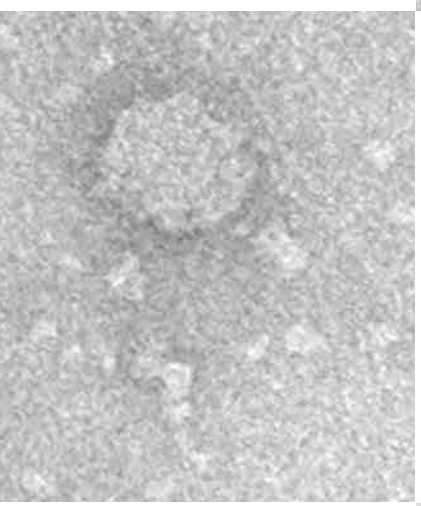
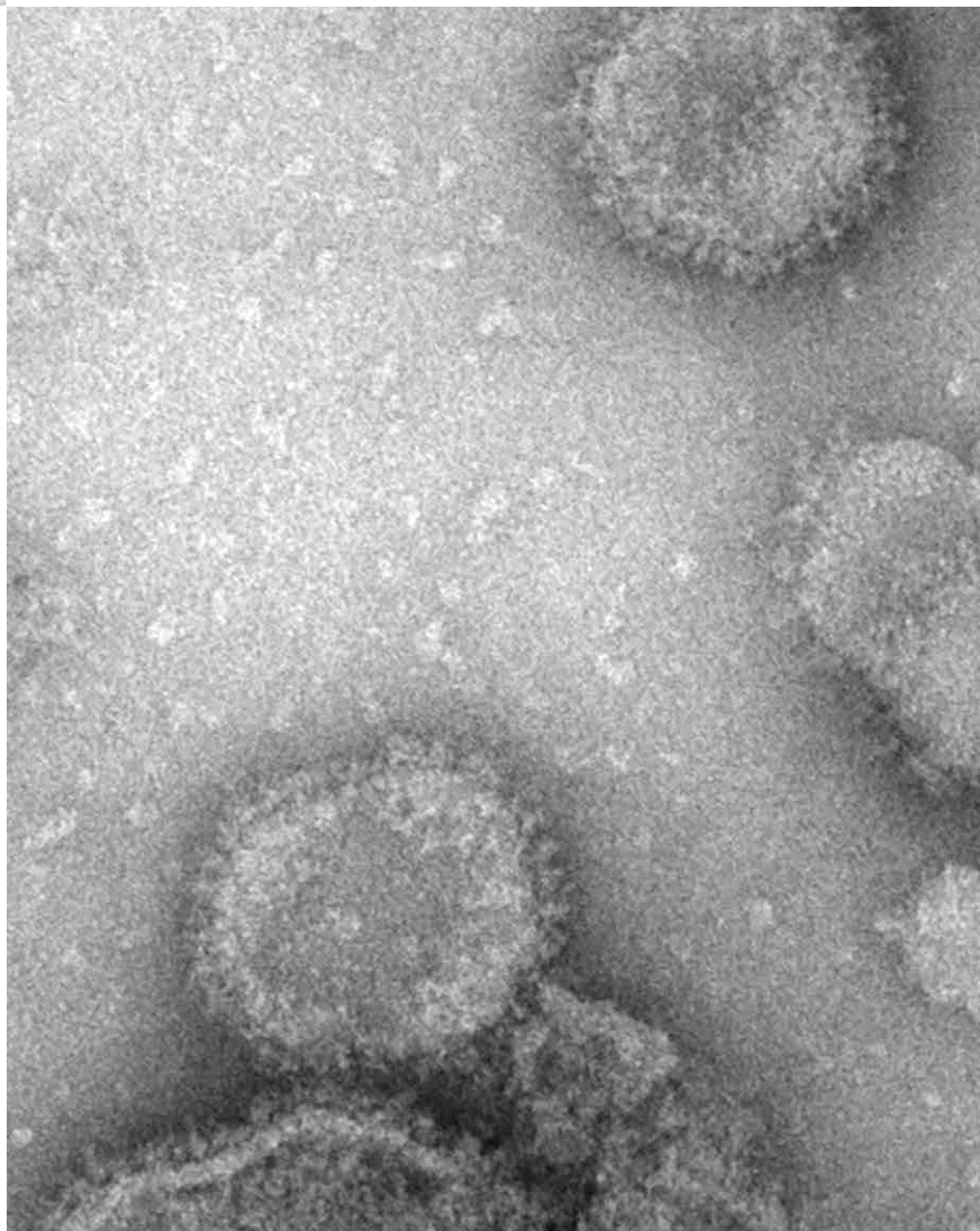


Viral replicon systems and their biosafety aspects

Inventory and description of viral replicon systems and characteristics relevant for risk assessment



CGM 2022-06

ONDERZOEKSRAPPORT

perseus report

Viral replicon systems and their biosafety aspects

Inventory and description of viral replicon systems and characteristics relevant for risk assessment

December 2022

Final_COGEM Report CGM 2022-06

Karen VAN DER MEULEN & Patrick L.J. RÜDELSHEIM
PERSEUS BV

perseus

Ordering information

COGEM report No CGM 2022-06
E-mail: info@cogem.net
Phone: +31 30 274 2777

Postal address: Netherlands Commission on Genetic Modification (COGEM),
P.O. Box 578, 3720 AN Bilthoven, The Netherlands

When ordering this report (free of charge), please mention title and number.

Advisory Committee

The authors gratefully acknowledge the members of the advisory committee for the valuable discussions and patience.

Chair: Prof. Dr. Jeroen Kortekaas (Wageningen University & Research)

Members: Dr. Ir. Gorben Pijlman (Wageningen University & Research)
Prof. Dr. Toos Daemen (University Medical Center Groningen/University of Groningen)
Dhr. Harmen Kloosterboer (National Institute for Public Health and the Environment)
Dr. Clara Posthuma (COGEM secretariat)
Dr. Ir. Eveline Ultee (COGEM secretariat)

Acknowledgements

The authors like to thank the following persons for sharing their technical insights and experience:

- Prof. Dr. J. Neyts (Rega Institute for Medical Research, KU Leuven)
- Dr. E. Heylen (Rega Institute for Medical Research, KU Leuven)

On the cover: Transmission electron micrograph of alphavirus-like particles. Image by Sandra Abbo

Disclaimer

perseus

Foreword

Replicons are incomplete viral genomes, generally comprised of RNA, that are capable of replication inside target cells, while being incapable of forming infectious particles. Replicon systems have proven invaluable tools in fundamental and applied research, by facilitating research on highly pathogenic viruses under lowered containment levels, and by allowing the development of highly efficacious candidate vaccines and therapeutics. The incapacity to form infectious particles strongly reduces any theoretical or perceived risks of pathogenicity and spreading into the environment. The inability to form infectious particles results from the partial or complete deletion of at least one gene encoding an essential structural protein from the viral genome. Replicons can be introduced into cells via electroporation, transfection or infection. The latter is only possible when the deleted structural gene, or a heterologous gene with complementing function, is provided in trans, resulting in the formation of so-called viral replicon particles (VRP). The gene or genes deleted from the viral genome may be replaced by a foreign gene of interest. Such foreign genes may be pathogen- or host derived, and applied for various applications, including vaccination and therapy. The feasibility of rapidly introducing foreign genes, combined with generally high expression levels attributed to the replicating genome, renders replicon systems highly promising platforms for vaccine development. Importantly, the inability of replicons to form progeny particles autonomously, suggests that replicons are intrinsically safe.

Replicon systems have been developed by using reverse genetics systems of RNA viruses, with members of the family Togaviridae, genus Alphavirus, being used most frequently. In recent years, COGEM has advised about environmental risks of diverse replicon-based systems. Factors that were taken into account include the possibility to form replication-competent virus resulting from recombination, and the formation of so-called virus-like vesicles. Due to the rapidly increasing interests in viral replicon systems with an anticipated increase in permit applications, scientific advice, and requests for downscaling, COGEM is considering a generic advice on the environmental risks of these systems. To this end, COGEM commissioned a research project to inventory current viral replicon systems that can serve as a basis for the decision if a generic advice on the development and application of these systems is feasible. The project was carried out by Perseus B.V. with support from an advisory committee and external experts Prof. Dr. Johan Neyts and Dr. Elisabeth Heylen from the Rega Institute for Medical Research, KU Leuven. The Advisory Committee praised the research of Perseus and the external experts, resulting in this comprehensive review of the most relevant viral replicon systems and their biosafety aspects.

Prof. Dr. J. Kortekaas

Chair of the Advisory Committee

perseus

Summary

Viral RNA replicons are defined as self-amplifying RNA molecules of viral origin and originate from single-stranded, positive- or negative-sense RNA viruses. Due to their self-amplifying nature, genome replication and transcription will occur in the replicon-containing cell, generally resulting in higher expression levels of the transgenes in comparison with replication-defective vectors. Over the past decades, viral RNA replicons have thus gained much attention in R&D as well as clinical applications, such as gene therapy and vaccination.

Viral RNA replicons are generated by deleting the genetic information of one or multiple structural proteins of a wild-type virus. The remaining viral RNA can either be used as such, i.e. as naked replicon, it can be packaged into (synthetic) nanoparticles, or it can be packaged into a viral replicon particle (VRP) thereby supplying the missing structural proteins. VRPs are produced either by co-transfecting cells with genetic sequences encoding for the missing proteins or by using cells stably expressing the missing proteins. Alternatively, not the viral RNA, but the complementary DNA can be delivered to the cells after which the cDNA is converted into viral RNA in the cell. The latter approach is referred to as DNA-launched replicons or DREPs.

Taking into account that replicons originate from wild type viruses of which most are able to induce disease, a careful risk consideration is crucial to decide on the safety of the replicons for workers and environment. Since 2000, The Netherlands Commission on Genetic Modification (COGEM) performed risk assessments and provided recommendations for multiple studies involving replicon systems. Hereby, the possibility to downscale the biosafety level of activities in contained facilities (laboratories, animal facilities) with replicons was an important consideration of the risk assessment. In order to facilitate future assessments and potential downscaling, it is aimed to work towards a more general COGEM advice that could be applied to multiple viral replicon systems. Therefore**, this report provides an inventory of replicon systems that are currently available or in development, and summarizes their biosafety characteristics.**

A literature review was undertaken compiling scientific information related to the developments and use of replicons, as well as their potential risks. The setup was done according to the typical consecutive steps of a systematic review and was based on a pre-specified protocol, including research question, search strategy, inclusion/exclusion criteria for the articles and methods for the analysis. Based on the scientific publications obtained, also an extensive secondary search was performed to retrieve additional relevant publications. Hereby, most publications presented applications of a previously generated construct, referring to a common original paper in which that construct was initially described. While some studies described features that are claimed to be beneficial for biosafety, none had the intention to study biosafety as such. Apart from the extensive literature review, additional risk considerations were identified in advices and recommendations previously issued by COGEM, as well as on the websites of (inter)national organizations related to (bio)safety, including but not limited to ZKBS, EBSA, ABSA, NIH, CORDIS, CDC and WHO.

Based on the literature review, it became evident that viral RNA replicons have found a wide range of applications. In R&D, they are a valuable tool to study viral genome replication of those viruses that do not grow sufficiently well in cell culture and to study highly infectious viruses of pathogenicity class (or risk group) 3 and 4 at lower containment level. Also, they allow to test large libraries of compounds for their antiviral activity in a high-throughput approach and can be used for cell modification and differentiation, e.g. for the generation of stem and progenitor cells for future treatment of genetic and degenerative disorders. In human and veterinary medicine, viral RNA replicons have been exploited in several ways to prime the immune system in view of vaccine development and were shown to result in transient expression of the gene of interest, strong immune responses in various animal models, and protection against viral challenge. Instead of delivering genes encoding proteins of infectious agents, replicons can also be used to deliver other genes of interest such as in the context of treating genetic disorders, cancer or other long-term diseases. Due to their cytoplasmic localization and self-replicating nature, significantly elevated

and prolonged levels of transgene expression can be achieved. Several replicon systems intended to be used as vaccine against infectious disease and as anti-cancer therapy are currently being explored in clinical trials.

When it comes to the assessment of the risks of replicons for workers and environment, data are more limited. However, based on the scientific literature, advices, recommendations and guidelines, relevant risk considerations were identified. For naked replicons, considerations were focused on the risk of genome integration, persistence in the host cell, generation of virus-like vesicles (VLV), and effects on cells not targeted by the replicons. For VRP, the main risk consideration was the formation of primary replication competent virus particles (RCV) with (potentially) the same characteristics as the wild type virus, when bringing together all genetic elements of the virus. For both systems, also the risk of secondary RCV was identified. Such secondary RCV may be formed when replicon RNA is recombined with, or complemented by a(n) (un)related wild type virus.

In order to increase the safety of a replicon system and potentially allow for downscaling of activities with the replicon system, several modifications and measures have been described to limit or avoid the above-mentioned risks. Most are aiming at reducing the likelihood of primary RCV formation e.g. by ensuring limited sequence homology between different genetic elements or by providing the coding sequences via multiple genetic elements, thereby reasoning that multiple recombination events are less likely to occur than a single event. Also, reducing the potential impact in case RCV would be formed has been addressed e.g. by modifying the genes coding for nonstructural and/or structural proteins or by starting from attenuated virus strains, and as such attenuating the virus particle formed in case of recombination. In view of secondary RCV formation, measures to avoid simultaneous presence of replicon and (un)related wild type viruses are highlighted, such as testing of hosts for the presence or absence of (un)related wild type viruses.

Taking all of the above into account, following conclusions are made:

- Despite differences in origin and design of replicon systems, some common elements have been identified in the risk assessment;
- The formation of primary RCV when generating VRPs is the most important risk consideration. Despite multiple approaches developed to reduce the likelihood of primary RCV formation, scientific uncertainty on the actual contribution of these measures as well as limitations to test the effectiveness of the measures in a validated manner, may still limit (radical) downscaling. On the other hand, even though each individual measure may not (yet) be proven leak tight, using multiple measures on different aspects of the system may create a solid barrier;
- While secondary RCV formation based on recombination with an (un)related virus co-present in the host cannot be ruled out, it seems to be mostly a theoretical concern, unproven under real application conditions. The potential for recombination can be further reduced by avoiding simultaneous presence of the (un)related virus;
- Current risk assessments start from the pathogenicity class of the recipient virus. However, as design and application of replicons is a rapidly evolving field, chimeric forms and even fully designed elements instead of those based on existing viruses are possible next steps. In such case reference to a pathogenicity class may need to be revised. Risk considerations identified in the current study can be used to support such pathogenicity class assignment, starting at the lowest level which would be applicable for self-amplifying RNA i.e. a naked replicon without a transgene.

perseus

Samenvatting

Virale RNA replicons worden gedefinieerd als zelf-replicerende RNA moleculen van virale oorsprong. Ze zijn afgeleid van positief of negatief enkelstrengs RNA-virussen. Omwille van hun zelf-replicerende eigenschappen, zullen genoomvermeerdering en transcriptie gebeuren in een replicon bevattende cel, wat kan resulteren in hoge expressieniveaus van een transgen. De expressieniveaus zijn doorgaans hoger dan deze die verkregen worden met replicatie-deficiënte vectoren. Gedurende de laatste decennia is er dan ook veel aandacht uitgegaan naar virale RNA replicons, zowel in R&D als in klinische toepassingen, bijvoorbeeld voor gentherapie en vaccinatie.

Virale RNA replicons worden gemaakt door de genetische informatie van één of meerdere structurele eiwitten van het wild type virus te verwijderen uit het virale genoom. Het resterende virale RNA kan dan als zodanig gebruikt worden, i.e. naakte replicons, het kan worden verpakt in (synthetische) nanopartikels, of het kan verpakt worden in zogenaamde virale replicon partikels (VRP). VRP worden gemaakt door de coderende sequenties voor de ontbrekende structurele eiwitten aan te bieden tijdens een cotransfectie van cellen, of door de ontbrekende structurele eiwitten stabiel tot expressie te brengen in de cellen. Als alternatief kan niet zozeer het virale RNA worden toegediend aan de cel, maar het complementaire DNA waarna dit cDNA zal worden omgezet in viraal RNA eens in de cel. Bij deze laatste benadering wordt gesproken van zogenaamde 'DNA-launched replicons' of DREPs.

Aangezien virale replicons afgeleid zijn van wild type virussen waarvan er vele een (ernstige) ziekte kunnen veroorzaken, is een goede risicoanalyse cruciaal om de veiligheid van de replicons voor de humane gezondheid en de omgeving correct te kunnen inschatten. De Nederlandse Commissie Genetische Modificatie (COGEM) heeft sinds het jaar 2000 dergelijke risicoanalyses uitgevoerd en adviezen opgesteld voor verschillende studies met repliconsystemen. Hierbij was de mogelijkheid voor het omlaagschalen van het inperkingsniveau van de activiteiten in laboratoria en proefdierfaciliteiten een belangrijke overweging in de risicobeoordelingen. Om in de toekomst de risicobeoordeling en een eventuele omlaagschaling van het inperkingsniveau te vereenvoudigen, zou COGEM willen toewerken naar een meer generiek advies dat toegepast zou kunnen worden voor verschillende repliconsystemen. Een eerste stap hierbij is **deze voorbereidende studie met als doel om de verschillende repliconsystemen die momenteel beschikbaar of in ontwikkeling zijn te invertariseren en hun bioveiligheids karakteristieken in kaart te brengen.**

Hiertoe werd een literatuurstudie uitgevoerd waarbij de wetenschappelijke informatie gerelateerd aan de ontwikkeling en het gebruik van virale replicons, evenals de risico's, werd verzameld. De literatuurstudie werd uitgevoerd volgens een vooraf opgesteld protocol inclusief de definitie van een onderzoeksvraag, een strategie voor het opzoeken van literatuur, inclusie en exclusie criteria en methodes voor analyse van de gevonden literatuur. Na het uitvoeren van de eerste uitgebreide literatuurstudie werd bijkomend gezocht naar relevante publicaties. De meeste publicaties beschreven de toepassing van een eerder ontwikkeld repliconsysteem, al dan niet met verwijzing naar een gemeenschappelijke publicatie waarin het replicon voor de eerste keer werd beschreven. Hoewel sommige studies de mogelijke eigenschappen van een repliconsysteem aanhaalden die kunnen bijdragen aan een grotere (bio)veiligheid, had geen van de studies de intentie om de bioveiligheid van het replicon systeem an sich te onderzoeken. Naast de wetenschappelijke literatuur werd bijkomend gezocht naar risico-overwegingen in adviezen en aanbevelingen eerder uitgevaardigd door COGEM, evenals in de informatie beschikbaar via de websites van (inter)nationale organisaties die betrokken zijn bij bioveiliheid, zoals ZKBS, EBSA, ABSA, NIH, CORDIS, CDC en de WHO.

Op basis van de literatuur is het duidelijk dat virale RNA replicons een brede toepassing hebben gevonden. In R&D worden ze onder meer gebruikt om de replicatie van het virale genoom te bestuderen van virussen die moeilijk vermeerderen in celcultuur. Daarnaast vinden ze hun toepassing in de studie van virussen met een hoge pathogeniciteitsklasse (3 of 4), omdat de replicons activiteiten bij een lager inperkingsniveau toelaten. Ook kunnen ze gebruikt worden om

aan de hand van een ‘high-throughput’ systeem de antivirale werking van nieuw ontwikkelde producten te onderzoeken. Tenslotte worden de virale replicons gebruikt om cellen te modificeren en differentiëren met het oog op een latere toepassing in onder meer stamceltherapie. In de humane en de diergeneeskunde werden virale replicons al onderzocht voor hun mogelijke toepassing als vaccin. De bekomen resultaten tonen aan dat een transgen, tot expressie gebracht met behulp van een replicon, een krachtige immuunrespons kan induceren in verschillende proefdiermodellen die de dieren beschermt tegen een daaropvolgende infectie met het wild type virus. Naast genen coderend voor eiwitten van infectieuze agentia kunnen replicons ook gebruikt worden om andere genen in het lichaam te brengen, bijvoorbeeld in de context van de behandeling van genetische aandoeningen, kanker of andere chronische ziektes. Voor elk van de toepassingen zijn er inmiddels verschillende repliconsystemen in klinische ontwikkeling.

De (wetenschappelijke) gegevens in verband met de risicobeoordeling van replicons voor de humane gezondheid en de omgeving zijn beperkter beschikbaar. Toch kon op basis van de literatuur, de adviezen, de aanbevelingen en de richtlijnen, een aantal risico-overwegingen worden geïdentificeerd. Voor naakte replicons waren relevante overwegingen onder meer het risico op integratie in het genoom, persistentie in de gastheer(cel), vorming van virus-achtige vesikels (VLV) en de effecten op niet-doelwit cellen. Voor VRP was de mogelijke vorming van (primaire) replicatie competente virus partikels (RCV), al dan niet met dezelfde karakteristieken als het wild type virus, de belangrijkste risico-overweging, in het bijzonder tijdens hun productie wanneer alle virale genetische elementen worden samengevoegd. Zowel voor de naakte replicons als voor de VRP ging ook de nodige aandacht naar het risico op secundaire RCV. Deze kunnen gevormd worden wanneer het replicon RNA zou recombineren met of gecomplementeerd worden door een (niet-)verwant wild type virus. Om de veiligheid van repliconsystemen te verbeteren en bijgevolg een omlaagschaling toe te laten van de activiteiten met de repliconsystemen, zijn verschillende strategieën ontwikkeld. De meeste van deze strategieën hebben als doel om de vorming van primaire RCV te reduceren, bijvoorbeeld door het verminderen van de sequentiehomologie tussen de verschillende genetische elementen of door het splitsen van de genetische elementen over aparte RNAs. De redenering hierbij is dat wanneer meerdere recombinaties nodig zijn om een RCV te verkrijgen, de kans kleiner is dat dit gebeurt dan wanneer slechts één recombinatie nodig is. Daarnaast richtten verschillende strategieën zich op het beperken van de impact van eventueel gevormde RCV, bijvoorbeeld door de genen coderend voor de virale eiwitten te modificeren of door het replicon te ontwerpen op basis van een verzwakte virusstam. Met het oog op de vorming van secundaire RCV werd vooral het belang benadrukt dat de gastheer(cel) vrij dient te zijn van (niet-)verwante wild type virussen.

Uit bovenstaande kunnen volgende conclusies worden gemaakt:

- Ondanks verschillen in oorsprong en opbouw van de beschreven repliconsystemen, kunnen bepaalde gemeenschappelijke risico-overwegingen worden geïdentificeerd;
- De vorming van primaire RCV bij de productie van VRP is de belangrijkste risico-overweging. Ondanks het feit dat er verschillende strategieën beschreven zijn om de kans op vorming van RCV te reduceren, is het onvoldoende duidelijk wat de werkelijke bijdrage is van deze strategieën tot de veiligheid van de repliconsystemen. Bovendien zijn de testmogelijkheden voor RCV beperkt en/of niet gevalideerd. Dit vormt een mogelijke belemmering bij het (radicaal) omlaagschalen van de activiteiten met replicons. Anderzijds, ook al is er voor de verschillende individuele strategieën nog geen waterdicht bewijs wat hun impact is op de veiligheid, het gelijktijdig implementeren van meerdere strategieën gericht op verschillende facetten van het repliconsysteem zou mogelijk wel een afdoende inperking van de risico’s kunnen geven;
- Ook al kan de vorming van secundaire RCV als gevolg van recombinatie met of complementatie door een (niet-)verwant wild type virus niet volledig uitgesloten worden op basis van de huidig beschikbare kennis, het lijkt veeleer een theoretische overweging, waarvan het optreden in praktijk niet bewezen is. De mogelijkheid dat het optreedt kan verder gereduceerd worden door aanwezigheid van (niet-)verwante wild type virussen in de gastheer te vermijden;

- De huidig beschikbare risicobeoordelingen starten steeds vanuit de pathogeniciteitsklasse van het oudervirus. Het ontwikkelen en toepassen van replicons is echter een snel evoluerende discipline, waarbij onder meer chimere replicons of volledig synthetisch ontworpen replicons een mogelijke volgende stap vormen. In die gevallen is het refereren naar een pathogeniciteitsklasse van een oudervirus mogelijk niet (meer) relevant. De risico-overwegingen zoals geïdentificeerd in de huidige studie kunnen helpen om een risicoclassificatie van replicons op te bouwen, waarbij gestart kan worden vanuit het laagst mogelijke risico, namelijk deze van een naakt replicon zonder transgen.

perseus

Table of contents

perseus

Abbreviations

ABSA	American Biological Safety Association
aPDL1	Anti-programmed death ligand 1
ATPase	Adenosine triphosphatase
BHK	Baby hamster kidney
BSC	Biosafety cabinet
BSL	Biosafety level
C	Capsid protein
Cas	CRISPR-associated protein
CCHFV	Crimean-Congo hemorrhagic fever virus
CCVB	Canadian Centre for Veterinary Biologicals
CDC	Centers for Disease Control and Prevention
cDNA	Copy DNA
COGEM	Commission on Genetic Modification, The Netherlands
CORDIS	Community Research and Development Information Service
CRISPR	Clustered regularly interspaced short palindromic repeat
CSFV	Classical swine fever
DENV	Dengue Virus
DM	*Dierverblijf met micro-organismen* (animal facility with microorganisms)
DNA	Deoxyribonucleic acid
DREP	DNA-launched replicon
ds	Double stranded
EBSA	European Biosafety Association
EEEV	Eastern equine encephalitis virus
FOEN	Federal Office for the Environment, Switzerland
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCV	Hepatitis C virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HYSV	Huaiyangshan banyang virus
iPSC	Induced pluripotent stem cell
IRES	Internal ribosome entry site sequences
IU	Infectious units
JEV	Japanese encephalitis virus
KU	Katholieke Universiteit
KUNV	Kunjin virus
m.o.i.	Multiplicity of infection
MERS-CoV	Middle East Respiratory Syndrome coronavirus
miRNA	MicroRNA
ML	Microbiologisch laboratorium (microbiological laboratory)
MRE	miRNA recognition element
mRNA	Messenger RNA
MV	Measles virus
NIH	National Institutes of Health, USA

NP	Nucleoprotein
nrRNA	Non-replicating mRNA
NS	Nonstructural
ORF	Open reading frame
PCR	Polymerase chain reaction
PEDV	Porcine epidemic diarrhea virus
PFU	Plaque forming units
qPCR	Quantitative PCR
RCV	Replication competent virus particle
RdRp	RNA-dependent RNA polymerase
R&D	Research and development
RVFV	Rift Valley fever virus
RG	risk group
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RSV	Respiratory syncytial virus
S	Structural
SACGM	Scientific Advisory Committee on Genetic Modification
SARS-CoV	Severe acute respiratory syndrome corona virus
SeV	Sendai virus
SFV	Semliki forest virus
SGP	Sub-genomic promoter
SINV	Sindbis virus
siRNA	Small interfering RNA
SPDV	Salmon pancreas disease virus
sRNA	Small RNA
TBEV	Tick-borne encephalitis virus
TCID	tissue culture infectious dose
UK	United Kingdom
USA	United States of America
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis virus
VLV	Virus-like vesicle
vRNA	Viral RNA
VRP	Virus replicon particle
VSIV	Vesicular stomatitis Indiana virus
VSV	Vesicular stomatitis virus
WEEV	Western equine encephalitis virus
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus
ZKBS	*Zentrale Kommission für die Biologische Sicherheit* (Central Committee on Biological Safety, Germany)

Glossary

Gene therapy	Clinical research in humans either involving activities with genetically modified organisms (GMO), or whereby genetically modified cells can be created in the human body, or whereby changes are made to the genetic material of human cells. (definition according to Gene Therapy Office, https://www.loketgentherapie.nl/en/gene-therapy-office)
Infectious	Capable of entering a host cell followed by gene expression.
Productive infection	Viral infection of a cell that produces infectious progeny.
Replication competent virus (RCV)	Virus particles capable of infecting cells and replicating to produce additional infectious virus particles.
- Primary RCV	RCV generated during the production of VRP due to recombination between the various genetic elements coding for structural and nonstructural proteins.
- Secondary RCV	RCV generated during the application of naked replicons or VRP due to recombination with / complementation by (un)related wild type viruses that thus supply missing genetic elements.
Virus like vesicle (VLV)	Virus-like vesicles are membrane-enclosed vesicles that resemble enveloped viruses in organization but lack the viral capsid.
Viral replicon particle (VRP)	A viral replicon enveloped / encapsidated by a protein coat. A viral replicon particle can infect a cell only once as it lacks the structural genes to build the coat proteins.
Viral RNA replicon	A self-amplifying RNA molecule derived from an RNA virus. Genetic information for parts or all of the regions coding for structural proteins of a wild-type virus are deleted. Genes for nonstructural proteins enable genome replication. Genes for structural proteins may be replaced by foreign genes.

