs “compete” for the available replicase complexes. If the helper RNA replicates excessively, it can interfere with the replicon's replication, reducing the overall quality of the VRP stock (34). VRP production is also sensitive to temperature. Most cells are grown at 37 degrees, optimised VRP production often occurs at 32 degrees. And even though cell growth is slower at lower temperatures, the overall quality and quantity of the VRP stock are higher (3).

2 Aims of the thesis

In this work, we aimed to generate VRPs for six alphaviruses and confirm their biosafety, enabling their subsequent use for safe research on the corresponding infectious viruses. This way, the research would be conducted on the closest analogues of the infectious viruses with identical infection cycles, but without safety hazards, since the VRPs are capable of only a single cycle of infection.

The following steps were performed subsequently in the scope of this thesis:

1. The necessary plasmids were generated based on the existing cloning plan.
2. The constructed plasmids were transcribed *in vitro* to produce replicon and helper RNAs, which were then transfected into the BHK21 mammalian cell line in a set of replicon, helper E and helper C, to generate VRPs.
3. The generated VRPs were titrated to confirm the concentration of the infectious units.
4. A second round of infection was performed to confirm that no viruses were generated and that the VRPs are indeed single-cycle particles.

3 Experimental part

In this research, VRPs are quantified by creating reporter assays. The titration process involves infecting a monolayer of cells with different dilutions of the VRP stock and counting cells expressing the reporter proteins – in our case, mCherry (red) and ZsGreen (green).

In the previous work in our laboratory, *in silico* plasmid maps for the replicons of the six viruses (CHIKV, ONNV, MAYV, BFV, RRV, and SINV) and the helper E and helper C plasmids were designed.

3.1 Materials and methods

3.1.1 Cloning of plasmid constructs

General cloning strategy

Plasmid constructs encoding alphavirus replicons and helper RNAs were generated using restriction enzyme-based cloning and PCR-based modification. Synthetic fragments encoding viral structural components or reporter genes were obtained commercially from GenScript and cloned into appropriate plasmid backbones.

Restriction digestion

The restriction digestions were performed to obtain the vector and insert fragments. Vector fragments were digested in reactions containing: 5 μ L of 10 $\times$ FastDigest Clear buffer (Thermo Fisher Scientific), 3 μ g of DNA, 1 μ L of each FastDigest restriction endonuclease (Thermo Fisher Scientific) and water up to 49 μ L. The reaction mixtures were incubated at 37°C for 1 hour. After incubation, FastAP enzyme was added to prevent the vector from self-ligating. The mixture was incubated for another 30 minutes. The vector fragment was purified from the reaction mixture using the ZymoClean DNA Purification Kit according to the manufacturer's instructions.

The inserts were digested in reactions containing: 2 μ L of 10 $\times$ FastDigest Green buffer (Thermo Fisher Scientific), 3 μ g of DNA, 1 μ L of each FastDigest restriction endonuclease, and water to a final volume of 20 μ L (Thermo Fisher Scientific). The reaction mixtures were incubated at 37°C for 1 hour. The insert was run on a 0.8% Tris-Acetate-EDTA (TAE) agarose gel for 15-20 minutes, after which the desired DNA band was excised and purified using the ZymoClean Gel Purification Kit according to the manufacturer's instructions.

Ligation

The purified vector and insert fragments were ligated in a 1:1 ratio using T4 DNA ligase (Thermo Fisher Scientific) according to the manufacturer's protocol. The reactions were incubated overnight at 12°C to improve ligation efficiency.

Transformation of bacteria

10 µL of the ligation mixture was mixed with 100 µL of *E. coli* strain XL-10 competent cells. The mixture was incubated on ice for 20 minutes and underwent a heat shock (2 minutes at 42°C and 2 minutes on ice), after which 1 mL of SOB media supplemented with 0.4% glucose was added to the mixture. The final mixture was incubated at 37°C for 45 minutes with shaking at 180 rpm, then plated onto lysogeny broth (LB) agar plates supplemented with either kanamycin (25 µg/mL) or ampicillin (100 µg/mL). The plates were incubated at 37°C overnight.

Plasmid DNA purification

The bacteria were grown at 37°C in SOY (Tryptic soy broth, Becton Dickinson) medium with shaking at 180 revolutions per minute for propagation. The medium was supplemented with either ampicillin or kanamycin to a final concentration of 100 µg/mL (ampicillin) or 25 µg/mL (kanamycin). The plasmid DNA was extracted and purified using the FavorPrep Plasmid Extraction Mini Kit (Favorgen) according to the manufacturer's instructions. The plasmid DNA concentration was confirmed using a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific). The received plasmid DNA constructs were validated using restriction digestion with FastDigest restriction endonucleases (Thermo Fisher Scientific).

Cloning of SINV helper constructs

Helper 1

To create Helper E and Helper C, Helper 1 for SINV was first generated, which combines the capsid and envelope protein-coding sequences. Helper 1 was generated by cutting a synthetic DNA fragment containing the sequence of SINV structural proteins with *HindIII* and *AatII*. The generated fragment was ligated to a fragment of the infectious complementary DNA (icDNA) of SINV (TOTO1101), which had been digested with the same enzymes to generate complementary ends.

Helper E

Helper E was generated by digesting the SINV Helper 1 with *CpoI* and *NcoI* enzymes and ligated with a DNA fragment containing the sequence for the SG promoter, capsid enhancer and envelope

protein derived from a synthetic fragment. The synthetic fragment was digested with the same enzymes as Helper 1 to generate complementary ends.

Helper C

Helper C was constructed by digesting SINV Helper 1 with *NcoI* and *EcoRI* enzymes and inserting the sequence of the SG promoter and capsid protein by digesting a synthetic fragment with the same enzymes to generate complementary ends.

Cloning of replicon constructs

Insertion of mCherry and ZsGreen reporter genes

Previously in our lab, replicons for MAYV, BFV, CHIKV, and ONNV had been generated, each containing the CD20 protein sequence as the GOI. The mCherry/ZsGreen replicons for the same viruses were generated by replacing the CD20 protein sequence with ZsGreen and mCherry, derived from the SP6-CHIKV-icDNA-ZsGreen/mCherry constructs. Plasmids containing CD20 were digested with *XmaI* and *MssI*, while reporter gene fragments were excised using the same enzymes and ligated into the replicon backbone.

Generation of SINV replicon backbone

The SINV replicon backbone was generated from its icDNA (pTOTO1101) by polymerase chain reaction (PCR) amplification to remove structural genes. The primers were designed to anneal to regions flanking the structural genes and to contain a *PacI* restriction site, generating complementary ends for ligation of the PCR product following purification. PCR was performed using Platinum SuperFi DNA polymerase with the following reaction composition: 5 µL of 5× Platinum SuperFi buffer (Thermo Fisher Scientific), 0.1 mM dNTPs mix, 1 mM forward primer (Microsynth SEQLAB), 1 mM reverse primer (Microsynth SEQLAB), 20 ng of template DNA, 0.2 µL of Platinum SuperFi polymerase (Thermo Fisher Scientific) and water up to 25 µL.

The thermal cycling conditions were: initial denaturation: 98°C for 2 minutes; denaturation: 98°C for 20 seconds, primer annealing: 60°C for 20 seconds, extension: 72°C for 5 minutes (30 cycles); final extension: 72°C for 5 minutes.

PCR products were purified using the Clean and Concentrator kit (Zymo Research), followed by digestion with *PacI* to generate compatible ends and *DpnI* to remove the template plasmid DNA. The digested product was purified again before ligation overnight at 12°C.

Cloning of SINV replicon reporter constructs

SINV replicons with reporter genes (mCherry, ZsGreen) were generated by digesting the SINV replicon generated in the previous step with *XbaI* and *PmeI* restriction enzymes. CHIKV replicon-Zsgreen/mCherry constructs were digested with the same enzymes to generate compatible fragments containing the reporter proteins. The digested SINV replicon and the digested CHIKV replicons containing the reporter protein coding sequences were ligated.

Cloning Helper E for ONNV, BFV, and MAYV

Previously in our lab, constructs for ONNV, BFV and MAYV Helper E had been generated. However, a cloning error resulted in final sequences containing a truncated capsid enhancer. To generate Helper E constructs with a functional capsid enhancer, synthetic fragments containing the SG promoter, capsid enhancer and envelope sequences were ordered from the GeneScript company. The synthetic constructs were digested with *ApaI* and *BssHI* (ONNV), *SpeI* and *NotI* (BFV), and *SanDI* and *XhoI* (MAYV).

3.1.3 *In vitro* transcription

Plasmid DNA linearisation

All replicon-ZsGgreen/mCherry and helper C/E constructs obtained during cloning were linearised and transcribed *in vitro*. The linearisation mix was prepared as follows: 2 µL of 10× buffer, 1.5 µL of restriction enzyme, 3 µg of DNA, and RNase-free water to a final volume of 20 µL.

The restriction mixtures were incubated at 37°C for 30 minutes and purified using the Clean and Concentrator kit (Zymo Research). The purified DNA was eluted with 10 µL of the kit's elution buffer, and the concentration of linearised DNA was determined using a NanoDrop spectrophotometer.

***In vitro* transcription**

In vitro transcription (IVT) was performed using the mMESSAGE mMACHINE SP6 Kit (Thermo Fisher Scientific) with the following mixture: 5 µL of 2× NTP/CAP mixture, 2 µL of 10× buffer, 3 µL of linearised DNA template, and 1 µL of SP6 enzyme mix.

The reaction mixes were incubated at 37°C for 2 hours. The size of the *in vitro* transcribed RNA was confirmed by gel electrophoresis on a 1% TAE agarose gel, with 0.5 µL of the IVT mixed with 2 µL of RNA loading dye (Thermo Fisher Scientific).

3.1.4 Production of viral replicon particles (VRPs)

Before transfection, BHK-21 cells were grown to 80-90 % confluency in GMEM (PAN-Biotech) media supplemented with 7.5% FCS (Fetal calf serum, PAN Biotech), 20 mM HEPES, 10% TPB (Tryptose Phosphate Broth, Thermo Fisher Scientific), 100 IU/ml Penicillin, 100 µg/ml Streptomycin (Thermo Fisher Scientific). The cells were maintained at 37°C and 5% CO₂ before the experiment. One 100 mm plate was used for two electroporations. The cells were washed with 4 ml of PBS, trypsinised with 2 ml of trypsin, and after 30-40 seconds, trypsinisation was stopped by adding 8 ml of GMEM medium.

The cells were collected and centrifuged at 200× g for 5 minutes. The supernatant was aspirated, and the cells were resuspended in 800 µL of ice-cold PBS (Gibco) for each electroporation.

800 µL of PBS-cell mixture, 9.5 µL of replicon IVT mixture, 9.5 µL of helper C IVT mixture, and 9.5 µL of helper E IVT mixture RNAs were added to the pre-cooled 4 mm electroporation cuvettes (BioRad). Electroporation was performed using two pulses at 850 V and 25 µF, with a 10-15-second delay, at room temperature.

Finally, the cells were transferred to a new 60 mm dish containing 4 mL of fresh GMEM medium and incubated for 48 or 72 hours at 28°C and 5% CO₂.

3.1.5 Titration of VRPs using fluorescent microscopy

Cell seeding

BHK-21 cells were seeded in 24-well plates 1 day before infection to achieve approximately 100% confluency at the time of titration. One full plate of BHK-21 cells was used per 24-well plate.

Preparation of serial dilutions

Ten-fold dilutions of VRPs were prepared in a 96-well plate using viral growth medium (VGM) (GMEM supplemented with 0.2% bovine serum albumin (BSA)). 180 µL of VGM was added to each well, and 20 µL of viral stock was added to the first dilution well. A 10-fold serial dilution series was generated by transferring 20 µL in sequence across wells, resulting in dilutions from 10⁻¹ to 10⁻⁵.

150 µL of each dilution was added to the corresponding wells on the 24-well plates. Cells were incubated at 37°C for 1 hour to allow VRP entry. Following incubation, 300 µL of fresh medium was added to each well.

