ZKBS
Zentrale Kommission
für die Biologische Sicherheit

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General position statement of the ZKBS on frequently carried out genetic engineering operations based on the criteria of comparability:

Genetic engineering work with RNA viruses derived from Minigenomes, replicons and virus-like particles for introduction into human or animal cells

1. Description of viral minigenomes, viral replicons and virus-like particles

1.1. General introduction

Numerous plasmid-based reverse genetics systems have been established in recent decades for research into RNA viruses. If replication-competent[1] virus particles are produced during the application of these systems, they can only be handled with complex safety measures. Therefore, research approaches that do not require a complete run through the viral replication cycle often rely on systems that only reflect individual aspects of the viral life cycle. Such replication-defective systems can usually be handled with significantly reduced security measures. Depending on the research approach or field of application, the focus is on the ability to autonomously synthesize viral RNA, on the expression and interaction of viral proteins or on the formation and release of virus-like particles and their infectivity. This allows a differentiation of the systems into different groups.

1.2. Viral minigenomes

Viral minigenomes are linear single-stranded RNA molecules containing the *cis*-regulatory sequences of the corresponding viral genome and, in the simplest case, a single reporter gene. In special cases, additional foreign genes without reporter function may also be cloned in. The *cis*-regulatory sequences are regions at the 5' and 3' ends of the genome that control its transcription and propagation by virally encoded factors. However, the open reading frames (ORFs) of these viral factors are not present in the minigenome, so that transcription and propagation of viral RNA occurs only when these factors are made available in target cells. This can be ensured by transfection of expression plasmids or mRNA, transduction by viral vectors or even infection of the cell with a helper virus. If, in addition, viral structural proteins are also expressed in the cells, this can lead to the release of replication-defective virus-like particles, which can subsequently transmit the minigenome to further cells (see point 1.4.). Minigenomes thus represent the most reduced system for studying viral RNA synthesis and identifying the factors that interfere with this process. Minigenomes of highly pathogenic negative-strand RNA viruses are frequently used, for example, Ebolaviruses [1] .

1.3. Viral replicons

Like viral minigenomes, viral replicons possess all the *cis*-regulatory sequences necessary for viral RNA synthesis. However, the linear single-stranded RNA molecule also contains the

[1] For the sake of clarity, 'replication competent' in this opinion refers to the ability to complete the viral replication cycle, including the repeated delivery of infectious viral particles after infection of cells. Viral particles that are unable to do so because of the RNA they contain are referred to as 'replication defective'.

ORFs of all proteins essential for RNA synthesis. The RNA can thus replicate independently in susceptible cells without the help of other proteins expressed *in trans.* However, because the RNA still lacks at least one ORF of a protein involved in the formation of infectious viral particles, no infectious viral particles are formed. This defect can be compensated by expression of the missing structural proteins *in trans*. In this case, so-called 'single round infectious particles' are formed, which can infect a target cell once, but do not lead to the renewed formation of infectious particles in this cell. The RNA contained in the particle, however, is replicated and translated again. Replicon particles produced in this way are classified as virus-like particles (see point 1.4.).

A foreign gene, typically a reporter gene, is usually cloned into the site of the deleted ORF of the structural protein. Thus, in cells transfected with replicon RNA or a plasmid with a corresponding cDNA construct, expression of the reporter protein and viral proteins occurs. This expression is typically of longer duration than when a non-replicative mRNA is transfected. However, because replication of the viral RNA and encoded proteins can lead to a cytopathic effect due in part to intracellular immune responses, protein expression using a replicon is also time-limited. For example, with the commonly used alphaviral replicons, expression can be achieved for three to five days [2] . However, with newer replicon systems, the insertion of specific point mutations or deletions attempts to reduce the cytopathic effect of the replicon and thus increase its persistence in cells. Expression of the reporter gene of a Sendaivirus replicon developed using this approach lasted for more than six months in cell culture [3] . Similarly, insertion of a selection marker, such as a puromycin resistance gene, can lead to prolonged persistence in cells under appropriate selection pressure. Another way to increase persistence is to express immunosuppressive proteins using separate expression plasmids or directly via the replicon. However, integration of the replicon into cellular DNA is not expected even with increased persistence, since the RNA replicon is not reverse transcribed unless this is explicitly induced by insertion of a nucleic acid segment encoding a reverse transcriptase. The most commonly used replicons are based on alphaviruses (e.g. *Semliki Forest virus*, *Sindbis virus*, *Venezuelan equine encephalitis virus*), flaviviruses (e.g. *Kunjin virus*, *West Nile virus*, *Dengue virus*), paramyxoviruses (e.g. measles virus, sendaivirus) or rhabdoviruses (e.g. vesicular stomatitis virus, rabies virus) (Figure 1).