Introduction

Viral RNA replicons are defined as self-amplifying RNA molecules of viral origin. They are generated by deleting the genetic information of one or multiple structural proteins of a wild-type virus. Several techniques are used to allow a replicon particle to enter a target cell. E.g. the viral replicon RNA can be brought into the cells by transfection or electroporation or a viral replicon particle (VRP) can be generated by simultaneously providing RNA coding for the missing protein components to the cell, after which particles are formed which are able to infect a cell, but are unable to subsequently form infectious virus particles due to the absence of the coding sequences for (part of) the structural proteins.

Due to their self-amplifying nature, RNA replicons have gained much attention in R&D as well as clinical applications, such as gene therapy and vaccination. By removing the genes coding for the structural proteins and replacing them with transgenes of interest, they form ideal vectors to deliver genes to tissues of interest or to help present specific proteins to the immune system. Since genome replication and transcription will occur in the replicon-containing cell, higher expression levels of the transgenes can be obtained in comparison with replication-defective vectors.

Since 2000, The Netherlands Commission on Genetic Modification (COGEM) performed risk assessments and provided recommendations for multiple studies involving replicon systems. Hereby, the possibility to downscale the biosafety level (BSL) of activities in contained facilities (laboratories, animal facilities) with replicons was an important part of the risk assessment. In order to facilitate future assessments and potential downscaling, it is aimed to work towards a more general COGEM advice that could be applied to multiple viral replicon systems.

For this purpose, this preparatory study aims to provide an inventory of replicon systems originating from RNA viruses that are currently available or in development, and to summarize their biosafety characteristics. Based on this inventory, it can be explored whether a more general advice can be set up and, if so, what information might be crucial herein.

It is highlighted that this study focusses on the intrinsic characteristics of specific replicons and how these characteristics might influence risk classification. The final risk classification of a specific construct also depends on other components, e.g. on the function and hazards of the inserted sequences.

1 Methods

1.1 Literature study

A literature review was undertaken compiling scientific information related to the developments and use of replicons, as well as their potential risks.

In order to formulate **search strings**, the following keywords were selected:

- Replicon / Vector
- Virus / Replication competent / RCV
- Biosafety / Risk / Adverse effect

After selecting keywords, a typical search string was composed using Boolean operators: (VIR* OR "REPLICATI* COMPET*") AND (REPLICON OR VECTOR) AND (BIOSAFE* OR RISK OR "ADVERSE EFFECT" OR SAFE* OR ENVIRONMENT*) AND (RNA).

Two electronic bibliographic multi-disciplinary databases were chosen to **search for relevant publications**: Web of Science™ core collection[1], and Scopus®[2]. Web of Science™ core collection consists of six online databases indexing scholarly books, peer reviewed journals, original research articles, reviews, editorials, chronologies, abstracts, as well as other items. Disciplines included in this index are agriculture, biological sciences, engineering, medical and life sciences, physical and chemical sciences, and many others. The database contains 1.4 billion cited references going back to 1900. Scopus® by Elsevier is an abstract and citation database of peer-reviewed literature, including scientific journals, books and conference proceedings, covering research topics across all scientific and technical disciplines, ranging from medicine and social sciences to arts and humanities. Scopus® is updated daily and includes over 71 million records and over 1.4 billion cited references after 1970.

The search was expected to result in the identification of publications in English or, if in another language, having a title, abstract or keywords in English. The searches were not limited in time. Initially, only original articles were searched for. In case the number of articles on a specific topic was large, relevant review articles were consulted.

In the first stage of **selection**, the title, keywords and abstract of the retrieved references were screened. This resulted in an important reduction of the number of potentially relevant publications. Of the selected references an attempt was made to retrieve a full text document, after which the full content was examined. The references of the included studies were manually screened to search for further papers. No language or publication restrictions were applied, and studies were not selected based on quality.

An **extraction** was performed of high-level data and the final set of publications was selected. The key findings of the selected, full text papers were then summarized including, but not limited to, information on the virus on which the replicon system was based, construction of de replicon itself (e.g. deletions, mutations, substitutions), and the likelihood of replication competent virus particle (RCV) formation.

The literature search was concluded on January 25, 2021 and resulted in 1391 publications. Additional publications were retrieved until April 30, and were based on reference lists of publications identified in the primary literature study, on reference lists in COGEM advices, and on internet searches using terms relevant for the current study.

Most publications presented applications of a previously generated construct, referring to a common original paper in which that construct was initially described. While some described features that are claimed to be beneficial for biosafety, none had the intention to study biosafety.

[1] https://clarivate.com/products/web-of-science/databases/
[2] https://www.scopus.com

1.2 Precedents in risk assessment

Further insight in risk assessments underlying activities with viral RNA replicons was obtained through an analysis of **advices and recommendations previously issued by COGEM**. For that, a search based on the term 'replicon' was performed through the publications on the COGEM website (https://cogem.net/en/publicaties/?str=replicon). In total, 27 advices were identified of which 26 were consulted. One advice was not available for public consultation (see Table 1).

Table 1. Overview of COGEM advices related to viral RNA replicons sorted per replicon system and year of publication

Replicon system(s)		Title (Dutch)	Year of publication	COGEM reference
Flaviviridae	Dengue virus (DENV) / Yellow fever virus (YFV) (chimeric)	Pseudo-infectieuze virusdeeltjes; een modelsysteem voor het bestuderen van infectieprocessen van flavivirussen	2005	(CGM/050615-01)
Flaviviridae	Zika virus (ZIKV)	Advies classificatie en inschaling werkzaamheden (genetisch gemodificeerd) Zika virus	2016	(CGM/160307-01)
Flaviviridae	Tickborne encephalitis virus	Advies omlaagschaling werkzaamheden met SRIPs afgeleid van TBEV	2019	(CGM/190725-02)
Hepeviridae	Hepatitis E virus (HEV)	Inschaling werkzaamheden met Hepatitis E virus	2012	(CGM/121221-01)
Nairoviridae	Crimean-Congo hemorrhagic fever virus (CCHFV)	Classificatie van en inschaling van werkzaamheden met *Crimean-Congo hemorrhagic fever virus*	2011	(CGM/110321-01) *Not publicly available*
Paramyxoviridae	Sendai virus (SeV)	Inschaling werkzaamheden gg-Sendai virus	2013	(CGM/130329-01)
Phenuiviridae	Huaiyangshan banyangvirus	Inschaling werkzaamheden met gg-Huaiyangshan virus	2013	(CGM/130502-01)
Phenuiviridae	Rift Valley fever virus (RVFV)	Werkzaamheden met *Rift Valley fever virus*	2008	(CGM/080313-05)
		Advies inschaling productie genetisch gemodificeerd *Rift Valley fever virus*	2011	(CGM/110322-01)
		Inschaling werkzaamheden met gg-*Rift Valley fever virus*	2012	(CGM/120716-02)
Togaviridae	Semliki forest virus (SFV)	Combinatie vaccins voor HIV	2003	(CGM/030604-02)
		Advies werkzaamheden met een recombinante Semliki forest virus vector	2007	(CGM/070723-02)
		Klinische studie met een Semliki forest virus vaccin tegen baarmoederhalskanker	2011	(CGM/111222-02)
Togaviridae	Sindbis virus (SINV)	Advies werkzaamheden met gg-Sindbis virus replicons met het 'amyloid precursor protein' (APP) gen	2019	(CGM/190415-01)
Togaviridae	Venezuelan equine encephalitis virus (VEEV)	Werkzaamheden met VEE virus gebaseerde replicondeeltjes	2009	(CGM/090603-01)
		Advies inschaling van werkzaamheden met replicons afgeleid van het Venezuelan equine encephalitis virus	2015	(CGM/150715-01)
		Advies inschaling van werkzaamheden met gg-replicondeeltjes afgeleid van Venezuelan equine encephalitis virus	2016	(CGM/160815-01)
		Vervolgadvies omschaling van werkzaamheden met gg-VEEV replicondeeltjes	2016	(CGM/160906-02)
		Advies omlaagschaling werkzaamheden met gg-VEEV RNA replicons	2017	(CGM/170224-01)
		Vervolgadviesvraag VEEV replicons en noodzaak RCV test	2017	(CGM/170322-03)
		Werkzaamheden met gg-VEEV en gg-SPDV replicons met aquatische donorsequenties	2017	(CGM/171030-03)

Replicon system(s)		Title (Dutch)	Year of publication	COGEM reference
Togaviridae	SINV / VEEV (chimeric)	Constructie en productie van alphavirusvectoren	2004	(CGM/040212-02)
		Vaccinatie van apen met recombinante chimere alphavirusvectoren ten behoeve van vaccinonderzoek	2006	(CGM/060314-01)
Togaviridae	SFV, SINV and Middle East Respiratory Syndrome coronavirus (MERS-CoV)	Inschaling werkzaamheden met gg-Middle East Respiratory Syndrome coronavirus (MERS-CoV)	2013	(CGM/130822-01)
Togaviridae	SFV, SINV and VEEV	Werkzaamheden met gg-alphavirus-replicons (SFV, SINV en VEEV) met donorsequenties van griepvirussen, Human respiratory syncytial virus, en Marburg- en Ebolavirussen	2017	(CGM/171024-01)
Togaviridae	Salmon pancreas disease virus (SPDV)	Werkzaamheden met gg-VEEV en gg-SPDV replicons met aquatische donorsequenties	2017	(CGM/171030-03)
Togaviridae	/	Vervolgadvies over vorming van 'virus-like vesicles' (VLVs) tijdens werkzaamheden met gg-replicons	2018	(CGM/180313-02)

Apart from the COGEM advices on specific projects, also following **COGEM research reports** were consulted:

- Assessment of preclinical gene therapy studies worldwide (Verhagen et al., 2015);
- Recombinant and chimeric viruses: Evaluation of risks associated with changes in tropism (Peeters, 2005).

Additionally, advices and recommendations were searched using the term ‘replicon’ on the websites of **(inter)national organizations related to (bio)safety**, including but not limited to the European Biosafety Association (EBSA), ABSA, National Institutes of Health (NIH), and WHO. Following sources were identified as specifically referring to the risk assessment of replicons:

- Guidance from the UK Scientific Advisory Committee on Genetic Modification - ‘The SACGM Compendium of guidance - Part 2: Risk assessment of genetically modified microorganisms (other than those associated with plants) [3]
- USA Centers for Disease Control and Prevention (CDC) ‘Biosafety in Microbiological and Biomedical Laboratories - 6th Edition’ [4]
- Canadian Centre for Veterinary Biologics
- Dutch Research Council Project: Environmental safety of synthetic replicon particle vaccines - risk for RNA recombination with wildtype viruses (RepliSAFE)[5]
- Community Research and Development Information Service (CORDIS)[6]
- Central Committee on Biological Safety (Zentrale Kommission für die Biologische Sicherheit, ZKBS)

1.3 Expert consultations

The study was further supported by scientific guidance and critical review by Prof. Dr. Johan Neyts and Dr. Elisabeth Heylen of the Laboratory of Virology and Chemotherapy of the Rega Institute of the Katholieke Universiteit (KU) Leuven[7].

In addition, the Advisory Committee provided useful suggestions on studies and publications.

[3] https://www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp/part2.pdf
[4] https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf
[5] https://www.nwo.nl/en/projects/15791
[6] https://cordis.europa.eu/en
[7] https://rega.kuleuven.be/cmt/jn

2 Single-stranded RNA viruses, the backbone for RNA replicons

2.1 Introduction

RNA viruses are characterized by their capacity of highly efficient self-amplification of RNA in host cells. This makes them attractive vehicles for delivery of transgenes of interest for vaccination or for treating genetic disorders, cancer or other long-term diseases. The current report focuses on the application of, as well as the biosafety considerations for replicons originating from positive- and negative-sense, single-stranded RNA viruses. Application of double-stranded (ds) RNA viruses, which so far has only been explored sporadically, as well as of single-stranded RNA viruses that use reverse transcriptase (i.e. retroviruses) remain outside the scope of the current study.

2.2 RNA virus replication cycle

The RNA virus life cycle contains various steps, all of which are required for producing viral progeny. The first step of the viral replication cycle is the attachment of the virus to the host cell membrane by means of interacting with one or more cellular receptors. Upon attachment, the entire virus particle or parts of it are taken up by the host cell by means of endocytosis. Subsequent interaction with the cellular environment then results in a release of the viral genome into the cytoplasm. The viral genome is then translated and transcribed using the host cellular machinery. Finally, the various viral components are assembled after which the virus particle is released from the cell by means of cell lysis, budding or exocytosis.

Different types of RNA viruses are identified based on specific variations to this general replication cycle, as further introduced in the following sections.

2.2.1 Positive-sense, single-stranded RNA viruses

Representative examples of positive-sense, single-stranded RNA viruses include, but are not limited to *Caliciviridae*, *Coronaviridae*, *Flaviviridae* and *Togaviridae* (see also Section 2.3).

An overview of their replication cycle is provided in Figure 1.

For positive-sense, single-stranded RNA viruses, the viral genome itself is infectious and serves as the viral messenger (m)RNA. In brief, upon entry and uncoating of the virus in the host cell (1), the viral (v)RNA is released into the cytoplasm where it uses the cellular machinery to translate its genome. Among the first proteins to be synthesized (2) are those needed to synthesize additional genomes and mRNAs. These proteins involved are called nonstructural proteins and include, but are not limited to, the RNA-dependent RNA polymerase (RdRp), RNA helicase and adenosine triphosphatase (ATPase). RdRp, in association with other proteins required for genome replication is often called the replicase complex. Some nonstructural proteins require co- or post-translational cleavage before they become active (2b). Cleavage occurs by viral proteases. Nonstructural proteins will not be assembled into newly formed virus particles.

The viral mRNA also serves as the template for genome replication and synthesis of additional viral RNAs (3). Hereby, a dsRNA genome is synthesized from the genomic positive-sense, single-stranded RNA. The dsRNA genome is then transcribed/replicated thereby providing viral mRNAs, and new positive-sense, single-stranded RNA genomes.

Proteins that are only required later in the viral replication cycle are encoded by the newly formed viral or subgenomic mRNA (4, 4b). Subgenomic RNAs will not be assembled into newly formed virus particles, as they do not comprise a packaging signal.

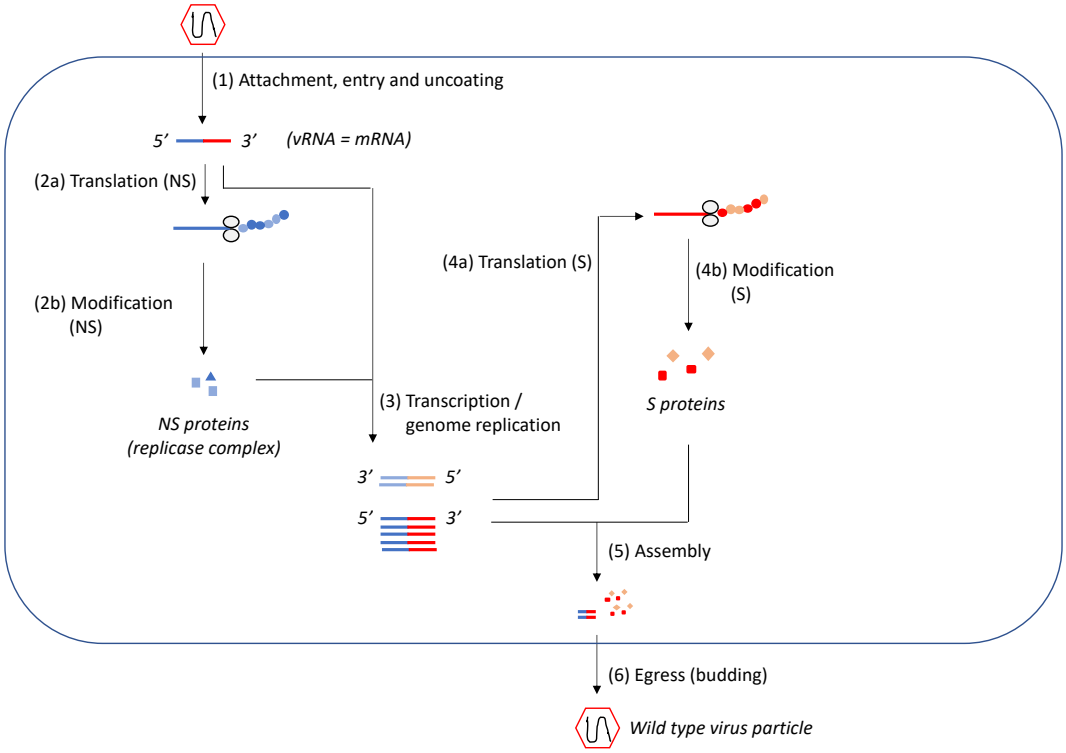


Figure 1. Replication of an enveloped, positive-sense, single-stranded RNA viruses (vRNA: viral RNA; mRNA: messenger RNA; NS: nonstructural; S: structural)

For some positive-sense, single-stranded RNA viruses, such as *Flaviviridae* and *Picornaviridae*, the viral genome is translated into one polyprotein, which is subsequently cleaved into nonstructural and structural proteins.

Proteins and genetic material are finally assembled (5) and released (6) from the cell as described above.

2.2.2 Negative-sense, single-stranded RNA viruses

Representative examples of non-segmented negative-sense, single-stranded RNA viruses include *Bornaviridae*, *Filoviridae*, *Rhabdoviridae* and *Paramyxoviridae*. Examples of segmented negative-sense, single-stranded RNA viruses include *Arenaviridae*, *Phenuiviridae* and *Hantaviridae* (see also Section 2.3).

An overview of the replication cycle of negative-sense, single-stranded RNA viruses is provided in Figure 2.

For negative-sense, single-stranded RNA viruses, the viral genome itself (i.e. naked RNA) is not infectious. Due to its polarity, it cannot serve as viral mRNA and it cannot be translated. Upon entry and uncoating of the virus in the host cell (1), viral RNA first needs to be transcribed (2). Therefore, viral proteins present in the virus particle are required. The proteins are present in a ribonucleoprotein (RNP) complex that usually consists of the viral RNA, multiple monomers of RNA-binding nucleoprotein, RdRp and other accessory proteins. Whereas non-segmented negative-sense RNA viruses have their genome packaged in one RNP, segmented negative-sense RNA viruses have a distinct RNP for each genomic RNA segment that is functionally independent for transcription and replication. For most negative-sense RNA viruses, the RNP complex is released into the cytoplasm for further processing. For some viruses, such as those belonging to the *Bornaviridae*, the RNP complex is transferred to the nucleus.

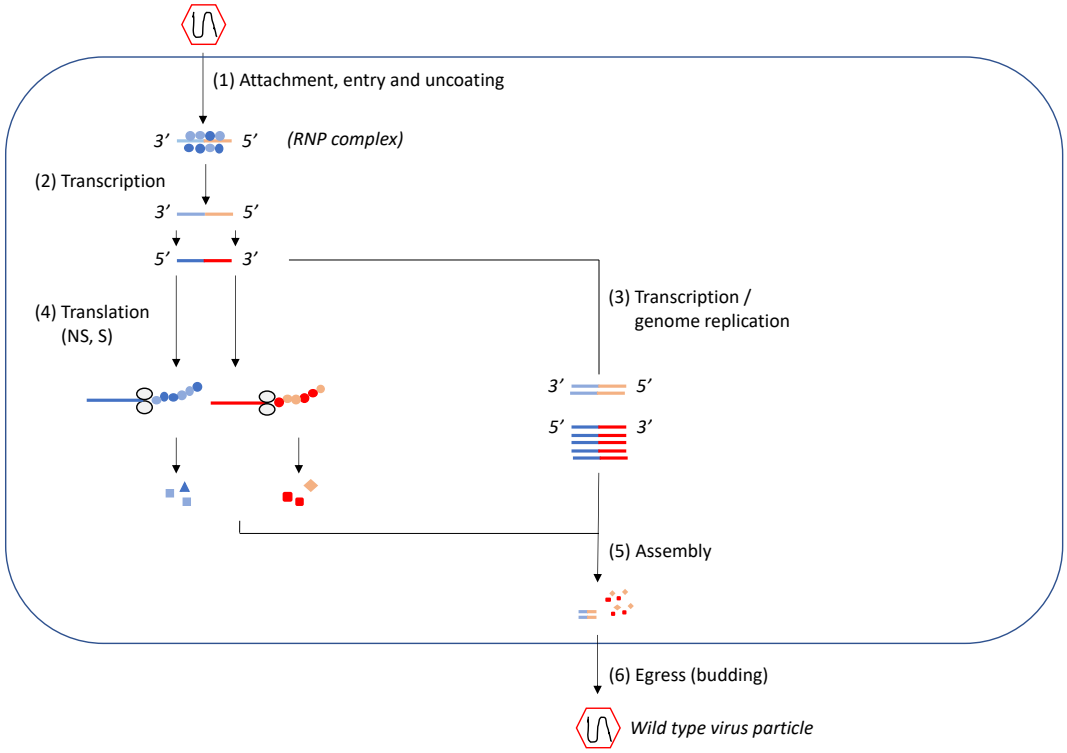


Figure 2. Replication of an enveloped, negative-sense, single-stranded RNA viruses (RNP: ribonucleoprotein; NS: nonstructural; S: structural)

Actual transcription (3) may differ between the single-stranded, negative-sense virus families. Some viruses have a strict negative-sense and thus their complete genome will be transcribed into (positive-sense) mRNA by RdRp. Others, such as members of the *Arenaviridae* and *Phenuiviridae*, are ambisense or contain both negative-sense and ambisense genome segments, respectively. In other words, their RNA is partly of positive and partly of negative polarity (Nguyen and Haenni, 2003). The 5' part of an ambisense RNA is of positive polarity containing an open reading frame (ORF) that can theoretically be directly translated into protein(s). The 3' part of this same RNA is of negative polarity. It contains an ORF, but in the complementary strand. Interestingly, most ambisense viruses are not strictly ambisense, but contain negative as well as ambisense RNA segments.

For strictly negative-sense, as well as for ambisense viruses, mRNA is sequentially translated into different proteins (4). Proteins and genetic material are finally assembled (5) and released (6) from the cell as described above.

2.3 Risk group classification of RNA viruses

RNA viruses have been associated with multiple diseases in vertebrates [8]. Table 2 lists the different viral families and species from which replicons have been generated, their host range, whether they are known to cause disease in humans and/or animals, as well as the risk classification, in first instance based on the assessments as published by COGEM. In case no indication could be found in the listings of COGEM, other risk classifications were verified such as the Belgian Service of Biosafety and Biotechnology. In case the Belgian listings did not mention the virus, additional databases such as the Central Committee on Biological Safety, Germany, and the Federal Office for the Environment, Switzerland (FOEN), were consulted.