Infected cells were incubated at 37°C and 5% CO₂ for 24 hours to allow expression of the reporter markers.

Quantification of infected cells

After 24 hours, the cells were visualised under a fluorescent microscope and counted using the following steps:

- Wells with a countable number of cells were selected
- The number of positive cells (cells expressing the fluorescent protein) was recorded from 20 fields of vision in each well across 3 replicates
- The average of these numbers was recorded
- The titre was calculated using the formula:

$$\text{IF titre} = \text{average number of fluorescent cells} \times \text{constant} \times \text{dilution factor} \times K$$

Where:

- Constant = dish surface (mm²) / field surface (mm²)
- Field surface = $\pi \times r^2$
- Field diameter = magnification of the ocular/magnification of the objective
- K = 1000 µL / 150 µL (volume of VRPs used for infection) = 6.67

It is important to note that the infected cells (cells bearing fluorescent marker) were counted manually, assuming 1 infected cell = 1 IU, so minor inconsistencies in the titre calculations are possible.

3.1.6 Second round of infection

Creation of the P1 stock

In this experiment, cells were prepared for titration. To create P1 stock, 20 µL of the VRP stock was added to the cells and after 1 hour of incubation, the VRPs were aspirated off from the cells. 100 µL of fresh media was added to the wells, then aspirated to wash away any remaining VRP stock. After that, 100 µL of fresh media was added to the cells, which were incubated at 37°C and 5% CO₂ for 24 hours, and the resulting P1 stock was collected.

Second round of infection

The P1 stock, along with the VRP stock, was used to infect the cells for a side-by-side comparison. 20 µL of each stock was added to BHK-21 cells and incubated for 1 hour. The stocks were then aspirated, 100 µL of fresh media was added to the wells, and the wells were aspirated again to

wash away any remaining VRP stock. Then 100 μ L of fresh medium was added to the cells. The cells were incubated at 37°C and 5% CO₂ for 24 hours.

Imaging the results

To verify that no infection occurred after cells were infected with the P1 stock and to double-check that the VRP stock was fully functional, side-by-side images of the corresponding cells were taken. Images of the full wells were captured on an EVOS M5000 (Thermo Fisher Scientific).

3.2 Result and discussion

3.2.1 Generation of plasmid constructs encoding alphavirus replicons and helpers

The plasmids required for the replicon split-helper system were successfully generated by molecular cloning. The replicon, capsid and glycoprotein helper sequences were cloned into separate expression vectors. Constructs were verified by restriction digestion analysis and long-read sequencing chemistry from Oxford Nanopore Technologies, which confirmed the expected insert sequences and orientations. The validated plasmids were subsequently used to produce single-cycle alphavirus vectors. The constructs generated in this study and those existing in the lab are shown in Table 1.

Table 1. Overview of alphavirus replicon and helper constructs used in this study. The table summarises the availability and origin of the constructs used for VRP production for SINV, MAYV, CHIKV, BFV, RRV, and ONNV. For each virus, the table indicates whether the corresponding replicon backbone, reporter replicons (Replicon-ZsGreen and Replicon-mCherry), and helper constructs (Helper 1, Helper E, Helper C, and Helper E without enhancer) were either already available in the laboratory or generated during this study. Dashes indicate constructs that were not included or not applicable for that virus in the experimental design. SINV Helper 1 was generated to clone SINV Helper C and E.

	SINV	MAYV	CHIKV	BFV	RRV	ONNV
Replicon	Generated in this study	Existing in the lab	Existing in the lab	Existing in the lab	Existing in the lab	Existing in the lab
Replicon-Zsgreen	Generated in this study	Generated in this study	Generated in this study	Generated in this study	Generated in this study	Generated in this study
Replicon-mCherry	Generated in this study	Generated in this study	Generated in this study	Generated in this study	Existing in the lab	Generated in this study
Helper 1	Generated in this study	–	–	–	–	–
Helper E	Generated in this study	Generated in this study	–	Generated in this study	Existing in the lab	Generated in this study
Helper C	Generated in this study	Existing in the lab	Existing in the lab	Existing in the lab	Existing in the lab	Existing in the lab
Helper E no enhancer	–	Generated in this study	Existing in the lab	Generated in this study	Generated in this study	Generated in this study

In this study, as illustrated in Figure 7, the production system utilises a tripartite split-helper system comprising of a replicon and two separate helpers (Helper C and Helper E). To enable quantitative and qualitative assessment of VRP infectivity, the viral structural proteins in the replicon were replaced with heterologous reporter genes, ZsGreen and mCherry, in the replicon backbones. To investigate the species-specific requirements for translational control, two versions of the Helper E constructs were generated for the majority of the virus panel: one with the native capsid enhancer sequence (downstream loop, DLP) and one lacking it (Figure 7).

The precise molecular architecture of these plasmids is critical for generating single-cycle VRPs. Each component presented in Figure 7 plays a distinct role in that. The SP6 promoter is essential for IVT to generate RNA transcripts (3). The linearisation site is a unique restriction site located immediately after the poly(A) tail. Linearising the plasmid ensures the production of a clean transcript with a precise 3' terminus, which is important for the stability and replicability of the resulting RNA (9). An antibiotic resistance cassette, encoding resistance to ampicillin or kanamycin, enables the selective maintenance and large-scale propagation of plasmids in *E. coli* during cloning.

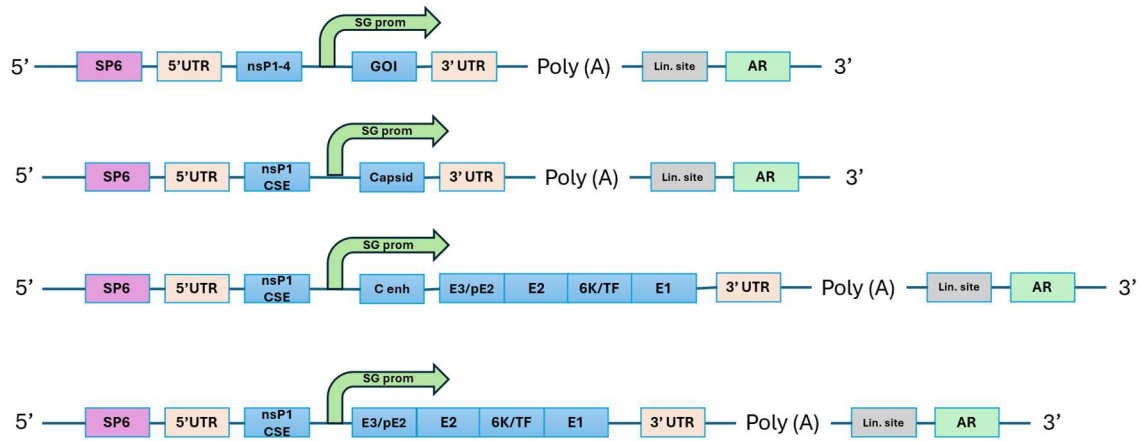


Figure 7. Schematic representation of the replicon and helper plasmid constructs used for VRP production.

The plasmids contain an SP6 promoter followed by alphavirus 5' UTR elements and either the replicase region/nsP1 CSE or helper-derived structural gene regions. The subgenomic promoter drives expression of the gene of interest, capsid, or structural proteins. Constructs include helper plasmids with and without the capsid enhancer sequence (C enh). The 3' UTR, poly(A) region, linearization site, and antibiotic resistance (AR) cassette are also indicated.

3.2.2 Generation of VRPs

Virus-like replicon particles (VRPs) were generated for CHIKV, MAYV, ONNV, RRV, SINV, and BFV using a three-component system: replicon RNA (encoding either mCherry or ZsGreen reporter protein under the SG promoter), and two helper RNAs encoding for envelope glycoproteins and capsid protein of the respective virus. Some differences in fluorescence intensity were observed between the cells transfected with replicons derived from different viruses. In Figure 7, we can clearly see that the fluorescent signal intensities from CHIKV, MAYV, and ONNV exceed those from RRV and BFV (Figure 7). The different levels of fluorescent protein expression across different alphavirus replicons are a direct indicator of the integrated efficiency of their viral replication machinery. This efficiency is demonstrated by the kinetics of the alphavirus life cycle, which is characterised by the rapid expansion of virus-specific RNAs and the massive production of progeny virions within hours of infection (4,5). Within a few hours of entry, SG RNA synthesis can accumulate up to 10^6 RNA molecules per cell (4). However, the timeline for reaching peak replication and VRP production varies across different viruses. For example, previous laboratory experience shows that while most viruses require 72 hours to produce the maximum number of VRPs, SINV-based VRPs are better when collected after 48 hours post-transfection.

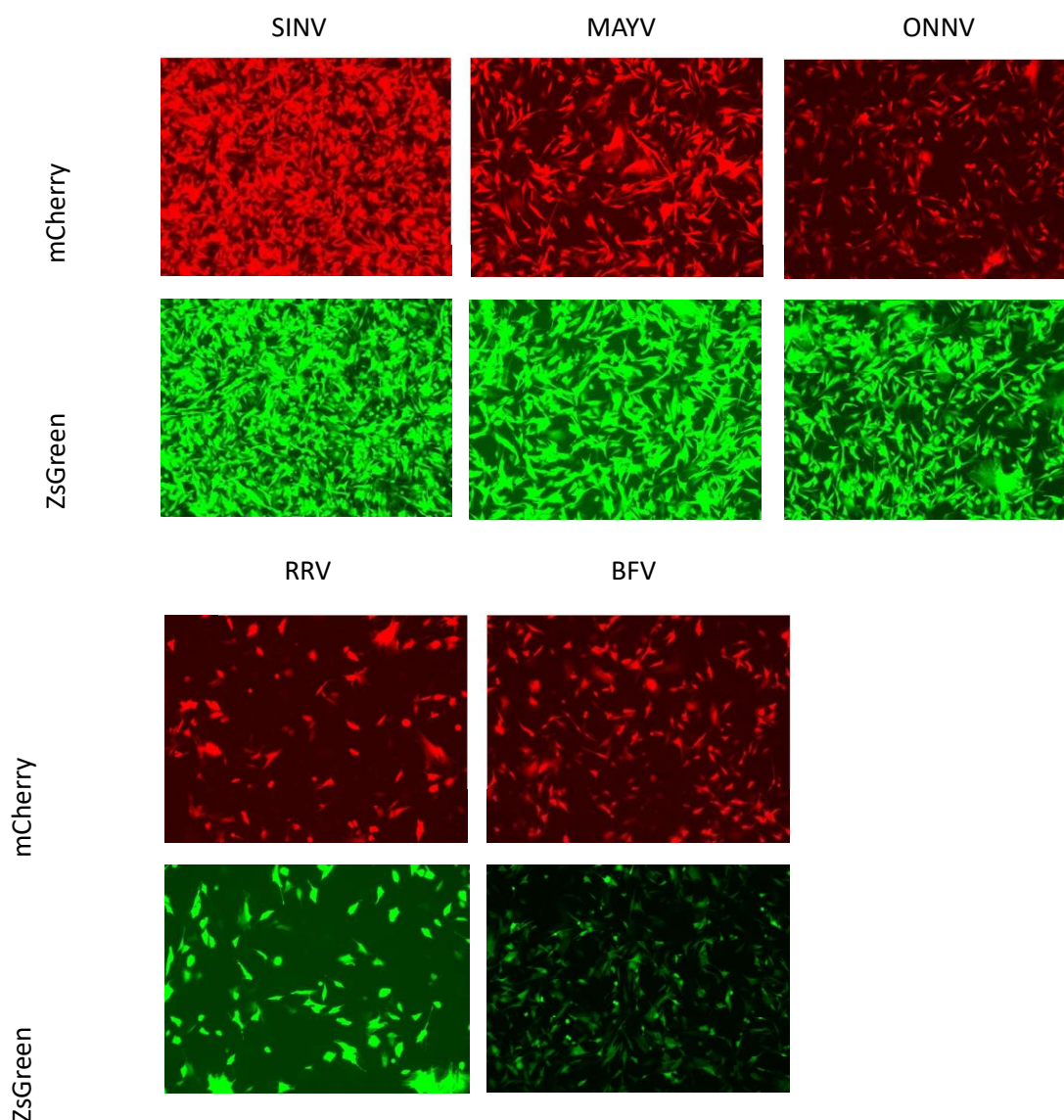


Figure 8. Fluorescence microscopy analysis of reporter expression from alphavirus replicon constructs. Cells were examined by fluorescence microscopy after transfection/production of replicon systems derived from SINV, MAYV, ONNV, RRV, and BFV carrying either mCherry or ZsGreen reporter genes. Red and green fluorescence indicate expression of the corresponding reporter proteins.

