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To the Minister for the Environment
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DATE	23 December 2022
REFERENCE	CGM/221223-01
SUBJECT	Generic advice on alphavirus and flavivirus replicons

Dear Ms Heijnen,

Further to the outcome of a research project commissioned by COGEM on viral replicon systems and their biosafety aspects, COGEM hereby notifies you of the following.

Summary:

Much research is being done on the development and application of 'viral replicons'. Viral replicons are derived from viruses (virus vectors) and are used, among other things, in research on vaccines and cancer. After infecting a cell, replicons are still able to replicate their viral genome, but are not able to form new virus particles and are therefore unable to spread further. This is because one or more genes encoding for proteins that make up the virus particle (the structural proteins) are deleted or replaced by a transgene (the gene of interest).

The structural proteins can be externally added during production of the replicons, resulting in the generation of packaged replicons, the viral replicon particles. These replicon particles can infect a cell once, but thereafter are incapable of forming new virus particles because the structural genes are not present in the genome. Examples of replicons are the self-amplifying mRNA vaccines against COVID-19 and influenza currently under development.

In view of the increase in the number of requests for advice on assigning containment levels for work with replicons, COGEM commissioned a research project on various aspects of replicon systems. Based in part on the research results and with the aim of streamlining the authorisation procedures, COGEM has drawn up generic advice on containment levels for work with viral replicons. This generic environmental risk assessment is limited to replicons derived from alphaviruses (genus *Alphavirus*) and flaviviruses (genus *Flavivirus*).

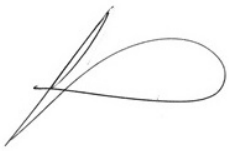
COGEM is of the opinion that given the characteristics of these replicons and replicon particles, generic downscaling of containment requirements is possible. Depending on which genes have been deleted, COGEM advises the following:

- Laboratory work with ‘naked’ alphavirus or flavivirus replicons can be carried out at containment level I, or at one level lower than the pathogenicity class of the parental virus.
- Laboratory work with replicon particles can be carried out at one level lower than the pathogenicity class of the parental virus.
- Generic conditions apply to work with both naked replicons and replicon particles: the cells used must contain no related alphaviruses or flaviviruses, and the transgenes used must not restore the removed functions.
- In addition, replicon particle preparations must not contain any virus generated during production that is capable of replicating and spreading.

When the above conditions are met, COGEM considers that the environmental risks of carrying out work with alphavirus and flavivirus replicons at the stated containment levels are negligible.

The attached report contains COGEM’s advice and a discussion of the underlying reasoning.

Yours sincerely,



Professor Sybe Schaap
Chair of COGEM

c.c. - Drs. Y. de Keulenaar, head of the GMO Office
- Ministry of Infrastructure and Water Management, Environmental Safety and Risks Directorate, Directorate-General for the Environment and International Affairs

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Generic downscaling of containment requirements for work with viral replicons derived from alphaviruses and flaviviruses

COGEM Advisory Report CGM/220311-01

1. INTRODUCTION

There is considerable interest in the development and application of viral replicons in fundamental and other laboratory research as well as in medical applications in vaccine and cancer research. These include the 'self-amplifying' mRNA vaccines currently being investigated as vaccines against COVID-19[1,2,3,4,5] or influenza.[6] Viral replicons are virus vectors that are able to replicate the viral genome in the infected cell, but are unable to form new virus particles and spread further. This is because one or more genes encoding for essential structural proteins are deleted or replaced by a transgene, such as a heterologous viral protein that elicits an immune response. By providing the structural proteins *in trans*, viral replicon particles (VRPs) are generated that can infect cells just once.

COGEM commissioned a research project to inventory viral replicon systems, both those that are being used and those under development, and the biosafety aspects of viral replicons. The project was carried out by Dr Karen van der Meulen and Dr Patrick Rüdelsheim of Perseus and the research results are described in the report *Viral replicon systems and their biosafety aspects – Inventory and description of viral replicon systems and characteristics relevant for risk assessment* (CGM/2022-06). The research results form the basis of this advisory report, which advises a generic downscaling of containment requirements for work with viral replicons derived from viruses from the genera *Alphavirus* and *Flavivirus*.

1.1 General information on viral replicons

Viral replicons are RNA or DNA molecules that can replicate independently and are derived from RNA or DNA viruses. This advice concerns only RNA replicons derived from positive-stranded RNA viruses. Viral RNA replicons have various applications, including for research into viral genome replication, as potential vaccines, and as possible cancer immunotherapies or gene therapies.[7,8,9] In the production of replicons a distinction is made between 'naked' viral replicons and 'viral replicon particles' (VRPs).

Naked viral replicons have one or more essential structural genes deleted from the viral genome. The non-structural genes and RNA sequences necessary for genome replication, such as untranslated regions (UTRs) and non-coding regions (NCRs), are still present, which means that the replicon RNA is capable of independent replication. Because the structural genes have been deleted, virus particles cannot be produced.

'Naked' viral replicons can be delivered into the cell as an RNA molecule by electroporation or transfection. Naked RNA can also be packaged into non-viral or synthetic particles, such as nanoparticles (lipid nanoparticles: LNPs), which can be taken up by the cell by means of endocytosis. Another way of administering viral replicons is to produce an RNA replicon from a DNA plasmid delivered into the cell for this purpose, called DNA-launched RNA replication (DREP). The plasmid is

transported to the nucleus, where the replicon RNA is transcribed, for example by a CMV promoter on the plasmid. Translation and replication of the replicon RNA takes place in the cytoplasm following the release of the RNA molecule from the cell nucleus. If the replicon also contains a selection gene, such as the gene that encodes neomycin resistance, stable replicon cell lines can be selected in which the replicon RNA remains in the cells.