[8] Description of RNA viruses in plants (e.g. *Nodaviridae*) remains outside the scope of this study.

perseus

Table 2. Overview of host range, disease and risk group classification of viruses (wild type and, if relevant, vaccine strain) from which replicons have been derived (non-limitative list) [9]

Classification	Family	Virus	Host range	Disease		Risk group
				Human	Animal	
Positive-sense, non-segmented	*Astroviridae*	Human astrovirus	Humans	✓	-	2*
	Caliciviridae	Norovirus (Norwalk virus)	Humans; rodents; felines; canines; sea lions; pigs; sheep; cattle; bats	✓	-	2
	Coronaviridae	Middle East Respiratory Syndrome coronavirus	Humans; dromedary; camel; bats (?)	✓	✓	3
		Severe acute respiratory syndrome corona virus	Humans; bats; civets	✓	✓	3
		Severe acute respiratory syndrome corona virus 2	Humans; bats; minks; cats	✓	✓	3
	Flaviviridae	Bovine viral diarrhoea virus	Even-toed ungulates	-	✓	2
		Classical swine fever	Pigs	-	✓	4
		Dengue virus	Humans; mosquitoes	✓	-	3
		Hepatitis C virus	Humans; non-human primates; mosquitoes	✓	-	2
		Japanese encephalitis virus	Humans; mosquitoes and vertebrate hosts, primarily pigs and wading birds	✓	✓	3
		Kunjin virus	Humans; mosquitoes; birds; horses	✓	✓	2***
		Niénokoué virus	Mosquitoes	-	-	1**
		Tick-borne encephalitis virus	Humans; ruminants; birds; rodents; horses; carnivores; ticks	✓	-	3
		West Nile virus	Humans; non-human primates; equines; dogs; cats; sheep; llamas; alpacas; alligators; birds; squirrel; chipmunk; rabbits	✓	✓	3
		Yellow fever virus	Humans	✓	✓	3
		Yellow fever virus - Vaccine strain 17D				2
		Zika virus	Humans; monkeys; sheep; goats; horses; cows; ducks; rodents; bats; orangutans; carabaos; mosquitoes	✓	-	3
	Hepeviridae	Hepatitis E virus	Humans; fish; amphibians; moose; kestrels	✓	-	2
	Picornaviridae	Mengovirus	Humans; rodents; pigs; monkeys	✓	-	2*
		Poliovirus	Humans	✓	-	2

[9] The table only refers to vaccine strains for which a risk group classification has been published by COGEM or international databases

Classification	Family	Virus	Host range	Disease		Risk group
				Human	Animal	
		Foot-and-mouth disease virus	Cloven-hoofed animals (including bovids)	-	✓	4
		Enterovirus 71	Humans	✓	-	2
		Coxsackie virus	Humans	✓	-	2
		Hepatitis A virus	Humans; non-human primates	✓	-	2
		Human rhinovirus	Humans; primates	✓	-	2
	Togaviridae	Salmon pancreas disease virus	Atlantic salmon (farmed salmonid fish)	-	✓	2
		Chikungunya virus	Humans; non-human primates; rodents; birds; mosquitoes	✓	✓	3
		Semliki forest virus	Humans; mosquitoes; wild birds; rodents; domestic animals and non-human primates	✓	✓	2
		Sindbis virus	Humans; mammals; birds; insects; amphibians	✓	✓	2
		Venezuelan equine encephalitis virus	Humans; horses; burros; bats; and terrestrial mammals	✓	✓	3
		Venezuelan equine encephalitis virus - Vaccine strain TC83				2
		Western equine encephalitis virus	Humans; reptiles; bats; pheasants; wild birds; mosquitoes; horses; dogs; rodents	✓	✓	3
		Eastern equine encephalitis virus	Humans; reptiles; bats; pheasants; wild birds; mosquitoes; horses; dogs; rodents	✓	✓	3
Negative-sense, non-segmented	*Bornavirida*	Bornavirus	Horses; ruminants; dogs; squirrels; foxes	✓	✓	2 H; 3 A*
	Filoviridae	Ebolavirus	Humans; chimpanzees; gorillas, baboons; duikers; rodents; shrew; bats	✓	✓	4
		Marburg virus	Humans; non-human primates	✓	✓	4
	Paramyxoviridae	Measles virus	Humans	✓	-	2
		Parainfluenza virus	Humans; birds	✓	✓	2
		Nipah virus	Humans; pigs; bats	✓	✓	4
		Human metapneumovirus	Humans;	✓	-	2
		Respiratory syncytial virus	Humans; cattle	-	✓	2
		Newcastle Disease Virus	Birds	-	✓	3
		Newcastle Disease Virus - Vaccine strain LaSota				2
		Sendai virus	Rodents (mice)	-	✓	2

Classification	Family	Virus	Host range	Disease		Risk group
				Human	Animal	
	Rhabdoviridae	Vesicular stomatitis virus	Humans; horses; cattle; pigs; mules; sand flies; grasshoppers; rodents	✓	✓	3
Negative-sense, segmented	*Arenaviridae*	Lassavirus	Humans; Mastomys natalensis (multimammate rat)	✓	✓	4
		Junin virus	Humans; rodents	✓	✓	4
		Tacaribe virus	Humans; bats	✓*	✓	2
		Machupo virus	Rodents; humans; ticks; mosquitoes	✓	✓	4
	Hantaviridae	Hantaan virus	Humans; rodents	✓	-	3*
	Nairoviridae	Crimean-Congo hemorrhagic fever virus	Humans; cattle; sheep; goats; birds; ticks	✓	✓	4
	Phenuiviridae	Huaiyangshan banyangvirus (also known as 'severe fever with thrombocytopenia virus')	Humans; goats; sheep; cattle; dogs; chickens, rodents, pigs; ticks	✓	✓	4
		Rift Valley fever virus	Humans; ruminants; camelids; mosquitoes	✓	✓	3
		Uukuniemi uukuvirus	Humans; ticks; blackbirds; mosquitoes	✓*	✓	2

* based on classification published by the Belgian Service of Biosafety and Biotechnology (SBB)[10]
** based on classification published by the Central Committee on Biological Safety, Germany (ZKBS)[11]
*** based on classification published by the Federal Office for the Environment, Switzerland (FOEN)[12]

[10] https://www.biosafety.be/content/tools-belgian-classification-micro-organisms-based-their-biological-risks
[11] https://zag.bvl.bund.de/organismen/index.jsf;jsessionid=bawi1SnukahqqNfXcysWnG5lQ0ocmlcTmNeo0Oi5.subs208?dswid=7044&dsrid=934
[12] https://www.bafu.admin.ch/bafu/en/home/topics/biotechnology/publications-studies/publications/classification-of-organisms.html

3 Overview of replicon systems and their application

3.1 Viral RNA replicons: an introduction

As mentioned above, single-stranded RNA viruses are ideal candidates to transiently deliver genetic information in basic research and clinical therapy, provided their pathogenic properties can be removed. Such is the case for viral RNA replicons.

Viral RNA replicons are defined as self-amplifying RNA molecules of viral origin. They are generated by retaining the untranslated regions (UTRs), genes coding for the nonstructural proteins and, when present, a sub-genomic promoter (SGP) of the parent virus to allow genome replication. By deleting genes coding for one or more of the structural proteins, progeny virus particles cannot be produced. Structural genes can then be replaced by genes of experimental interest (e.g. reporter genes) or by genes of therapeutic interest.

Viral RNA-based replicons have clear safety advantages as compared with DNA-based systems, avoiding the potential risk for insertional mutagenesis (see also Section 4). Cytoplasmic replication also contributes to higher expression efficiencies. However, RNA molecules are more fragile than DNA molecules as a result of their sensitivity to ribonucleases.

3.1.1 Naked RNA replicons

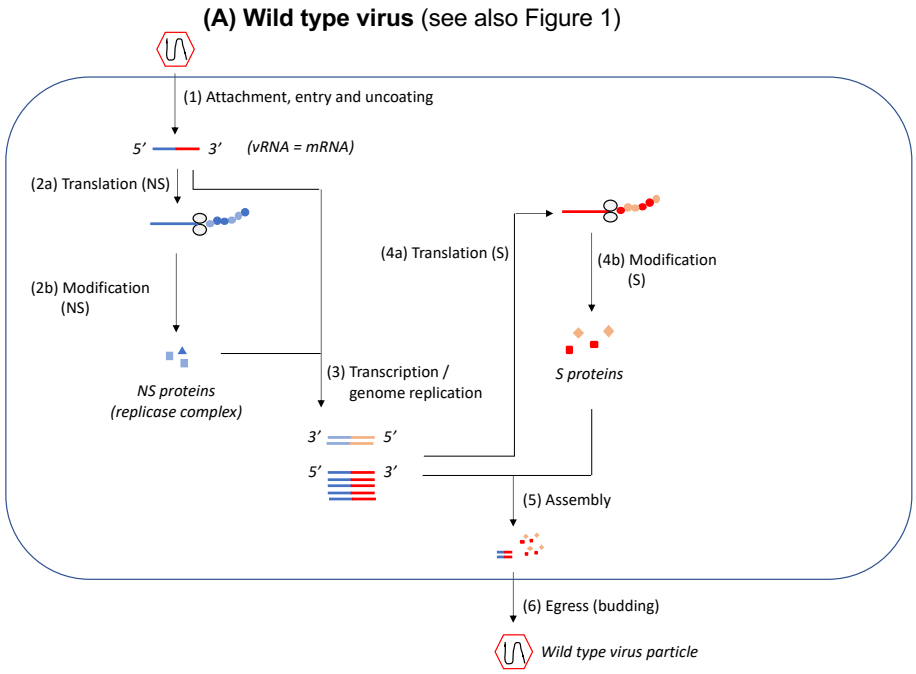
Naked RNA is the simplest form in which the replicon can be delivered into the target cell. In brief, an ***in vitro* transcribed 'naked' viral replicon RNA** will, as such, be transfected into susceptible cells, e.g. by electroporation. Once introduced into the cell, the replicon RNA hijacks the host cell's translation machinery to produce nonstructural proteins, and, where relevant, proteins as encoded by a transgene of interest. Newly transcribed, nonstructural proteins then assemble to form the replicase complex together with host cell factors, allowing replicon RNA to replicate.

Figure 3 provides a schematic comparison of a typical transfection of cells with a viral replicon based on a positive-sense RNA virus, as compared to a wild type positive-sense RNA virus (see also Figure 1).

An alternative approach is based on synthetic cDNA containing DNA polymerase promotors and terminators, the viral sequences required for transcription and replication, and if relevant, the transgene of interest. Upon delivery of the cDNA into host cells containing a DNA polymerase, cDNA will be converted into RNA for subsequent transcription and translation of the RNA replicon, which is referred to as a **DNA-launched replicon (DREP)**.

The use of naked RNA replicons allows to study genome replication and, consequently, the impact of e.g. viral inhibitors or cellular factors on genome replication. They do not allow the study of other steps in the viral life cycle such as viral entry or egress.

Although direct electroporation or transfection of cells with naked RNA is feasible in *in vitro* circumstances, it is less suitable when applying the replicon *in vivo*, taking the high sensitivity of RNA to destruction into account. In that case, naked RNA replicons can be packaged in non-viral (synthetic) particles such as lipid nanoparticles, that will help to ensure that the RNA is protected from nucleases, and ultimately will increase the likelihood that the replicon reaches the target tissue (reviewed by Rodriguez-Gascon et al. (2014)).



	(A) Infection of a cell with a wild type virus	(B) Transfection of a cell with a naked replicon
1	Attachment, entry and uncoating of the virus	Transfection of RNA
2	Translation of vRNA (~ mRNA) into nonstructural proteins	Translation of vRNA (~ mRNA) into nonstructural proteins
3	Genome replication and synthesis of additional viral RNAs coding for structural and nonstructural proteins	Genome replication and synthesis of additional viral RNAs coding for nonstructural proteins
4	Translation of RNA into structural proteins	/
5	Assembly of viral proteins and RNA into a new viral particle	/
6	Egress of the infectious virus particle	/

Figure 3. Schematic representation of a wild type infection with an enveloped, positive-sense RNA virus (A), compared to the transfection of cells with a viral replicon based on a positive-sense RNA virus (B). (vRNA: viral RNA; mRNA: messenger RNA; NS: nonstructural; S: structural)

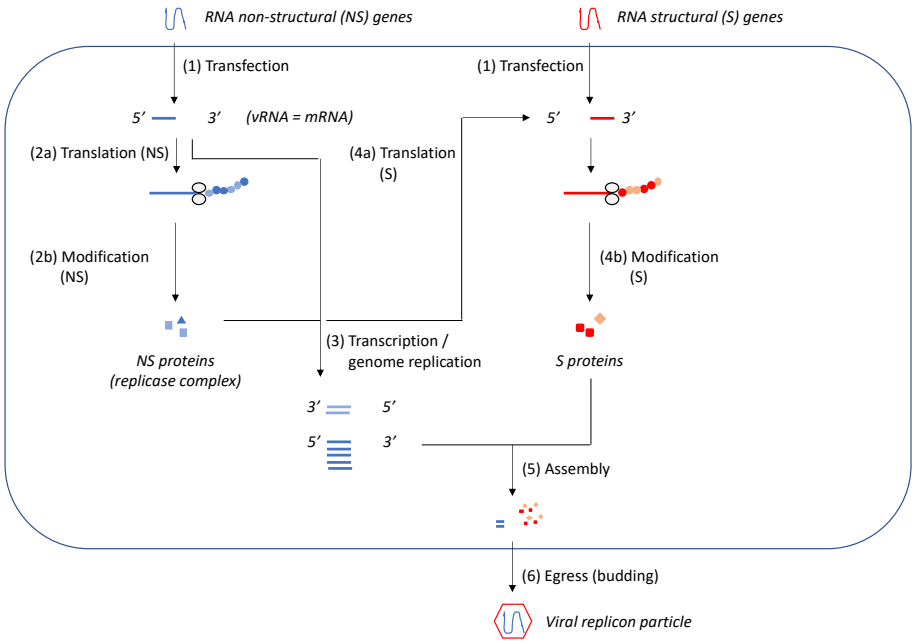
3.1.2 Viral replicon particles

By providing the genetic elements coding for missing structural proteins *in trans*, either by transfecting the cells with the RNAs (or cDNAs) coding for the structural proteins (Figure 4B), or by using cells previously transfected to express the structural proteins (Figure 4B), replicon RNA can be packaged into **viral replicon particles or VRP** [13]. By modifying the factors involved in packaging of the genetic elements coding for the structural proteins, packaging of these particular RNAs may be prevented (see Section 4.2.1).

Upon release, the VRPs are able to subsequently infect a host cell but cannot generate infectious progeny viruses as they lack the genetic elements encoding the structural proteins. Figure 5 provides a schematic comparison of a typical infection of cells with a VRP based on a positive-sense RNA virus, as compared to a wild type positive-sense RNA virus (see also Figure 1).

VRPs allow to study genome replication, but also other steps in the viral life cycle such as viral entry or egress can be addressed.

(A1) VRP production - Non-replicating helper RNA



[13] The use of heterologous viruses to complement for missing structural proteins remains outside the scope of the current report

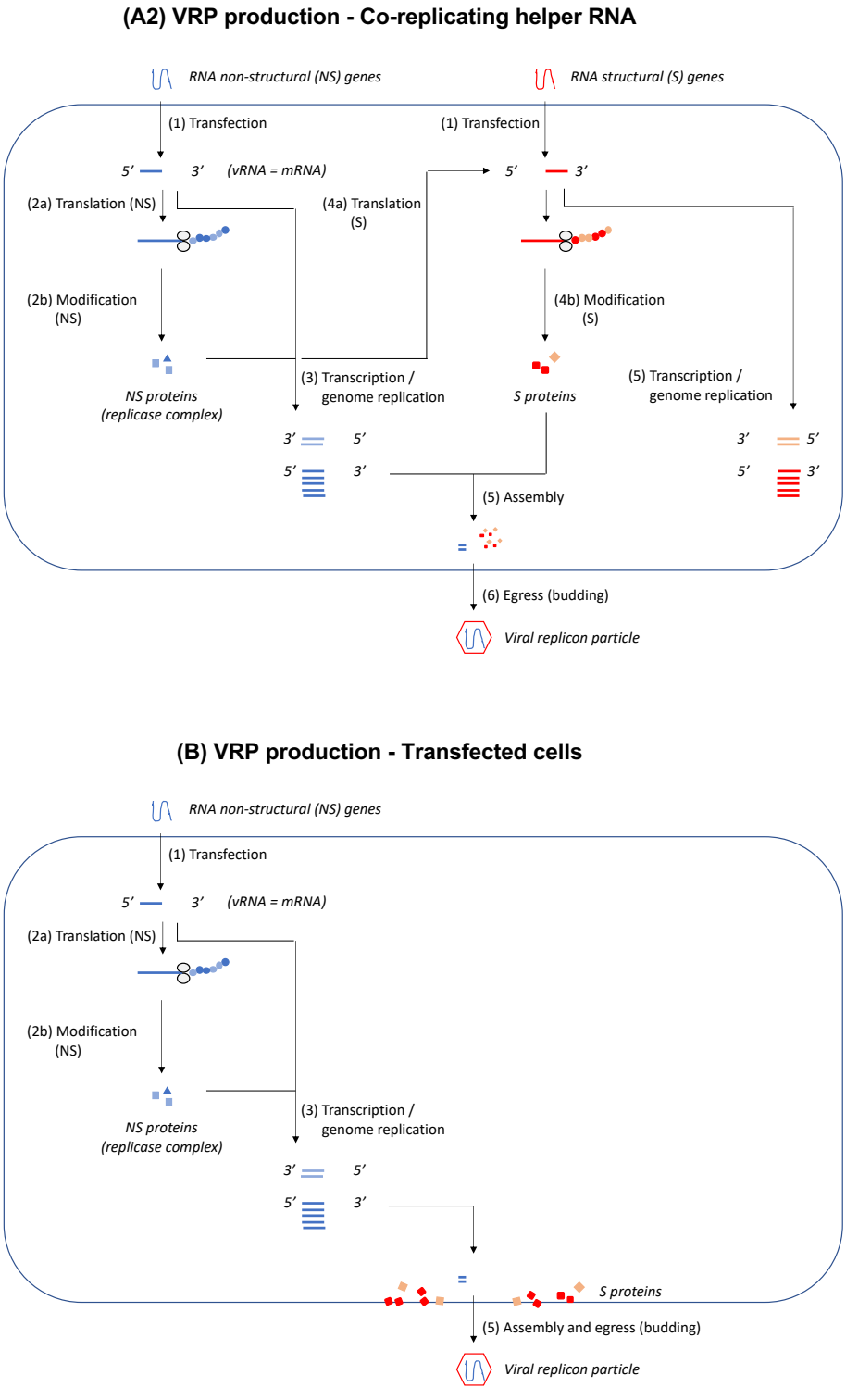
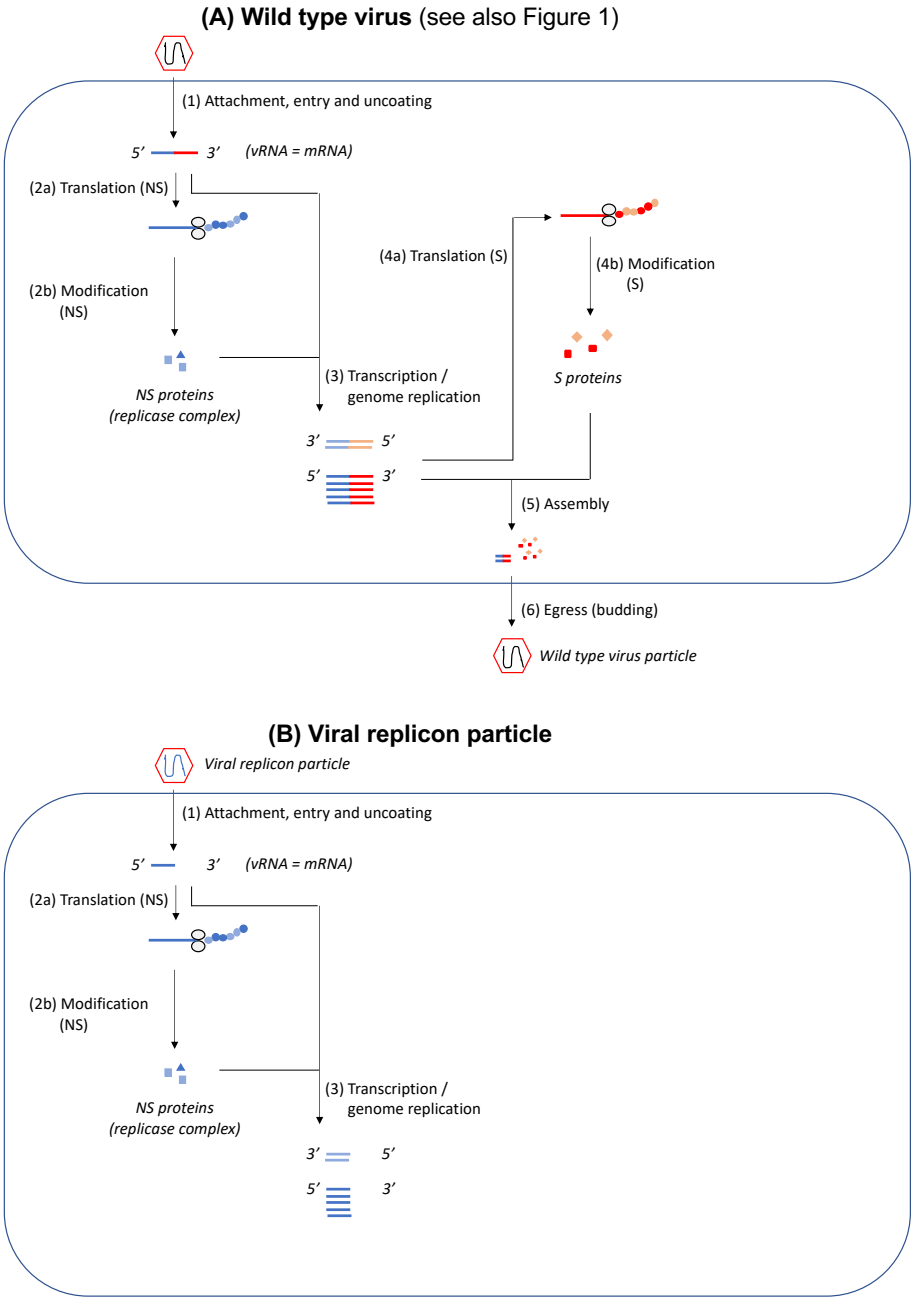


Figure 4. Schematic examples of the production of VRPs based on an enveloped, positive-sense RNA virus using non-replicating (A1) or co-replicating (A2) helper RNA, or using cells expressing structural proteins (B). (vRNA: viral RNA; mRNA: messenger RNA; NS: nonstructural; S: structural)



	(A) Infection of a cell with a wild type virus	(B) Infection of a cell with a viral replicon particle
1	Attachment, entry and uncoating of the virus	Attachment, entry and uncoating of the VRP (transduction)
2	Translation of vRNA (~ mRNA) into nonstructural proteins	Translation of vRNA (~ mRNA) into nonstructural proteins
3	Genome replication and synthesis of additional viral RNAs coding for structural and nonstructural proteins	Genome replication and synthesis of additional viral RNAs coding for nonstructural proteins
4	Translation of RNA into structural proteins	/
5	Assembly of viral proteins and RNA into a new viral particle	/
6	Egress of the infectious virus particle	/

Figure 5. Schematic representation of an infection with a wild type positive-sense RNA virus (A), compared to the infection of cells with a viral replicon particle based on a positive-sense RNA virus (B) (vRNA: viral RNA; mRNA: messenger RNA; NS: nonstructural; S: structural)

3.2 Application of viral RNA replicons

3.2.1 Replicons in research

3.2.1.1 Study viral (genome) replication

Several viruses do not amplify efficiently in cell culture. To overcome the need to use wild type virus particles to study genome replication, a self-replicating naked RNA replicon of the virus can be created and transfected in a suitable cell line.

These systems have been developed for e.g. poliovirus (Kaplan and Racaniello, 1988), Semliki Forest virus (SFV) (Liljeström and Garoff, 1991), Kunjin virus (KUNV) (Khromykh and Westaway, 1997), hepatitis C virus (HCV) (Lohmann et al., 1999), hepatitis E virus (HEV) (Cao et al., 2018a; Cao et al., 2018b) and norovirus (Chang et al., 2006). The resulting cell line, stably expressing the replicon RNA, can subsequently be used to study the effect of viral gene expression on intracellular events, as well as to study antiviral drug discovery, in particular on substances that affect viral genome replication. In these cellular systems, no structural proteins are provided *in trans*, as they do not aim to generate VRPs.

3.2.1.2 Study of highly infectious viruses at lower containment level

Due to their high pathogenicity and the limitations in the prevention or treatment of infection, viruses of pathogenicity class (or risk group) 4 can only be handled in BSL-4 containment conditions. In order to allow research on these highly pathogenic agents at a lower containment level, various replicon systems have been generated (reviewed by Hoenen et al. (2011)). This is the case for multiple human RG4 viruses belonging to e.g. *Arenaviridae*, *Phenuiviridae, Hantaviridae, Paramyxoviridae* and *Filoviridae*. For RG4 viruses of veterinary importance, RNA replicons (sometimes also referred to as minigenomes [14]) have been designed for food and mouth diseases virus and classical swine fever virus (CSFV).

Also for risk group 3 agents, such as for *Flaviviridae* (reviewed in Kummerer (2018)) and, very recently for the severe acute respiratory syndrome corona virus type 2 (SARS-CoV-2) (Zhang et al., 2021), RNA replicon systems have been developed to allow research at a lower containment level.