1.4. Virus-like particles

Overexpression of certain proteins of RNA viruses in eukaryotic cells can lead to spontaneous assembly of particles. These can be pure protein complexes or membrane vesicles in which viral membrane proteins are incorporated. Typical proteins exhibiting this property are viral (nucleo)capsid, matrix and envelope proteins. Because of their morphological similarity to natural viral particles, such particles are called virus-like particles. If one or more of these proteins are overexpressed in a cell in the presence of biological macromolecules or, for example, pharmaceutical agents, these (macro)molecules can also be entrapped in the lumen of the particles. RNA molecules and proteins are particularly suitable for this purpose, provided that their size does not exceed the virus-specific packaging capacity of the particles. Virus-like particles with components for specific receptor binding and entry into a target cell can thus serve for the targeted transfer of the enclosed (macro)molecules to cells. The efficiency of formation of such particles is high when one of the expressed viral proteins specifically binds the (macro)molecule. Such an interaction may be naturally possible, as in the case of nucleocapsid proteins and the matching viral packaging signal. However, it can also be artificially generated by modification of the viral protein and/or the desired binding partner.

Virus-like particles are becoming increasingly important, especially in tumor therapy and vaccine development [5] .

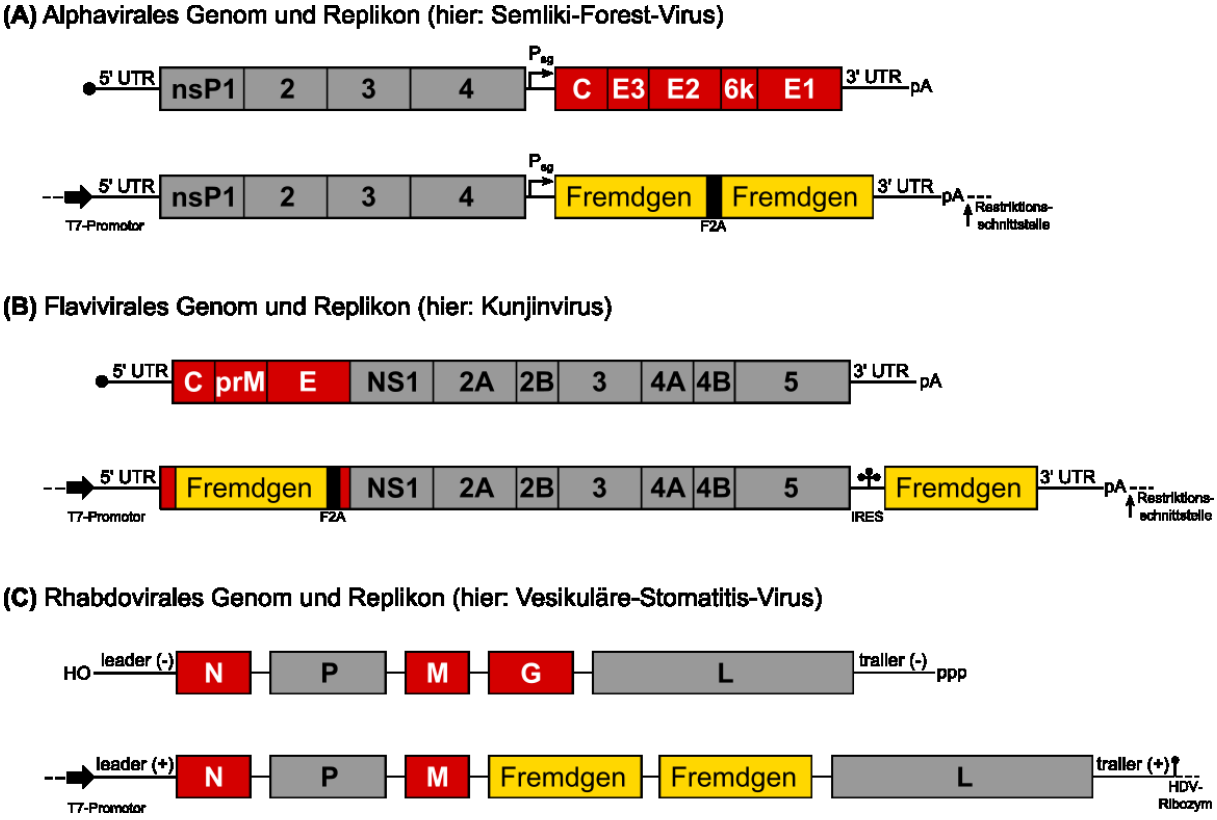

Figure 1: Schematic representation of typical replicon constructs (each in its plasmid form) and the viral genomes from which they are derived. ORFs of non-structural proteins are shown in grey, of structural proteins in red and foreign genes in yellow. Transcription of viral RNA is usually under the control of a phage promoter. To obtain the correct viral 3' end, either a restriction site (for *in vitro* transcription) or a Hepatitis delta virus (HDV) ribozyme (for *in vivo* transcription) is cloned downstream of the viral cDNA. **(A)** Alphaviral replicon: foreign genes are typically inserted downstream of the subgenomic promoter (P_{sg}) in place of the structural ORF. Insertion of additional transcription units under the control of another subgenomic promoter is possible. If multiple foreign proteins are to be encoded in an ORF, they are separated by a picornaviral 2A peptide sequence (F2A) to allow expression of separate proteins. **(B)** Flaviviral replicon: foreign genes may be inserted in place of the coding region of the structural proteins or as separate ORFs upstream *of* the non-coding region at the 3' end. In this case, an internal ribosome entry site (IRES) is inserted for retranslation initiation. Due to overlapping regulatory sequences or the signal peptide of NS1, portions of the C and E coding regions must be conserved. **(C)** Rhabdoviral replicon: foreign genes in replicons of viruses of the order *Mononegavirales* are often inserted in place of the gene of the protein mediating receptor binding and/or fusion (here both G). Other insertion sites of complete transcription units between the remaining genes are possible. In contrast to replicons of plus-stranded RNA viruses, the proteins of the replication complex must be provided in *trans for* the initial start of viral RNA synthesis. However, subsequent RNA synthesis occurs autonomously. Figure adapted from [4] .

Note on the classification under genetic engineering law:

According to Section 3 No. 1 GenTG, "organisms" are defined as biological entities that are capable of reproducing or transferring genetic material. Virus-like particles that do not contain nucleic acids, e.g. consist exclusively of proteins, therefore do not fulfil the definition of an "organism" and consequently cannot be "genetically modified organisms" (GMOs) within the meaning of the GenTG. If, on the other hand, a virus-like particle contains nucleic acids, the legal classification of these particles depends on the type of nucleic acid. Non-coding RNAs, such as guide RNAs or siRNAs, and mRNAs (except for replicable mRNA of plus-strand RNA and retroviruses) are not the heritable nucleic acids of an organism. Thus, these RNAs are not genetic material in the strict sense. Likewise, these RNAs are not capable of replication in a cell. Virus-like particles containing such RNAs are therefore not GMOs.