To assess the effect of the translational enhancer on the generation of VRPs, helper E constructs without the capsid enhancer element were also generated and tested for ONNV, MAYV, BFV, and RRV. Usually, CHIKV helper E is engineered without a capsid enhancer – this system has been shown to work best in previous experiments in our lab (3). Therefore, it was decided to implement the same logic in the mentioned viruses to determine whether it is possible to boost VRP production. SINV, on the other hand, as a model virus, was used with an enhancer, as this structure had performed best in previous lab research.

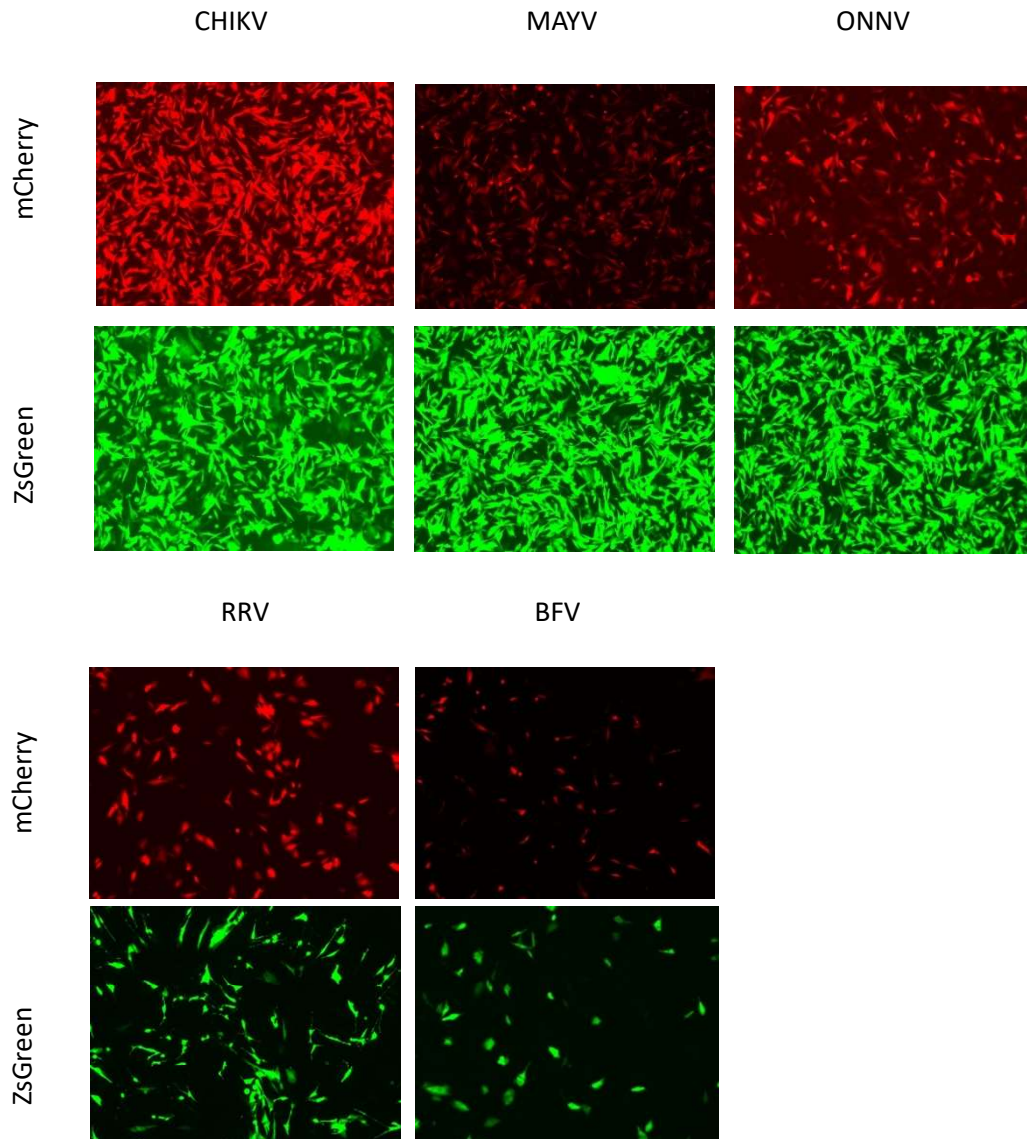


Figure 9. Fluorescence microscopy analysis of reporter expression from alphavirus replicon systems and helpers with no capsid enhancer of the Helper E. Cells were visualised by fluorescence microscopy following the use of replicon constructs derived from CHIKV, MAYV, ONNV, RRV, and BFV carrying either mCherry or ZsGreen reporter genes. Red and green fluorescence indicate expression of the corresponding reporter proteins.

3.2.3 First round infection (titration of VRPs)

To quantify VRP production, titration was performed in BHK-21 cells. For that, BHK-21 cells were infected with VRP stocks. Titration was performed by observing green or red cells using a fluorescent microscope. The fluorescence images were obtained from the same cells used to calculate the titers, providing a direct visual correlation between VRP genome replication in the cells, as assessed by fluorescence intensity, and the generation of infectious particles, assessed by the titration results.

A surprising difference was found between mCherry and ZsGreen constructs of ONNV VRPs with the capsid enhancer. While ONNV-ZsGreen produced high VRP titers, the ONNV mCherry version consistently yielded no detectable titre across all three replicates (Table 2). Notably, the reporter's expression was clearly detectable in the transfected cells for both constructs. Most likely, this is due to experimental error, since ZsGreen has worked and mCherry constructs were functional for every other VRP. In particular, CHIKV mCherry constructs were functional, and since these viruses are very similar, it is safe to assume that ONNV mCherry would have worked if not for experimental error.

Table 2. Average titers of alphavirus VRPs carrying mCherry and ZsGreen reporter sequences. The table summarises the calculated average titers and standard deviations of VRP preparations generated from different alphavirus replicons, including RRV, ONNV, MAYV, BFV, and SINV, carrying either mCherry or ZsGreen reporters. Titers are presented as infectious units (iu) per millilitre, with standard deviation calculated from three replicate measurements.

VRP	Average titer (iu/mL)	Standard deviation
RRV mCherry	$7,76 \times 10^4$	$5,20 \times 10$
RRV ZsGreen	$1,57 \times 10^4$	$2,81 \times 10^3$
ONNV mCherry	0	0
ONNV ZsGreen	$1,68 \times 10^4$	$1,39 \times 10^3$
MAYV mCherry	$6,41 \times 10^5$	$5,18 \times 10^4$
MAYV ZsGreen	$6,51 \times 10^5$	$1,55 \times 10^4$
BFV mCherry	$3,40 \times 10^5$	$3,40 \times 10^2$
BFV ZsGreen	$2,49 \times 10^3$	$2,98 \times 10^2$
SINV mCherry	$2,70 \times 10^6$	$2,19 \times 10^5$
SINV ZsGreen	$3,37 \times 10^6$	$5,35 \times 10^6$

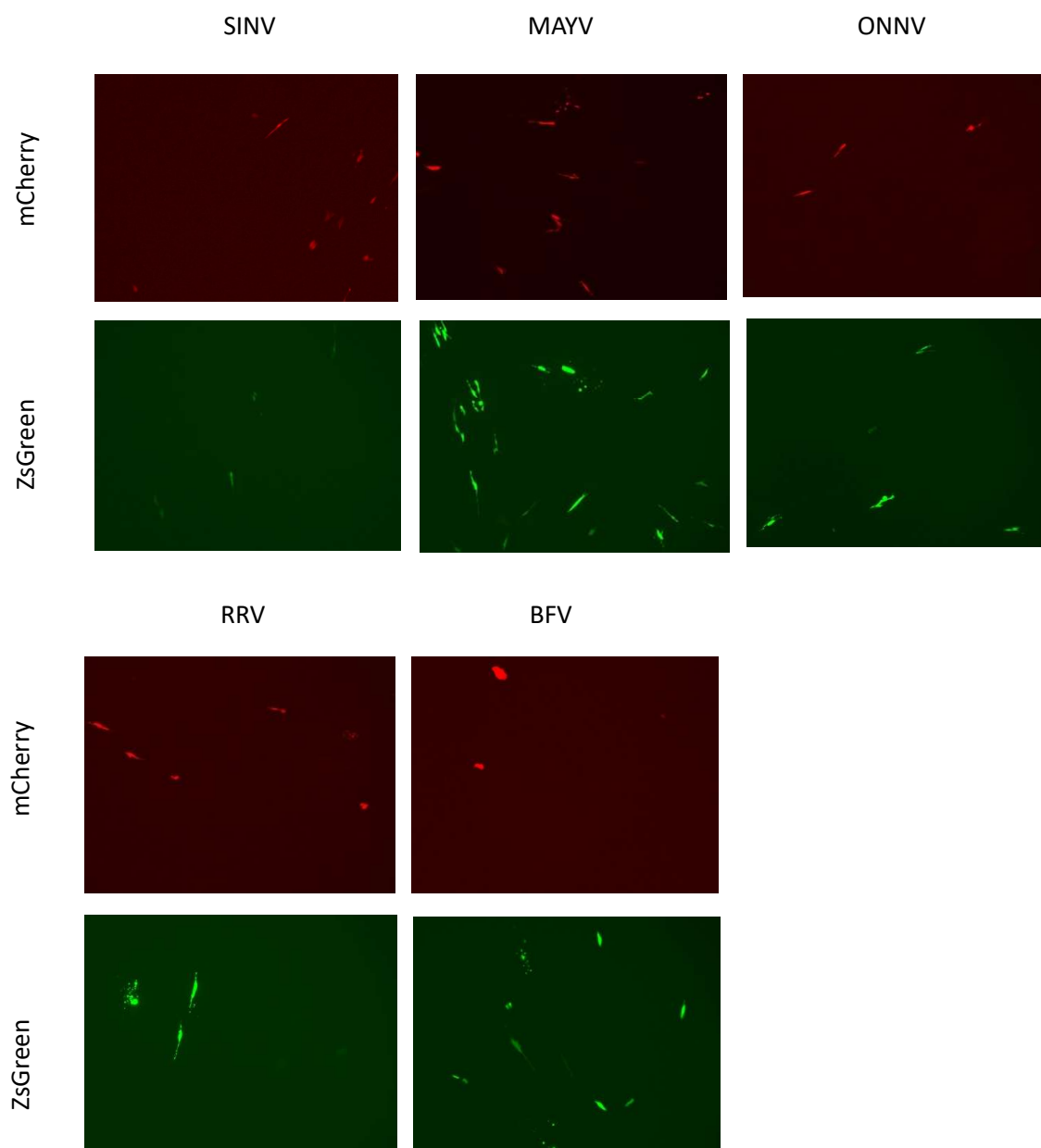


Figure 10. Fluorescence microscopy images from VRP titration assays. Representative wells from titrations of SINV, MAYV, ONNV, RRV, and BFV VRPs carrying either mCherry or ZsGreen reporter genes are shown. Red- or green-fluorescent cells indicate successful delivery and expression of the replicon RNA after VRP-mediated infection. The images were taken from wells corresponding to the same stock dilution used later for titer calculation.

The effect of removing the capsid enhancer was virus-dependent. ONNV, particularly the ZsGreen reporter construct, showed higher titers in the absence of the capsid enhancer in the Helper E (Table 3). This improvement suggests that this virus, which is evolutionarily closer to CHIKV than to model organism SINV, does not naturally require the specialised stalling mechanism of the capsid enhancer sequence (13,33).