Naked RNA packaged by viral structural proteins are called VRPs. During the production of VRPs the missing structural proteins are provided *in trans*, for example in a cell line in which these proteins are expressed stably or by using one or more helper RNAs or plasmids. VRPs are capable of infecting cells, but the lack of structural genes in the replicon RNA means that no new VRPs can be produced. As infection occurs just once, further spread is prevented.

1.2 The research report

In recent years much research has been done on various viral replicons derived from viruses from different families of RNA viruses.[9] The research report contains an inventory of the wide diversity of viral replicon systems and describes the characteristics of these systems that are of interest for the environmental risk assessment. The report focuses on describing replicon systems derived from positive- and negative-sense single-stranded RNA viruses. A distinction is made between 'naked' replicons and replicon systems that produce VRPs. The characteristics of the transgene are not covered by the research report because the risk considerations are different.

The report shows that despite the wide variety of replicon systems, several common aspects relating to potential environmental risks can be identified. The most important of these is the formation of replication-competent virus (RCV) during the production of VRPs. The possibility of RCV being formed if recombination takes place with related (wild type) viruses that may be present in the host cells is also discussed. The report describes various strategies for limiting these risks. Finally, it includes a schematic representation of aspects to be taken into account when considering downscaling containment requirements for viral replicon systems.

1.3 The scope of this advice

Based on the research findings, COGEM sees possibilities for generic downscaling of containment requirements for work with naked replicons and VRPs derived from viruses from the genera *Alphavirus* and *Flavivirus*, under contained use. The first replicon systems derived from alphaviruses and flaviviruses were reported many years ago[10,11,12,13,14,15,16] and are frequently used in research, including clinical trials.[17,18,19] Because the risks of these replicon systems have been clearly identified, it is possible to set generic containment levels for these replicons. For other viral replicon systems there is too little information available at this stage to be able to carry out a generic environmental risk assessment and assign appropriate containment levels.

2. REPLICONS DERIVED FROM ALPHAVIRUSES

2.1 Replication of alphaviruses and the structure of virus particles

Alphaviruses (genus *Alphavirus;* family *Togaviridae*) have a wide host range and are capable of infecting birds, mammals, fish and insects.[20,21] They can spread via bloodsucking insects, especially mosquitoes.[20] The genus *Alphavirus* has 32 species,[21] including various pathogenic species in humans and/or animals, such as *Chikungunya virus* (CHIKV), *Ross River virus* (RRV), *Semliki Forest virus* (SFV), *Sindbis virus* (SINV) and *Venezuelan equine encephalitis virus* (VEEV).[22]

Alphavirus particles have a diameter of about 70 nm and contain a positive single-stranded RNA genome of 9.7 to 11.8 kb.[21] Replication of the virus genome takes place in the cytoplasm of the host cell. The genome contains two large open reading frames (ORFs). The non-structural proteins (nsP1–nsP4) are read from the ORF at the 5'-end of the genome as a polyprotein; the individual proteins are formed following cleaving by the viral protease in nsP2. These proteins are involved in the replication of the viral RNA.[22,23] The ORF at the 3'-end of the genome encodes the structural proteins. This ORF is expressed from a subgenomic mRNA, which is transcribed from an internal promoter, 26S, situated in the area between the structural and non-structural ORF.[20,23] Viral and cellular proteases cleave the structural polyproteins in the individual proteins (C, E3, E2, 6K and E1). In some cases, (-1) frame shifting during the translation leads to the production of an extra protein called TF.[24] The non-coding regions are at both ends of the genome and the viral RNA has a 5'-cap structure and a poly(A) tail at the 3'-end.

The RNA in the virus particle is surrounded by the capsid protein (C) and together they form the nucleocapsid. The protein coat is surrounded by a lipid membrane containing the glycoproteins (envelope proteins, E1 and E2) involved in binding and infection of the host cell.[25] The glycoproteins therefore have a direct influence on the host range.[21] The assemblage and budding of new virus particles takes place on the plasma membrane of the host cell.[26]

2.2 Naked replicons and VRPs derived from alphaviruses

In naked replicons derived from alphaviruses the ORF encoding the structural genes is deleted. A transgene may be inserted at the position of the structural ORF and expressed from the subgenomic 26S promoter. In some replicons a second 26S promoter is introduced into the replicon genome so that multiple (trans)genes can be expressed. SFV, SINV and VEEV in particular are used in the construction of replicon systems.[27]

For the production of the VRPs, the structural genes are provided *in trans*. Co-transfection of the replicon RNA is mostly done with one or more helper constructs, from which the structural proteins are translated. The helper constructs are often also derived from alphaviruses from which the coding sequence for the non-structural proteins has been removed. A distinction is made between single helper strategies, in which the structural genes are provided on a single helper RNA, and split helper systems. In the latter production systems, the C gene and the genes encoding the glycoproteins are situated on two different helper RNAs.[13,74] As far as COGEM knows, production systems in which the structural

genes are provided from plasmids have not been described. Stable cell lines that produce the structural proteins have been described, but are little used.[28]