3.2.1.3 Antiviral drug discovery

A replicon-based assay allows for a high-throughput approach to test large libraries of compounds. In general, structural genes are replaced by reporter genes within the replicon RNA, after which cells are transfected with naked replicons or infected with a VRP to allow genome replication and, in case structural proteins are provided *in trans,* viral egress and a subsequent single cycle of infection. A potential inhibitory effect of drug substances can be assessed by means of reduced expression of the reporter protein.

It remains outside the scope of this report to describe each system in detail. For that, the reader is referred to scientific reviews such as, but not limited to, Hoenen et al. (2011) and Fernandes et al. (2020a).

3.2.1.4 Cell modification in vitro

Viral RNA replicons have also been used for cell modification and differentiation *in vitro*. Replicons originating from *Paramyxoviridae* have been used to make induced pluripotent stem cells (iPSCs). In brief, a measles virus (MV)-based replicon has been generated to introduce reprogramming factors such as OCT4, SOX2, KLF4, or cMYC in cells. Replicon genomes are subsequently eliminated from the derived iPSCs, taking their transient nature into account, leaving a viral free cell system for research (Wang et al., 2019).

Also, Sendai virus (SeV) vectors have been shown to efficiently generate human iPSCs from human fibroblasts (Fusaki et al., 2009) and human blood cells (Seki et al., 2012). Here, the issue was raised of the sustained cytoplasmic replication of the Sendai viral vectors after the iPSCs have been

[14] Whereas the terms ‘replicon’ and ‘minigenome’ have been used interchangeably in literature, minigenomes refer to self-amplifying RNA as well as non-amplifying RNA, whereas RNA replicons by default refer to self-amplifying RNA.

established. Several approaches have been explored to overcome this sustained replication, as discussed further in Section 4.1.3.

Since replicons are able to modify cells but do not bear the risk of insertional mutagenesis, as compared to their DNA counterparts, they are promising tools in the generation of stem and progenitor cells for future treatment of genetic and degenerative disorders.

3.2.2 Human and verterinary medicinal applications of replicons

3.2.2.1 Vaccination against infectious disease

Protection against infection relies on the priming of the immune system towards components of the infectious agent. Replicons can be exploited in several ways to prime the immune system. Their main advantage over conventional (i.e. nonreplicating) mRNA is that antigen expression is proportional to the number of RNA transcripts whereby conventional mRNA may thus require large doses or repeated administrations. RNA replicons overcome this limitation.

Administration of replicon RNA can be achieved either as VRPs, liposome-encapsulated particles (e.g. liposomes, dendrimers), naked RNA replicons or synthetic cDNA. Such vaccination has resulted in transient expression of the gene of interest, strong immune responses in various animal models, and protection against viral challenge.

A detailed overview of all replicon systems used for vaccine development remains outside the scope of this report. For that, the reader is referred to scientific reviews such as, but not limited to, Bloom et al. (2020), Hikke and Pijlman (2017), and Lundstrom (2020). Several replicon systems intended to be used as vaccine against infectious disease are currently being explored in clinical trials (reviewed by Blakney et al. (2021)).

3.2.2.2 Gene therapy and anti-cancer treatment

Instead of delivering genes encoding proteins of infectious agents, replicons can also be used to deliver other genes of interest such as in the context of gene therapy and anti-cancer treatment. Due to their cytoplasmic localization and self-replicating nature, significantly elevated and prolonged levels of transgene expression can be achieved without the risk of genome integration.

Most current applications are related to cancer treatment. For example, viral replicons can be used to deliver transgenes that, in turn, can affect tumour growth and/or attract cytolytic effector cells. This has been explored for an SFV-based DREP encoding human papillomavirus (HPV) early proteins E6 and E7 (van de Wall et al., 2018). Using intradermal delivery followed by electroporation, it was demonstrated that the replicon induced effective, therapeutic antitumor immunity (van de Wall et al., 2018). Also, injection of SFV based replicons coding for anti-programmed death ligand 1 (aPDL1) monoclonal antibodies (Ballesteros-Briones et al., 2019) or a Sendai or KUNV-based replicon encoding mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hoang-Le et al., 2009; Inoue et al., 2008) resulted in a potent anti-tumour response in *in vivo* models.

Finally, a mengovirus-based replicon has been used to modify tumour environment in the liver by inducing local recruitment of innate immunity effectors to the tumour which complemented the effect of peptide-based vaccines in tumour regression (Couty et al., 2008).

Apart from delivering a gene of interest, replicons can also be used as delivery vehicles for small RNAs (sRNAs) that target specific promotor regions. Hereby, their non-integrating nature and possibility of inducing short-term sRNA expression is of major importance. The sRNAs will in turn induce long-term transcriptional gene regulation mechanisms, including transcriptional gene silencing or transcriptional gene activation, with subsequent long-term therapeutic effects for various diseases. Alternatively, clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9) can be delivered by RNA replicons with the same aim of genome editing. The application of replicons as delivery vehicle for sRNAs and CRISPR/Cas has been reviewed by Baltusnikas et al. (2018, 2019).

In view of cancer treatment, some replicon systems can also be used because of their intrinsic oncolytic properties. For example, RNA replicons derived from poliovirus, showed a direct oncolytic effect on tumour cells of various origin *in vitro* based on their cytopathic effects (Ansardi et al., 2001). The oncolytic effect was confirmed *in vivo* where it was additionally shown that the replicons not only exerted an effect at the site of inoculation but were also able to reach tumour cells that had metastasized from the initial site of implantation. The effective distribution after *in vivo* inoculation

establishes the therapeutic potential of poliovirus replicons for a variety of cancers. Also, for vesicular stomatitis virus (VSV) replicons, an intrinsic oncolytic effect was demonstrated without vector-associated toxicities. Moreover, a preferred genome replication in tumour cells, due to significantly attenuated antiviral responses in these cells, further supports the safety of the VSV replicon application (Shinozaki et al., 2004; Shinozaki et al., 2005). Also, for recombinant SFV particles (recSFV) and naked viral RNA replicon promising results have been obtained in view of cancer therapy because they ensure a high level of transgene expression, a rapid and strong cytopathic effect (Vasilevska et al., 2012), and an upregulation of interferon-stimulated genes (Ballesteros-Briones et al., 2019).

Several replicon systems intended to be used as anti-cancer therapy are currently being explored in clinical trials (reviewed by Lundstrom (2019) and Blakney et al. (2021); Komdeur et al. (2021)).

3.3 Summary

In the sections described above, examples were provided on the different applications of replicons. It is clear that replicons can cover a broad range of techniques and applications, all aiming to harness the power of replication competency while limiting the potential hazards associated with the wild type virus. As a conclusion of this section, we present a summary of applications of the different replicons in Table 3**.**

Table 3. Application of viral RNA replicons (non-limitative overview)

Classification	Family	Virus [15]	Study viral (genome) replication	Study of highly infectious viruses	Antiviral drug discovery	Cell modification *in vitro*	Vaccination against infectious disease	Gene therapy / anti-cancer therapy	References (non-limitative list of (review) articles)
							(Pre-) Clinical*	(Pre-) Clinical*	
Positive-sense, non-segmented	*Astroviridae*	Human astrovirus	✓						Lulla and Firth (2020)
	Caliciviridae	Norovirus (Norwalk virus)	✓		✓				Chang et al. (2006); Fernandes et al. (2020b); Hanneman (2020)
	Coronaviridae	Middle East Respiratory Syndrome coronavirus		✓	✓				Chen et al. (2021)
		Severe acute respiratory syndrome corona virus	✓	✓	✓				Hertzig et al. (2004); Wang et al. (2008); Fernandes et al. (2020)
		Severe acute respiratory syndrome corona virus 2		✓	✓				Kotaki et al. (2020); Zhang et al. (2021)
	Flaviviridae	Bovine viral diarrhoea virus	✓				✓		Behrens et al. (1998); Hikke and Pijlman (2017)
		Classical swine fever		✓		✓	✓✓		Hikke and Pijlman (2017); Bloom et al. (2020); Lundstrom (2020); Blakney et al. (2021)
		Dengue virus	✓	✓	✓	✓			Kummerer (2018); Fernandes et al. (2020); Hannemann (2020)
		Hepatitis C virus	✓	✓	✓	✓			Fernandes et al. (2020); Ward et al. (2020)
		Japanese encephalitis virus	✓	✓	✓	✓	✓✓		Li et al. (2013); Yun et al. (2016); Kummerer (2018)
		Kunjin virus		✓	✓		✓✓	✓✓	Khromykh and Westaway (1997); Hanneman (2020); Lundstrom (2020)

[15] Wild type and/or vaccine strain, see Table 2

Classification	Family	Virus [15]	Study viral (genome) replication	Study of highly infectious viruses	Antiviral drug discovery	Cell modification *in vitro*	Vaccination against infectious disease	Gene therapy / anti-cancer therapy	References (non-limitative list of (review) articles)
							(Pre-) Clinical*	(Pre-) Clinical*	
		Niénokoué virus		✓					Kummerer (2018)
		Tick-borne encephalitis virus		✓			✓✓		Bloom et al. (2020)
		West Nile virus		✓	✓	✓	✓✓		Scholle et al. (2004); Hikke and Pijlman (2017); Fernandes et al. (2020)
		Yellow fever virus		✓	✓	✓		✓✓	Kummerer (2018); Fernandes et al. (2020): Lundstrom (2020)
		Zika virus		✓	✓	✓			Kummerer (2018); Fernandes et al. (2020)
	Hepeviridae	Hepatitis E virus	✓		✓	✓			Lohmann et al. (1999); Emerson et al. (2004); Fernandes et al. (2020a)
	Picornaviridae	Mengovirus	✓					✓	Couty et al. (2008)
		Poliovirus	✓		✓			✓	Kaplan and Racaniello (1988); Ansardi et al. (2001); Fernandes et al. (2020)
		Foot-and-mouth disease virus		✓					Tulloch et al. (2014)
		Enterovirus 71	✓						Xiong et al. (2017); Fernandes et al. (2020)
		Coxsackie virus			✓				Fernandes et al. (2020)
		Hepatitis A virus			✓				Fernandes et al. (2020)
		Human rhinovirus			✓				Fernandes et al. (2020)
	Togaviridae	Salmon pancreas disease virus					✓		Hikke and Pijlman (2017); Fernandes et al. (2020)
		Chikungunya virus		✓	✓		✓✓		Pohjala et al. (2011); Lundstrom (2020)
		Semliki forest virus	✓				✓	✓✓	Zhang et al. (2014); Hikke and Pijlman (2017);

Classification	Family	Virus [15]	Study viral (genome) replication	Study of highly infectious viruses	Antiviral drug discovery	Cell modification *in vitro*	Vaccination against infectious disease	Gene therapy / anti-cancer therapy	References (non-limitative list of (review) articles)
							(Pre-) Clinical*	(Pre-) Clinical*	
									Bloom et al. (2020); Lundstrom (2019); Lundstrom (2020); Komdeur et al. (2021)
		Sindbis virus	✓				✓	✓	Pan et al. (2005); Hikke and Pijlman (2017); Lundstrom (2020)
		Venezuelan equine encephalitis virus		✓			✓✓	✓✓	Hikke and Pijlman (2017); Bloom et al. (2020); Lundstrom (2019); Lundstrom (2020); Blackney et al. (2021)
		Western equine encephalitis virus		✓	✓		✓		Hannemann (2020); Lundstrom (2020)
		Eastern equine encephalitis virus		✓			✓		Lundstrom (2020)
	Chimeric	Sindbis virus/ Venezuelan equine encephalitis virus					✓✓		Bloom et al. (2020); Blackney et al. (2021)
		Sindbis virus/ Western equine encephalitis virus(/Eastern equine encephalitis)					✓		Atasheva et al. (2009)
		Vesicular stomatitis virus/ Chikungunya virus						✓	Lundstrom (2020)
Negative-sense, non-segmented	*Bornavirida*	Bornavirus	✓			✓			Daito et al. (2011); Ikeda et al. (2016)
	Filoviridae	Ebolavirus	✓	✓					Hoenen et al. (2011); Tao et al. (2017)
		Marburg virus	✓	✓					Mühlberger et al. (1998); Hoenen et al. (2011)
	Paramyxoviridae	Measles virus	✓			✓	✓	✓✓	Wang et al. (2019); Lundstrom (2019); Lundstrom (2020)
		Parainfluenza virus					✓		Hara et al. (2013)
		Nipah virus		✓	✓				Freiberg et al. (2008)

Classification	Family	Virus [15]	Study viral (genome) replication	Study of highly infectious viruses	Antiviral drug discovery	Cell modification *in vitro*	Vaccination against infectious disease	Gene therapy / anti-cancer therapy	References (non-limitative list of (review) articles)
							(Pre-) Clinical*	(Pre-) Clinical*	
		Human metapneumovirus	✓						de Graaf et al. (2008)
		Respiratory syncytial virus	✓		✓				Duvall et al. (2016); Hanneman (2020)
		Newcastle Disease Virus					✓		Viktorova et al. (2018)
		Sendai virus	✓			✓		✓	Bitzer et al. (2003); Inoue et al. (2008); Ban et al. (2011); Park et al. (2016)
	Rhabdoviridae	Vesicular stomatitis virus	✓				✓✓	✓	Pattnaik et al. (1992); Hikke and Pijlman (2017); Lundstrom (2019); Lundstrom (2020);
Negative-sense, segmented	*Arenaviridae*	Lassavirus	✓	✓	✓		✓		Hass et al. (2004); Hoenen et al. (2011); Lundstrom (2020)
		Junin virus	✓	✓					Hoenen et al. (2011)
		Tacaribe virus	✓	✓					Hoenen et al. (2011)
		Machupo virus	✓	✓					Hoenen et al. (2011)
	Hantaviridae	Hantaan virus		✓					Flick et al. (2003)
	Nairovirodae	Crimean-Congo hemorrhagic fever virus	✓	✓	✓				Hoenen et al. (2011)
	Phenuiviridae	Huaiyangshan banyangvirus (also known as 'severe fever with thrombocytopenia virus')	✓	✓					(CGM/130502-01); Hoenen et al. (2011); Brennan et al. (2015)
		Rift Valley fever virus	✓	✓			✓	✓	Oreshkova et al. (2015); Hikke and Pijlman (2017)
		Uukuniemi uukuvirus	✓						Hoenen et al. (2011)

* ✓: Pre-clinical trials only; ✓✓ Clinical trials

4 Risk considerations for replicon systems

This section focuses on specific considerations that (may) affect the risk of replicon systems for the user and/or the environment. While reviewing the different considerations, it must be stressed that for each consideration, the relevance for the risk assessment will depend on the type of replicon system and its application.

Additionally, it is noted that this report focusses on the hazards of the replicon system itself. When transgenes are inserted in the replicon RNA, these transgenes and their expression products may additionally affect the hazards of the system (e.g. oncogenes, toxins). The assessment of the transgene, however, is not part of the current report.

4.1 Naked replicons

Naked replicons (see also Section 3.1.1) comprise the genetic information for the nonstructural proteins and, if relevant, for some of the structural proteins in case they contain important elements necessary for efficient RNA replication and/or other sequences required for nonstructural protein- or transgene expression. Consequently, transfection of cells with naked RNA allows for genome replication, but not for generation of replicon particles.

4.1.1 Genome integration

Upon cell infection, some viruses integrate their genome into the host chromosome, either as part of their life cycle (e.g. retroviruses), or accidentally. Genome integration may not only potentially promote long-term persistence of a virus in the cell, it could also lead to drastic consequences for the host cell, such as gene disruption, insertional mutagenesis and/or cell death. For DNA viruses, the genome forms a potential substrate for host genome integration, without the need for prior processing. However, most RNA viruses are not able to deliberately integrate their genome into the host chromosome, as their genetic information resides in RNA molecules and not DNA. The only exception are retroviruses, because they possess the ability to transcribe their viral RNA genome into DNA, a mandatory step for productive retroviral infection. For all other RNA viruses, genome integration has not been reported or has been reported only as an incidental event (Desfarges and Ciuffi, 2012). No data were found on the actual consequences of such rare integration events. Furthermore, no data were found on the likelihood for and the potential consequences of integration of replicons based on cDNA (DREP).

4.1.2 Persistence in the host cell

Several RNA viruses have developed specific mechanisms, e.g. at the level of viral transcription and replication, that could lead to the establishment of persistent infection. To follow-up viral genome replication and to screen drug compounds *in vitro*, this feature has been exploited in RNA replicon systems based on, amongst others, HCV (Lohmann et al., 1999), SARS-CoV (Hertzig et al., 2004), norovirus (Chang et al., 2006) and HEV (Cao et al., 2018a). In brief, cells are stably transfected with replicon RNA carrying a drug resistance gene, after which RNA genome replication is maintained in the cell.

For other applications, for example when cells will be used for additional applications such as stem cell therapy, or when subsequent activities have to be performed at a lower containment level, prolonged presence of the RNA replicon is undesired. In these cases, conditional RNA replicon replication provides a means to control replicon expression and potential persistence in the cell. Different approaches have been described, as illustrated below.

Ban et al. (2011) described a modified SeV replicon system in which temperature-sensitive mutations were introduced, so that remaining viral replicon-related genes are removed when maintaining cells at non-permissive temperatures. Also, the use of miRNA or small interfering (si)RNA provides an interesting means to conditionally express replicon RNA. For example, a SeV-based replicon was

extended with a sequence encoding a micro (mi)RNA targeting the RNA-polymerase L gene (coding for RNA polymerase L) (CGM/130329-01).

To control replicon replication, additionally the use of riboswitches has been studied. In brief, a riboswitch is a regulatory segment of a mRNA molecule that binds a specific small molecule, such as a therapeutic compound, resulting in a change in production of the proteins encoded by the RNA. For example, for alphaviruses Bell and colleagues (Bell et al., 2015) incorporated a riboswitch into the replicon 3' UTR. By doing so, they could successfully regulate expression of DNA-launched and VRP-packaged replicons. When integrating riboswitches into the 3' and 5' UTR of the subgenomic RNA region of the TC-83 strain of Venezuelan equine encephalitis virus (VEEV), replication could be regulated at an even greater fold. The results clearly indicate that riboswitches hold promise for development of novel vaccination strategies, whereby gene expression can be controlled in such a way that only one injection will allow for priming and boosting of the immune response. Also for gene therapy or cancer treatment, regulated expression of replicons will have clear benefits (Ketzer et al., 2014).

Rather than including an element for conditional replication in the replicon system itself, also external control factors can be used. These include but are not limited to treatment of replicon-infected cells with siRNA as was described for SeV-based replicons (CGM/130329-01). Alternatively, cells can be treated with specific small molecules that can eliminate replicons from the cells, as was described for Borna virus-based replicons (Komatsu et al., 2019).

4.1.3 Generation of virus-like vesicles

Above it is mentioned that the risk of the transgene remains outside the scope of this study. However, using a sequence of certain viral envelope proteins as transgene in a naked replicon is still considered relevant for this report, since it may change the characteristics of the replicon itself. Indeed, it may allow for the formation of so-called virus-like vesicles (VLV).

In brief, upon their expression, the envelope proteins support the formation of vesicles in which the replicon RNA is packaged and that can spread from cell to cell. This has been described when using a transgene coding for the G protein of VSV, a member of the *Rhabdoviridae*, in VEEV-based replicons or SFV-based replicons (*Togaviridae*). VLVs with RNA replicon genomes were generated that could spread throughout a cell culture (Rolls et al., 1994; Rose et al., 2014; van den Pol et al., 2017). The majority of the infectious particles were smaller and less dense than either VEEV or SFV particles. Characterization by electron microscopy showed membrane-enveloped vesicles that contained the VSV-G protein. Infectious particles were apparently generated by budding of vesicles containing VSV-G protein and the RNA replicon (Rolls et al., 1994). Similarly, VLVs were generated when an envelope protein of murine leukemia virus was expressed by a SFV replicon (Rolls et al., 1994). From these studies, it is concluded that formation of VLVs cannot be excluded when the coding sequence for (homologous or heterologous) envelope proteins are used as transgene (ZKBS, 2017).

4.1.4 Effects on non-targeted cells

Replicons could potentially exert (negative) effects on cells that are not targeted for the delivery of the gene of interest. In order to limit the replicon genome replication and transgene expression to targeted cells or tissues, different approaches have been described. One such approach is by making use of miRNAs, which are involved in silencing gene expression. miRNAs bind to their cognate target RNA which possesses a miRNA-binding sequence generally referred to as the miRNA Recognition Element or MRE. Subsequently, the miRNA will silence the target RNA. Lee et al. (2010) used this process to engineer a DENV-based replicon that could not replicate in the liver. Hereby a liver-specific MRE was inserted into the viral mRNA. Binding of tissue-specific miRNA, as present in the liver, with the MRE incorporated in the replicon RNA conferred an inhibitory effect on the replicon RNA.

4.2 Viral replicon particles

The risk considerations as mentioned for naked replicons are also relevant for VRP. The sections below elaborate on additional risk considerations that have to be taken into account when producing and/or applying VRP.

4.2.1 Generation of replication competent viruses during production: 'primary' RCV

In the creation/production of VRP there is a stage in which different viral genetic elements come together to assemble the infectious particle. In that stage, there is a potential risk for recombination with the formation of 'primary' RCV. For non-segmented single-stranded RNA viruses, recombination may theoretically occur between replicon RNA and co-replicating helper RNA (recombination is even less likely in case of non-replicating helper RNA). For segmented, single-stranded negative-sense RNA viruses, recombination may theoretically occur between the transcription plasmids and the expression plasmids when homologous sequences are present. Depending on the virus family, the likelihood for recombination may differ. Based on that, different strategies have been developed to reduce the likelihood of such recombination, as explained below.

A. Using bipartite helper RNA system

For those replicon systems where structural genes are provided via helper RNA, such as but not limited to those generated from *Togaviridae*, the likelihood of recombination can be reduced by dividing the genetic elements coding for the structural proteins over multiple helper RNAs. Hereby, the rationale is that increasing the number of helper RNAs, increases the number of recombination events that are required to generate a full, functional viral genome. This is clearly illustrated in a study by Pushko et al. (1997) where a monopartite and a bipartite helper RNA system were used for a VEEV replicon. In the monopartite system, where complementing genes were present on only one helper RNA, plaque formation indicative for recombination was found for different types of helper RNAs used, even when only very limited sequence homology was present with the replicon RNA. On the other hand, when using the bipartite system where the complementing genes were divided over two helper RNA, no infectious virus was detected in the plaque assay.

Similar observations were made for Sindbis virus (SINV), where no recombinants were detected using one helper RNA coding for the capsid protein and a second helper RNA coding for viral membrane proteins (Frolov et al., 1997). These results were further confirmed in later studies by Hyvärinen et al. (2013). It is highlighted that in a bipartite system, single recombinations may still occur but these will not result in plaque forming virus since only either capsid or membrane proteins will be included (Pushko et al., 1997).

Beissert and colleagues (2020) developed a novel bipartite vector system using trans-amplifying RNA. In brief, an alphavirus replicon encoding a particular transgene is used, but rather than including all nonstructural genes of the alphavirus, the genetic sequence encoding replicase is deleted from the replicon. Replicase activity is subsequently provided *in trans* by an optimized non-replicating mRNA (nrRNA). In case the replicon would be complemented with genes encoding structural proteins, it would still lack replicase activity and thus be unable to induce a productive infection in cells without the nrRNA.

Blakney et al. (2018) developed a system called splitzicon. Hereby, the genes encoding the nonstructural proteins and the gene of interest are located on separate RNA molecules, but still exhibit the self-amplification properties of replicon RNA.

B. Limitation of sequence homology between genetic elements

Whether or not recombination will occur, at least in part depends on the amount of sequence homology between the genetic elements. As a rule of thumb, the higher the homology between sequences, the higher the likelihood for recombination. Therefore, limiting sequence homology will reduce the likelihood of recombination. Depending on the replicon system, limitation of sequence homology can be situated at different levels. Representative examples are described below.

For replicons based on VEEV, as an example of positive-sense, single-stranded RNA viruses, it was shown that the rate of recombination in a monopartite helper RNA system was inversely related to the size of deletion in the nonstructural protein genes in the helper RNA (Pushko et al., 1997). However, even when the helper RNA contained less than 1 kb of nonstructural gene sequence, recombinants were detected, as evidenced by the generation of plaques in a plaque assay. The authors suggested that the likelihood for recombination was not only determined by the amount of sequence homology, but that also specific sequences in the helper RNA may have affected the rate

of nonhomologous recombination independently of deletion size, although the sequences of the recombinants were not determined.

Sequence homology can also be diminished by introducing mutations in coding sequences for the structural genes. In this perspective, Widman et al. (2008) used a VEEV replicon to supplement the genetic information for the C protein in a West Nile virus (WNV) genome containing a 70-codon deletion in the C protein. With the aim of producing VRPs but avoiding recombination between the modified WNV genome and the VEEV replicon, 36 silent mutations were incorporated in the C gene, thereby generating a sequence that was no longer complementary to the WNV genome and thus preventing replication of a recombinant genome.

For negative-sense, single-stranded segmented RNA viruses such as the *Phenuiviridae*, recombination at the level of the UTR is a specific risk consideration. Their UTRs direct replication and transcription of viral RNA and are sufficient to allow encapsidation of viral RNA into ribonucleoprotein complexes (Barr et al., 2003; Dunn et al., 1995; Osborne and Elliott, 2000). Their terminal panhandle sequences are conserved between the different genome segments (Lowen et al., 2005). In the context of replicons, it is to be considered that when segments previously and deliberately made devoid of their UTR reobtain a homologous or heterologous UTR by recombination, such may result in autonomous replicating virus particles. For example, in an RVFV-based replicon system where an expression plasmid coding for the M ORF (no M-UTR) is co-transfected with transcription plasmids coding for the S or L segment, recombination may allow for the M ORF to obtain a UTR of the S or L segment and consequently allow replication and encapsidation of the M segment. The above-mentioned recombination events have never been described in nature, taking into account that the likelihood for a single cross-over in two RNA segments is extremely low. Moreover, in the unlikely and mainly theoretical event that such recombination would occur, the resulting virus which harbors two genome segments with the same UTR, is likely attenuated, as was demonstrated in experimental studies using reverse genetics for Bunyamwera virus (Lowen et al., 2005).