MAYV and RRV produced lower titers in the absence of the enhancer, indicating that the DLP is essential for resuming structural protein synthesis during translational shutdown in the host cell

(Table 3). These viruses tend to maintain structural protein synthesis, similar to that of SINV and SFV, rather than that of CHIKV or ONNV.

BFV constructs lacking the enhancer yielded no detectable titers for either reporter, despite clear fluorescence during VRP generation. The failure of BFV constructs to produce a titer indicates that packaging was unsuccessful. Once again, this shows the special nature of the BFV replicase. BFV is the sole representative of its serological complex and is genetically distant from other viruses in this study (8,9). The presence of fluorescent signal from the reporter proteins during VRP generation (transfection of BHK-21 cells) indicates that the BFV replicon was translationally functional and replication-competent, producing the reporter proteins, but probably the translation of the capsid and envelope proteins from the helper RNAs without the enhancer was not enough for the assembly of the VRPs (7). This suggests that the BFV structural proteins may be even more sensitive to the host cell's translational shutoff than MAYV or RRV (5). Consequently, BFV might require a translational enhancer sequence – as does SINV – to support the production and assembly of viral replicon particles.

The results regarding the capsid enhancer highlight the molecular strategies alphaviruses use to regulate translation of their structural proteins. In model viruses SINV and SFV, the expression of structural proteins is heavily dependent on the translational enhancer, which is a stable RNA hairpin or downstream loop (DLP) structure located at the 5' terminal of the capsid coding sequence (3). This element allows these viruses to bypass host-induced translational shutoff by maintaining efficient SG RNA translation, even when eIF2 α is phosphorylated (13). Consequently, without these enhancers, SINV-based systems are often significantly less effective (3). So, while for some viruses removing the capsid-encoding sequence results in lower protein expression and VRP yields, for others it improves yields. This increase in titer means that these viruses, just like CHIKV, do not naturally require this specialised mechanism (3). In these cases, the presence of additional structures may act as a structural impediment, interfering with host ribosomal function and hindering the translation of structural proteins, thereby reducing the number of packaged viral particles (3).

The observed inefficiencies in VRP production for certain backbones, most notably BFV and RRV, suggest that the use of wild-type non-structural sequences may limit overall yield by rapidly inducing cytopathic effects. A potential alternative to the RNA-based transfection used in this study is the application of CMV (human cytomegalovirus)-driven DNA plasmids (5,31). While in vitro transcribed RNA replicons generally provide superior transgene expression due to their immediate self-amplifying nature, a DNA-layered system in which the replicon is placed under a

DNA-dependent RNA polymerase II promoter might have significantly altered the results (1) (12). Such systems can provide a more sustained production phase, producing more structural proteins from the helpers before the cell shuts down (12).

Because this study utilised wild-type replicase sequences, nsP2-mediated shutoff likely narrowed the window for VRP assembly. To optimise these systems in the future, non-cytopathic mutations could be introduced into nsP2. These mutations abolish nsP2's ability to degrade the cellular transcription machinery, thereby reducing toxicity and enabling persistent replication in mammalian cells (1,5). By extending the lifespan of the producer cells, such mutations could provide the necessary time for structural proteins to reach the critical threshold required for efficient packaging of genetically distant or sensitive viruses like BFV (5).

In conclusion, these results highlight differences in alphavirus systems for VRP production. The fact that ONNV thrives in the absence of a translational enhancer, whereas MAYV and RRV are considerably less functional and BFV fails without it, suggests a major difference in how these viruses interact with the mammalian host cell translation machinery (ribosomes). This again shows that regulatory elements optimized for well-studied model alphaviruses cannot be automatically assumed to work the same way across all alphaviruses, especially in less-studied, more genetically distant viruses such as BFV.

Table 3. Average titers of alphavirus VRPs produced using Helper E constructs lacking the capsid enhancer.

The table shows the calculated average titers and standard deviations of VRPs generated from different alphavirus replicon systems carrying either mCherry or ZsGreen reporters. In these constructs, the corresponding Helper E RNA lacks the capsid enhancer element. CHIKV is included as a positive/control system because it efficiently produces VRPs even in the absence of the enhancer. The results allow comparison of VRP production efficiency between alphavirus backbones in the absence of the capsid enhancer, with variation observed between both virus systems and reporter constructs. Blank entries indicate samples for which titers were not determined or could not be calculated.

VRP	Average titer (iu/mL)	Standard deviation
CHIKV mCherry	$8,86 \times 10^6$	$8,73 \times 10^5$
CHIKV ZsGreen	$3,71 \times 10^7$	$3,40 \times 10^6$
RRV mCherry no enhancer	$1,36 \times 10^3$	$9,00 \times 10^2$
RRV ZsGreen no enhancer	$7,08 \times 10^2$	$1,77 \times 10^2$
ONNV mCherry no enhancer	$2,21 \times 10^4$	$4,59 \times 10^3$
ONNV ZsGreen no enhancer	$3,45 \times 10^5$	$7,12 \times 10^4$
MAYV mCherry no enhancer	$3,28 \times 10^4$	$3,54 \times 10^3$
MAYV ZsGreen no enhancer	$1,42 \times 10^5$	$9,81 \times 10^3$
BFV mCherry no enhancer	0	0
BFV ZsGreen no enhancer	0	0

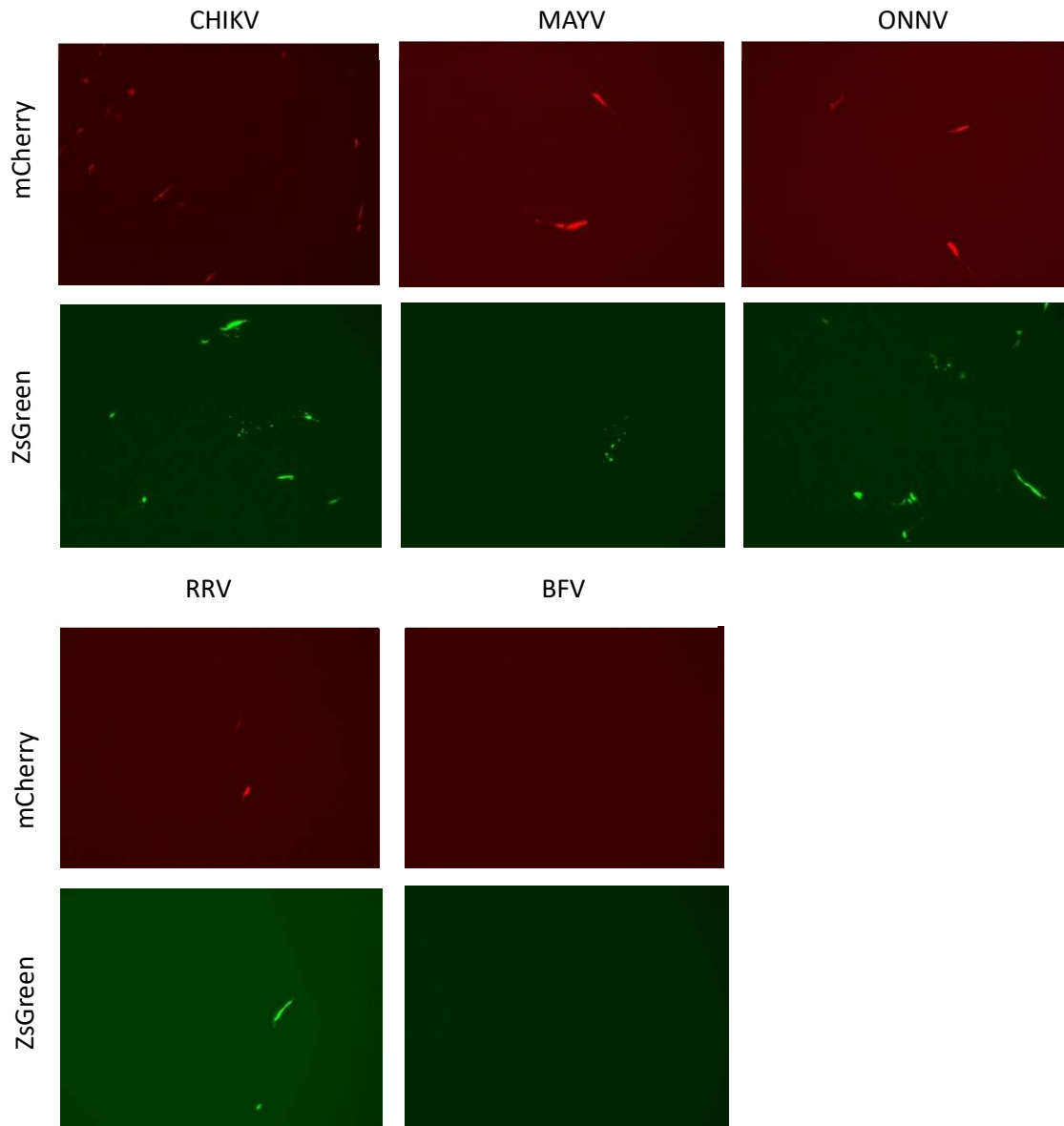


Figure 11. Fluorescence microscopy images from VRP titration assays created with no capsid enhancer in the Helper E construct. Representative wells from titrations of CHIKV, MAYV, ONNV, RRV, and BFV VRPs carrying either mCherry or ZsGreen reporter genes are shown. Fluorescent cells indicate successful delivery of the replicon RNA and subsequent reporter expression after VRP-mediated infection. The images were taken from wells containing the same stock dilution used later for titer calculation, allowing for qualitative and quantitative evaluation.

3.2.4 Second round of infection

To confirm that we have not generated a fully functional virus but VRPs that are only single-cycle, a second round of infection was performed. Because no packaging occurs after the first infection, the P1 (first passage) stock used to infect new cells should not contain any infectious particles; hence, no reporter protein should be expressed in the newly infected cells.

As shown in Figure 12, which has representative images for the whole panel of viruses, the rest of which can be found in the appendix section, cells infected during P1 exhibited robust reporter protein expression, confirming that the VRPs successfully delivered the replicon genome and initiated RNA replication and subgenomic transcription of the reporter proteins (3). For the second round (P2), the cell culture supernatant from P1-infected cells was collected and used to inoculate a fresh monolayer of BHK-21 cells. In this case, as evident from Figure 11, no detectable fluorescence was observed in any of the replicates across the VRP panel. This result was consistent with the VRPs generated with Helper E, both those containing the capsid enhancer and those lacking it. The absence of reporter expression in the P2 passage confirms that no new infectious particles were produced after the initial infection (4,12).

The primary objective of using an alphavirus replicon system is to exploit its high levels of transient gene expression while ensuring biological safety, given that VRPs cannot spread to adjacent cells or produce new progeny (6). The success of the passaging experiment demonstrates that the structural proteins required to package the replicon were present only during the initial production phase, when they were introduced in trans by Helper RNAs and were not encoded within the replicon genome (30).

VRPs that showed unexpected results were BFV and RRV (Appendix A5, A6). In BFV samples, only a minimal fluorescent signal was observed after passaging. In the condition with the capsid enhancer, a few isolated weak ZsGreen- and mCherry-positive signals were detected, whereas without the capsid enhancer, fluorescence was essentially absent. These results are consistent with titration results showing that BFV titers were very low with the enhancer and non-existent without it. The presence of the reporter protein during VRP generation indicates functional replicase production; however, the failure to generate detectable infectious titers, especially in the absence of a capsid enhancer, indicates a significant deficiency at the assembly or packaging stage. One reason could be that BFV is genetically distant from all other viruses in this study and, as a result, may be more sensitive to host-cell translational shutoff (9). As a result, the host's defense mechanism likely inhibited the production of capsid and envelope protein, making packaging impossible. To address this issue, the next logical step would be to transition from the split-helper system to a single helper system (Helper 1) (3). While the current split helper strategy offers high biosafety, it appears inefficient. The primary limitation in this regard is that the split helper system requires the simultaneous delivery and successful replication of three independent RNA species (the replicon, Helper E, and Helper C) within a single producer cell (3). Thus, the statistical probability of successful co-expression is smaller than that of a two-component system. Therefore,

adopting a single helper system may improve overall packaging and integration efficiency while still maintaining an acceptable level of biosafety.