2.3 The recently reported self-amplifying mRNAs are naked alphavirus replicons

Recently, results of clinical trials with self-amplifying (sa)mRNA vaccines against COVID-19 have been made known.[1,2,3,5] These vaccines consist of the naked replicon genome of the VEEV vaccine strain TC-83 packaged in LNP particles. The gene encoding the Spike protein of SARS-CoV-2 is inserted at the position of the structural genes. The advantage of using samRNA vaccines over standard mRNA vaccines is that a lower dose can be used, because the mRNA itself can replicate in the host cell. A comparable vaccine against influenza is being studied which is based on the naked replicon genome of SFV.[1]

3. REPLICONS DERIVED FROM FLAVIVIRUSES

3.1 Replication of flaviviruses and the structure of virus particles

Flaviviruses (genus *Flavivirus*, family *Flaviviridae*) are arboviruses and can be transmitted by mosquitoes (especially *Culex* spp. or *Aedes* spp.) and other bloodsucking arthropods (e.g. ticks). Mammals and birds are the primary hosts. The genus *Flavivirus* contains 53 species,[29] including species that can cause diseases in humans and animals. The species with the most health implications for humans worldwide are *West Nile virus* (WNV), *Yellow fever virus* (YFV), *Japanese encephalitis virus* (JEV), *Dengue virus* (DENV), *Zika virus* (ZIKV) and *Tick-borne encephalitis virus* (TBEV).[30,31] With the exception of TBEV, which is transmitted by ticks, WNV, YFV, JEV, DENV and ZIKV are transmitted to humans by mosquitoes.

Flaviviruses have a positive single-stranded RNA genome of about 11 kb and are surrounded by a lipid membrane.[30,31,32] Replication of the virus genome takes place in the cytoplasm of the host cell. The RNA encodes a single polyprotein, the ORF being flanked by untranslated regions (UTRs).[33] The polyprotein is split into a number of different structural proteins (C, (pr)M and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).[29,32] The RNA is surrounded by the nucleocapsid (C). The structural proteins (pr)M and E are envelope proteins, which are found in the lipid membrane surrounding the nucleocapsid. (pr)M and E are involved in binding the virus to the cellular receptor, the fusion of the viral membrane (the envelope) with the membrane of the host cell, and immunity against the virus.[32] The NS proteins have various functions and are involved in RNA replication and polyprotein processing.[34] The new virus particles are assembled in the endoplasmic reticulum (ER) of the host cell and then released from the cell by budding.[35]

3.2 Naked replicons and VRPs derived from flaviviruses

Several replicons derived from different flaviviruses have been described in the literature. These replicons are used for studying viral replication, in screening for antiviral drugs or to enable research into pathogenic viruses at a lower containment level. There are also clinical trials with flavivirus replicons as vaccines against infectious diseases or as cancer treatments.

Naked replicons derived from flaviviruses from the genus *Flavivirus* usually have a large in-frame deletion in the region of the structural gene sequences (C-(pr)M-E). The 5' part of the coding sequence of the C protein (to about 40 codons) should then be retained because of the presence of cis-acting elements needed for efficient RNA replication.[12,36,37] Also, a sequence of 24 to 30 codons on the 3' end of the E gene is needed in order to retain the signal sequence for the NS protein.[12,38,39] Replicons have been reported in which just part of the structural genes have been deleted, such as deletion only in C,[40,41] (pr)M and a large part of E,[11,42,43] or only part of the E gene.[44]

Transgenes, selector genes or reporter genes are usually inserted in the position of the deletion. This can be done in frame, whereby the produced protein at the C or N terminus is fused with the remaining parts of the flavivirus C and E proteins. But some replicons have been constructed in which the transgene (or the non-structural gene) is expressed from an internal ribbon entry site (IRES) or in which the activity of the 2A peptide of the Foot and mouth disease virus[45] ensures the correct N terminus. Use of the IRES or the 2A peptide makes it possible to express multiple transgenes from this position. In bicistronic replicons, an expression cassette consisting of an IRES and a transgene is inserted into the replicon genome downstream of the non-structural genes.[12,46]

As in alphavirus replicons, flavivirus VRPs can be produced by providing the deleted structural genes *in trans*. Systems have been described that make use of stable cell lines that produce the structural proteins, plasmids that carry the structural genes, or alphavirus replicon RNAs that express the flavivirus structural proteins.[47,48,49]

4. CONSIDERATIONS FOR DOWNSCALING CONTAINMENT REQUIREMENTS FOR REPLICONS

When working with replicons there is a risk that RCV will be produced or that replicons could spread in another way. Below we take a more in-depth look at these risks of working with replicons derived from viruses from the genera *Alphavirus* and *Flavivirus*.

4.1 Replicons cannot spread as long as functional structural proteins are absent

In replicons substantial deletions are made in the sequences encoding essential structural proteins. Given that the non-structural genes and the replication signals are still present in the replicon genome, the replicon RNA can be replicated, but new infectious virus particles can no longer be produced.

The C protein and the envelope proteins are essential structural proteins of alphaviruses and flaviviruses necessary for producing infectious virus particles. The C protein is necessary for producing the nucleocapsid, because the RNA genome can be packaged by a complex of C proteins.[50,51] By means of budding on the host cell membrane the nucleocapsid is surrounded by a viral envelope containing the viral envelope proteins.