If virus-like particles, which are not GMOs themselves, are used to transduce eukaryotic cells, the RNAs, proteins and/or other (macro)molecules transferred with them are transiently detectable in the cell. Transferred non-replicable mRNAs are also transiently translated. However, there is usually no change in the genetic material of the transduced cells. In these cases, the transfer of the RNAs described or of proteins and other (macro)molecules does not represent a procedure for altering the cellular genetic material. Originally non-recombinant cells transduced with the described virus-like particles are not GMOs. Provided that the recombinant cells used for production have been separated from the described virus-like particles by filtration or centrifugation, the handling of these particles therefore does not constitute genetic engineering work.

The use of **virus-like particles containing proteins or mRNAs coding for them that** can **directionally modify the genome of the target cell** constitutes a mutagenesis procedure. On 25 July 2018, the European Court of Justice (ECJ) ruled that organisms produced using new mutagenesis techniques that were mainly developed after the adoption of the 2001/18/EC Release Directive in 2001 are GMOs within the meaning of the Release Directive. The decision as to whether this ruling is also to be applied within the scope of Directive 2009/41/EC on the contained use of micro-organisms to organisms modified by such mutagenesis processes is a matter for the respective competent authorities of the German federal states.

2. Summary of relevant criteria for the safety classification of genetic engineering work with viral minigenomes, viral replicons and virus-like particles

2.1 Potential hazards of genetic engineering work with *Escherichia coli*

To produce viral minigenomes, viral replicons and virus-like particles, subgenomic nucleic acid segments of RNA viruses are first amplified in their cDNA form using vectors in *E. coli* K12 derivatives. The expression of the viral nucleic acid segment is usually controlled by a T7 or SP6 phage promoter. Alternatively, viral or eukaryotic promoters can be used. These promoters are usually not active in *E. coli*. Moreover, the viral nucleic acid segments do not possess any hazard potential of their own. Nor is it to be assumed that the viral genome is completed by recombination with the genome of *E. coli.* Provided that the vector-receptor system used is a biological safety measure, genetic engineering work involving the introduction of plasmids with subgenomic viral nucleic acid segments into *E. coli* K12 derivatives of risk group 1 therefore has no hazard potential for humans, animals or the environment.

2.2 Hazard potential of human and animal cells with viral minigenome or replicon

For the safety assessment of human and animal cells of risk group 1 to which a viral minigenome or replicon has been transferred by transfection or transduction, the possibility of the formation of viral or virus-like particles is of crucial importance. Unless a viral structural protein (or combination of structural proteins) is expressed in the cell to enable particle formation, neither replication-defective virus-like particles nor replication-competent virus particles can be delivered. If no gene of a prion or toxin has been transferred, there is no potential hazard to humans, animals or the environment when handling such cells.

If, on the other hand, after co-transfection of further plasmids or mRNAs or due to transduction of the cell line or its infection with a helper virus, viral structural proteins are expressed which are in principle suitable for packaging the minigenome or replicon specifically or non-specifically, or if this cannot be ruled out, the particles which may have been released determine the hazard potential of the cells. This must also be taken into account in particular if the transfected cells are assigned to at least risk group 2, since such cells generally contain replication-competent viral genomes including structural genes. In the case of such cells, an interaction between the viral proteins or nucleic acid segments may also have to be evaluated.