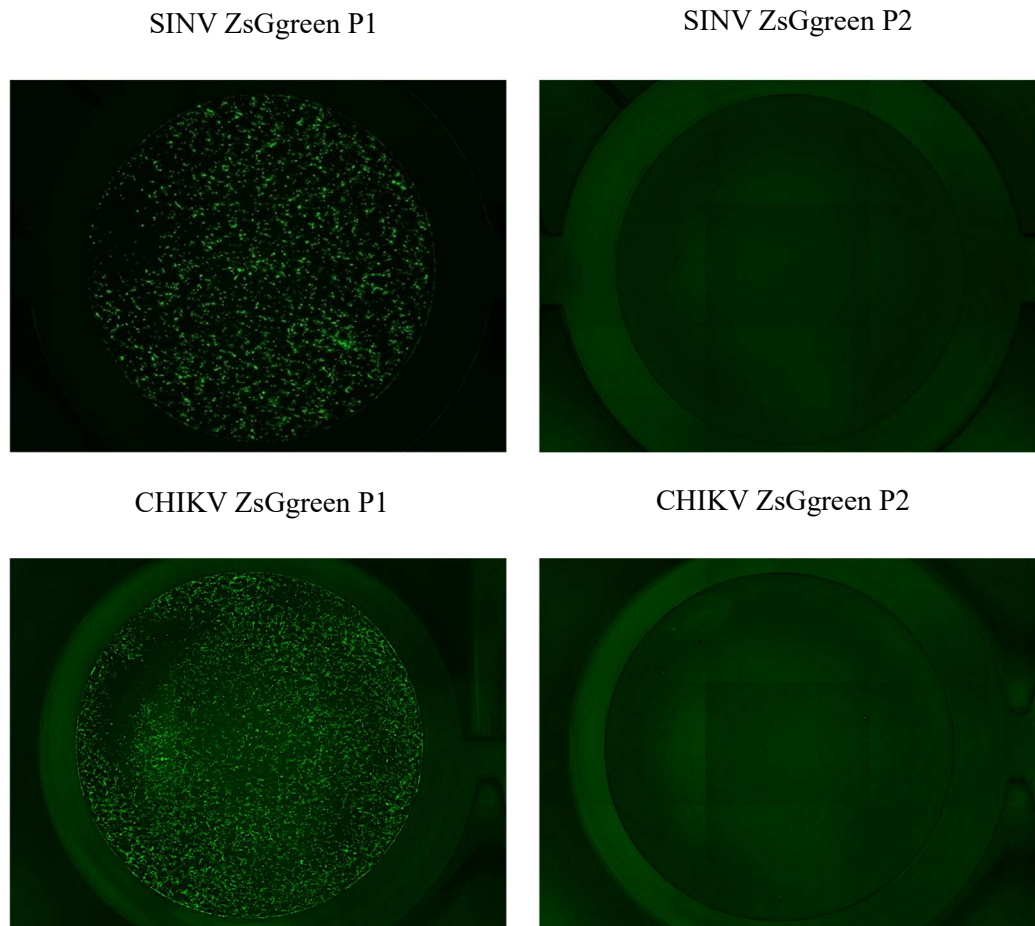


Figure 12. Assessment of single-cycle infectivity of CHIKV and SINV ZsGreen VRPs by passaging. Cells infected with the original VRP stock (P1) showed reporter expression, whereas cells infected with material collected from the P1 infection (P2) showed no detectable fluorescence. Since VRPs are designed to be propagation-defective, the absence of reporter expression in P2 is consistent with the lack of secondary infectious particle production. The shown example is representative of the passaging results obtained for the VRP panel. CHIKV ZsGreen is shown as a representative construct for VRPs produced without the capsid enhancer, while SINV ZsGreen is shown as a representative construct for VRPs produced with the enhancer.

Summary

This study successfully developed and evaluated VRP production systems for a panel of arboviral alphaviruses, providing insights into the molecular requirements for packaging understudied alphaviruses. By comparing viruses such as ONNV and BFV with established models such as SINV, this research underscores the significant biological diversity within the genus and challenges the assumption that regulatory tools are universally interchangeable.

A central finding of this thesis is the virus-dependent role of the capsid enhancer (downstream loop, or DLP). In model viruses like SINV, this element is essential for maintaining the translation of structural proteins when the host cell attempts to shut down protein synthesis via eIF2 α phosphorylation (1). While for some viruses, like BFV, MAYV, and RRV, this structure proved extremely important for VRP generation, similarly to the model virus SINV, for others, like ONNV it proved an impediment, lowering VRP production efficiency.

The split-helper system employed in this study proved to be a robust tool for maintaining high biosafety standards. The second infection run (P2 passage) confirmed the strictly single-cycle nature of the generated VRPs (1). The lack of secondary infection across the virus panel demonstrates that the packaging signal remained restricted to the replicon genome and that no replication-competent viruses (RVPs) were generated through homologous recombination (1). This validation confirms that these systems are safe for downstream applications in a lower-containment (BSL-2) setting.

In conclusion, the varying success of VRP production across the panel demonstrates that the rules applied to model organisms like SINV do not apply genus-wide. The high productivity of ONNV without the enhancer element suggests it is well-adapted to the mammalian translation machinery, while BFV's failure highlights its unique and potentially more rigid molecular requirements.

To overcome the packaging inefficiencies observed in BFV and RRV, the next logical step is to reduce the system to a two-component co-transfection. The probability of producing sufficient structural proteins to package the replicon RNA increases significantly (1). While this approach carries a higher risk of generating RVPs, it may be necessary to initially establish a viable packaging protocol for these genetically distant, under-researched viruses (1). Ultimately, this study provides a foundational framework for tailoring alphavirus vectors to their specific molecular phenotypes, advancing their utility in vaccine and antiviral research.

References

1. Ahola T, McInerney G, Merits A. Alphavirus RNA replication in vertebrate cells. *Adv Virus Res.* 2021;111:111–56. doi:10.1016/bs.aivir.2021.07.003 PubMed PMID: 34663497.
2. Volk SM, Chen R, Tsetsarkin KA, Adams AP, Garcia TI, Sall AA, et al. Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. *J Virol.* 2010 Jul;84(13):6497–504. doi:10.1128/JVI.01603-09 PubMed PMID: 20410280; PubMed Central PMCID: PMC2903258.
3. Gläsker S, Lulla A, Lulla V, Couderc T, Drexler JF, Liljeström P, et al. Virus replicon particle based Chikungunya virus neutralization assay using Gaussia luciferase as readout. *Virol J.* 2013 Jul 15;10:235. doi:10.1186/1743-422X-10-235 PubMed PMID: 23855906; PubMed Central PMCID: PMC3718613.
4. Lin HC, Chiao DJ, Shu PY, Lin HT, Hsiung CC, Lin CC, et al. Development of a Novel Chikungunya Virus-Like Replicon Particle for Rapid Quantification and Screening of Neutralizing Antibodies and Antivirals. *Microbiol Spectr.* 2023 Apr 13;11(2):e0485422. doi:10.1128/spectrum.04854-22 PubMed PMID: 36856407; PubMed Central PMCID: PMC10101068.
5. Dominguez F, Palchevska O, Frolova EI, Frolov I. Alphavirus-based replicons demonstrate different interactions with host cells and can be optimized to increase protein expression. *J Virol.* 2023 Nov 30;97(11):e0122523. doi:10.1128/jvi.01225-23 PubMed PMID: 37877718; PubMed Central PMCID: PMC10688356.
6. Lundstrom K. Alphavirus vectors for gene therapy applications. *Curr Gene Ther.* 2001 May;1(1):19–29. doi:10.2174/1566523013349039 PubMed PMID: 12109136.
7. Petrakova O, Volkova E, Gorchakov R, Paessler S, Kinney RM, Frolov I. Noncytopathic replication of Venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in Mammalian cells. *J Virol.* 2005 Jun;79(12):7597–608. doi:10.1128/JVI.79.12.7597-7608.2005 PubMed PMID: 15919912; PubMed Central PMCID: PMC1143662.
8. Powers AM, Brault AC, Shirako Y, Strauss EG, Kang W, Strauss JH, et al. Evolutionary relationships and systematics of the alphaviruses. *J Virol.* 2001 Nov;75(21):10118–31. doi:10.1128/JVI.75.21.10118-10131.2001 PubMed PMID: 11581380; PubMed Central PMCID: PMC114586.
9. Omler A, Mutso M, Vaher M, Freitas JR, Taylor A, David CT, et al. Exploring Barmah Forest virus pathogenesis: molecular tools to investigate non-structural protein 3 nuclear localization and viral genomic determinants of replication. *mBio.* 2024 Aug 14;15(8):e0099324. doi:10.1128/mbio.00993-24 PubMed PMID: 38953633; PubMed Central PMCID: PMC11323547.
10. Strauss JH, Strauss EG. The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev.* 1994 Sep;58(3):491–562. doi:10.1128/mr.58.3.491-562.1994 PubMed PMID: 7968923; PubMed Central PMCID: PMC372977.
11. Kamrud KI, Alterson K, Custer M, Dudek J, Goodman C, Owens G, et al. Development and characterization of promoterless helper RNAs for the production of alphavirus replicon

- particle. *J Gen Virol.* 2010 Jul;91(Pt 7):1723–7. doi:10.1099/vir.0.020081-0 PubMed PMID: 20181749; PubMed Central PMCID: PMC2888770.
12. Zimmer G. RNA replicons - a new approach for influenza virus immunoprophylaxis. *Viruses.* 2010 Feb;2(2):413–34. doi:10.3390/v2020413 PubMed PMID: 21994644; PubMed Central PMCID: PMC3185613.
 13. Rupp JC, Sokoloski KJ, Gebhart NN, Hardy RW. Alphavirus RNA synthesis and non-structural protein functions. *J Gen Virol.* 2015 Sep;96(9):2483–500. doi:10.1099/jgv.0.000249 PubMed PMID: 26219641; PubMed Central PMCID: PMC4635493.
 14. Long S, Guzyk M, Perez Vidakovics L, Han X, Sun R, Wang M, et al. SARS-CoV-2 N protein recruits G3BP to double membrane vesicles to promote translation of viral mRNAs. *Nat Commun.* 2024 Dec 5;15(1):10607. doi:10.1038/s41467-024-54996-3 PubMed PMID: 39638802; PubMed Central PMCID: PMC11621422.
 15. Skidmore AM, Bradfute SB. The life cycle of the alphaviruses: From an antiviral perspective. *Antiviral Res.* 2023 Jan;209:105476. doi:10.1016/j.antiviral.2022.105476 PubMed PMID: 36436722; PubMed Central PMCID: PMC9840710.
 16. Tong Jia Ming S, Tan Yi Jun K, Carissimo G. Pathogenicity and virulence of O'nyong-nyong virus: A less studied Togaviridae with pandemic potential. *Virulence.* 2024 Dec;15(1):2355201. doi:10.1080/21505594.2024.2355201 PubMed PMID: 38797948; PubMed Central PMCID: PMC11135837.
 17. Sokoloski KJ, Karki D, Isom CM, Moni S. The pathogenicity and virulence of Sindbis virus. *Virulence.* 2026 Dec;17(1):2609389. doi:10.1080/21505594.2025.2609389 PubMed PMID: 41442410; PubMed Central PMCID: PMC12758272.
 18. Mousavi SA, Malerød L, Berg T, Kjekken R. Clathrin-dependent endocytosis. *Biochem J.* 2004 Jan 1;377(Pt 1):1–16. doi:10.1042/BJ20031000 PubMed PMID: 14505490; PubMed Central PMCID: PMC1223844.
 19. Tan YB, Chmielewski D, Law MCY, Zhang K, He Y, Chen M, et al. Molecular architecture of the Chikungunya virus replication complex. *Sci Adv.* 2022 Dec 2;8(48):eadd2536. doi:10.1126/sciadv.add2536 PubMed PMID: 36449616; PubMed Central PMCID: PMC9710867.
 20. Hawman DW, Fox JM, Ashbrook AW, May NA, Schroeder KMS, Torres RM, et al. Pathogenic Chikungunya Virus Evades B Cell Responses to Establish Persistence. *Cell Rep.* 2016 Aug 2;16(5):1326–38. doi:10.1016/j.celrep.2016.06.076 PubMed PMID: 27452455; PubMed Central PMCID: PMC5003573.
 21. Bettis AA, L'Azou Jackson M, Yoon IK, Breugelmans JG, Goios A, Gubler DJ, et al. The global epidemiology of chikungunya from 1999 to 2020: A systematic literature review to inform the development and introduction of vaccines. *PLoS Negl Trop Dis.* 2022 Jan;16(1):e0010069. doi:10.1371/journal.pntd.0010069 PubMed PMID: 35020717; PubMed Central PMCID: PMC8789145.
 22. Tan YB, Law MCY, Luo D. Targeting the alphavirus virus replication process for antiviral development. *Antiviral Res.* 2023 Feb;210:105494. doi:10.1016/j.antiviral.2022.105494 PubMed PMID: 36574906.