The alphavirus envelope protein E2 is responsible for binding the virus particle to the host receptor, while the envelope protein E1 is involved in fusing the viral envelope with the host cell membrane.[52] In flaviviruses the envelope protein E is responsible for both receptor binding and fusion with the host cell membrane.

PrM is considered to be a chaperone protein for facilitating the correct folding of the E protein and, together with the E protein, initiating budding on the ER membrane. When released from the cell, prM is cleaved by a cellular protease to produce infectious flavivirus particles. The remaining M protein is positioned under the E protein and very rarely comes to the surface of the viral envelope.[53]

In both alphaviruses and flaviviruses the ectodomains of the envelope proteins determine the binding and fusion of the virus particles to the host cell receptors. The deletion of these domains removes the ability of the viruses to spread.

Alphavirus and flavivirus replicons from which only the C protein sequences have been deleted can, in many cases, still spread. For example, alphavirus ΔC replicons can spread to adjacent cells via infectious virus-like vesicles (VLVs). Flavivirus replicons should retain part of the C protein to allow the replicon to function (see section 4.1.2). Depending on the size of the deletion in the C protein, this leads to different phenotypes. C deletions have been described that lead to attenuation of the flaviviruses and/or to the generation of compensatory mutations.[40,41,54,64]

When the sequences of both the C protein and the ectodomains of the envelope proteins are deleted, replicons are no longer able to spread and are biologically contained.

4.1.1 Working with alphavirus replicons may lead to the formation of virus-like vesicles (VLVs)

Working with alphavirus replicons may lead to the formation of VLVs. VLVs are spherules on the plasma membrane where replication complexes may be assembled.[55] These VLVs are infectious when the deletion in the structural genes is limited to the C gene and the alphavirus envelope proteins (glycoproteins E1 and E2) remain intact. The generation of infectious VLVs is much more inefficient than the production of virus particles, however these VLVs are able – to a limited extent – to infect other cells.[56,57]

When envelope protein donor sequences from alphaviruses, rhabdoviruses or retroviruses are used in alphavirus replicon systems, infectious VLVs may also be produced. It has been reported that expression of the G protein of the Vesicular stomatitis Indiana virus (VSIV, *Vesiculovirus Indiana*, from the *Rhabdoviridae* family) in an SFV replicon system from which all the structural genes have been removed leads to the formation of infectious VLVs.[55,58] The formation of infectious VLVs has also been reported in SFV and VEEV replicon systems in which the structural genes have been replaced by the glycoprotein of the Rabies virus (RABV-G, *Lyssavirus rabies*).[59,60] Expression of the envelope protein of Murine leukaemia virus (MLV) in the SFV replicon also led to the formation of infections VLVs.[61]

However, VLVs are not only seen as an unwanted by-product. Research is being done on the application of VLVs in animals as vaccines against various infectious diseases.[59,60,62,63] Repeated passaging of VLVs in cell cultures can cause mutations in the non-structural genes that lead to more efficient production of the VLVs.[55]

4.1.2 The flavivirus C coding sequence contains essential replication signals

Cis-acting sequences for RNA replication are present in the first 18 to about 40 codons of the C gene in the flavivirus genome. As a consequence, about 40% of the total C protein (which is made up from

around 100 amino acids in the mature form) should be retained to make efficient replication of the replicon possible. For this reason, all functional flavivirus replicons still contain the 5' part of the C coding sequence. The central hydrophobic domain is essential for the functioning of the C protein in assembling virus particles.[64,65,66] Deletion of at least 50% of the C coding sequence, including the part encoding the central hydrophobic domain, inactivates the C protein, but replication of the genome is still possible.

4.2 Spreading by complementation and recombination

4.2.1 Complementation by transgenes in the replicon can lead to spreading

Replicons are frequently used to express 'foreign' genes, such as selector genes and reporter genes and, in replicon vaccines, antigens of pathogenic organisms. Often transgenes are inserted in the position of the deleted structural genes. In alphavirus replicons, transgenes can also be expressed from a second subgenomic mRNA if a second 2S6 promoter has been cloned in the genome. If transgenes encode proteins that can complement the removed functions of the C protein or the envelope proteins, the replicon may spread.

4.2.2 Complementation or recombination with related viruses can lead to spreading

If related alphaviruses or flaviviruses are present in the cells or in laboratory animals, there is a risk that the functions removed from the replicon will be restored by complementation or recombination. This can lead to unintended spread of the replicon or, in the case of recombination, to the generation of RCV. This risk applies during work with both naked replicons and VRPs.

Recombination can occur between overlapping sequences of the replicon RNA and the genome of the related viruses. This can lead to restoration of the deletions, and possibly also to the generation of 'new' viruses. Recombination of both alphaviruses and flaviviruses in nature has been reported, and in some cases this has led to the creation of new species.[67,68,69]

However, in vaccine studies recombination between wild type flaviviruses with attenuated vaccine strains has not been observed,[70] although recombination can be induced under laboratory conditions. In an experimental setting, low-frequency recombination led to the formation of highly attenuated recombinant replication-competent flaviviruses.[71]

Recombination between non-homologous sequences of flaviviruses can occur in rare cases.[72,73] To prevent the generation of replication-competent viruses during work with replicons, it is important to work with cell lines in which no related alphaviruses or flaviviruses are present.