Of subordinate relevance for the risk assessment is generally whether the minigenome or replicon and any further subgenomic viral nucleic acid segments were transferred as *in vitro*-transcribed RNA or whether this is only transcribed in the cell after transfer of a plasmid with the viral cDNA. It should only be noted that due to the mechanistic differences between homologous RNA and homologous DNA recombination, different recombination products can be expected.

2.3 Hazard potential of virus-like particles

When assessing the hazard potential of virus-like particles delivered by cells in which a viral minigenome, viral replicon or, where applicable, other packable RNA is present, three aspects must be considered in addition to the properties of the foreign gene contained (see point 2.4.): the infectivity of the particles, the possibility of abrogating their replication defect and possible contamination with replication-competent helper viruses.

Virus-like particles are replication-defective particles. They may or may not be able to transfer the RNA they contain to a target cell, i.e. virus-like particles may be infectious or non-infectious.

A replication defect is always present in the absence of infectivity and is due to the absence of viral proteins that mediate binding to a cellular receptor and/or subsequent entry or fusion of the particle into or with the cell. Non-infectious virus-like particles have no hazard potential for humans, animals or the environment, regardless of the RNA they contain.

However, most virus-like particles are infectious. If human or animal cells are transduced with these particles, however, there is no further release of these particles. This can always be assumed if the packaged RNA is either not replicated in the cell or if no viral proteins are present in the transduced cell that enable the RNA to be repackaged into infectious particles. It should be noted that in the presence of structural proteins of heterologous viruses, complementation often cannot be excluded. The hazard potential of infectious virus-like particles depends on the RNA they contain.

Depending on the manufacturing system, human and animal cells with a viral mini-genome or replicon can deliver replication-competent virus particles in addition to replication-defective virus-like particles, if applicable. These may result from RNA or DNA recombination with the helper constructs used for packaging. In terms of risk assessment, the likelihood of such recombination events occurring and leading to a correct, replication-competent genome structure is of importance. It can be assumed that a correct genome structure results primarily from homologous recombination events. In contrast, the probability that a replication-competent genome will result from at least two illegitimate (i.e., non-homologous) recombination events should be considered very low. The possibility of illegitimate recombination is therefore only considered in the risk assessment for viruses in risk group 4,

due to the severity of the resulting damage. In order to reduce the likelihood of genome reconstitution by recombination, helper functions should, where possible, be introduced into production cells on separate nucleic acid segments. In addition, homologous regions between the minigenome or replicon and the helper constructs should be avoided as much as possible. Finally, the generation of replication-competent virus particles can also be prevented by removing coding or regulatory nucleic acid segments that are not required for the specific research purpose. However, it must be taken into account that in some cases incomplete viral genomes can also lead to the formation of replication-competent, possibly attenuated viral particles (e.g. in the case of alphaviral replicons). If, due to the inserted foreign gene or the combination of minigenome or replicon and helper constructs, it cannot be excluded that replication-competent viral particles may be delivered after recombination, these determine the hazard potential of the mixture of viral particles. It should be noted that structural proteins of heterologous viruses may also functionally replace a missing structural protein and thus cancel the replication defect.

If virus-like particles are produced with the aid of a replication-competent helper virus, the presence of a mixture of replication-defective virus-like particles and replication-competent virus particles in the cell culture supernatant must be assumed in principle. The hazard potential of this mixture is determined by the replication-competent virus particles, except in the case of a helper virus of risk group 1. In this case, too, the recombination possibilities between the viral helper genome and an introduced minigenome or replicon must be taken into account.

2.4 Hazard potential of the foreign gene

Since the described minigenomes and replicons are truncated viral genomes that cannot independently perform certain functions within the viral replication cycle, when inserting foreign, usually viral, nucleic acid segments, it is important to consider whether the replication defect of the minigenome or replicon, or of virus-like particles containing them, can be abrogated. This is particularly true when nucleic acid segments encoding **foreign structural proteins** are inserted into replicons and thereby enable or could enable the formation of replication-competent virus particles.

Virus-like particles that can transduce human cells and contain RNA with **neoplastic transforming potential** may have a hazard potential for humans. This can always be assumed if, as a result of transduction in a human cell, there is prolonged overexpression of an oncogene, prolonged suppression of a tumour suppressor gene or targeted mutation of a proto-oncogene or tumour suppressor gene or their mRNA. A long-lasting expression of the proteins responsible for this can generally only be expected in the case of transduction with virus-like particles containing a replicon. The reason for this is the low stability of RNA in biological media, which manifests itself in short half-lives of a few hours. Since replicons can also proliferate independently of complementation in native susceptible cells, transduction of such RNA can be expected to result in prolonged expression of the encoded proteins despite the short half-life of the individual RNA molecules. For virus-like particles containing a replicon including a nucleic acid segment with neoplastic transforming potential, a low hazard potential for humans is therefore to be assumed as a precautionary measure. A low hazard potential of virus-like particles for humans can also not be excluded if the particles contain a protein or a non-replicative mRNA encoding such a protein, which can lead to the transformation of a cell by mutation or epigenetic modification even when expressed in the short term. Such proteins include those that specifically adversely modify a cellular proto-oncogene or tumor suppressor gene or permanently increase the expression of an oncogene or permanently decrease the expression of a tumor suppressor. Examples include, in particular, reprogramming factors and complexes between the endonuclease Cas9 and a guide RNA directed against a tumor suppressor. In contrast, a minigenome is neither translated nor transcribed nor replicated in the absence of complementing factors. Virus-like particles containing a minigenome, including a nucleic acid segment with neoplastic transforming potential, therefore have no hazard potential for humans, animals or the environment.