23. Richardson JS, Anderson DM, Mendy J, Tindale LC, Muhammad S, Loreth T, et al. Chikungunya virus virus-like particle vaccine safety and immunogenicity in adolescents and adults in the USA: a phase 3, randomised, double-blind, placebo-controlled trial. *Lancet*. 2025 Apr 19;405(10487):1343–52. doi:10.1016/S0140-6736(25)00345-9 PubMed PMID: 40158526.
24. Marinho MDS, Ferreira GM, Grosche VR, Nicolau-Junior N, Campos T de L, Santos IA, et al. Evolutionary Profile of Mayaro Virus in the Americas: An Update into Genome Variability. *Viruses*. 2024 May 20;16(5):809. doi:10.3390/v16050809 PubMed PMID: 38793690; PubMed Central PMCID: PMC11126029.
25. Caicedo EY, Charniga K, Rueda A, Dorigatti I, Mendez Y, Hamlet A, et al. The epidemiology of Mayaro virus in the Americas: A systematic review and key parameter estimates for outbreak modelling. *PLoS Negl Trop Dis*. 2021 Jun;15(6):e0009418. doi:10.1371/journal.pntd.0009418 PubMed PMID: 34081717; PubMed Central PMCID: PMC8205173.
26. Morrison TE, Whitmore AC, Shabman RS, Lidbury BA, Mahalingam S, Heise MT. Characterization of Ross River virus tropism and virus-induced inflammation in a mouse model of viral arthritis and myositis. *J Virol*. 2006 Jan;80(2):737–49. doi:10.1128/JVI.80.2.737-749.2006 PubMed PMID: 16378976; PubMed Central PMCID: PMC1346871.
27. Harley D, Sleigh A, Ritchie S. Ross River virus transmission, infection, and disease: a cross-disciplinary review. *Clin Microbiol Rev*. 2001 Oct;14(4):909–32, table of contents. doi:10.1128/CMR.14.4.909-932.2001 PubMed PMID: 11585790; PubMed Central PMCID: PMC89008.
28. Tsetsarkin KA, Chen R, Sherman MB, Weaver SC. Chikungunya virus: evolution and genetic determinants of emergence. *Curr Opin Virol*. 2011 Oct;1(4):310–7. doi:10.1016/j.coviro.2011.07.004 PubMed PMID: 21966353; PubMed Central PMCID: PMC3182774.
29. Vanlandingham DL, Tsetsarkin K, Hong C, Klingler K, McElroy KL, Lehane MJ, et al. Development and characterization of a double subgenomic chikungunya virus infectious clone to express heterologous genes in *Aedes aegypti* mosquitoes. *Insect Biochem Mol Biol*. 2005 Oct;35(10):1162–70. doi:10.1016/j.ibmb.2005.05.008 PubMed PMID: 16102421.
30. Lundstrom K. Alphavirus-based vaccines. *Viruses*. 2014 Jun 16;6(6):2392–415. doi:10.3390/v6062392 PubMed PMID: 24937089; PubMed Central PMCID: PMC4074933.
31. Lundstrom K. Plasmid DNA-based Alphavirus Vaccines. *Vaccines*. 2019 Mar 8;7(1):29. doi:10.3390/vaccines7010029 PubMed PMID: 30857255; PubMed Central PMCID: PMC6466081.
32. Perri S, Greer CE, Thudium K, Doe B, Legg H, Liu H, et al. An alphavirus replicon particle chimera derived from venezuelan equine encephalitis and sindbis viruses is a potent gene-based vaccine delivery vector. *J Virol*. 2003 Oct;77(19):10394–403. doi:10.1128/jvi.77.19.10394-10403.2003 PubMed PMID: 12970424; PubMed Central PMCID: PMC228391.
33. Ventoso I, Berlanga JJ, Toribio R, Díaz-López I. Translational Control of Alphavirus-Host Interactions: Implications in Viral Evolution, Tropism and Antiviral Response. *Viruses*. 2024

Jan 30;16(2):205. doi:10.3390/v16020205 PubMed PMID: 38399981; PubMed Central PMCID: PMC10893052.

34. Bredenbeek PJ, Frolov I, Rice CM, Schlesinger S. Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *J Virol.* 1993 Nov;67(11):6439–46. doi:10.1128/JVI.67.11.6439-6446.1993 PubMed PMID: 8411346; PubMed Central PMCID: PMC238079.
35. Polo JM, Belli BA, Driver DA, Frolov I, Sherrill S, Hariharan MJ, et al. Stable alphavirus packaging cell lines for Sindbis virus and Semliki Forest virus-derived vectors. *Proc Natl Acad Sci U S A.* 1999 Apr 13;96(8):4598–603. doi:10.1073/pnas.96.8.4598 PubMed PMID: 10200308; PubMed Central PMCID: PMC16378.

Appendix

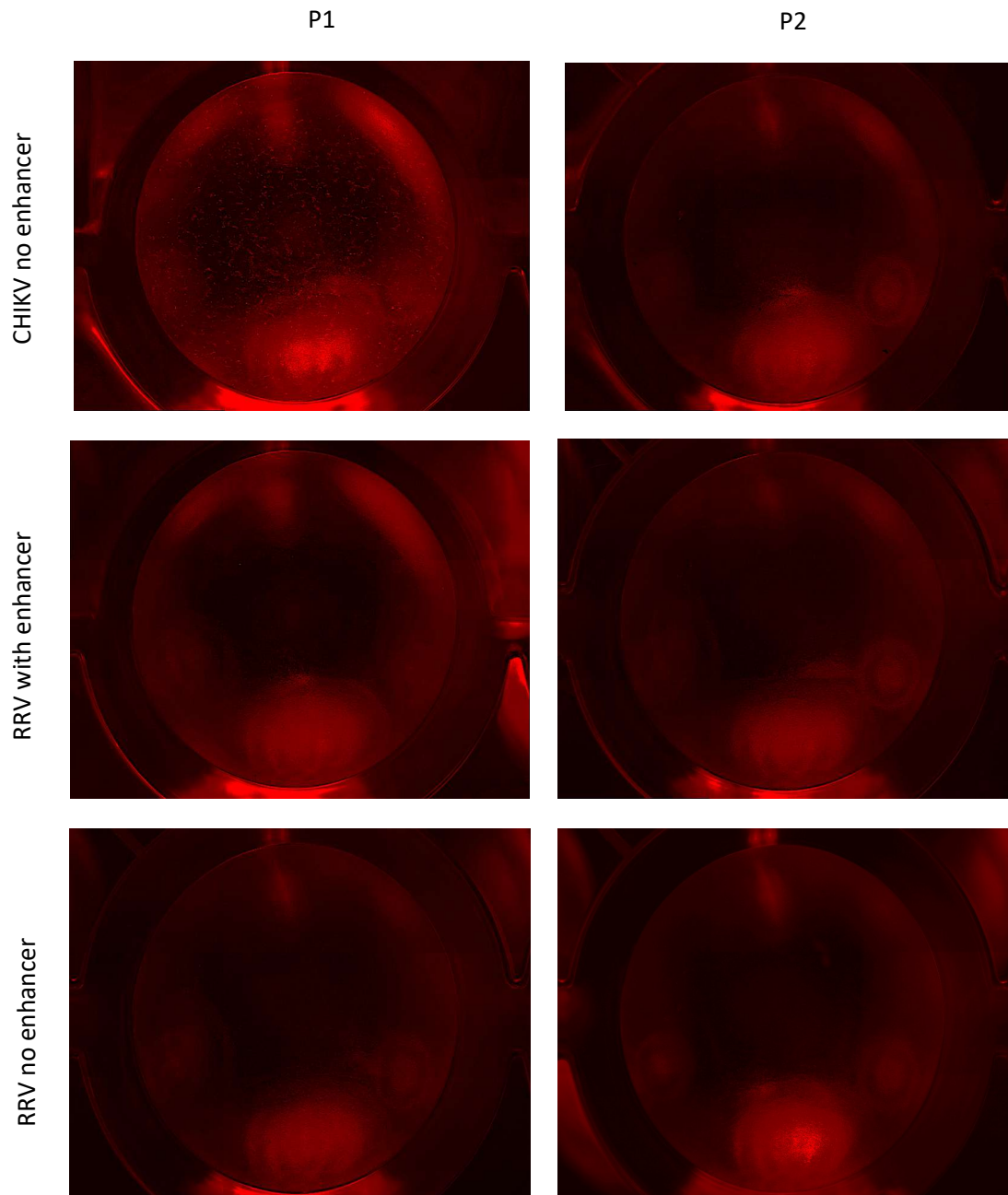


Figure A1. Fluorescence microscopy analysis of cells infected with CHIKV and RRV mCherry P1 and P2 VRP stocks. Representative fluorescence images are shown for CHIKV without capsid enhancer, RRV with capsid enhancer, and RRV without capsid enhancer. The left column shows cells infected with the first collected VRP stock (P1), while the right column shows cells infected with the passaged stock (P2). CHIKV without enhancer shows visible red fluorescence in P1, indicating successful reporter delivery by the produced VRPs. In contrast, both RRV samples, with and without the capsid enhancer, show little to no detectable fluorescence in P1, suggesting inefficient or unsuccessful VRP production under these conditions. No clear fluorescence is observed in P2 for any construct, which is consistent with the single-cycle nature of VRPs and indicates that infectious progeny were not produced after passage. Each image shows a single well from a 96-well plate.