4.3 Replication-competent virus may be generated during the production of VRPs

In the production of VRPs, the structural proteins whose coding sequences have been removed from the replicon genome are presented *in trans* to ensure the replicon RNA is packaged. In the process, infectious particles (VRPs) are produced which are able to infect cells once. When there are overlapping sequences between the replicon genome and the constructs for the expression of the structural proteins, RCV may be generated by recombination.

In the production of alphavirus VRPs, in particular, the formation of RCV is a risk, because co-replicating alphavirus helper RNA constructs are usually used, which increases the probability of recombination. In the helper RNAs at least the sequences encoding the non-structural proteins nsP1-4 are deleted, including the packaging signals. In many cases the 26S promoter is also removed from the helper RNAs. However, the 5' UTR and the 3' UTR of the alphavirus genome, which contains the signals necessary for the (co-)replication of the helper RNAs, remain present, which means that homologous recombination with the replicon genome cannot be ruled out.

Various strategies are used to minimise the probability of RCV being formed as a result of recombination during the production of alphavirus VRPs. These include the use of split helper systems in which the C gene and the genes encoding the glycoproteins are located on two different helper RNAs.[13,74,75] When split helper production systems are used in laboratories, the formation of RCV is greatly reduced, because multiple recombination events should occur.[13,74] The probability of homologous recombination occurring can be further reduced by reducing the length of identical sequences between the replicon RNA and the helper constructs or by recoding overlapping sequences.[13] Additional mutations, for example in the structural genes on the helper construct, can also ensure that any RCV that is formed will be less virulent than the parental virus.[13,74,76] In the same way, the risk associated with any RCV that is formed can be reduced by using structural genes from a less pathogenic alphavirus, as long as the attenuated phenotype is caused by the structural genes.[77]

The probability of RCV being formed when producing flavivirus VRPs is much smaller, because the structural genes are not provided by co-replicating (flavivirus) helper RNAs. The deleted structural genes are complemented in trans by using stable cell lines that produce the structural proteins or by using plasmids that carry the structural proteins. There are also production systems in which the flavivirus structural systems are expressed from alphavirus replicon RNAs. Split helper systems have not been described for flavivirus replicons.

As far as is known, the generation of RCV during the production of flavivirus VRPs has never been reported.[78] However, little research has been done into the possibility of RCV formation in flavivirus production systems.

4.4 The presence of replication-competent virus can be assessed with an RCV test

The presence or absence of RCV in a batch of produced VRPs can be demonstrated by means of an RCV test. The literature contains little information on carrying out and validating RCV tests for alphavirus and flavivirus replicons.

In the assays that are described, the produced VRP preparations are tested by infecting sensitive cells with the VRPs; the presence or absence of infectious virus (RCV) in the preparation is then assessed by microscopic analysis of the cells (cytopathic effect (CPE) or syncytia) or by a plaque assay of the supernatant.[13,74,78,79,80,81] In the assays described, $1x10^8$ to $4x10^8$ VRPs were tested each time.[13,74,79,81] In some cases the assays are validated by including infectious virus as a positive control.[74,81] The absence of RCV can also be plausibly demonstrated by observing whether injection of VRPs in suckling mice

causes disease or not. It has been reported that administering $5x10^5$ to $5x10^7$ VRP infectious units (IU) did not lead to disease,[13,78] in contrast to administering 1 particle forming unit of the parental virus.[78]

Sometimes a VRP preparation is passaged once or twice on sensitive cells before doing the RCV test. One or more passages on sensitive cells increases the sensitivity of the assay, because a selection of the replicating RCV cells is made and the potentially disruptive replication-incompetent VRPs are diluted. At the same time the repeated passages allow a distinction to be made between any VLVs present and infectious virus (RCV) because VLVs are formed much less efficiently than virus particles.[55]

5. ADVICE: CONTAINMENT REQUIREMENTS FOR RESEARCH WITH ALPHAVIRUS AND FLAVIVIRUS REPLICONS CAN BE DOWNSCALED

Based in part on a research project commissioned by COGEM on the biosafety aspects of replicon systems, the present advisory report examines the possibility of setting a generic containment level for work with naked replicons and VRPs.

Replicons derived from viruses from the genera *Alphavirus* and *Flavivirus* have long been used in the laboratory and in clinical trials, and the environmental risks of these replicons have been extensively researched. For this reason, COGEM is of the opinion that a generic downscaling of containment requirements for these replicons is possible under certain conditions. These are explained below.

5.1 General conditions for downscaling containment requirements for work with alphavirus and flavivirus replicons

The general conditions for downscaling containment requirements for work with alphavirus and flavivirus replicons are, first, that the cells used must not contain any related alphaviruses or flaviviruses in order to prevent the possibility of removed functions in the replicons being restored through complementation or recombination. Second, no transgenes should be used that are able to complement the removed functions. This means that sequences that encode the envelope proteins of viruses from the families *Togaviridae*, *Rhabdoviridae* and *Retroviridae* are excluded from use as transgenes in alphavirus replicons because they can lead to the formation of infectious VLVs (see section 5.2.3).

The genome of some animal cells contains endogenous retroviruses (ERVs). Under certain laboratory conditions these ERVs, or parts of them, can be activated. Envelope proteins of the ERVs may then be expressed, which could lead to complementation of the removed envelope proteins of the described replicons. For this reason, this advice excludes cell lines which are known to express functional endogenous envelope proteins and which can lead to complementation of replicons.[a]

[a] A literature study of the available scientific knowledge on ERVs in human and animal cells is currently being carried out for COGEM to obtain a better understanding of the possible risks to human health and the environment associated with the presence or use of these endogenous viral sequences in work with GMOs.