A further criterion in the risk assessment of virus-like particles that can transduce human cells is whether an RNA present in the particle can be reverse-transcribed and the resulting DNA integrated into the human genome. In the case of accidental exposure of the experimenter to such virus-like particles, insertional mutagenesis cannot be completely ruled out. Therefore, virus-like particles that can transduce human cells and contain a **reverse transcriptase and integrase** or lead to the expression of these enzymes can be assumed to have a low hazard potential for humans.

If **prions or toxins** are expressed with the aid of viral minigenomes or replicons, or if the corresponding genes are transferred by means of virus-like particles, the hazard potential of this genetic engineering work depends, among other things, on the specific properties of the prions and toxins and the expected level of expression. An assessment of the hazard potential of this work must therefore be carried out by the ZKBS on a case-by-case basis.

3. Criteria for comparability of genetic engineering work with viral minigenomes, viral replicons and virus-like particles

The following summarises general comparability criteria for genetic engineering operations involving viral minigenomes, viral replicons and virus-like particles derived from an RNA virus. Genetic engineering operations involving GMOs that meet the above criteria should be comparable with each other and assigned to the safety level corresponding to the risk group of the GMO. **This opinion does not apply to genetic engineering operations involving the *Hepatitis D virus* and other viruses of the genus *Deltavirus***.

If **prions or toxins** are to be expressed or their genes transferred, a case-by-case evaluation by the ZKBS is required.

If nucleic acid segments with a **neoplastic transforming potential** are to be transferred, the instructions listed under point 4 must be observed.

If replication-competent **helper viruses** are used for the propagation of minigenomes or the production of virus-like particles, their hazard potential must be taken into account in the risk assessment of the genetic engineering work.

The following definitions of terms are used:

- **Minigenome:** subgenomic nucleic acid segment of an RNA virus containing all *cis*-regulatory viral sequences necessary for its replication and possibly transcription; at least one viral protein necessary for RNA replication is not encoded; in addition, one or more foreign genes may be present.
- **Replicon:** subgenomic nucleic acid segment of an RNA virus that contains all *cis*-regulatory viral sequences and encodes all viral proteins necessary for its replication and possibly transcription (In the case of minus-strand RNA viruses, the proteins of the replication complex must be made available *in trans* for the initial start of viral RNA synthesis. However, subsequent RNA synthesis occurs autonomously); at least one of the proteins required for particle formation, binding to a cellular receptor, **or** transfer of viral RNA to a cell is not encoded; in addition, one or more foreign genes may be included.

  Note: The replication defect must be due to a deletion of a functionally significant portion of the viral nucleic acid segment encoding the protein that allows particle formation, receptor binding**, or** entry or fusion of the viral particle into or with the target cell. However, to preserve regulatory functions, nucleic acid segments encoding non-functional portions of such a structural protein may be included where appropriate. However, due to the high mutation rate of viral RNA polymerases, a stable replication defect cannot be assumed if it is based exclusively on the insertion of inactivating point mutations, artificial stop codons or frameshift mutations.

- **Virus-like particles:** replication-defective protein complexes or membrane vesicles derived from an RNA virus with embedded proteins, which may contain RNA and may be capable of transferring it to human or animal cells.
- **Helper plasmids and equivalent nucleic acid segments:** Eukaryotic expression plasmids or nucleic acids with subgenomic viral nucleic acid segments which, after transfection or transduction in the cell, provide viral proteins necessary for the propagation of a minigenome or replicon (only in the case of minusstrand RNA viruses) or the packaging of an RNA; no viral *cis*-regulatory sequences and packaging signals are included, except those overlapping with the coding regions necessary for the functions described.

Transfer of plasmids with subgenomic viral nucleic acid segments to E. coli K12 derivatives

3.1. *E. coli* K12 derivatives of **risk group 1** including a plasmid containing subgenomic nucleic acid segments of an RNA virus and, where appropriate, other nucleic acid segments are GMOs of **risk group 1**. If the nucleic acid segments include an oncogene, the GMOs are to be assigned to **risk group 1,** provided that the vector-receptor system used is a biosafety measure.

Transfection of a minigenome or replicon into human or animal cells

3.2. Human and animal cells of **risk group 1** to which a minigenome has been transferred by transfection are **risk group 1** GMOs unless the replication defect of the minigenome is expected to be abolished. There is no shedding of infectious virus-like particles or replication-competent virus particles.