C. Avoiding packaging of genetic elements coding for structural proteins in VRPs

Depending on the virus family, different elements are involved to ensure that all genetic elements are packaged into new virus particles such as but likely not limited to specific packaging signals (reviewed by e.g. Mendes and Kuhn (2018), and Twarock and Stockley (2019)), specific RNA-protein interactions (reviewed by e.g. Hornak et al. (2016)) or UTRs (Osborne and Elliott, 2000). During infection with a wildtype virus, these elements ensure that the various viral components are included in the virus particle, thereby releasing a fully infectious virus able to enter and replicate in new host cells. As a biological containment measure for replicons, it is reasoned that when the elements are removed or modified, the related RNAs will not be packaged into the VRP, and, consequently, the VRP itself will not harbor these RNAs and thus cannot express the encoded proteins. Depending on the replicon system, interference with the elements involved in packaging can be situated at different levels. Representative examples are described below.

For those replicon systems where structural genes are provided via a co-replicating helper RNA, the likelihood of packaging of the helper RNA into the VRP can be reduced by not providing a packaging signal in the co-replicating helper RNA. That modifying packaging signals may result in hampered packaging, has been demonstrated for RNA originating from SFV. Liljeström and Garoff (1991) described defective helper RNA, derived from SFV RNA, that lacked a large portion of the nonstructural region, including the region corresponding to the packaging signal identified for SINV. This defective helper was able to package SFV replicons but was not packaged itself. Similar results were obtained by White et al. (1998). On the other hand, for replicons generated from SINV, it was shown that even a co-replicating helper RNA lacking a packaging sequence is still co-packaged at a rate of about 1/300 of that of single RNAs containing a packaging sequence (Frolova et al., 1997; Lu and Silver, 2001). Of particular interest herein is the C protein. Packaging of the co-replicating helper RNA encoding the C protein without the packaging signal may, at least in part, be the result of the presence of other sequences in the C protein that support packaging (Frolova et al., 1997). Even when using a C protein from a different alphavirus, formation of particles containing the co-replicating helper RNA could not be prevented (Frolov et al., 1997)

As explained above, for negative-sense, single-stranded segmented RNA viruses such as the *Phenuiviridae*, it is particularly relevant to remove the UTRs of the genome segments that are not to

be packaged into the VRP, taking into account that presence of the UTR is sufficient to allow encapsidation of that genome segment into ribonucleoprotein complexes (Osborne and Elliott, 2000).

D. Reducing the hazard of potentially formed RCV

Whereas the previous approaches address the likelihood of recombination, safety of the replicon can be enhanced by modifying the nonstructural and/or structural proteins in such a way that in the unlikely event an RCV is formed, it does not exhibit hazardous characteristics.

For example, for SFV-based replicons, mutations were introduced that result in removal of protease activity of the capsid protein, so that in case of recombination of helper and replicon RNAs, the newly formed viral particle remains replication defective (CGM/111222-02). Also for SFV-based replicons, mutations were introduced in the cleavage site of the precursor protein p62 (Berglund et al., 1993). The rationale was to construct a cleavage-deficient variant of the precursor protein that would not be cleaved into envelope proteins without external addition of chemotrypsin and thus would render the particles non-infectious unless chemotrypsin is externally added. The resulting RCV indeed turned out to be largely non-infectious, due to reduced binding and uptake into endosomes as well as the impaired ability to induce membrane fusion in the endosome. Based on these results the authors concluded that a p62 cleavage-deficient helper function could be used to enhance the biosafety of the SFV replicon system.

Apart from introducing mutations in the structural proteins, an alternative approach is the use of structural proteins of a less pathogenic virus homologue. Provided that the less pathogenic phenotype is associated with attenuating mutations in genes encoding structural proteins, the VRPs generated are likely to display the biological properties of that virus, rather than that of the more pathogenic virus. For a VEEV-based replicon, Pushko et al. (1997) described the use of helper glycoprotein genes derived from V3014, a recombinant VEE vaccine strain, which contain two strongly attenuating mutations. V3014 is avirulent upon subcutaneous inoculation of mice (Grieder et al., 1995) and horses (Smith et al., unpublished observations), and therefore, any recombinant generated during VRP packaging is very likely to be at least as attenuated as V3014.

Similarly, structural genes of less pathogenic heterologous viruses can be used, thereby generating chimeric viruses. As an example, it is referred to a VEEV-based replicon, whereby structural proteins of SINV were provided *in trans* (CGM/060314-01; Perri et al., 2003). In comparison to VEEV, SINV is less pathogenic to humans. Also, despite extensive use, no laboratory acquired infections with SINV have been described that could be assigned to airborne transmission, whereas this has been described for VEEV (Pedrosa and Cardoso, 2011; Rusnak et al., 2018).

Finally, also the genetic sequence of nonstructural genes can be modified to ensure that RCV, if formed, are not hazardous. Such was described for e.g. RVFV, whereby the coding sequence for the NSs protein in the S genome segment was deleted (Kortekaas et al., 2011). NSs is a non-essential protein, but it is involved in the blockage of interferon gene expression and thus plays an important role in virulence (Billecocq et al., 2004; Bouloy et al., 2001; Ikegami et al., 2006).

E. Conditional generation of VRPs

As mentioned for the naked replicons, also production of VRPs can be made conditional, as illustrated below.

Harvey et al. (2004) generated a stable BHK packaging cell line, carrying a Kunjin structural gene cassette under the control of a tetracycline-inducible promoter. In the presence of tetracycline, no structural proteins were produced. Withdrawal of tetracycline from the medium resulted in production of Kunjin structural proteins that were capable of packaging transfected and self-amplified Kunjin replicon RNA into secreted virus-like particles. The same cells were also capable of packaging replicon RNA from closely (WNV) and distantly (DENV-2) related flaviviruses.

For alphavirus replicons it was shown that by incorporating miRNA-specific target sequences (tissue-specific MRE) into the helper RNAs, VRP were found to be efficiently produced if miRNA-specific inhibitors were introduced into cells. However, in the absence of such inhibitors, cellular miRNAs were capable of downregulating helper RNA replication and consequently inhibited VRP production (Kamrud et al., 2010).

4.2.2 Testing for the absence of 'primary' RCV

Formation of RCV during production of VRP is a major risk consideration. Still, only limited data are available on assays and/or limits of detection to provide evidence for successfully avoiding the presence of RCV. Table 4 provides an overview of the assays described to determine whether RCV have been generated, as identified during the current study. In brief, data on RCV testing were identified for 5 out of 34 identified replicon systems based on positive-sense, single-stranded RNA viruses (including 1 chimeric system), 1 out of 14 identified replicon systems based on non-segmented, negative-sense, single-stranded RNA viruses, and 1 out of 10 identified replicon systems based on segmented, negative-sense, single-stranded RNA viruses.

The amount of data is rather scarce as compared to those for replication-deficient viruses, such as retro/lentiviruses and adeno-associated viruses, for which multiple guidelines have been developed. In brief, the FDA Guidance for Industry "Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up", provides recommendations regarding the testing for RCV during the manufacture of retroviral vector-based gene therapy products, and during follow-up monitoring of patients who have received retroviral vector-based gene therapy products. Recommendations include the identification and amount of material to be tested as well as general testing methods.

The requirement for testing adenovirus vectors for presence of replication competent adenovirus (RCA) is described in Ph. Eur. 5.14 and the FDA guidance document "Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) - Guidance for Industry" (2020). No acceptance criteria are specified in Ph. Eur. 5.14, but the FDA guidance document and the FDA BRMAC meeting number 30 (Adenovirus Titer Measurements and RCA levels, 2001) clearly state that the test is reported as "no RCA detected" if it complies with the acceptance criterion of <1 RCA/3 $\times$ 10^{10} VP.

Table 4. Examples of assays for the verification of RCV presence (non-limitative list)

Classification	Family	Virus [16]	Assay for RCV	Results	Detection limit	Reference
Positive-sense, non-segmented	*Flaviviridae*	Kunjin virus	Microscopy (detection of E protein by immunofluorescence during 2 passages in Vero cells)	No fluorescence detected	Not described	Harvey et al. (2004)
			Intracranial inoculation of suckling mice	No clinical signs upon inoculation with 4 x 10^6 IU	Not described	
	Picornaviridae	Poliovirus	Intraspinal or intracranial inoculation of mice	No neurovirulence	Not described	Bledsoe et al. (2000a); Bledsoe et al. (2000b)
	Togaviridae	Semliki Forest virus	Microscopy (infect BHK-21 cells and titrate supernatant in a PFU assay)	No PFU found in 4.6 x 10^9 particles from 13 independent SFV-lacZ recombinant stocks (frequency of recombination < 4.6 x 10^9)	Not described	Smerdou and Liljestrom (1999)
		Venezuelan equine encephalitis virus	Microscopy (detection of syncytia upon 2 passages in Vero cells)	No RCV detected	1 RCV / 1x10^{10} gg- replicon particles	(CGM/170224-01)
			Microscopy (detection of syncytia upon 2 passages in Vero cells)	No RCV detected	< 100 RCV / 1x10^8 gg-replicon particles	Pushko et al. (1997)
	Chimeric	Sindbis virus/ Venezuelan equine encephalitis virus	Microscopy (detection of syncytia upon 5 passages in BHK cells)	No RCV detected	Not described	(CGM/060314-01)
			Reporter assay (test supernatant of 5 passages in BHK cells for β-galactosidase expression)	No RCV detected in 40 batches	Not described	
Negative-sense, non-segmented	*Paramyxoviridae*	Sendai virus	Microscopy (detection of syncytia), and	Absence of syncytia	Not described	(CGM/130329-01)
			qPCR (detection of NP mRNA), and	Negative	2 - 3 positive cells	

[16] Wild type and/or vaccine strain, see Table 2

Classification	Family	Virus [16]	Assay for RCV	Results	Detection limit	Reference
			Fluorescence microscopy (detection of fluorescent labeled antibodies to NP protein)	No fluorescence detected	Not described	
Negative-sense, segmented	*Phenuiviridae*	Rift Valley fever virus	Microscopy (detection of fluorescence during 2 passages in BHK cells)	No fluorescence detected upon inoculation of cells at an m.o.i. of 1	Not described	Kortekaas et al. (2011)
			Inoculation of suckling mice	No clinical signs upon inoculation with 1.0×10^4 $TCID_{50}$	Not described	Dodd et al. (2012)

E: envelop; IU: infectious units; BHK: baby hamster kidney; PFU: plaque forming units; RCV: replication competent virus particles; qPCR: quantitative PCR; NP: nucleoprotein; mRNA: messenger RNA; m.o.i.: multiplicity of infection; TCID: tissue culture infectious dose

4.2.3 Generation of virus-like vesicles

When using a sequence of certain viral envelope proteins in helper RNA (or cDNA), generation of VLVs is a risk consideration during the production of VRPs, similar as described for the naked replicons (see Section 4.1.3).

4.3 Generation of replication competent viruses during application: 'secondary' RCV

Also secondary events could lead to generation of RCV, more specifically recombination with or complementation by related viruses and/or unrelated viruses present in the host *in vitro* or *in vivo* during application of replicons.

In nature, recombination events do not appear to be a hallmark of negative-sense, single-stranded RNA viruses (Han and Worobey, 2011). While sporadic authentic examples indicate that homologous recombination does occur, recombination seems to be generally rare or even absent in most negative-sense RNA viruses, and most of the homologous recombination events reported in the literature were likely generated artificially. Natural recombination has, however, been described for positive-sense, single-stranded RNA viruses, where it is an important mechanism for promoting genetic variation. For example, for flaviviruses, recombination has been described under natural circumstances for DENV (e.g. Chen et al. (2008); Tolou et al. (2001); Worobey et al. (1999)). For Western equine encephalitis virus (WEEV) it is believed that it is a naturally occurring recombinant between SINV and Eastern equine encephalitis virus (EEEV) (Hahn et al., 1988). Also, natural recombination amongst enteroviruses (Combelas et al., 2011; Muslin et al., 2019) and amongst coronaviruses (Su et al., 2016) is frequently observed. Hereby, recombination of enteroviruses, in particular poliovirus, contributed to the emergence of pathogenic circulating vaccine-derived polioviruses that have been complicating the World Health Organization (WHO) program for the global eradication of poliomyelitis (Muslin et al., 2019), whereas recombination of coronaviruses may have contributed to the emergence of the pandemic SARS-CoV-2 virus (Zhu et al., 2020).

Experimental conditions using cellular systems, have confirmed and provided further insights in the recombination events. However, it is important to notice that during the systemic literature search only one publication was identified in which recombination events were mimicked for viral RNA replicons (Taucher et al., 2010). The authors described a system based on self-replicating subgenomic RNAs (replicons) derived from tick-borne encephalitis virus (TBEV), WNV, and Japanese encephalitis virus (JEV) for investigating the ability of the flavivirus genomes to recombine. They showed that, despite the fact that the replicon pairs shared approximately 600 nucleotides of identical sequence where a precise homologous crossover event would have yielded a wild-type genome, this was not observed. Only two aberrant recombination events were detected, both of which yielded unnatural genomes containing duplications. Moreover, infectious clones of these genomes yielded viruses with impaired growth properties. For TBEV and WNV, their systems did not yield any viable recombinant genomes at all. These data indicate that even in the case of high sequence homology, recombination was a rare event and resulted in impaired viruses only. The impaired character of chimeric viruses was also demonstrated for *Togaviridae* (Kuhn et al., 1996; Paessler et al., 2003; Schoepp et al., 2002) .

Taking the above into account, recombination between replicon and (un)related viruses cannot be excluded. However, for recombination to happen, several conditions have to be fulfilled. For example, replicon RNA and wild type virus have to be present in the same host cells. Therefore, if replicon and wild type viruses enter different host cells, either because of differences in tropism or differences in route of application (e.g. injection in view of vaccination versus natural infection), it is unlikely that recombination or complementation will occur. The likelihood of recombination or complementation is even further diminished if genome replication or the RNA replicon takes part in different cellular compartments as compared to the wild type virus replication. Finally, even if replicon RNA and wild type virus are able to infect the same host cell and replicate in the same cellular compartment, a phenomenon called super-infection exclusion (also referred to as trans-inhibition or homologous interference) may prevent replication of the wild type virus if the cell is already infected by the replicon. The phenomenon has been described for various single-stranded RNA viruses that are used for viral replicon design, including poliovirus (Kaplan and Racaniello, 1988), alphaviruses (Karpf et al., 1997), MV, and HCV. The mechanism of exclusion has been observed at various steps

of the viral life cycle, including attachment, entry, viral genomic replication, transcription, and exocytosis.

Even though the data above indicate that the likelihood of recombination with or complementation by (un)related viruses is low, it may not be able to exclude the event. Therefore, precautionary measures have to be implemented mainly aiming at minimizing the risk of contact between replicon-containing materials or individuals on the one hand and the (un)related viruses on the other hand. In brief, measures can include but are not necessarily limited to selection of cells, experimental animals and/or clinical trial subjects free of the (un)related viruses as tested by means of serological testing and/or polymerase chain reaction (PCR).

5 Risk classification and containment

5.1 Case studies

5.1.1 Positive-sense, single-stranded RNA virus: VEEV replicons

The case study on risk classification and containment for VEEV replicons as a representative example of positive-sense, single-stranded RNA viruses, is based in 8 advices by COGEM (see Table 1). The evolution of the assessment over the years provides a unique illustration of the factors that are deemed important for defining the risk classification and for the conditions that are imposed for safe work.

Wildtype VEEV belongs to pathogenicity class 3, as it is a mosquito-borne viral pathogen organism affecting all equine species (horses, donkeys, and zebras), as well as humans. COGEM (CGM/190905-02) classified the VEEV strain TC-83 in pathogenicity class 2 based on the fact that the strain was attenuated by *in vitro* cultivation in guinea pig heart cells and deployed as a vaccine (Berge et al., 1961).

The first opinion (CGM/090603-01) covered the use of so-called null-replicon particles based on VEEV, further defined as containing only genes coding for nonstructural proteins and not carrying any transgene. During production of the null-replicon particles, which was not performed in the Netherlands and therefore not subject of the opinion, structural proteins were provided by two helper plasmids. The helper plasmids were devoid of packaging signals and could therefore not be assembled in the null-replicons. Finally, some of the structural proteins carried mutations resulting in attenuation of VEEV.

COGEM specifically considered:

- recombination during production resulting in primary RCV, in particular the fact that non-homologous recombination sporadically had led to RCV;
- dispersal via the natural vector and via aerosols;
- complementation or recombination with a related virus resulting in secondary RCV.

Since the risk for humans and the environment of the intended activities was deemed to be negligibly small, the COGEM advised to allow downscaling the containment requirements to BSL 2 (according to classification in the Netherlands ML-II and DM-II), with some specific additional measures to minimize possible exposure.

In 2015, in response to a proposal for generating “iPS cells by a synthetic Self-Replicative RNA”, the COGEM further refines its advice **(CGM/150715-01)**. In this case the replicons are produced via *in vitro* transcription from a DNA template. Only the sequences coding for the four nonstructural VEEV proteins and for human transcription factors were present. Furthermore, the replicon had two internal ribosome entry site sequences (IRES) and a puromycin resistance gene. There were no packaging signals. Reviewing the previous assessment, COGEM highlighted:

- the negligible risk for complementation or recombination with a related virus;
- the unlikely persistence of the replicon in the transfected cells;
- the unlikely occurrence of dispersal in the cell culture via exosomes.

For this example of a naked RNA replicon, COGEM advised downscaling the containment requirements to BSL 1 (according to classification in the Netherlands ML-I and DM-I).

The next evaluation (CGM/160815-01) concerned the production and experimental use of a VEEV-replicon in which a protein of the porcine epidemic diarrhea virus (PEDV) was introduced with the intention to vaccinate pigs. The attenuated vaccine strain TC-83 was used in which the genes coding for structural proteins were replaced either by a gene coding for Green fluorescent protein (GFP) or for a gene coding for the S protein of PEDV. The replicons were subsequently produced by co-transfecting animal cells with the replicon RNA and two helper RNAs, one with the gene coding for the C protein of VEEV and the other with genes coding for glycoproteins E1 and E2 of VEEV. The helper RNAs did not contain genes coding for nonstructural proteins or the packaging signal. Furthermore, molecular adaptations were made to the helper RNAs including removal of the 26S

promoter element, introduction of stop codons at the 5' and 3' UTRs, as well as introduction of a mutation in the capsid gene. Finally, the production cells were free of wild-type VEEV.

In this case, the COGEM stressed:

- the presence of the S protein potentially influences the tropism of the VRP;
- the low probability of recombination during production resulting in primary RCV, given the limited sequence homology between the different RNAs and the additional molecular modifications;
- the RCV test results confirm that the likelihood is low, yet the test had not been properly described (e.g. detection limit);
- the negligible risk for complementation or recombination with a related virus resulting in secondary RCV;
- the negligible risk for complementation or recombination with wildtype PEDV.

Given that the absence of RCV had not been demonstrated convincingly, COGEM advised to conduct the activities at BSL 2 (according to classification in the Netherlands ML-II and DM-II), provided that the production cells would be free of VEEV and related viruses. Furthermore, COGEM also indicated that further downscaling could be envisaged when more information on the RCV testing was provided. Given the large number of tests already conducted (over 2000 batches had been assessed showing RCV absence) COGEM considered that it would not be necessary to test each batch provided the production process remained unchanged.

In an additional advice on the same application requested by the "Bureau GGO" (CGM/160906-02), COGEM highlighted:

- the lower intrinsic pathogenicity of the vaccine VEEV strain TC-83;
- the fact that the replicons are not capable of producing infectious particles;
- the very low probability of primary RCV formation during production;
- the fact that if RCV would be produced, they would be highly attenuated as compared to wild type VEEV.

In 2017, the same replicon was assessed based on additional information provided by the applicant (CGM/170224-01). This advice was also expected to be relevant for other applications (e.g. other inserts) of the same platform technology. Further information had been provided on the RCV-test based on two passages on Vero-cells, the validation of this RCV test and on the absence of biodistribution and shedding of a similar vaccine (VEEV replicon with a gene from swine influenza virus).

Based on this information, COGEM:

- was satisfied with the demonstrated quality of the RCV test, thereby allowing downscaling to BSL-1 conditions;
- accepted the biodistribution and shedding information to justify the downscaling of the housing of treated pigs from level DM-II to DM-I (and, after 7 days post injection, D-I);
- confirmed that even a challenge with PEDV of vaccinated animals 14 days after vaccination would not result in simultaneous presence of the replicon and the challenge agent.

Importantly, COGEM also indicated that the risk for the occurrence of RCV is not related to the type of insert, the animal species or the application route. Of course, the cells and animals have to be free of VEEV and related viruses.

In contrast to the previous advices, COGEM seemed to stress the need for demonstrating the absence of RCV by an RCV-test on every batch. This was further clarified in an additional advice **(CGM/170322-03)**, in which COGEM pointed out that such tests are common practice for 'quality control' and 'good practice', but that in order to ensure safety for humans and the environment with the specific VEEV-replicon vector system sufficient evidence had been provided and further testing was not necessary to allow the downscaling to BSL 1 (according to classification in the Netherlands ML-I and DM-I). It must be kept in mind that these levels still impose certain restrictions to safeguard humans and the environment.

Finally, this additional advice specifies that the downscaling is only allowed as long as the inserts do not revert the limiting effect of the deletions in the replicon. This could be the case for sequences of virus species of the *Togaviridae* or the *Rhabdoviridae*. E.g. expression of the G-protein of VSV or

Vesicular stomatitis Indiana virus (VSIV) in similar alphavirus replicon systems can lead to formation of VLV capable of distribution from cell to cell.

The different elements discussed in the above cases are summarized in Figure 6.

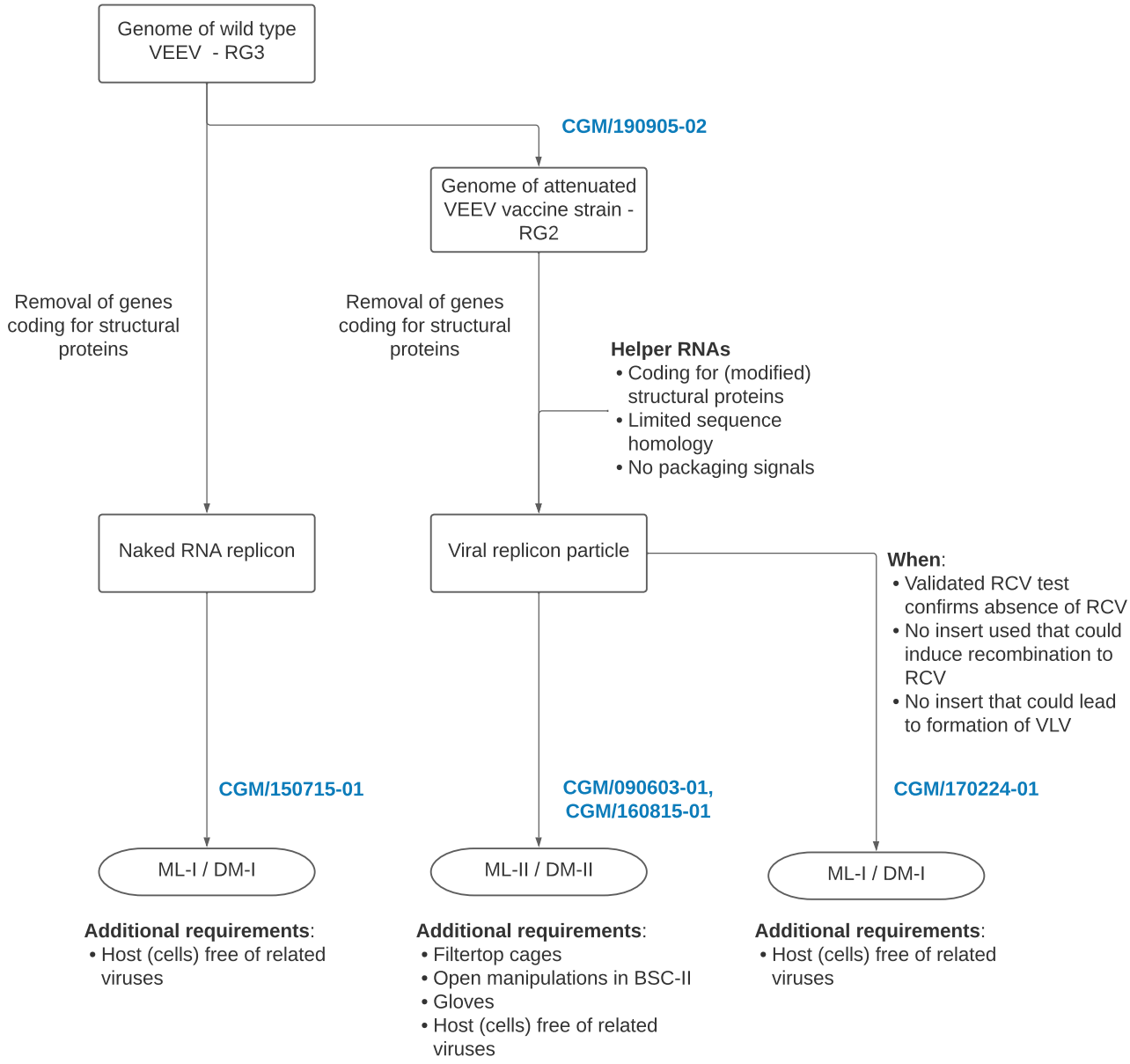


Figure 6. Schematic representation of elements taken into account by COGEM for downscaling of containment measures (ML/DM) for different applications of VEEV replicons (VEEV: Venezuelan equine encephalitis virus; RG: risk group; RCV: replication competent virus particle; ML: microbiologisch laboratorium; DM: dierverblijf met micro-organismen; BSC: biosafety cabinet)

The conclusions of the VEEV-replicon opinions are further exploited in COGEM advice (CGM/171030-03), which deals a.o. with VEEV-replicon based on attenuated strain TC-83 with “aquatic” donor sequences, referring to sequences coding for proteins of pathogenic viruses of fish such as the Infectious salmon anemia virus, Tilapia lake virus, Red sea bream iridovirus, Scale drop disease virus, Piscine orthoreovirus and the betanodaviruses.