5.2 Containment requirements for work with naked alphavirus and flavivirus replicons can be downscaled

As naked replicons do not have the essential genetic information needed to generate new virus particles, they are biologically contained and are unable to spread further. COGEM is therefore of the opinion that the containment requirements for naked replicons derived from viruses from the genera *Alphavirus* and *Flavivirus* can be downscaled, on the condition that certain measures are taken to reduce the risk of the replicon spreading or the generation of replication-competent virus.

For assigning containment levels for work with naked replicons, COGEM makes a distinction between replicons from which the coding sequences for both the C and envelope proteins have been removed and replicons from which the coding sequences of only the C proteins or only the envelope proteins have been removed.

5.2.1 Containment levels for work with naked alphavirus replicons

COGEM is of the opinion that work with naked alphavirus ΔC/ΔE1/ΔE2 replicons in which the essential structural proteins are no longer functional can be carried out at containment level I, because these replicons are biologically contained and are no longer able to spread. This downscaling applies to replicons derived from alphaviruses in pathogenicity classes 2 and 3. To qualify for the downscaled containment level, at least 70% of the coding sequence of the C protein and, at the minimum, the sequences encoding both ectodomains of the E1 and E2 proteins must be deleted.

For work with alphavirus replicons in which only the C protein has been inactivated by deletion of all or a large part of the C coding sequence, ΔC replicons, infectious VLVs can be formed as a result of the presence of the genes encoding the envelope proteins. For this reason, alphavirus ΔC replicons are excluded from this generic advice.

Alphavirus ΔE1/ΔE2 replicons in which both envelope proteins are no longer functional are biologically contained and do not have the capacity to spread. The replicon RNA can still be packaged in the C proteins, but the absence of functional envelope proteins removes the capacity to spread.

Given the pace of developments in the field and the new and unknown constructs that may be produced in future for launching alphavirus replicons, COGEM advises that, for the time being, work with ΔE1/ΔE2 replicons should be downscaled to one containment level below the level required for the parental virus. This would allow work with naked ΔE1/ΔE2-replicons derived from alphaviruses from pathogenicity class 3 to be carried out at ML II and work with replicons derived from alphaviruses from pathogenicity class 2 to be carried out at ML I. For these replicons to be eligible for downscaling, the minimum requirement is that the sequences encoding both ectodomains of the E1 and E2 proteins must be deleted.

5.2.2 Containment levels for work with naked flavivirus replicons

COGEM is of the opinion that work with naked ΔC/Δ(pr)M/ΔE replicons can be carried out at containment level I. Naked replicons are biologically contained and unable to spread because they do

not have the essential genetic information needed to produce new virus particles. This downscaling applies to replicons derived from flaviviruses in pathogenicity classes 2 and 3. To qualify for downscaling, the flavivirus ΔC/Δ(pr)M/ΔE replicons must meet the following conditions:

i) Only the C sequences essential for replication of the replicon may be retained and at least 50% of the coding sequence must be removed, including the central hydrophobic domain.
ii) Also, at the minimum, the sequences encoding the ectodomains of the (pr)M and E proteins must be deleted.

Regarding the flavivirus ΔC-replicons, COGEM has previously noted (section 4.1) that small to medium-sized deletions in the C protein of flaviviruses can lead to different phenotypes. Depending on the position and size of the deletions, (attenuated) virus may be formed or compensatory mutations may occur at other positions in the C gene. These mutations can restore the hydrophobic characteristics of the protein, followed by the production of infectious virus particles.[40,54,64] Larger deletions will prevent any further spreading of the replicon.[41,54]

The presence of replication signals in the coding sequence of the C protein means it is not possible to delete the whole gene. There are numerous possible replicon constructs with variations in deletions in the C protein. It is not possible to predict in advance whether or not particles can be formed, for one thing because similar deletions in different flaviviruses can lead to different phenotypes. For this reason, COGEM is of the opinion that, for the time being, containment levels for work with flavivirus ΔC replicons should be decided on a case-by-case basis.

Δ(pr)M/ΔE replicons are biologically contained if the ectodomains of the envelope proteins are deleted and do not have the capacity to spread. The replicon RNA can still be packaged in the C proteins, but the absence of functional envelope proteins removes the capacity to spread. Given the rapid pace of developments in the field and the new and unknown constructs that may be produced in future, COGEM advises that, for the time being, work with Δ(pr)M/ΔE replicons should be downscaled to one containment level below the level of the parental virus. This would allow work with naked Δ(pr)M/ΔE replicons derived from flaviviruses from pathogenicity class 3 to be carried out at ML II and work with replicons derived from flaviviruses from pathogenicity class 2 to be carried out at ML I. To be eligible for downscaling, the minimum requirement is that the sequences encoding the ectodomains of the (pr)M and E proteins must be deleted.