3.3 Human and animal cells of **risk group 1 to** which a replicon and, where appropriate, one or more helper plasmids or equivalent nucleic acid segments encoding the proteins of the viral replication complex have been transferred by transfection (only in the case of minus strand RNA viruses) are GMOs of **risk group 1,** unless the replication defect of the replicon is expected to be abolished. The release of non-infectious virus-like particles without hazard potential may occur.

Notice: The virus-like particles that may be released are not GMOs within the meaning of § 3 GenTG, as they cannot infect cells and thus cannot transfer genetic material or reproduce.

3.4. Human and animal cells of **risk group 1**, **2**, **3**** or **3,** to which a minigenome or replicon has been transferred by transfection and in which an abrogation of the replication defect of the viral RNA due to a foreign gene cannot be excluded, are to be evaluated as GMOs on a **case-by-case basis** by the ZKBS. The delivery of replication-competent virus particles cannot be excluded.

Propagation of minigenomes in human or animal cells

3.5. Human and animal cells of **risk group 1** in which viral non-structural proteins are expressed after exclusive transfer of helper plasmids or equivalent nucleic acid fragments are **risk group 1** GMOs, as appropriate. There is no shedding of virus-like particles or replication-competent virus particles.

3.6. Human and animal cells of **risk group 1** to which a minigenome and one or more helper plasmids or equivalent nucleic acid segments encoding viral non-structural proteins have been transferred are GMOs of **risk group 1** unless the replication defect of the minigenome is expected to be abolished and not all structural proteins necessary for the formation of infectious particles are encoded in the minigenome. There is no release of virus-like particles or replication-competent virus particles.

3.7. For human and animal cells of **risk group 1** to which a minigenome has been transferred and which have been infected with a replication-competent helper virus, the

risk group of the helper virus determines the risk group of the GMO. Possible recombination between the genome of the helper virus and the minigenome must be taken into account. The release of replication-competent particles of the helper virus occurs.

3.8. Human and animal cells of **risk group 1**, **2**, **3**** or **3 to** which a minigenome and one or more helper plasmids or equivalent nucleic acid segments encoding viral non-structural proteins have been transferred, and in which an abrogation of the replication defect of the minigenome due to homologous recombination cannot be excluded, shall be evaluated as GMOs on a **case-by-case basis** by the ZKBS. The delivery of replication-competent virus particles cannot be excluded.

Generation of virus-like particles

3.9. Human and animal cells of **risk group 1** in which viral structural proteins are expressed after exclusive transfer of helper plasmids or equivalent nucleic acid segments may be **risk group 1** GMOs. Non-infectious or infectious virus-like particles without hazard potential may be released.

Notice: The virus-like particles that may be released are not GMOs within the meaning of § 3 GenTG, as they do not contain any genetic material and cannot reproduce.

3.10. Human and animal cells of **risk group 1 to** which a replicon of a virus of **risk group 1**, **2**, **3**** or **3,** which does not contain an oncogene and does not contain functions for reverse transcription and integration of its reverse-transcribed DNA, and one or more helper plasmids or equivalent nucleic acid segments encoding viral structural and, where appropriate, non-structural proteins have been transferred, are GMOs of risk group 1, unless the replicon's replication defect has been abolished due to homologous recombination or because the replicon does not contain functions for reverse transcription and integration of its reverse-transcribed DNA. The DNA transferred is a **risk group 1** GMO, unless the replication defect of the replicon is expected to be abolished by homologous recombination or by a foreign gene. The release of infectious virus-like particles occurs. These are **risk group 1** GMOs.

3.11. Human and animal cells of **risk group 1 to** which a replicon of a virus of **risk group 1**, **2**, **3**** or **3** containing an oncogene or functions for reverse transcription and integration of its reverse-transcribed DNA and one or more helper plasmids or equivalent nucleic acid segments encoding viral structural and, where appropriate, non-structural proteins have been transferred are GMOs of risk group 2, unless the replicon's replication defect has been abrogated due to homologous recombination or integration of its reverse-transcribed DNA. non-structural proteins are **risk group 2** GMOs, unless the replication defect of the replicon is expected to be abolished due to homologous recombination or a foreign gene. The release of infectious virus-like particles occurs. These are **risk group 2** GMOs.

3.12. Human and animal cells of **risk group 1** to which a replicon of a virus of **risk group 4** and one or more helper plasmids or equivalent nucleic acid segments encoding viral structural and, where applicable, non-structural proteins have been transferred are GMOs of **risk group 3,** provided that illegitimate (i.e. (i.e. non-homologous) recombination with a single helper construct could abolish the replication defect of the replicon and it cannot be assumed that the replication defect of the replicon is abolished due to homologous recombination or a foreign gene. The release of infectious virus-like particles occurs. These are GMOs of **risk group 1** or **2**. However, with a very low probability, replication-competent virus particles of **risk group 4** can also be produced after at least two-fold illegitimate recombination. Due to the very low probability of occurrence of this event, **level 3 safety measures** are considered sufficient for this work.