The risk for RCV creation during production of the VEEV replicon is deemed to be negligibly low, provided that the cells are free of VEEV and related viruses. Therefore, it is also not necessary to

conduct RCV-test from a safety perspective. The creation of VLVs is also deemed highly unlikely. In consequence, these activities can be conducted at BSL 1 (according to classification in the Netherlands ML-I and DM-I).

In the same period, COGEM was asked to advise on an application for alphavirus-replicons (including VEEV) with donor sequences of influenza viruses, human respiratory syncytial virus (RSV), Marburg- and Ebolaviruses **(CGM/171024-01)**. In this evaluation, COGEM addresses the likelihood and consequences of the creation of VLV. Both are highly speculative and the COGEM repeated that BSL 1 (according to classification in the Netherlands ML-I and DM-I) would be adequate. Nevertheless, COGEM agreed that the containment proposed by the applicant, i.e. BSL2 (according to classification in the Netherlands ML-II and DM-II), is a suitable precautionary action. Some additional conditions included using gloves, performing "open" manipulation in a biosafety cabinet (BSC) type II, ensuring that the material is free of wildtype VEEV and related viruses, and housing animals in filter top cages.

5.1.2 Positive-sense, single-stranded RNA viruses: SFV and SINV replicons

The case study on risk considerations for SFV and SINV replicon systems as another representative example of positive-sense, single-stranded RNA viruses, is based on an opinion by ZKBS (2017). Also their assessment provides a unique illustration of the factors that are deemed important for defining the risk classification and for the conditions that are imposed for safe work.

Wildtype SFV and SINV belong to pathogenicity class 2. Both viruses are spread mainly by mosquito bites (arboviruses). There is so far no evidence for human-to-human transmission. SFV is able to cause a lethal encephalitis in rodents but generally only causes mild symptoms in humans. SINV may cause a rash-arthritis syndrome in humans referred to as Pogosta disease (Finland), Ockelbo disease (Sweden), or Karelian fever (Russia).

In order to classify replicon systems based on SFV and SINV, ZKBS took following risk considerations into account:

- Formation of replication-competent VLV

As mentioned in Section 4.1.3 and 4.2.3, expression of envelope proteins, either encoded via the transgene or via helper RNA (or cDNA), allow for the formation of replication-competent VLV in which the replicon RNA is packaged and that can spread from cell to cell.

ZKBS reasoned that in the absence of coding sequences for the E (precursor) protein(s) from SFV or SINV, the risk that VLVs are formed is negligible. Thus, provided that the transgene does not code for an E (precursor) protein, naked replicons can be classified as RG1 and activities can be performed at a BSL1 containment level. On the other hand, for replicon systems that do code for an E (precursor) protein the risk of VLV formation cannot be excluded and the replicons are therefore classified as risk group 2. Activities have to be performed at BSL2.

- Formation of replication-competent infectious virus particles (RCV)

In case cells are transfected with replicon RNA and helper RNA coding for all structural proteins, recombination between replicon and helper RNA may result in infectious RCV. Therefore, such activities are to be performed in a BSL2 environment.

ZKBS allowed for downscaling to BSL1 in following situations:

- When a 3-fold mutation has been introduced in the coding sequence of the E precursor protein, that ensures that the mutated precursor protein can no longer be cleaved in envelope proteins unless chemotrypsin is actively added to the host cells. In case recombination would occur, the RCV will be non-infectious.
- When coding sequences for C and E (precursor) protein(s) are split over two helper RNAs that do not show sequence homology and thus have a negligible risk for recombination.
- For SFV only, when coding sequences for C and E (precursor) protein(s) are split over two helper RNAs that do show sequence homology, since experimental data demonstrate that the particles that may be formed upon recombination are non-pathogenic (Ruiz-Guillen et al., 2016). Since the latter has not been demonstrated for SINV, no such downscaling is warranted for similar SINV-based replicon systems.

A summary of the risk considerations and their impact on risk classification and subsequent containment measures is provided in Figure 7.

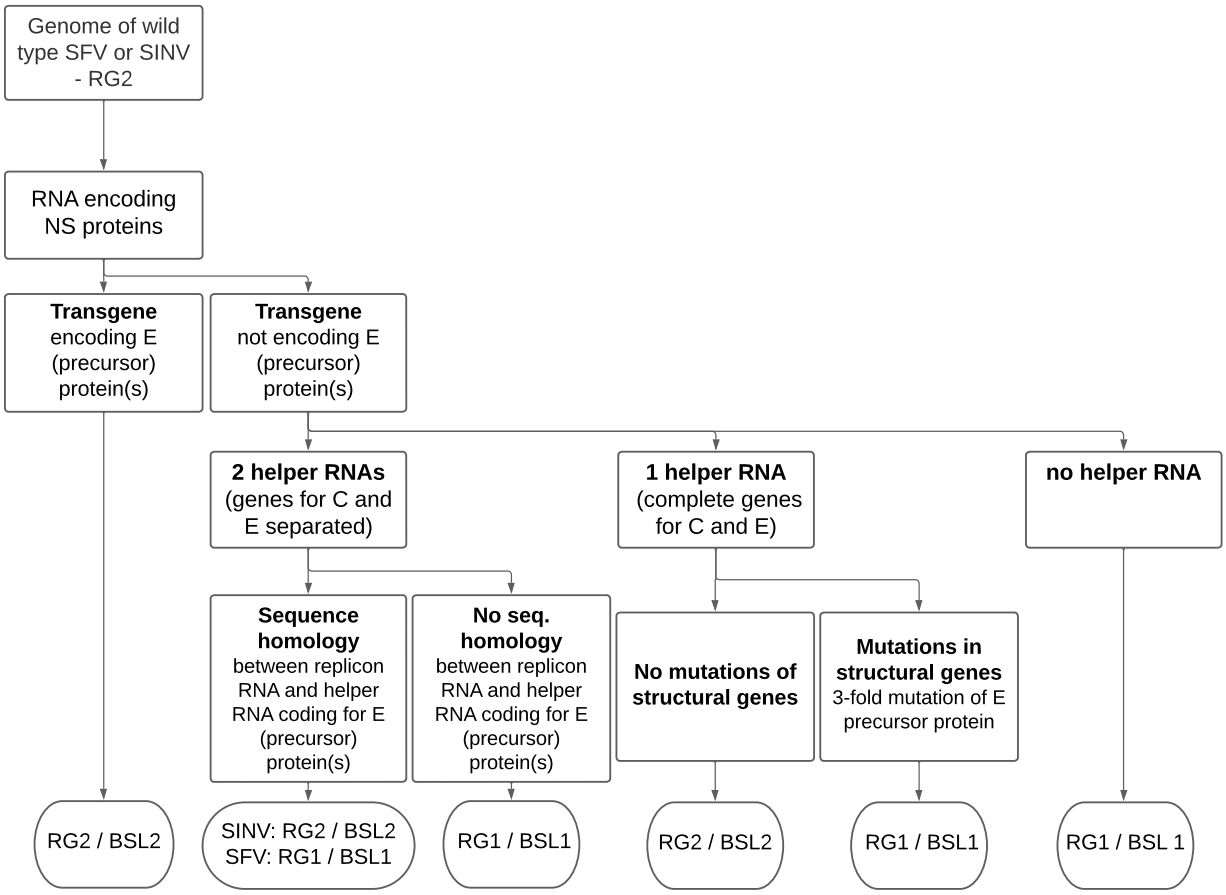


Figure 7. Schematic representation of elements taken into account by ZKBS for downscaling of risk group classification containment measures for different applications of SFV and SINV replicons (SFV: Semliki Forest virus; SINV: Sindbis virus; RG: risk group; NS: nonstructural; E: envelope; C: capsid; BSL: biosafety level)

In case the formation of VLV or RCV is identified as a risk consideration, but it can be demonstrated that no VLV or RCV have actually been formed, ZKBS highlights that a downscaling is possible. However, no recommendations are described on how to demonstrate the absence of VLV or RCV.

An additional risk consideration, not included in the table above, that was taken into account by ZKBS, was the risk group of the cells used for trans- or infection. In case the replicon itself is classified as RG 1, but the cells used to trans- or infect are of risk group 2, the containment level remains at (BS)L2. Also the impact of the transgene (other than the one described above) was considered, but remains outside the scope of the current study.

5.1.3 Negative-sense, single-stranded segmented RNA virus: RVFV replicons

The case study on risk considerations for RVFV replicons as a representative example of negative-sense, single-stranded, segmented RNA viruses, is based in 3 advices by COGEM (see Table 1) and is illustrated in Figure 8.

Wildtype RVFV belongs to pathogenicity class 3 (CGM/080313). Infection is most commonly seen in domesticated animals in sub-Saharan Africa, such as cattle, buffalo, sheep, goats, and camels. Infection of humans may occur through contact with blood, body fluids, or tissues of infected animals, or through bites from infected mosquitoes. Spread from person to person has not been documented. Although RVFV infection often causes severe disease in animals, human infection is subclinical or

accompanied with self-limiting febrile disease. Only in a minority of cases, more severe symptoms develop.

The first advise (CGM/080313-05) covered the production of RVFV genome segments in mammalian and insect cells. Hereby, maximum two out of three (incomplete or complete) genome segments, each encoded by a separate transcription plasmid, were simultaneously produced.

COGEM specifically considered:

- risk of reconstructing infectious fully virulent virus;
- risk of complementation or recombination with a related virus.

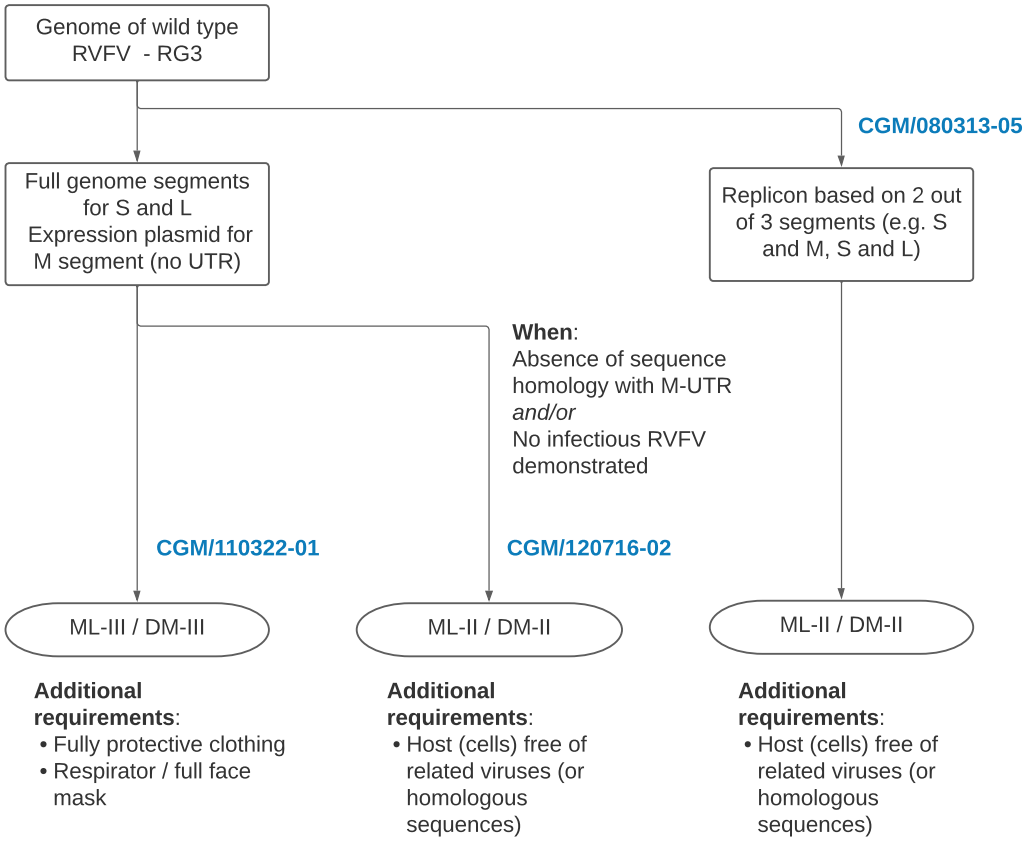


Figure 8. Schematic representation of elements taken into account by COGEM for downscaling of containment measures (ML/DM) for different applications of RVFV replicons (RVFV: Rift Valley fever virus; RG: risk group; UTR: untranslated region; ML: microbiologisch laboratorium; DM: dierverblijf met micro-organismen)

Taking into account that maximum two out of three genome segments were being used, and provided that host cells are free of related viruses that could potentially allow for recombination, the risk for humans and the environment of the intended activities was deemed to be negligibly small. Consequently, the COGEM advised to allow downscaling the containment requirements to BSL 2 (ML-II).

A second advice was issued in 2011 (CGM/110322-01). Activities described were twofold. First, it was intended to transfect animal cells with L, S or M minigenomes, as well as with a minigenome containing the coding sequence for the ectodomain of influenza virus HA or NA. Subsequently, it was aimed to vaccinate rats, mice and sheep with the produced VRPs. Second, animal cells were transfected with the L and S segment and an expression plasmid containing the coding sequence for the M segment or of unrelated glycoproteins of various viruses, with the aim to study whether these unrelated glycoproteins can complement for the M protein. The expression plasmids did not

contain a UTR and, consequently, the respective genome segments can only be expressed but not be replicated or packaged into a particle.

In case of experiments including the M segment, however, COGEM highlighted the risk for homologous recombination and subsequent generation of infectious, fully virulent RVFV. In brief, the risk could not be excluded during the proposed activities, since an M minigenome segment is used whereby it is not clear whether the M segment encoding sequences are flanked by sequences that show overlap with the M-UTRs. Since no data were provided on either the absence of sequence homology or the absence of infectious RVFV, no downscaling of containment requirements was allowed for. Experiments were to be performed at BLS3 with some specific additional measures in case of *in vivo* activities to minimize possible exposure.

It was also highlighted that there is a theoretical risk of heterologous recombination (Heinze et al., 2003; Iroegbu and Pringle, 1981). However, such recombination will most likely result in an attenuated virus (Lowen et al., 2005).

The third advice (CGM/120716-02) was related to the transfection of animal cells with the L and S segment, an expression plasmid coding for the M ORF but without the M-UTRs, as well as a minigenome containing the coding sequence for the ectodomain of influenza virus HA or NA. For these studies, the COGEM agreed to downscale to a containment level BSL2 based on the following arguments and provided that no source of the M genome segment or M-UTR is present during the experiments:

- taken the absence of the M-UTR in the expression plasmid into account, the M segment can only be expressed, not replicated or packaged into a virus particle;
- since the M segment cannot be replicated or packaged, no infectious fully virulent virus can be created;
- data are available showing lack of sequence homology that could facilitate recombination;
- data are available demonstrating the absence of infectious RVFV particles after production.

Although not an absolute requirement for downscaling, is it also described that a modified S segment is used wherein the coding sequence for NSs is deleted. Consequently, even if recombination would occur, the resulting virus will be attenuated due to the absence of NSs.

5.2 Other precedents

5.2.1 COGEM

Beside the advices on VEEV and RVFV, COGEM also provided advice on other replicon systems. An overview of the relevant advices, organized per replicon system, and the risk considerations taken into account, is provided in Table 5.

Table 5. Overview of risk considerations for various replicon systems, as included in COGEM advices

Classification	Family	Virus [17]	Applica-tion	Risk consideration in view of primary RCV formation					Other risk considerations	Down-scaling [a]	COGEM reference
				1 or 2 helper plasmids	Reduce sequence homology	Modified genes for (non) structural proteins [b]	Avoid packaging of genes for structural proteins	RCV testing			
Positive-sense, non-segmented	*Coronaviridae*	MERS CoV	VRP	*N.a. (structural proteins by cell line)*	Not specified	No	**Yes**	No	Sec. RCV	Yes (↓)	(CGM/130822-01)
	Flaviviridae	DENV	VRP	1	**Yes**	**Yes**	**Yes**	**Yes** (but unvalidated)	Sec. RCV	Yes (↓)	(CGM/050615-01)
		TBEV	VRP	**2**	**Yes**	Not specified	**Yes**	No	[c]	Yes (↓)	(CGM/190725-02)
		YFV	VRP	1	**Yes**	**Yes**	**Yes**	**Yes** (but unvalidated)	Sec. RCV	Yes (↓)	(CGM/050615-01)
		ZIKA	Naked	*N.a. (naked replicon)*	*N.a. (naked replicon)*	**Yes**	*N.a. (naked replicon)*	*N.a. (naked replicon)*	Sec. RCV	Yes (↓)	(CGM/160307-01)
	Hepeviridae	HEV	Naked	*N.a. (naked replicon)*	*N.a. (naked replicon)*	No	*N.a. (naked replicon)*	*N.a. (naked replicon)*	Sec. RCV	Yes (↓)	(CGM/121221-01)
	Togaviridae	SPDV	VRP	**2**	Not specified	**Yes**	**Yes**	No need in view of environment / human health	VLV; routes of transmission	Yes (↓↓)	(CGM/171030-03)
		SFV	VRP	1	Not specified	**Yes**	**Yes**	Literature-based	/	No	(CGM/070723-02)
			VRP	**2**	Not specified	Not specified	Not specified	Literature-based	/	Yes (↓) [d]	(CGM/030604-02)
			Naked	*N.a. (naked replicon)*	*N.a. (naked replicon)*	No	*N.a. (naked replicon)*	*N.a. (naked replicon)*	VLV	No	(CGM/170224-01)
		SINV	Naked	*N.a. (naked replicon)*	*N.a. (naked replicon)*	No	*N.a. (naked replicon)*	*N.a. (naked replicon)*	VLV	No	(CGM/170224-01)
			VRP	**2**	Not specified	**Yes**	**Yes**	**Yes** (validated)	Sec. RCV; routes of transmission	Yes (↓↓)	(CGM/190415-01)

[17] Wild type and/or vaccine strain, see Table 2

Classification	Family	Virus [17]	Applica-tion	Risk consideration in view of primary RCV formation					Other risk considerations	Down-scaling [a]	COGEM reference
				1 or 2 helper plasmids	Reduce sequence homology	Modified genes for (non) structural proteins [b]	Avoid packaging of genes for structural proteins	RCV testing			
		VEEV	VRP	**2**	Not specified	**Yes**	**Yes**	**Yes**	Sec. RCV; routes of transmission	Yes (↓)	(CGM/090603-01)
			Naked	*N.a. (naked replicon)*	*N.a. (naked replicon)*	No	*N.a. (naked replicon)*	*N.a. (naked replicon)*	Sec. RCV	Yes (↓↓)	(CGM/150715-01)
			VRP	**2**	Not specified	**Yes**	**Yes**	**Yes** (but inconclusive)	Sec. RCV	Yes (↓)	(CGM/160815-01; CGM/160906-02)
			VRP	**2**	Not specified	**Yes**	**Yes**	**Yes** (validated)	Sec. RCV; routes of transmission	Yes (↓↓)	(CGM/170224-01)
			VRP	**2**	Not specified	**Yes**	**Yes**	No need in view of environment / human health	VLV	Yes (↓↓)	(CGM/170322-03)
			VRP	*N.a. (naked replicon)*	*N.a. (naked replicon)*	**Yes**	*N.a. (naked replicon)*	*N.a. (naked replicon)*	VLV	Yes (↓)	(CGM/171024-01; CGM/180313-02)
			VRP	**2**	Not specified	**Yes**	**Yes**	No need in view of environment / human health	VLV	Yes (↓↓)	(CGM/171024-01; CGM/180313-02)
	Chimeric	SINV/VEEV	VRP	**2**	**Yes**	**Yes**	**Yes**	**Yes** (but inconclusive)	Routes of transmission	Yes (↓) [e]	(CGM/040212-02)
			VRP	**2**	**Yes**	**Yes**	**Yes**	**Yes** (but inconclusive)	Sec. RCV	Yes (↓) [e]	(CGM/060314-01)
Negative-sense, non-segmented	*Paramyxoviridae*	SeV	VRP	Not specified	Not specified	Not specified	Not specified	**Yes**	Persistent infection	No	(CGM/130329-01)
Negative-sense, segmented	*Phenuiviridae*	HYSV	VRP	*N.a.*	**Yes**	No	**Yes**	Not addressed	Sec. RCV	Yes (↓)	(CGM/130502-01)
		RVFV	VRP	*N.a.*	Not specified	No	*N.a. (max. 2 of 3 genome segments used)*	*N.a. (max. 2 of 3 genome segments used)*	Sec. RCV	Yes (↓)	(CGM/180313-02)
			VRP	*N.a.*	Not specified	No	**Yes**	No	Sec. RCV	No	(CGM/110322-01)
			VRP	*N.a.*	**Yes**	**Yes**	**Yes**	**Yes**	Sec. RCV	Yes (↓)	(CGM/120716-02)

[a] As compared to parental (non-attenuated) virus **and** provided the host (e.g. cells, animals) is free of (un)related wild type viruses to avoid formation of secondary RCV

[b] Modified either in terms of an attenuated parental virus strain as starting material or by means of deliberately modifying wt RNA

[c] As VRPs are used in virus-neutralization assays with heat-inactivated blood, there is no risk for secondary RCV

[d] Risk assessment concerned the host to which replicons have been administered and not the replicon system itself. Additionally, advice CGM111222-02 (not included in the table), addressed the potential risks of a clinical trial in humans with an SFV replicon system (downscaling of containment not relevant).

[e] As compared to the pathogenicity class of VEEV

5.2.2 Other

Most of the additional advices and recommendations, as referred to in Section 1.2, did address replicon systems, but did not focus on their risk assessment.

Most relevant information was extracted from 'The SACGM Compendium of guidance - Part 2: Risk assessment of genetically modified microorganisms (other than those associated with plants)', Section 2.12 [18], as well as from the Canadian Centre for Veterinary Biologics (CCVB) of the Canadian Food Inspection Agency, in particular from the environmental assessments of replicon-based products [19]. In general, their risk considerations were very similar to those used by COGEM (i.e. pathogenicity of parental virus, RCV formation (in R&D or when applied *in vivo*), potential impact of heterologous genes on replicon characteristics, off-target effects, and effects in non-target hosts).

In addition, SACGM and CCVB particularly referenced to the **limited genetic stability of viral RNA**. Indeed, single stranded RNA viruses are genetically unstable meaning that repeated replication of the genome can result in mutations that may result in an altered phenotype. Such is particularly relevant in *in vitro* studies using cells stably transfected with a naked RNA replicon or in case of prolonged expression in a host. Checking genetic stability was also addressed as a risk consideration in the context of the environmental assessments ensuring that the product administered to animals and potentially shed by animals is similar to the initially designed construct. Particularly the latter may, however, be less of a concern taking into account that the replicons allow for a single infection only and thus do not have the capacity to cause a productive infection with viral replication and spread. Consequently, the risk of genetic changes in the animals and subsequent selection of variants with altered characteristics seems negligible.

Also, reference was made to a potential **environmental impact** of the replicons, a topic less addressed in the COGEM advices, since the latter mainly focused on contained used applications (except for CGM/111222-02). Risk considerations in view of the environment were the potential shedding and dissemination of replicons, environmental persistence and the potential to enter the food chain.

The SACGM document furthermore highlighted following additional considerations:

- Modifications that result in attenuation *in vitro* do not necessarily reflect pathogenicity
- Particular attention should be paid to any modification or insert that may increase the virulence or pathogenic phenotype, and/or the ability to elicit or evade an immune response

Many of these considerations relate to worst case scenarios since factual information on frequencies of e.g. recombination are missing. Risk assessors may therefore take a precautionary position and overestimate the actual risk. In this respect the results of the ongoing Dutch Research Council Project 'Environmental safety of synthetic replicon particle vaccines - risk for RNA recombination with wildtype viruses (RepliSAFE)' [20] is expected to provide valuable information to further support the evaluation.

[18] https://www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp/part2.pdf
[19] https://www.inspection.gc.ca/animal-health/veterinary-biologics/environmental-assessments/merck-animal-health-s-rna-particle-prescription-pr/eng/1544130596594/1544130646137 and https://www.inspection.gc.ca/animal-health/veterinary-biologics/environmental-assessments/iped-vaccine/eng/1467040659842/1467040717031
[20] https://www.nwo.nl/en/projects/15791

6 Conclusion

This study was set up to collect information on replicon systems that are currently available or in development, and to summarize their biosafety characteristics. The overview should enable COGEM to develop a general advice that can be applied to multiple viral replicon systems, in particular allowing the risk classification and determination of the containment level of activities with replicons in contained facilities (laboratories, animal facilities).

The scientific literature illustrates that replicons have become a highly promising and widely applicable tool for basic research of pathogens with medical relevance, as well as for disease prevention and treatment. Several replicon approaches are being explored. While they differ based on the original virus (positive vs. negative strand, segmented vs. non-segmented), also the delivery mechanism as a naked replicon or as a VRP has an important impact on the risk considerations. Despite these differences, some common elements have been identified in the risk assessment as schematically represented in Figure 9.