5.2.3 Containment levels for work with alphavirus infectious VLVs should be assessed on a case-by-case basis

During some work with alphavirus replicons it is possible that infectious VLVs will be formed. This is the case when the C protein is absent, but the envelope proteins are still expressed (see section 5.2.1). Infectious VLVs may also be formed when complementation occurs because of the presence of envelope proteins of viruses from the families *Togaviridae*, *Rhabdoviridae* and *Retroviridae*. VLVs are less stable and their pathogenicity is limited, but they are still infectious. When repeatedly passaged in cell cultures, mutations can be selected that lead to more efficient production of the VLVs.[55]

COGEM is of the opinion that further research and more data are needed before it will be possible to advise a generic downscaling of containment requirements for work with replicons that can lead to the forming of infectious VLVs. That means, among other things, that additional conditions will have to be set concerning the transgenes to be used if the work involves replicons derived from alphaviruses: containment levels for work with alphavirus replicons with transgenes encoding envelope proteins of viruses from the families *Togaviridae, Rhabdoviridae* and *Retroviridae* should be determined on a case-by-case basis.

5.3 Downscaling containment requirements for work with alphavirus and flavivirus VRPs

During the production of VRPs, sequences encoding the structural genes deleted from the replicon are provided *in trans*, which means that there is a risk of the formation of RCV by recombination. As RCV is infectious and can have the same or similar characteristics as the parental virus, work with alphavirus or flavivirus VRP preparations which also contain, or can contain, RCV must be carried out at the same containment level as the parental virus.

5.3.1 Containment requirements for alphavirus VRPs produced with split-helper systems can be downscaled

A risk associated with the production of alphavirus VRPs is the formation of RCV. To reduce the probability of the formation of RCV, split-helper systems have been developed in which the C gene and the envelope genes are provided separately via different helper RNAs. Given the sequence homology in the 5' and 3' UTRs of the three co-replicating RNAs (the replicon RNA and the two helper RNAs), recombination cannot be completely ruled out. However, because the C and envelope sequences are divided between the different helper-RNAs, two independent recombination events are needed to generate RCV. Inducing additional mutations in the helper constructs can further attenuate any RCV that may be formed. Although these mutations cannot completely prevent the recombination events, they make the VRP production system safer.

A number of publications on split-helper systems describe tests for the formation of RCV during the production of the alphavirus VRPs. RCV has never been demonstrated in systems in which the C and envelope sequences are strictly separated on the two helpers.[13,74,75] None of the assays in tests for the presence of RCV on 2,060 batches of a split-helper system based on VEEV-TC83, on which COGEM has previously advised, were found to contain RCV.[81] It should be noted that in a number of these split-helper systems one or more additional mutations were induced in the structural genes to make the production systems safer.[13,74,81] These mutations have no influence on the recombination events, but weaken or inactivate any RCV that may be formed after recombination.[74]

If the C and envelope sequences are not strictly separated on both helper RNAs, RCV may occasionally be formed. This has been observed during the packaging of an SIV replicon with a split-helper system in which the envelope helper construct also contained part of the C gene of a related alphavirus.[82]

COGEM advises downscaling the containment level for work with alphavirus VRPs produced with a split-helper system to one level below that of the parental virus. This means that work with VRPs derived

from alphaviruses from pathogenicity class 3 can be carried out at ML II and work with replicons derived from alphaviruses from pathogenicity class 2 can be carried out at ML I. The following conditions apply:

- To prevent recombination of overlapping sequences, the sequences encoding C, E1 and E2 should be deleted in their entirety from the replicon genome.
- The VRPs should be produced with a split-helper system in which the C and envelope encoding sequences are strictly separated on two independent helper RNAs, with no overlap between the C and envelope sequences.
- Each alphavirus split-helper production system should be tested at least once in an RCV test (see section 5.4), with a negative result for the presence of RCV.

Regarding the above-mentioned split-helper system derived from VEEV-TC83 on which COGEM has previously advised,[81, 83] COGEM notes that the previous advice remains valid and no RCV test is necessary for work carried out at containment level ML I.

5.3.2 Containment requirements for flavivirus VRPs can be downscaled

The probability of RCV being formed by recombination of overlapping sequences during the production of flavivirus VRPs is considered to be smaller than for alphavirus VRPs.[84] The generation of RCV during the production of flavivirus VRPs has been investigated in two studies in which the replicon RNA was introduced into packaging cell lines that express the structural proteins. In both studies no RCV was detected after repeated passages in sensitive cells, despite the presence of overlapping sequences between the replicon RNA and the mRNAs from the packaging cell line.[78,84] No recombination was also observed in a production system in which the structural genes were provided via an alphavirus replicon.[78,85] In this system the C and (pr)M/E sequences were separately expressed from different 26S promoters.

COGEM is of the opinion that work with VRPs in which the ΔC/Δ(pr)M/ΔE flavivirus replicon is provided as an RNA molecule can be carried out at one containment level lower than the pathogenicity class of the parental virus. The structural proteins C, (pr)M and E can be expressed from plasmids, from an alphavirus (naked) replicon or in a stable cell line. Besides the general conditions (section 5.1), the following additional conditions apply:

- To prevent recombination of overlapping sequences, the sequences encoding the structural genes should be deleted as much as possible from the replicon genome. That means that only C sequences essential for the replication of the replicon may be retained, as long as at least 50% of the coding sequence has been removed, including the central hydrophobic domain. The sequences encoding the (pr)M and E proteins should be deleted in their entirety.
- Each flavivirus production system should be tested at least once in an RCV test (see section 5.4), with a negative result for the presence of RCV.