3.13. Human and animal cells of **risk group 1**, **2**, **3**** or **3 to which** a replicon and one or more helper plasmids or equivalent nucleic acid segments encoding viral structural and, where applicable, non-structural proteins have been transferred and in which a cancellation of the replication defect of the replicon due to homologous recombination or a foreign gene cannot be excluded, are to be evaluated as GMOs on a **case-by-case basis** by the ZKBS. Infectious virus-like particles are released. These are GMOs of **risk group 1** or **2**. In addition, the release of replication-competent virus particles cannot be excluded.

Note: The evaluation of Sindbis and Semliki Forest virus-derived replicon particles carried out in the general opinion of the ZKBS with ref. 6790-10-50 replaces a case-by-case evaluation, provided that the criteria mentioned therein are fulfilled.

3.14. Human and animal cells of **risk group 1** to which a minigenome of a virus of **risk group 1**, **2**, **3**** or **3** or a non-oncogenic packable RNA other than a minigenome or replicon and one or more helper plasmids or equivalent nucleic acid segments encoding viral structural and, where appropriate, non-structural proteins have been transferred are GMOs of risk group 1, where appropriate. The minigenome is a **risk group 1** GMO, if any, unless the replication defect of the minigenome is expected to be abolished by homologous recombination or a foreign gene and the minigenome does not contain functions for reverse transcription or integration of its reverse-transcribed DNA. The release of infectious virus-like particles occurs. These may be **risk group 1** GMOs.

3.15. Human and animal cells of **risk group 1** to which a minigenome of a virus of **risk group 1**, **2**, **3**** or **3** containing functions for reverse transcription and integration of its reverse-transcribed DNA and one or more helper plasmids or equivalent nucleic acid segments, encoding viral structural and non-structural proteins have been transferred are **risk group 2** GMOs, unless the replication defect of the minigenome is expected to be abolished due to homologous recombination or a foreign gene. The release of infectious virus-like particles occurs. These are **risk group 2** GMOs.

3.16. Human and animal cells of **risk group 1** to which a packable RNA, other than a minigenome or replicon, and one or more helper plasmids or equivalent nucleic acid segments encoding viral structural proteins have been transferred are GMOs of **risk group 2,** provided that the RNA encodes or is packaged together with a sequence-specific protein with a persistent oncogenic effect and confers its sequence specificity. Infectious virus-like particles with a low hazard potential are released.

Notice: The delivered virus-like particles are not GMOs in the sense of § 3 GenTG, as they do not contain genetic material and cannot reproduce.

3.17. Human and animal cells of **risk group 1 to** which a minigenome of a virus of **risk group 4** and one or more helper plasmids or equivalent nucleic acid segments encoding viral structural and non-structural proteins have been transferred are GMOs of **risk group 3,** provided that an illegitimate (i.e. non-homologous) recombination with a single helper construct could reverse the replication defect of the minigenome and does not assume the abrogation of the replication defect of the minigenome. (i.e. non-homologous) recombination with a single helper construct could abolish the replication defect of the minigenome and the replication defect of the minigenome is not expected to be abolished due to homologous recombination or a foreign gene. The release of infectious virus-like particles occurs. These are GMOs of **risk group 1** or **2**. However, with a very low probability, replication-competent virus particles of **risk group 4** can also be produced after at least two-fold illegitimate recombination. Due to the very low probability of occurrence of this event, **level 3 safety measures** are considered sufficient for this work.

3.18. Human and animal cells of **risk group 1**, **2**, **3**** or **3 to** which a minigenome and one or more helper plasmids or equivalent nucleic acid segments encoding viral structural and non-structural proteins have been transferred and in which a cancellation of the

replication defect of the minigenome due to homologous recombination or a foreign gene cannot be excluded, shall be evaluated as GMOs on a **case-by-case basis** by the ZKBS. Infectious virus-like particles are released. These are GMOs of **risk group 1** or **2**. In addition, the release of replication-competent virus particles cannot be excluded.

Transduction of human or animal cells with virus-like particles

3.19. Human and animal cells of **risk group 1** to which RNA has been transferred using virus-like particles of **risk group 1 are** GMOs of **risk group 1,** unless the replication defective particles are present in admixture with replication competent virus particles and the replication defect of the virus-like particles is abolished. In the absence of viral structural proteins, delivery of virus-like particles or replication-competent virus particles does not occur.

3.20. Human and animal cells of **risk group 1** to which RNA has been transferred using virus-like particles of **risk group 2** are GMOs of **risk group 1** after completion of transduction, provided that the replication-defective particles are not mixed with replication-competent virus particles and no viral structural proteins are expressed. There is no shedding of virus-like particles or replication-competent virus particles.

3.21. Human and animal cells of **risk group 2**, **3**** or **3** in which viral genomes are already present and to which RNA has been transferred by means of virus-like particles of **risk group 1** or **2** are GMOs and belong to the risk group of non-transduced cells, provided that the replication-defective particles are not present in admixture with replication-competent virus particles and homologous recombination between an already initially present viral RNA and the introduced viral RNA cannot be assumed. The release of virus-like particles or replication-competent virus particles may occur.