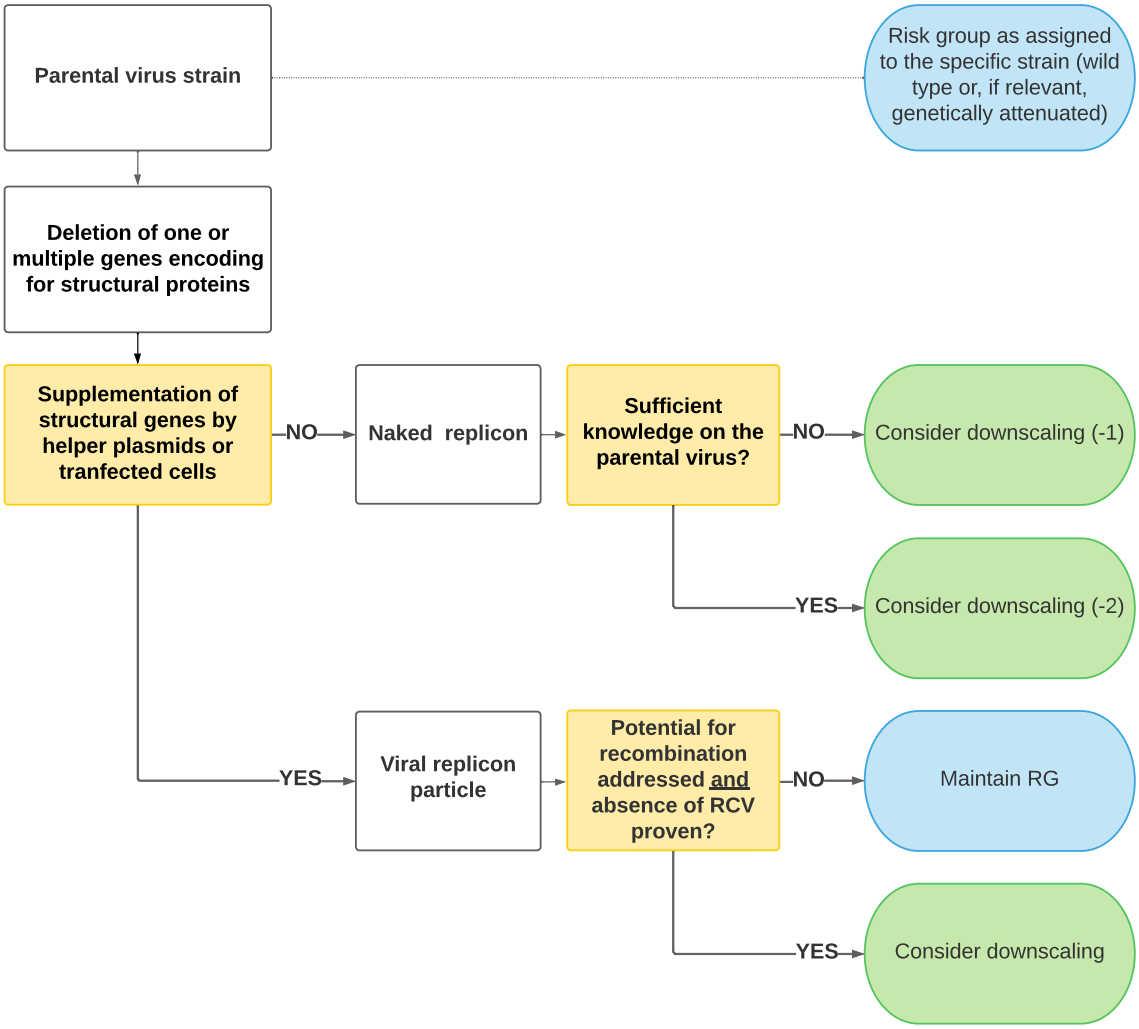


Figure 9. Summary of considerations justifying downscaling of the containment requirements of an activity with replicons (RG: risk group; RCV: replication competent virus particle)

As specified in Directive 2009/41/EC[21] and related national implementing legislation, one of the elements for assessment of the contained uses of a GMO must be the identification of any potentially harmful effect associated with the recipient organism. The importance of the hazardous features of the recipient organism results in the assignment of the **pathogenicity class of the recipient virus,** as illustrated in Table 2**.** When attenuated strains are used, their risk classification is typically lowered, due to the (highly) reduced pathogenic potential. Replicons are designed to combine additional safety features, thereby reducing even more the pathogenic nature of the original virus.

In the case of **VRP**, the coding sequences for the structural proteins and those for the nonstructural proteins are separated from each other. The resulting VRP can enter the target cell as the wild type virus would, however, while replication will occur, no new particles will be released from the cell. The risk classification of VRP *per se* can be downscaled substantially, since they have turned into single round expression systems without pathogenic characteristics. Their classification is hardly related to that of the original virus.

Nevertheless, uncertainty considerations may still limit radical downscaling:

- Potential formation of primary RCV

In the stage during the creation/production of VRP in which different viral genetic elements come together to assemble the infectious particle, there may be a theoretical (or known) possibility that recombination leads to the reconstitution of an RCV. Different approaches are discussed in the report for reducing the risk for primary RCV reconstitution. However, it remains to be highlighted that many considerations are of a theoretical nature. For example, different modifications in helper RNAs are expected to reduce the likelihood of recombination. Nonetheless, it is not clear what the exact impact is of each modification as such (e.g. partial or complete deletion of structural genes, no vs. limited sequence homology), as well as their (potential) cumulative nature (e.g. multiple plasmids and limited sequence homology). Moreover, this is likely to differ between various virus families.

- Proof of absence of RCV

Although recombination leading to primary RCV is theoretically possible, there is little evidence that this occurs at a significant frequency. Testing can provide further insights in the likelihood of recombination and the efficiency of the strategies for reducing the recombination potential.

Still, each test method has its limitations. Robust data on which test to perform and what detection limits to take into account are still limited to only a few of the viral species for which replicons have been designed.

One may question whether downscaling of the containment level for activities in which VRPs are produced, can be allowed without confirmatory testing the absence of RCV. Conversely, it can be questioned if repetitive testing is required, once it has been established that RCV formation is unlikely.

- High-risk recipient virus

While replicons provide a safe(r) approach for working with systems that are related to high-risk pathogens (in particular those belonging to RG4), the awareness of the high risk of the recipient may influence the willingness to downscale. Risk assessments are defined as an evaluation of likelihood and consequence. Even if the likelihood is low that recombination leads to a primary RCV, the possible consequences of handling a reconstituted RG4 virus at an inadequate containment level must be fully appreciated.

As COGEM observes the precautionary approach in its evaluations, scientific uncertainty must be taken into account and therefore downscaling activities in which VRPs are produced may remain limited.

In the case of **naked replicons**, there is no phase during which the coding sequences for the structural genes are present. Primary recombination is therefore excluded. When solely using a coding sequence for nonstructural genes in the absence of any complementing sequences or

[21] Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms. OJ L 125, 21.5.2009, p. 75–97

sequences that allow for the formation of VLVs, the risk of the replicon seems negligible (see Section 4.1). Independent of the consideration of any inserted sequence, the risk group of the naked replicon can be downscaled as it lacks all pathogenic features. A justification may be required in function of how well the recipient virus is known.

Although these elements provide a general argumentation, the specific nature of the replicon system (e.g. based on positive vs. negative-sense RNA, use of co-replicating vs. non-replicating helper RNA, segmented vs. non-segmented RNA) may have to be used to refine the downscaling effort (e.g. different elements are involved in packaging for positive- as compared to negative-sense RNA viruses, co-replicating helper RNAs are not used for negative-sense, segmented RNA viruses).

Whereas above we have highlighted the argumentation for downscaling the risk group of replicons, it must also be stressed that the situation differs depending on the type of activities performed with the replicon. For the worker handling the replicons, exposure is unintentional and unlikely even under low-level containment measures. In case of unintentional contact with the replicon, potential risks include genome integration, persistence, VLV generation, effects of non-targeted cells, and for VRP production also secondary RCV formation, as outlined in Chapter 4. When replicons are intentionally administered *in vivo* (e.g. deliberate administration in view of vaccination of animals or delivery of genes for the treatment of human diseases) other risks may occur, such as environmental risks. However, as the deliberate administration of the replicon is not leading to the production of fully infectious progeny virus, shedding will be limited in time and space. Therefore, all of the risk considerations are in a first instance relevant for and limited to the target cells. Cells or organisms treated with replicons can be handled in lower or no containment level once absence of replicon is demonstrated, as illustrated by CGM/030604 for an SFV-replicon and CGM/130329 for an SeV-replicon.

While secondary RCV formation based on recombination with an (un)related virus co-present in the host is a theoretical concern, its occurrence in reality remains unproven. The likelihood can be further reduced by avoiding simultaneous presence of the (un)related virus.

In conclusion, the most important concern is the primary production of RCV and in particular in the step where all genetic elements are present in the same cell. The recognition of this importance is illustrated by the fact that the majority of the publications address different strategies to reduce the risks for the formation of primary RCV. However, data on the actual need and / or efficiency of such measure remains scarcely documented. Even though it is not fully clear what the impact is of each of the measures, one might argue that the cumulative effect of different measures increases the safety profile. In his ‘Swiss cheese model’, James Reason (1997) visualises incidents as the result of the accumulation of multiple failures in defences (represented as the holes in slices of cheese) that unfortunately align, creating an open ‘hazard trajectory’ that results in harm. As long as enough layers of defence are in place, at least one with non-aligning holes, the probability of an accident can be minimized. For the risk measures of replicon systems, a similar reasoning could be proposed. Even though each individual measure is not (yet) proven leak tight, using multiple measures on different aspects of the system creates a solid barrier.

In the risk assessments reported in this study, the starting point has been the recipient virus. The combination of safety measures allows a downscaling of the risk group as well as the containment measures. It is not clear if this approach, so embedded in the GMO legislation, is and will remain adequate. In a case presented to COGEM (CGM/040212-02; CGM/060314-01), a chimeric replicon was presented combining VEEV nonstructural proteins with the SINV packaging system and structural proteins. With VEEV assigned to RG3 and SINV to RG2, the resulting chimeric replicon was assigned to RG2. Design and application of replicons is a rapidly evolving field. Other chimeric forms and even fully designed elements instead of those based on existing viruses are possible next steps. In such case reference to a RG determined for the recipient organism will not be possible, rather the risk classification will have to be developed *de novo*. The risk considerations identified in the current study can then be used to build up a ‘Swiss cheese model’ that would support such RG assignment, starting at the lowest level which would be applicable for self-amplifying RNA i.e. a naked replicon without a transgene.

7 References

Ansardi, D. C., Porter, D. C., Jackson, C. A., Gillespie, G. Y., and Morrow, C. D. (2001). RNA replicons derived from poliovirus are directly oncolytic for human tumor cells of diverse origins. *Cancer Research* **61**, 8470-8479.

Atasheva, S., Wang, E. Y., Adams, A. P., Plante, K. S., Ni, S., Taylor, K., Miller, M. E., Frolov, I., and Weaver, S. C. (2009). Chimeric alphavirus vaccine candidates protect mice from intranasal challenge with western equine encephalitis virus. *Vaccine* **27**, 4309-4319.

Ballesteros-Briones, M. C., Martisova, E., Casales, E., Silva-Pilipich, N., Bunuales, M., Galindo, J., Mancheno, U., Gorraiz, M., Lasarte, J. J., Kochan, G., Escors, D., Sanchez-Paulete, A. R., Melero, I., Prieto, J., Hernandez-Alcoceba, R., Hervas-Stubbs, S., and Smerdou, C. (2019). Short-Term Local Expression of a PD-L1 Blocking Antibody from a Self-Replicating RNA Vector Induces Potent Antitumor Responses. *Molecular Therapy* **27**, 1892-1905.

Baltusnikas, J., Satkauskas, S., and Lundstrom, K. (2018). Long-Term Transcriptional Gene Silencing by RNA Viruses. *Trends Biochem Sci* **43**, 397-401.

Baltusnikas, J., Satkauskas, S., and Lundstrom, K. (2019). Constructing RNA Viruses for Long-Term Transcriptional Gene Silencing. *Trends in Biotechnology* **37**, 20-28.

Ban, H., Nishishita, N., Fusaki, N., Tabata, T., Saeki, K., Shikamura, M., Takada, N., Inoue, M., Hasegawa, M., Kawamata, S., and Nishikawa, S. I. (2011). Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 14234-14239.

Barr, J. N., Elliott, R. M., Dunn, E. F., and Wertz, G. W. (2003). Segment-specific terminal sequences of Bunyamwera bunyavirus regulate genome replication. *Virology* **311**, 326-38.

Behrens, S.-E., Grassmann, C. W., Thiel, H.-J., Meyers, G., and Tautz, N. (1998). Characterization of an Autonomous Subgenomic Pestivirus RNA Replicon. *Journal of Virology* **72**, 2364-2372.

Beissert, T., Perkovic, M., Vogel, A., Erbar, S., Walzer, K. C., Hempel, T., Brill, S., Haefner, E., Becker, R., Tureci, O., and Sahin, U. (2020). A Trans-amplifying RNA Vaccine Strategy for Induction of Potent Protective Immunity. *Molecular Therapy* **28**, 119-128.

Bell, C. L., Yu, D., Smolke, C. D., Geall, A. J., Beard, C. W., and Mason, P. W. (2015). Control of alphavirus-based gene expression using engineered riboswitches. *Virology* **483**, 302-311.

Berge, T. O., Banks, I. S., and Tigertt, W. D. (1961). Attennuation of Venezuelan Equine Encephalomyelitis Virus by in vitro cultivation in guinea-pig heart cells. *American Journal of Epidemiology* **73**, 209-218.

Berglund, P., Sjöberg, M., Garoff, H., Atkins, G. J., Sheahan, B. J., and Liljeström, P. (1993). Semliki Forest Virus Expression System: Production of Conditionally Infectious Recombinant Particles. *Bio/Technology* **11**, 916-920.

Billecocq, A., Spiegel, M., Vialat, P., Kohl, A., Weber, F., Bouloy, M., and Haller, O. (2004). NSs Protein of Rift Valley Fever Virus Blocks Interferon Production by Inhibiting Host Gene Transcription. *Journal of Virology* **78**, 9798-9806.

Bitzer, M., Ungerechts, G., Bossow, S., Graepler, F., Sedlmeier, R., Armeanu, S., Bernloehr, C., Spiegel, M., Gross, C. D., Gregor, M., Neubert, W. J., and Lauer, U. M. (2003). Negative-strand RNA viral vectors: Intravenous application of Sendai virus vectors for the systemic delivery of therapeutic genes. *Molecular Therapy* **7**, 210-217.

Blakney, A. K., Ip, S., and Geall, A. J. (2021). An Update on Self-Amplifying mRNA Vaccine Development. *Vaccines (Basel)* **9**.

Blakney, A. K., McKay, P. F., and Shattock, R. J. (2018). Structural Components for Amplification of Positive and Negative Strand VEEV Splitzicons. *Front Mol Biosci* **5**, 71.

Bledsoe, A. W., Gillespie, G. Y., and Morrow, C. D. (2000a). Targeted foreign gene expression in spinal cord neurons using poliovirus replicons. *J Neurovirol* **6**, 95-105.

Bledsoe, A. W., Jackson, C. A., McPherson, S., and Morrow, C. D. (2000b). Cytokine production in motor neurons by poliovirus replicon vector gene delivery. *Nature Biotechnology* **18**, 964-969.

Bloom, K., van den Berg, F., and Arbuthnot, P. (2020). Self-amplifying RNA vaccines for infectious diseases. *Gene Therapy*.

Bouloy, M., Janzen, C., Vialat, P., Khun, H., Pavlovic, J., Huerre, M., and Haller, O. (2001). Genetic Evidence for an Interferon-Antagonistic Function of Rift Valley Fever Virus Nonstructural Protein NSs. *Journal of Virology* **75**, 1371-1377.

Brennan, B., Li, P., Zhang, S., Li, A., Liang, M., Li, D., and Elliott, R. M. (2015). Reverse genetics system for severe fever with thrombocytopenia syndrome virus. *J Virol* **89**, 3026-37.

Cao, D. J., Ni, Y. Y., and Meng, X. J. (2018a). Substitution of amino acid residue V1213 in the helicase domain of the genotype 3 hepatitis E virus reduces virus replication. *Virology Journal* **15**.
Cao, Y., Bing, Z., Guan, S., Zhang, Z., and Wang, X. (2018b). Development of new hepatitis E vaccines. *Human Vaccines and Immunotherapeutics* **14**, 2254-2262.
CGM/030604-02 (2003). Combinatie vaccins voor HIV. 2 pp.
CGM/040212-02 (2004). Constructie en productie van alphavirusvectoren. 4 pp.
CGM/050615-01 (2005). Pseudo-infectieuze virusdeeltjes; een modelsysteem voor het bestuderen van infectieprocessen van flavivirussen. 7 pp.
CGM/060314-01 (2006). Vaccinatie van apen met recombinante chimere alphavirusvectoren ten behoeve van vaccinonderzoek. 10 pp.
CGM/070723-02 (2007). Advies werkzaamheden met een recombinante Semliki forest virus vector. 8 pp.
CGM/080313-05 (2008). Inschaling van werkzaamheden met genetisch gemodificeerd Rift Valley fever virus (RVFV). 7 pp.
CGM/090603-01 (2009). Werkzaamheden met VEE virus gebaseerde replicondeeltjes. 9 pp.
CGM/110322-01 (2011). Inschaling werkzaamheden genetische gemodificeerd Rift Valley fever virus (RVFV).
CGM/111222-02 (2011). Klinische studie met een Semliki forest virus vaccin tegen baarmoederhalskanker. 11 pp.
CGM/120716-02 (2012). Inschaling van werkzaamheden met genetisch gemodificeerd Rift Valley fever virus.
CGM/121221-01 (2012). Inschaling werkzaamheden met Hepatitis E virus. 8 pp.
CGM/130329-01 (2013). Inschaling werkzaamheden gg-Sendai virus. 6 pp.
CGM/130502-01 (2013). Inschaling werkzaamheden met gg-Huaiyangshan virus. 8 pp.
CGM/130822-01 (2013). Inschaling werkzaamheden met gg-Middle East Respiratory Syndrome coronavirus (MERS-CoV). 12 pp.
CGM/150715-01 (2015). Advies inschaling van werkzaamheden met replicons afgeleid van het Venezuelan equine encephalitis virus. 6 pp.
CGM/160307-01 (2016). Advies classificatie en inschaling werkzaamheden (genetisch gemodificeerd) Zika virus. 14 pp.
CGM/160815-01 (2016). Advies inschaling van werkzaamheden met gg-replicondeeltjes afgeleid van Venezuelan equine encephalitis virus. 9 pp.
CGM/160906-02 (2016). Vervolgadvies omschaling van werkzaamheden met gg-VEEV replicondeeltjes. 5 pp.
CGM/170224-01 (2017). Advies omlaagschaling werkzaamheden met gg-VEEV RNA replicons. 8 pp.
CGM/170322-03 (2017). Vervolgadviesvraag VEEV replicons en noodzaak RCV test. 3 pp.
CGM/171024-01 (2017). Werkzaamheden met gg-alphavirus-replicons (SFV, SINV en VEEV) met donorsequenties van griepvirussen, Human respiratory syncytial virus, en Marburg- en Ebolavirussen. 13 pp.
CGM/171030-03 (2017). Werkzaamheden met gg-VEEV en gg-SPDV replicons met aquatische donorsequenties. 11 pp.
CGM/180313-02 (2018). Vervolgadvies over vorming van 'virus-like vesicles' (VLVs) tijdens werkzaamheden met gg-replicons. 4 pp.
CGM/190415-01 (2019). Advies werkzaamheden met gg-Sindbis virus replicons met het 'amyloid precursor protein' (APP) gen. 9 pp.
CGM/190725-02 (2019). Advies omlaagschaling werkzaamheden met SRIPs afgeleid van TBEV. 8 pp.
CGM/190905-02 (2019). Actualisatie van de pathogeniteitsclassificaties van een groot aantal humaan- en dierpathogene RNA en DNA virussen. 35 pp.
Chang, K. O., Sosnovtsev, S. V., Belliot, G., King, A. D., and Green, K. Y. (2006). Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. *Virology* **353**, 463-73.
Chen, J., Hu, B.-J., Zhao, K., Luo, Y., Lin, H.-F., and Shi, Z.-L. (2021). Development of A MERS-CoV Replicon Cell Line for Antiviral Screening. *Virologica Sinica*, 1-6.
Chen, S. P., Yu, M., Jiang, T., Deng, Y. Q., Qin, C. F., Han, J. F., and Qin, E. D. (2008). Identification of a recombinant dengue virus type 1 with 3 recombination regions in natural populations in Guangdong province, China. *Arch Virol* **153**, 1175-9.
Combelas, N., Holmblat, B., Joffret, M. L., Colbère-Garapin, F., and Delpeyroux, F. (2011). Recombination between poliovirus and coxsackie A viruses of species C: a model of viral genetic plasticity and emergence. *Viruses* **3**, 1460-84.
Couty, J. P., Crain, A. M., Gerbaud, S., Labasque, M., Marchiol, C., Fradelizi, D., Boudaly, S., Guettier, C., Vignuzzi, M., van der Werf, S., Escriou, N., and Viguier, M. (2008). Delivery of mengovirus-derived RNA replicons into tumoural liver enhances the anti-tumour efficacy of a peripheral peptide-based vaccine. *Cancer Immunology Immunotherapy* **57**, 1161-1171.

Daito, T., Fujino, K., Honda, T., Matsumoto, Y., Watanabe, Y., and Tomonaga, K. (2011). A Novel Borna Disease Virus Vector System That Stably Expresses Foreign Proteins from an Intercistronic Noncoding Region. *Journal of Virology* **85**, 12170-12178.

de Graaf, M., Herfst, S., Schrauwen, E. J. A., Choi, Y., van den Hoogen, B. G., Osterhaus, A. D. M. E., and Fouchier, R. A. M. (2008). Specificity and functional interaction of the polymerase complex proteins of human and avian metapneumoviruses. *Journal of General Virology* **89**, 975-983.

Desfarges, S., and Ciuffi, A. (2012). Viral Integration and Consequences on Host Gene Expression. *In* "Viruses: Essential Agents of Life" (G. Witzany, ed.), pp. 147-175. Springer Netherlands, Dordrecht.

Dodd, K. A., Bird, B. H., Metcalfe, M. G., Nichol, S. T., and Albarino, C. G. (2012). Single-Dose Immunization with Virus Replicon Particles Confers Rapid Robust Protection against Rift Valley Fever Virus Challenge. *Journal of Virology* **86**, 4204-4212.

Dunn, E. F., Pritlove, D. C., Jin, H., and Elliott, R. M. (1995). Transcription of a recombinant bunyavirus RNA template by transiently expressed bunyavirus proteins. *Virology* **211**, 133-43.

Duvall, J. R., VerPlank, L., Ludeke, B., McLeod, S. M., Lee, M. D., Vishwanathan, K., Mulrooney, C. A., Le Quement, S., Yu, Q., Palmer, M. A., Fleming, P., Fearns, R., Foley, M. A., and Scherer, C. A. (2016). Novel diversity-oriented synthesis-derived respiratory syncytial virus inhibitors identified via a high throughput replicon-based screen. *Antiviral Research* **131**, 19-25.

Emerson, S. U., Nguyen, H., Graff, J., Stephany, D. A., Brockington, A., and Purcell, R. H. (2004). In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. *J Virol* **78**, 4838-46.

Fernandes, R. S., Freire, M., Bueno, R. V., Godoy, A. S., Gil, L., and Oliva, G. (2020a). Reporter Replicons for Antiviral Drug Discovery against Positive Single-Stranded RNA Viruses. *Viruses-Basel* **12**.

Fernandes, R. S., Freire, M., Bueno, R. V., Godoy, A. S., Gil, L., and Oliva, G. (2020b). Reporter Replicons for Antiviral Drug Discovery against Positive Single-Stranded RNA Viruses. *Viruses* **12**.

Flick, K., Hooper, J. W., Schmaljohn, C. S., Pettersson, R. F., Feldmann, H., and Flick, R. (2003). Rescue of hantaan virus minigenomes. *Virology* **306**, 219-224.

Freiberg, A., Dolores, L. K., Enterlein, S., and Flick, R. (2008). Establishment and characterization of plasmid-driven minigenome rescue systems for Nipah virus: RNA polymerase I- and T7-catalyzed generation of functional paramyxoviral RNA. *Virology* **370**, 33-44.

Frolov, I., Frolova, E., and Schlesinger, S. (1997). Sindbis virus replicons and Sindbis virus: assembly of chimeras and of particles deficient in virus RNA. *Journal of virology* **71**, 2819-2829.

Frolova, E., Frolov, I., and Schlesinger, S. (1997). Packaging signals in alphaviruses. *J Virol* **71**, 248-58.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., and Hasegawa, M. (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proceedings of the Japan Academy Series B-Physical and Biological Sciences* **85**, 348-362.

Grieder, F. B., Davis, N. L., Aronson, J. F., Charles, P. C., Sellon, D. C., Suzuki, K., and Johnston, R. E. (1995). Specific restrictions in the progression of Venezuelan equine encephalitis virus-induced disease resulting from single amino acid changes in the glycoproteins. *Virology* **206**, 994-1006.

Han, G.-Z., and Worobey, M. (2011). Homologous recombination in negative sense RNA viruses. *Viruses* **3**, 1358-1373.

Hannemann, H. (2020). Viral replicons as valuable tools for drug discovery. *Drug Discovery Today* **25**, 1026-1033.