COGEM notes that when using a DNA launched replicon system (DREP) in which the replicon RNA and the complementary structural proteins are expressed by co-transfection of two DNA plasmids, recombination between the plasmids may occur. The deletions in the C gene and the (pr)M and E genes of the replicon are partial deletions, leaving overlapping sequences between the replicon plasmid and

the helper plasmid which contains the complete coding sequence of C-prM-E. In such a production system the probability that recombination will take place is very high. For this reason COGEM is of the opinion that a DNA launched flavivirus replicon system in combination with helper plasmids should be excluded from the generic advice and applications for the use of such systems should be assessed on a case-by-case basis.

5.4 Conditions for the RCV test

As a condition for downscaling containment requirements for work with alphavirus and flavivirus VRPs, COGEM proposes that the absence of RCV in a produced VRP preparation must first be demonstrated (section 5.3.1 and 5.3.2). Various methods have been described in the literature for carrying out an RCV test, many of which involve passaging a VRP preparation several times on sensitive cells before it is tested. The passaging ensures that any RCV present is selected and the replication-incompetent VRPs are diluted. Just one or two passages can achieve a level of sensitivity high enough to detect a single RCV in a preparation of $1x10^8$ to $4x10^8$ VRPs.[13,74,79,81]

COGEM is of the opinion that if the RCV test satisfies the following conditions, the sensitivity will be sufficient to demonstrate the presence of any RCV in a preparation:

- The produced RVP preparation must be passaged at least three times on sensitive cells. It is not necessary to use wild type parental virus as a positive control in the RCV assay as sufficient sensitivity can be achieved by passaging three times on sensitive cells.[b]
- Depending on the characteristics of the parental virus, RCV can be demonstrated by means of a validated qPCR test or by microscopic analysis (CPE, syncytia formation), plaque assay or antibody colouring.

5.5 Conclusion and summary

To summarise, COGEM is of the opinion that a generic downscaling of containment requirements for work with naked replicons and VRPs derived from viruses from the genus *Alphavirus* or *Flavivirus* is possible. This is subject to a number of conditions designed to limit the risk of the replicon spreading or replication-competent virus being generated. COGEM is of the opinion that when work with naked replicons and VRPs derived from viruses from the genus *Alphavirus* or *Flavivirus* meet the above conditions, the environmental risks of carrying out the activities at the stated containment levels are negligible. COGEM's advice on the appropriate containment levels for work with replicons derived from viruses from the genus *Alphavirus* and *Flavivirus* is summarised in Tables 1 and 2.

[b] Validating the RCV test and determining the detection limit is only possible if the parental virus is included as a positive control. Validation of the RCV test must be carried out at the containment level required for work with the parental virus.

Table 1. Containment levels for work with alphavirus replicons

Alphavirus	Deletion	Containment level	Additional conditions*
Naked replicons	ΔC, ΔE1, ΔE2	ML I	• Deletion of at least 70% of C and at least the ectodomains of E1 and E2
	ΔE1, ΔE2	1 level lower than the parental virus	• Deletion of at least the ectodomains of E1 and E2
VRPs	ΔC, ΔE1, ΔE2 (production with split helper) ǂ	1 level lower than the parental virus	• Deletion from the replicon genome of C, E1 and E2 in their entirety, because of possible recombination • The C and envelope encoding sequences should be strictly separated on the helper RNAs, with no overlapping sequences • Each production system should be tested at least once in an RCV test, with a negative result for the presence of RCV

** In addition to the two general conditions stated in section 5.1: the cells used must contain no related alphaviruses and the transgene used must not be able to complement the removed functions (including sequences encoding the envelope proteins of viruses from the families Togaviridae, Rhabdoviridae and Retroviridae).*

ǂ *Production systems which make no use of split helpers should be assessed on a case-by-case basis and fall outside the scope of this generic advice.*

Table 2. Containment levels for work with flavivirus replicons

Flavivirus	Deletion	Containment level	Additional conditions*
Naked replicons	ΔC, Δ(Pr)M, ΔE	ML I	• Only C sequences essential for the replication of the replicon may be retained, as long as at least 50% of the coding sequence has been removed, including the central hydrophobic domain. • Deletion of at least the ectodomains of (pr)M and E
	Δ(pr)M, ΔE	1 level lower than the parental virus	• Deletion of at least the ectodomains of (pr)M and E
VRPs	ΔC, Δ(pr)M, ΔE ǂ	1 level lower than the parental virus	• Only C sequences essential for the replication of the replicon may be retained in the replicon genome, as long as at least 50% of the coding sequence has been removed, including the central hydrophobic domain

			• Complete deletion of (pr)M and E from the replicon genome • Each production system should be tested at least once in an RCV test, with a negative result for the presence of RCV

** In addition to the two general conditions stated in section 5.1: the cells used must not contain any related flaviviruses and the transgene used must not be able to complement the removed functions.*

ǂ *Production systems which use a DNA launched flavivirus replicon in combination with helper plasmids should be assessed on a case-by-case basis and fall outside the scope of this generic advice.*

6. OBSERVATION FROM A HEALTH AND SAFETY PERSPECTIVE

Alphavirus and flavivirus VRPs are biologically contained and will not spread further. The VRPs have the capacity for a one-time infection of laboratory staff, which may lead to adverse effects caused by the transgenes used. COGEM therefore points out that in the interests of occupational health and safety, when working with VRPs additional measures geared to the specific infection route of the parental virus must be taken where required to prevent infection of laboratory staff.

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