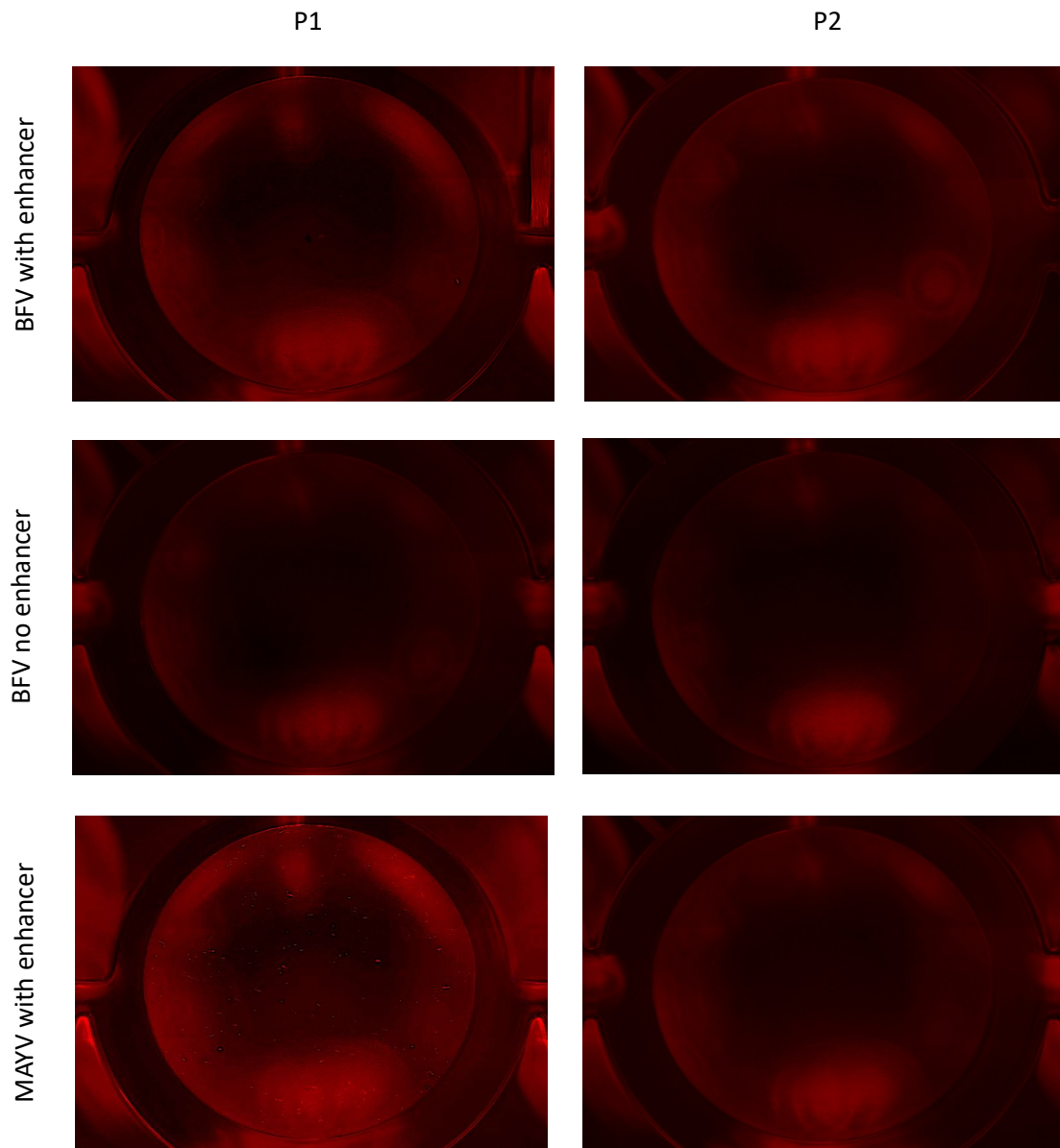


Figure A2. Fluorescence microscopy analysis of cells infected with BFV and MAYV mCherry P1 and P2 VRP stocks. Representative fluorescence images are shown for BFV with capsid enhancer, BFV without capsid enhancer, and MAYV with capsid enhancer. The left column shows cells infected with the first collected VRP stock (P1), while the right column shows cells infected with the passaged stock (P2). Both BFV constructs, with and without the capsid enhancer, show little to no clear red fluorescence in P1, suggesting inefficient or unsuccessful VRP production under these conditions. MAYV with enhancer shows a weak detectable signal in P1 compared with the BFV samples, indicating possible low-level reporter delivery, although the signal remains limited. In P2, no clear fluorescence is observed for any construct, which is consistent with the single-cycle nature of VRPs and indicates that no infectious progeny were generated after passage. Each image shows a single well from a 96-well plate.

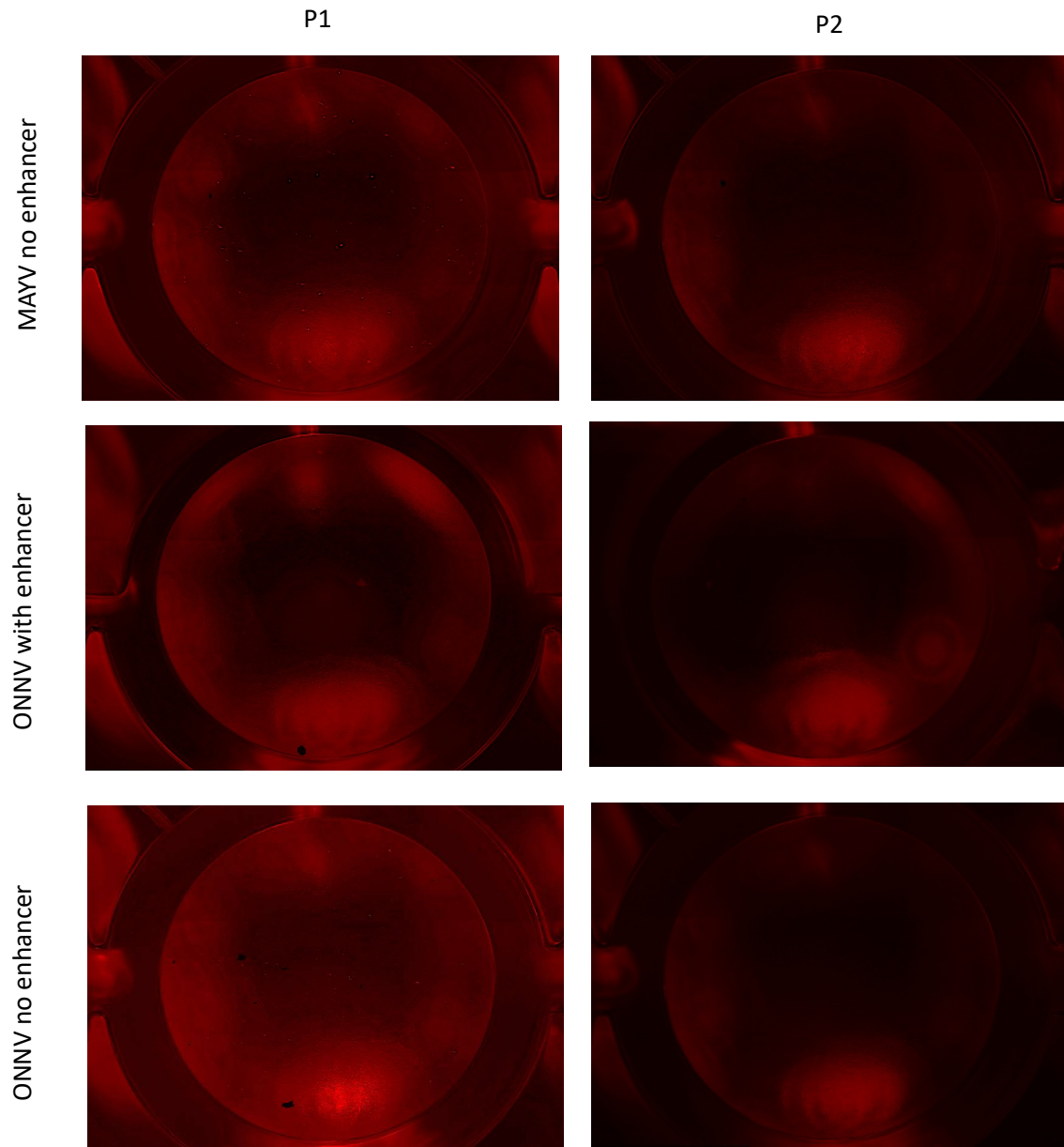


Figure A3. Fluorescence microscopy analysis of cells infected with MAYV and ONNV mCherry P1 and P2 VRP stocks. Representative fluorescence images are shown for MAYV without capsid enhancer, ONNV with capsid enhancer, and ONNV without capsid enhancer. The left column shows cells infected with the first collected stock (P1), while the right column shows cells infected with the passaged stock (P2). MAYV without enhancer shows only weak diffuse red signal in P1, without a clear population of reporter-positive cells, and no clear signal in P2. ONNV with enhancer shows little to no detectable fluorescence in either P1 or P2. ONNV without enhancer shows at most very weak signal in P1, but no clear fluorescence in P2. Overall, these results suggest inefficient or unsuccessful VRP production for these constructs under the tested conditions, while the lack of signal in P2 is consistent with the expected single-cycle nature of VRPs. Each image shows a single well from a 96-well plate.

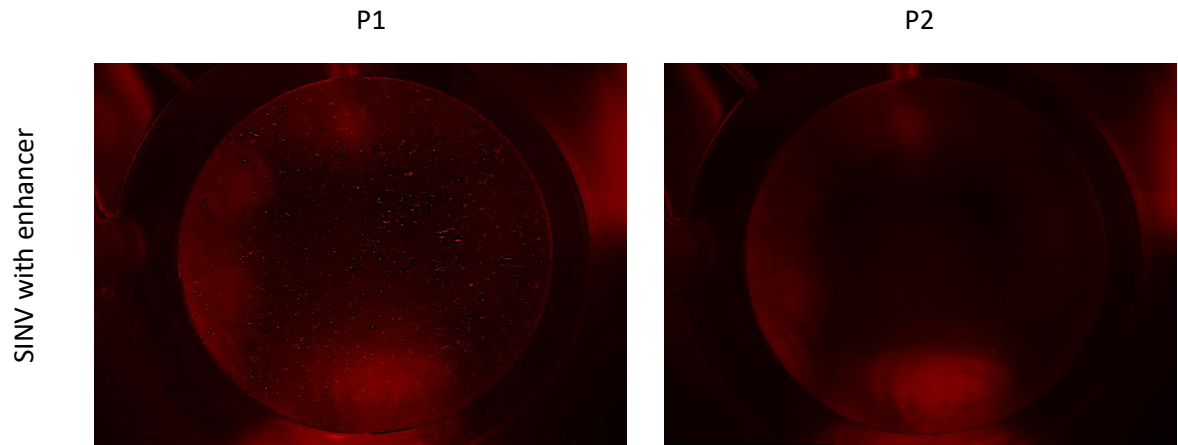


Figure A4. Fluorescence microscopy analysis of cells infected with SINV mCherry P1 and P2 VRP stocks. Representative fluorescence images of SINV with the capsid enhancer are shown. The left image shows cells infected with the first collected stock (P1), while the right image shows cells infected with the passaged stock (P2). In P1, red fluorescence is visible and multiple reporter-positive cells are observed, indicating successful delivery of the replicon and efficient VRP production. In contrast, P2 shows no clear reporter-positive cell population, only a background-level signal. This is consistent with the expected single-cycle nature of VRPs, in which particles can infect cells once but do not produce infectious progeny after passage. Each image shows a single well from a 96-well plate.