Hara, K., Fukumura, M., Ohtsuka, J., Kawano, M., and Nosaka, T. (2013). Human Parainfluenza Virus Type 2 Vector Induces Dendritic Cell Maturation Without Viral RNA Replication/Transcription. *Human Gene Therapy* **24**, 683-691.

Harvey, T. J., Liu, W. J., Wang, X. J., Linedale, R., Jacobs, M., Davidson, A., Le, T. T. T., Anraku, I., Suhrbier, A., Shi, P.-Y., and Khromykh, A. A. (2004). Tetracycline-Inducible Packaging Cell Line for Production of Flavivirus Replicon Particles. *Journal of Virology* **78**, 531-538.

Hass, M., Golnitz, U., Muller, S., Becker-Ziaja, B., and Gunther, S. (2004). Replicon system for Lassa virus. *Journal of Virology* **78**, 13793-13803.

Heinze, C., Willingmann, P., Schwach, F., and Adam, G. (2003). An unusual large intergenic region in the S-RNA of a Bulgarian tomato spotted wilt virus isolate. *Arch Virol* **148**, 199-205.

Hertzig, T., Scandella, E., Schelle, B., Ziebuhr, J., Siddell, S. G., Ludewig, B., and Thiel, V. (2004). Rapid identification of coronavirus replicase inhibitors using a selectable replicon RNA. *Journal of General Virology* **85**, 1717-1725.

Hikke, M. C., and Pijlman, G. P. (2017). Veterinary Replicon Vaccines. *In* "Annual Review of Animal Biosciences, Vol 5" (H. A. Lewin and R. M. Roberts, eds.), Vol. 5, pp. 89-109.

Hoang-Le, D., Smeenk, L., Anraku, I., Pijlman, G. P., Wang, X. J., de Vrij, J., Liu, W. J., Le, T. T., Schroder, W. A., Khromykh, A. A., and Suhrbier, A. (2009). A Kunjin replicon vector encoding granulocyte macrophage colony-stimulating factor for intra-tumoral gene therapy. *Gene Therapy* **16**, 190-199.

Hoenen, T., Groseth, A., de Kok-Mercado, F., Kuhn, J. H., and Wahl-Jensen, V. (2011). Minigenomes, transcription and replication competent virus-like particles and beyond: reverse genetics systems for filoviruses and other negative stranded hemorrhagic fever viruses. *Antiviral Res* **91**, 195-208.

Hornak, K. E., Lanchy, J. M., and Lodmell, J. S. (2016). RNA Encapsidation and Packaging in the Phleboviruses. *Viruses* **8**.

Hyvärinen, A., Yongabi, F., Mäkinen, K., Wahlfors, J., and Pellinen, R. (2013). Recombination of replicon and helper RNAs and the emergence of propagation-competent vectors upon Sindbis virus vector production. *Int J Mol Med* **32**, 410-22.

Ikeda, Y., Makino, A., Matchett, W. E., Holditch, S. J., Lu, B., Dietz, A. B., and Tomonaga, K. (2016). A novel intranuclear RNA vector system for long-term stem cell modification. *Gene Therapy* **23**, 256-262.

Ikegami, T., Won, S., Peters, C. J., and Makino, S. (2006). Rescue of infectious rift valley fever virus entirely from cDNA, analysis of virus lacking the NSs gene, and expression of a foreign gene. *J Virol* **80**, 2933-40.

Inoue, H., Iga, M., Nabeta, H., Yokoo, T., Suehiro, Y., Okano, S., Inoue, M., Kinoh, H., Katagiri, T., Takayama, K., Yonemitsu, Y., Hasegawa, M., Nakamura, Y., Nakanishi, Y., and Tani, K. (2008). Non-transmissible Sendai virus encoding granulocyte macrophage colony-stimulating factor is a novel and potent vector system for producing autologous tumor vaccines. *Cancer Science* **99**, 2315-2326.

Iroegbu, C. U., and Pringle, C. R. (1981). Genetic interactions among viruses of the Bunyamwera complex. *J Virol* **37**, 383-94.

Kamrud, K. I., Coffield, V. M., Owens, G., Goodman, C., Alterson, K., Custer, M., Murphy, M. A., Lewis, W., Timberlake, S., Wansley, E. K., Berglund, P., and Smith, J. (2010). In Vitro and In Vivo Characterization of MicroRNA-Targeted Alphavirus Replicon and Helper RNAs. *Journal of Virology* **84**, 7713-7725.

Kaplan, G., and Racaniello, V. R. (1988). Construction and characterization of poliovirus subgenomic replicons. *J Virol* **62**, 1687-96.

Karpf, A. R., Lenches, E., Strauss, E. G., Strauss, J. H., and Brown, D. T. (1997). Superinfection exclusion of alphaviruses in three mosquito cell lines persistently infected with Sindbis virus. *J Virol* **71**, 7119-23.

Ketzer, P., Kaufmann, J. K., Engelhardt, S., Bossow, S., von Kalle, C., Hartig, J. S., Ungerechts, G., and Nettelbeck, D. M. (2014). Artificial riboswitches for gene expression and replication control of DNA and RNA viruses. *Proc Natl Acad Sci U S A* **111**, E554-62.

Khromykh, A. A., and Westaway, E. G. (1997). Subgenomic replicons of the flavivirus Kunjin: construction and applications. *J Virol* **71**, 1497-505.

Komatsu, Y., Takeuchi, D., Tokunaga, T., Sakurai, H., Makino, A., Honda, T., Ikeda, Y., and Tomonaga, K. (2019). RNA Virus-Based Episomal Vector with a Fail-Safe Switch Facilitating Efficient Genetic Modification and Differentiation of iPSCs. *Molecular Therapy-Methods & Clinical Development* **14**, 47-55.

Komdeur, F. L., Singh, A., van de Wall, S., Meulenberg, J. J. M., Boerma, A., Hoogeboom, B. N., Paijens, S. T., Oyarce, C., de Bruyn, M., Schuuring, E., Regts, J., Marra, R., Werner, N., Sluis, J., van der Zee, A. G. J., Wilschut, J. C., Allersma, D. P., van Zanten, C. J., Kosterink, J. G. W., Jorritsma-Smit, A., Yigit, R., Nijman, H. W., and Daemen, T. (2021). First-in-Human Phase I Clinical Trial of an SFV-Based RNA Replicon Cancer Vaccine against HPV-Induced Cancers. *Molecular Therapy* **29**, 611-625.

Kortekaas, J., Oreshkova, N., Cobos-Jimenez, V., Vloet, R. P. M., Potgieter, C. A., and Moormann, R. J. M. (2011). Creation of a Nonspreading Rift Valley Fever Virus. *Journal of Virology* **85**, 12622-12630.

Kotaki, T., Xie, X., Shi, P.-Y., and Kameoka, M. (2020). A PCR amplicon–based SARS-CoV-2 replicon for antiviral screening. *bioRxiv*, 2020.08.28.267567.

Kuhn, R. J., Griffin, D. E., Owen, K. E., Niesters, H. G., and Strauss, J. H. (1996). Chimeric Sindbis-Ross River viruses to study interactions between alphavirus nonstructural and structural regions. *Journal of virology* **70**, 7900-7909.

Kummerer, B. M. (2018). Establishment and Application of Flavivirus Replicons. *In* "Dengue and Zika: Control and Antiviral Treatment Strategies" (R. Hilgenfeld and S. G. Vasudevan, eds.), Vol. 1062, pp. 165-173.

Li, S.-H., Li, X.-F., Zhao, H., Deng, Y.-Q., Yu, X.-D., Zhu, S.-Y., Jiang, T., Ye, Q., Qin, E. D., and Qin, C.-F. (2013). Development and characterization of the replicon system of Japanese encephalitis live vaccine virus SA14-14-2. *Virology journal* **10**, 64-64.

Liljeström, P., and Garoff, H. (1991). A New Generation of Animal Cell Expression Vectors Based on the Semliki Forest Virus Replicon. *Bio/Technology* **9**, 1356-1361.
Lohmann, V., Körner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-3.
Lowen, A. C., Boyd, A., Fazakerley, J. K., and Elliott, R. M. (2005). Attenuation of Bunyavirus Replication by Rearrangement of Viral Coding and Noncoding Sequences. *Journal of Virology* **79**, 6940-6946.
Lu, X., and Silver, J. (2001). Transmission of replication-defective Sindbis helper vectors encoding capsid and envelope proteins. *J Virol Methods* **91**, 59-65.
Lulla, V., and Firth, A. E. (2020). A hidden gene in astroviruses encodes a viroporin. *Nature Communications* **11**, 4070.
Lundstrom, K. (2019). Self-amplifying RNA virus vectors: clinical applications in cancer drug delivery. *Expert Opinion on Drug Delivery* **16**, 1027-1029.
Lundstrom, K. (2020). Self-Amplifying RNA Viruses as RNA Vaccines. *International Journal of Molecular Sciences* **21**.
Mendes, A., and Kuhn, R. J. (2018). Alphavirus Nucleocapsid Packaging and Assembly. *Viruses* **10**.
Mühlberger, E., Lötfering, B., Klenk, H.-D., and Becker, S. (1998). Three of the Four Nucleocapsid Proteins of Marburg Virus, NP, VP35, and L, Are Sufficient To Mediate Replication and Transcription of Marburg Virus-Specific Monocistronic Minigenomes. *Journal of Virology* **72**, 8756-8764.
Muslin, C., Mac Kain, A., Bessaud, M., Blondel, B., and Delpeyroux, F. (2019). Recombination in Enteroviruses, a Multi-Step Modular Evolutionary Process. *Viruses* **11**, 859.
Nguyen, M., and Haenni, A. L. (2003). Expression strategies of ambisense viruses. *Virus Res* **93**, 141-50.
Oreshkova, N., Spel, L., Vloet, R. P. M., Schreur, P. J. W., Moormann, R. J. M., Boes, M., and Kortekaas, J. (2015). Preliminary Evaluation of a Bunyavirus Vector for Cancer Immunotherapy. *Journal of Virology* **89**, 9124-9127.
Osborne, J. C., and Elliott, R. M. (2000). RNA binding properties of bunyamwera virus nucleocapsid protein and selective binding to an element in the 5' terminus of the negative-sense S segment. *J Virol* **74**, 9946-52.
Paessler, S., Fayzulin, R. Z., Anishchenko, M., Greene, I. P., Weaver, S. C., and Frolov, I. (2003). Recombinant Sindbis/Venezuelan Equine Encephalitis Virus Is Highly Attenuated and Immunogenic. *Journal of Virology* **77**, 9278-9286.
Pan, C. H., Valsamakis, A., Colella, T., Nair, N., Adams, R. J., Polack, F. P., Greer, C. E., Perri, S., Polo, J. M., and Griffin, D. E. (2005). Modulation of disease, T cell responses, and measles virus clearance in monkeys vaccinated with H-encoding alphavirus replicon particles. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 11581-11588.
Park, A., Hong, P., Won, S. T., Thibault, P. A., Vigant, F., Oguntuyo, K. Y., Taft, J. D., and Lee, B. (2016). Sendai virus, an RNA virus with no risk of genomic integration, delivers CRISPR/Cas9 for efficient gene editing. *Molecular Therapy-Methods & Clinical Development* **3**.
Pattnaik, A. K., Andrew Ball, L., LeGrone, A. W., and Wertz, G. W. (1992). Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69**, 1011-1020.
Pedrosa, P. B. S., and Cardoso, T. A. O. (2011). Viral infections in workers in hospital and research laboratory settings: a comparative review of infection modes and respective biosafety aspects. *International Journal of Infectious Diseases* **15**, e366-e376.
Peeters, B. P. H. (2005). Recombinant and chimeric viruses: Evaluation of risks associated with changes in tropism 117 pp.
Perri, S., Greer, C. E., Thudium, K., Doe, B., Legg, H., Liu, H., Romero, R. E., Tang, Z. Q., Bin, Q., Dubensky, T. W., Vajdy, M., Otten, G. R., and Polo, J. M. (2003). An alphavirus replicon particle chimera derived from Venezuelan equine encephalitis and Sindbis viruses is a potent gene-based vaccine delivery vector. *Journal of Virology* **77**, 10394-10403.
Pohjala, L., Utt, A., Varjak, M., Lulla, A., Merits, A., Ahola, T., and Tammela, P. (2011). Inhibitors of Alphavirus Entry and Replication Identified with a Stable Chikungunya Replicon Cell Line and Virus-Based Assays. *Plos One* **6**.
Pushko, P., Parker, M., Ludwig, G. V., Davis, N. L., Johnston, R. E., and Smith, J. F. (1997). Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology* **239**, 389-401.
Reason, J. (1997). "Managing the Risks of Organizational Accidents," Ashgate Publishing, Aldershot.
Rodriguez-Gascon, A., del Pozo-Rodriguez, A., and Solinis, M. A. (2014). Development of nucleic acid vaccines: use of self-amplifying RNA in lipid nanoparticles. *International Journal of Nanomedicine* **9**, 1833-1843.

Rolls, M. M., Webster, P., Balba, N. H., and Rose, J. K. (1994). Novel infectious particles generated by expression of the vesicular stomatitis virus glycoprotein from a self-replicating RNA. *Cell* **79**, 497-506.

Rose, N. F., Buonocore, L., Schell, J. B., Chattopadhyay, A., Bahl, K., Liu, X., and Rose, J. K. (2014). In vitro evolution of high-titer, virus-like vesicles containing a single structural protein. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 16866-16871.

Ruiz-Guillen, M., Gabev, E., Quetglas, J. I., Casales, E., Ballesteros-Briones, M. C., Poutou, J., Aranda, A., Martisova, E., Bezunartea, J., Ondiviela, M., Prieto, J., Hernandez-Alcoceba, R., Abrescia, N. G. A., and Smerdou, C. (2016). Capsid-deficient alphaviruses generate propagative infectious microvesicles at the plasma membrane. *Cellular and molecular life sciences : CMLS* **73**, 3897-3916.

Rusnak, J. M., Dupuy, L. C., Niemuth, N. A., Glenn, A. M., and Ward, L. A. (2018). Comparison of Aerosol- and Percutaneous-acquired Venezuelan Equine Encephalitis in Humans and Nonhuman Primates for Suitability in Predicting Clinical Efficacy under the Animal Rule. *Comparative medicine* **68**, 380-395.

Schoepp, R. J., Smith, J. F., and Parker, M. D. (2002). Recombinant chimeric western and eastern equine encephalitis viruses as potential vaccine candidates. *Virology* **302**, 299-309.

Scholle, F., Girard, Y. A., Zhao, Q. Z., Higgs, S., and Mason, P. W. (2004). Trans-packaged West Nile virus-like particles: Infectious properties in vitro and in infected mosquito vectors. *Journal of Virology* **78**, 11605-11614.

Seki, T., Yuasa, S., and Fukuda, K. (2012). Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a combination of activated T cells and Sendai virus. *Nat Protoc* **7**, 718-28.

Shinozaki, K., Ebert, O., Kournioti, C., Tai, Y. S., and Woo, S. L. C. (2004). Oncolysis of multifocal hepatocellular carcinoma in the rat liver by hepatic artery infusion of vesicular stomatitis virus. *Molecular Therapy* **9**, 368-376.

Shinozaki, K., Ebert, O., and Woo, S. L. C. (2005). Treatment of multi-focal colorectal carcinoma metastatic to the liver of immune-competent and syngeneic rats by hepatic artery infusion of oncolytic vesicular stomatitis virus. *International Journal of Cancer* **114**, 659-664.

Smerdou, C., and Liljestrom, P. (1999). Two-helper RNA system for production of recombinant Semliki Forest virus particles. *Journal of Virology* **73**, 1092-1098.

Su, S., Wong, G., Shi, W., Liu, J., Lai, A. C. K., Zhou, J., Liu, W., Bi, Y., and Gao, G. F. (2016). Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. *Trends in microbiology* **24**, 490-502.

Tao, W. Y., Gan, T. Y., Guo, M. Z., Xu, Y. F., and Zhong, J. (2017). Novel Stable Ebola Virus Minigenome Replicon Reveals Remarkable Stability of the Viral Genome. *Journal of Virology* **91**.

Taucher, C., Berger, A., and Mandl, C. W. (2010). A trans-Complementing Recombination Trap Demonstrates a Low Propensity of Flaviviruses for Intermolecular Recombination. *Journal of Virology* **84**, 599-611.

Tolou, H. J. G., Couissinier-Paris, P., Durand, J. P., Mercier, V., de Pina, J. J., de Micco, P., Billoir, F., Charrel, R. N., and de Lamballerie, X. (2001). Evidence for recombination in natural populations of dengue virus type 1 based on the analysis of complete genome sequences. *J Gen Virol* **82**, 1283-1290.

Tulloch, F., Pathania, U., Luke, G. A., Nicholson, J., Stonehouse, N. J., Rowlands, D. J., Jackson, T., Tuthill, T., Haas, J., Lamond, A. I., and Ryan, M. D. (2014). FMDV replicons encoding green fluorescent protein are replication competent. *Journal of Virological Methods* **209**, 35-40.

Twarock, R., and Stockley, P. G. (2019). RNA-Mediated Virus Assembly: Mechanisms and Consequences for Viral Evolution and Therapy. *Annual Review of Biophysics* **48**, 495-514.

van de Wall, S., Ljungberg, K., Ip, P. P., Boerma, A., Knudsen, M. L., Nijman, H. W., Liljestrom, P., and Daemen, T. (2018). Potent therapeutic efficacy of an alphavirus replicon DNA vaccine expressing human papilloma virus E6 and E7 antigens. *Oncoimmunology* **7**.

van den Pol, A. N., Mao, G., Chattopadhyay, A., Rose, J. K., and Davis, J. N. (2017). Chikungunya, Influenza, Nipah, and Semliki Forest Chimeric Viruses with Vesicular Stomatitis Virus: Actions in the Brain. *J Virol* **91**.

Vasilevska, J., Skrastina, D., Spunde, K., Garoff, H., Kozlovska, T., and Zajakina, A. (2012). Semliki Forest virus biodistribution in tumor-free and 4T1 mammary tumor-bearing mice: a comparison of transgene delivery by recombinant virus particles and naked RNA replicon. *Cancer Gene Therapy* **19**, 579-587.

Verhagen, J., Buijs, P., van den Hoogen, B., and van Eijck, C. H. J. (2015). Assessment of preclinical gene therapy studies worldwide. *COGEM Research Report 2015-03*, 151 pp.

Viktorova, E. G., Khattar, S. K., Kouiavskaia, D., Laassri, M., Zagorodnyaya, T., Dragunsky, E., Samal, S., Chumakov, K., and Belov, G. A. (2018). Newcastle Disease Virus-Based Vectored Vaccine against Poliomyelitis. *Journal of Virology* **92**.

Wang, J. M., Wang, L. F., and Shi, Z. L. (2008). Construction of a non-infectious SARS coronavirus replicon for application in drug screening and analysis of viral protein function. *Biochemical and Biophysical Research Communications* **374**, 138-142.

Ward, J. C., Bowyer, S., Chen, S. C., Campos, G. R. F., Ramirez, S., Bukh, J., and Harris, M. (2020). Insights into the unique characteristics of hepatitis C virus genotype 3 revealed by development of a robust sub-genomic DBN3a replicon. *Journal of General Virology* **101**, 1182-1190.

White, C. L., Thomson, M., and Dimmock, N. J. (1998). Deletion analysis of a defective interfering Semliki Forest virus RNA genome defines a region in the nsP2 sequence that is required for efficient packaging of the genome into virus particles. *J Virol* **72**, 4320-6.

Widman, D. G., Ishikawa, T., Fayzulin, R., Bourne, N., and Mason, P. W. (2008). Construction and characterization of a second-generation pseudoinfectious West Nile virus vaccine propagated using a new cultivation system. *Vaccine* **26**, 2762-71.

Worobey, M., Rambaut, A., and Holmes, E. C. (1999). Widespread intra-serotype recombination in natural populations of dengue virus. *Proc Natl Acad Sci U S A* **96**, 7352-7.

Xiong, Q., Wang, Y., Xie, B., Pei, X., and Peng, Y. (2017). Single-step construction of a picornavirus replicon RNA with precise ends. *Journal of Virological Methods* **248**, 87-91.

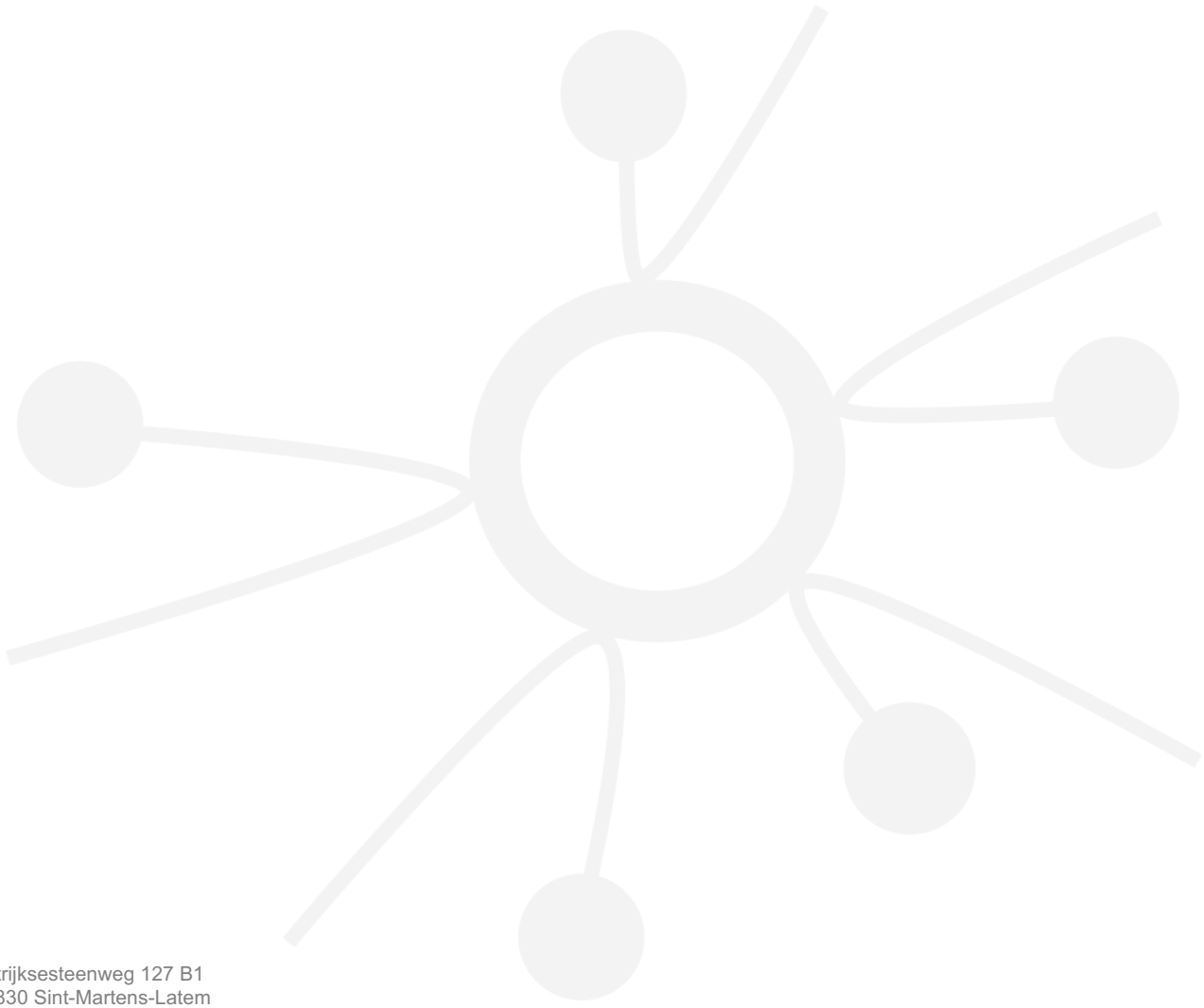
Yun, S.-I., Song, B.-H., and Lee, Y.-M. (2016). SA14-14-2Rep: A Replicon Vector Derived from the Live-Attenuated Japanese Encephalitis Vaccine SA14-14-2 Virus for Human and Veterinary Medicine. *Pediatric Infectious Diseases: Open Access* **01**.

Zhang, W. L., Hagedorn, C., Schulz, E., Lipps, H. J., and Ehrhardt, A. (2014). Viral Hybrid-Vectors for Delivery of Autonomous Replicons. *Current Gene Therapy* **14**, 10-23.

Zhang, Y., Song, W. H., Chen, S. Y., Yuan, Z. H., and Yi, Z. G. (2021). A bacterial artificial chromosome (BAC)-vectored noninfectious replicon of SARS-CoV-2. *Antiviral Research* **185**.

Zhu, Z., Meng, K., and Meng, G. (2020). Genomic recombination events may reveal the evolution of coronavirus and the origin of SARS-CoV-2. *Sci Rep* **10**, 21617.

ZKBS (2017). Allgemeine Stellungnahme der ZKBS zu häufig durchgeführten gentechnischen Arbeiten mit den zugrunde liegenden Kriterien der Vergleichbarkeit: Gentechnische Arbeiten mit dem Sindbis-Virusund dem Semliki-Forest-Virus-Expressionssystem. *Az. 6790-10-50* 13 pp. https://www.zkbs-online.de/ZKBS/SharedDocs/Downloads/01_Allgemeine%20Stellungnahmen/12_Stellungnahmen_Vergleichbarkeit/Sindbis-Virus_&_Semliki-Forest-Virus-Vektor_2017.html.



Kortrijksesteenweg 127 B1
B 9830 Sint-Martens-Latem
Belgium

T +32 9 321 07 05
F +32 9 321 07 05
info@perseus.eu
www.perseus.eu

IBAN BE08 0013 9730 5713
BIC GEBABEBB

RPR Gent
VAT BE 0480 089 325