3.22. Human and animal cells of **risk group 1**, **2**, **3**** or **3** to which RNA has been transferred using virus-like particles of **risk group 1** or **2**, in which mixing with replication-competent virus particles cannot be excluded due to homologous recombination or a foreign gene, are to be assessed as GMOs on a **case-by-case basis** by the ZKBS. The release of replication-competent virus particles cannot be excluded.

3.23. Human and animal cells of **risk group 1**, **2**, **3**** or **3 to** which RNA of a virus of **risk group 4** has been transferred using virus-like particles of **risk group 1** or **2,** in which mixing with replication-competent virus particles cannot be excluded due to illegitimate recombination, are GMOs of **risk group 3**. The release of replication-competent virus particles cannot be excluded. Due to the very low probability of the formation of replication-competent virus particles, **level 3 safety measures** are considered sufficient for this work.

4. Notes

Further explanations on specific viral minigenomes, viral replicons and/or virus-like particles can be found in the following general statements of the ZKBS:

- General statement of the ZKBS on frequently performed genetic engineering work with underlying criteria of comparability: stable and transient gene expression using γ-retroviral and lentiviral vectors (ref. 6790-10-41, updated February 2020).
- General statement of the ZKBS on frequently performed genetic engineering work with the underlying criteria of comparability: genetic engineering work with the *Sindbis virus* and the *Semliki Forest virus* expression system (ref. 6790-10-50, updated May 2017).
- Opinion of the ZKBS on the reclassification of replicon constructs of HCV in eukaryotic cells (ref. 6790-10-78, March 2003)
- ZKBS statement on the classification of recombinant rabies and vesicular stomatitis viruses (Ref. 45310.0117, updated September 2019).

When handling nucleic acids with neoplastic transforming potential, as well as *E. coli*, eukaryotic cells and virus-like particles in which these nucleic acids are present episomally or which contain them, the following additional safety measures must be observed:

- Disposable gloves must be worn and changed regularly.
- Persons with significant skin lesions (open eczema, wounds and infections) or with pronounced verrucosis (wart formation) should not perform any work with the above-mentioned nucleic acids, cells or particles.
- The use of sharp, pointed or fragile laboratory objects should be avoided whenever possible.
- Laboratory space and equipment that come into contact with the above-mentioned nucleic acids, cells or particles must be carefully cleaned after the end of the activity.
- Laboratory waste containing the above-mentioned nucleic acids, cells or particles must be denatured or inactivated by autoclaving or chemically.

In addition, the following safety precautions must be observed when handling virus-like particles that contain nucleic acids with neoplastic transforming potential and can transfer these to human cells:

- The safety cabinet in which this work is carried out must be marked accordingly.
- Vessels and equipment that are removed from the safety cabinet must first be disinfected from the outside with a suitable disinfectant.
- Cell culture flasks containing the virus-like particles shall preferably be closed with filter screw caps. If filter screw caps are not used, the cell culture flasks in which the virus-like particles are present shall be opened only sufficiently to ensure gas exchange. In addition, the aeration for gas exchange has to take place in the CO_2incubator first, in order to avoid the escape of culture liquid during transport.
- When handling virus-like particles that are not airborne, wear mouth and nose protection to prevent smear infection.
- When handling virus-like particles that can be transmitted via the air, respiratory protection with a retention capacity of class P3 must be worn. FFP3 respirators, respirators with P3 filters and TH3P respirator hoods, for example, have such a retention capacity. TH3P respirators are particularly suitable because they are less stressful for the wearer and there are fewer leakage problems.

Reference is also made to the following general comments of the ZKBS:

- Statement of the ZKBS: Precautionary measures when handling nucleic acids with neoplastic transforming potential (ref. 6790-10-01, updated December 2016)
- Opinion of the ZKBS: Evaluation of genetically modified organisms in which nucleic acid segments with neoplastic transforming potential have been introduced (ref. 6790-10-36, updated December 2014)

5. Literature

1. **Hoenen T, Groseth A, Kok-Mercado F de, Kuhn JH, 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**(2):195-208.
2. **Lundstrom K** (2016). Replicon RNA Viral Vectors as Vaccines. *Vaccines (Basel)* **4**(4):39.
3. **Schott JW, Morgan M, Galla M, Schambach A** (2016). Viral and synthetic RNA vector technologies and applications. *Mol Ther* **24**(9):1513-27.
4. **Fernandes RS, Freire MCLC, Bueno RV, Godoy AS, Gil LHVG, Oliva G** (2020). Reporter Replicons for Antiviral Drug Discovery against Positive Single-Stranded RNA Viruses. *Viruses* **12**(6):598.

5. **Roldão A, Silva AC, Mellado MCM, Alves PM, Carrondo MJT** (2019). Viruses and virus-like particles in biotechnology: fundamentals and applications. *Comprehensive Biotechnology* **2017**:633-56.