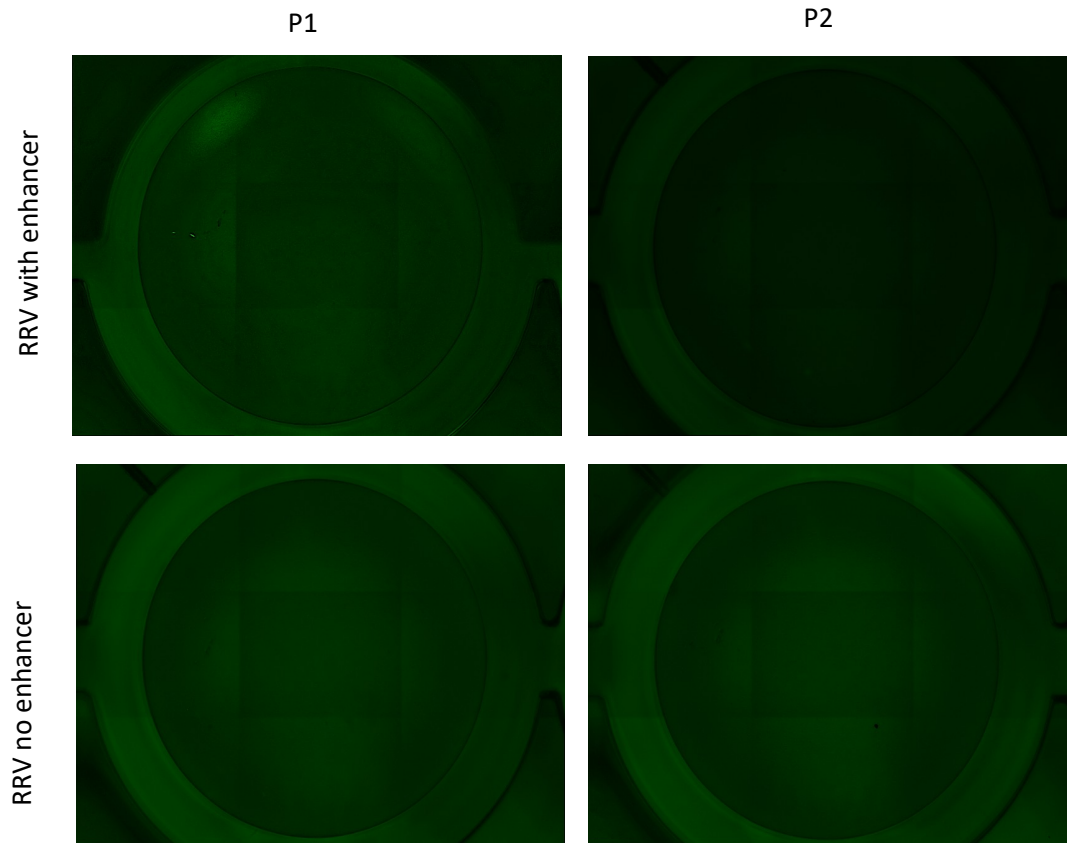


Figure A5. Fluorescence microscopy analysis of cells infected with RRV Zsgreen P1 and P2 VRP stocks. Representative green fluorescence images are shown for RRV with capsid enhancer and RRV without capsid enhancer. The left column shows cells infected with the first collected stock (P1), while the right column shows cells infected with the passaged stock (P2). Both RRV with and without enhancer show no clear population of reporter-positive cells in P1, with only weak, diffuse/background fluorescence visible. This suggests that RRV VRP production was inefficient or unsuccessful under the tested conditions, regardless of the presence of the capsid enhancer. No clear reporter signal is detected in P2 either, which is consistent with the absence of productive infectious progeny after passage. Each image shows a single well from a 96-well plate.

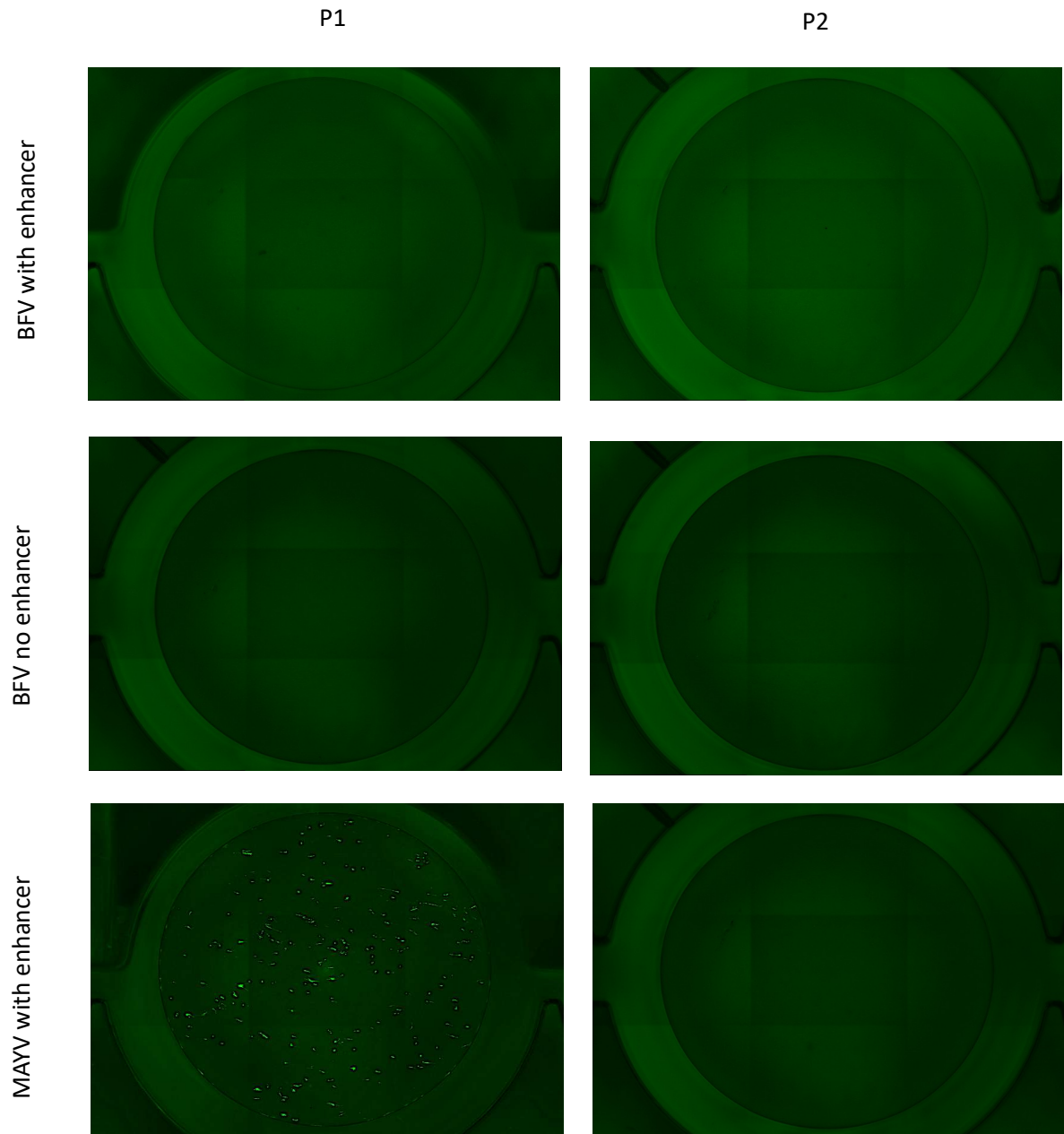


Figure A6. Fluorescence microscopy analysis of cells infected with BFV and MAYV Zsgreen P1 and P2 VRP stocks. Representative green fluorescence images are shown for BFV with capsid enhancer, BFV without capsid enhancer, and MAYV with capsid enhancer. The left column shows cells infected with the first collected stock (P1), while the right column shows cells infected with the passaged stock (P2). Both BFV samples show little to no detectable reporter-positive cells in P1, regardless of whether the capsid enhancer was present, suggesting inefficient or unsuccessful BFV VRP production under these conditions. In contrast, MAYV with an enhancer shows a clear population of green fluorescent cells in P1, indicating successful replicon delivery by the produced VRPs. No clear reporter-positive cell population is observed in P2 for any construct, consistent with the single-cycle nature of VRPs and suggesting that infectious progeny were not produced after passage. Each image shows a single well from a 96-well plate.

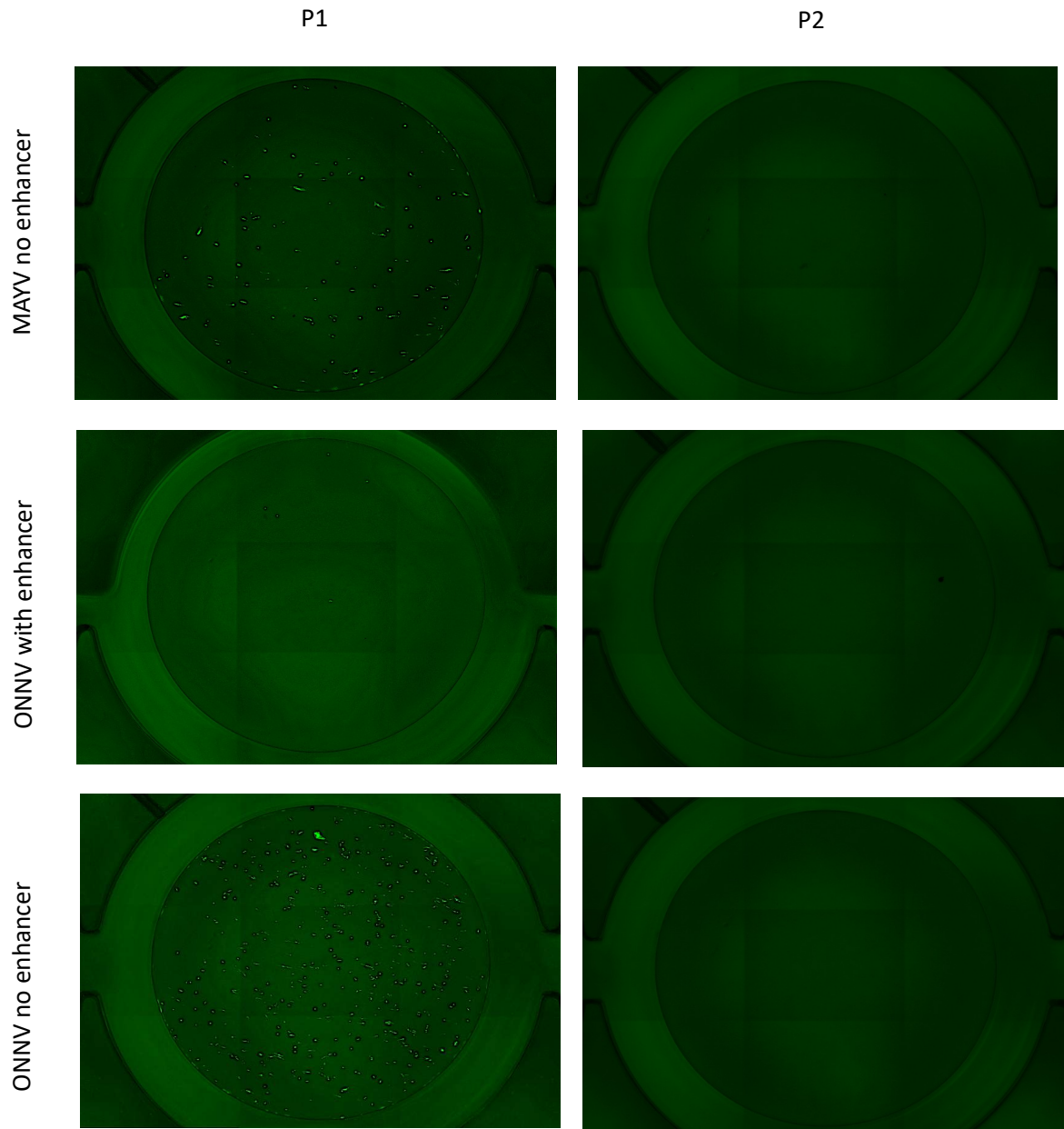


Figure A7. Fluorescence microscopy analysis of cells infected with MAYV and ONNV ZsGreen P1 and P2 VRP stocks. Representative green fluorescence images are shown for MAYV without capsid enhancer, ONNV with capsid enhancer, and ONNV without capsid enhancer. The left column shows cells infected with the first collected stock (P1), while the right column shows cells infected with the passaged stock (P2). MAYV without enhancer shows a visible population of green reporter-positive cells in P1, indicating successful replicon delivery by the produced VRPs. ONNV with enhancer shows little to no clear reporter-positive signal in P1, suggesting inefficient VRP production under these conditions. In contrast, ONNV without an enhancer shows a clear green fluorescent cell population in P1, indicating detectable VRP production and reporter delivery. No clear reporter-positive cells are observed in P2 for any construct, consistent with the single-cycle nature of VRPs and suggesting that infectious progeny were not produced after passage. Each image shows a single well from a 96-well plate.

Non-exclusive license to reproduce thesis and make thesis public

I, Natavan Aliyeva,

1. grant the University of Tartu a free permit (non-exclusive licence) to

reproduce, for the purpose of preservation, including for adding to the digital archives of the University of Tartu until the expiry of the term of copyright, my thesis

Engineering and Validation of Single-Cycle Alphavirus Replicon Particles,

supervised by Evelin Väljaots;

2. grant the University of Tartu a permit to make the thesis specified in point 1 available to the public via the web environment of the University of Tartu, including via the digital archives, under the Creative Commons licence CC BY NC ND 4.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work until the expiry of the term of copyright;
3. am aware of the fact that the author retains the rights specified in points 1 and 2;
4. confirm that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Natavan Aliyeva

19/05/2026