Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

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

Gene transfer using retroviral vectors

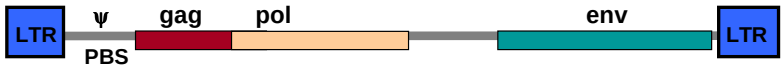
1. Description of the retroviral system

1.1 . General introduction

Retroviruses (family: *Retroviridae*) are uncoated RNA viruses that the International Committee on Taxonomy of Viruses (ICTV) has divided into two sub-families, the orthoretroviruses and spumaretroviruses, and seven types, based on their genetic similarities.

The genome of replication-competent retroviruses comprises two identical single-stranded RNA molecules with a length of 7-15 kb [1]. They replicate via a double-stranded DNA intermediate (provirus), which stably integrates into the genome of the infected cell. The three genes *gag*, *pol* and *env* are the basic elements of every retroviral genome (Fig. 1).

a)



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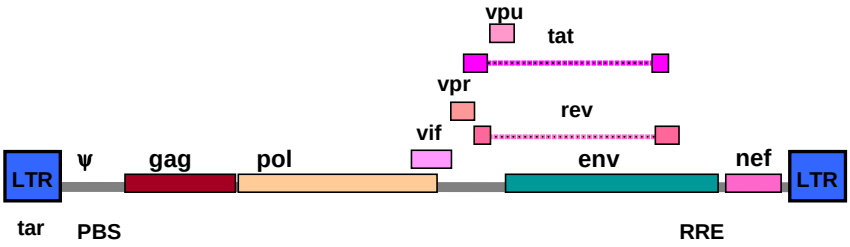

Figure 1:

a) Genetic map of a simple retrovirus using Moloney Mouse Leukaemia Virus provirus (MoMLV) as an example. PBS (primer binding site) is the binding site for the tRNA-primer; ψ indicates the packaging signal; gag (group specific antigen), pol (reverse transcriptase and integrase) and env (coat protein) are coding regions; LTR (long terminal repeats) contain repetitive sequences found at both ends of the DNA genome. The figure was modified from [1].

b) Genetic map of a complex retrovirus using the HIV provirus as an example. In addition to regulatory elements such as LTRs, packaging signal ψ, and the primer binding site (PBS), HIV possesses the tat-activation region (tar) and the rev-responsive element (RRE), as well as the genes gag, pol and env, and a number of additional reading frames coding for regulatory proteins. The figure was obtained and modified from: http://hiv-web.lanl.gov/MAP/landmark.html

A so-called LTR (long terminal repeat) with promoter and enhancer functions is located at both termini of the DNA genome (provirus). LTRs contain the repetitive sequences U_3RU_5, with the repetition R present at both termini of the RNA genome. U5 (unique sequence) represents a nucleotide sequence that is only present at the 5'-end of the RNA genome, and the sequence called U3 (unique sequence) is only present at the 3'-end of the RNA genome. These sequences are important for reverse transcription of the genome. In addition to these sequences, complex retroviruses contain various extra reading frames for gene products that perform regulatory functions (see Fig. 1).

1.2. Gene transfer using retroviral vectors

Recombinant retroviruses originally developed for gene transfer were based on murine retroviruses, particularly Molony Mouse Leukaemia Virus (MoMLV). They can only transduce actively dividing cells. Although non-dividing cells can take up retroviruses, the nucleocapsid is only able to pass through the nuclear membrane when this is degraded during cell division, allowing the nucleocapsid to enter the nucleus [2]. To stably transduce non-dividing or terminally differentiated cells retroviral vectors were constructed based on lentiviruses. While the early lentiviruses were derived from HIV-1 [3] and SIV (simian immunodeficiency virus), more recent vector systems have also been developed based on FIV (feline immunodeficiency virus) [4], EIAV (equine infectious anaemia virus) [5], CAEV (caprine arthritis encephalitis virus) [6], BIV (bovine immunodeficiency virus) [7] and MVV (Maedi/Visna virus) [8].

Murine retroviral vectors

The production of recombinant retroviruses derived from murine retroviruses involves two components: the retroviral vector and the packaging cell line (Fig. 2). The retroviral vector is a plasmid, usually derived from pBR328. This does not code for retroviral proteins, but only contains the packaging signal ψ, the primer binding site and the retroviral 5' and 3' LTRs, which flank an insertion site for the gene to be transferred, and if required, a selection marker. Expression of more than one transgene is achieved by a bicistronic expression cassette containing the IRES (internal ribosome entry site) sequence from picorna virus. Ribosome binding to IRES allows cap-independent translation of the proteins. The packaging cell line provides the retroviral proteins necessary for packaging the vector RNA and producing retroviral particles, and is created by introducing nucleic acids (helper genome) coding for retroviral structural proteins. The packaging signal ψ is deleted in this helper genome so that the viral RNA transcribed from the helper genome in the helper cell line is not packaged into virions. While in the first constructed packaging cell lines the structural genes were introduced into the cell together, now gag-pol and env are transfected separately into the cell to minimize recombination events during cell proliferation [9].

Depending on the kind of helper genome, coat proteins expressed in the helper cell lines are either ecotropic or amphotropic. The host range of ecotropic murine retroviruses is limited to cells from mice and rats. In contrast, amphotropic murine retroviruses have a wide host cell range that includes murine and non-murine – also human – cells. Increasingly, recombinant retrovirus development includes modifying or exchanging the coat protein of murine retroviruses to achieve a wide host range, a targeted host tropism for a certain cell type or higher stability of the virion (pseudotyping) [10]. A range of heterologously expressed coat proteins have already been used successfully for pseudotyping. Donors include arenaviruses [11], alphaviruses [12] and members of the families of *Hepadnaviridae* [13], *Flaviviridae* [14] and *Rhabdoviridae*. In particular, the vesicular stomatitis virus G-glycoprotein (VSV-G) is used in

many cases to provide murine retroviruses with a broad host range, including human cells. The glycoprotein in the packaged virus particle binds to specific receptors on the cell surface and initiates membrane fusion. The receptors for the most used retroviral coat proteins are only functionally expressed in polarized epithelial cells [15]. Thus, *in vivo* transduction of epithelial lung tissue cells with VSV-G pseudotyped retroviral vectors from the apical side could only be shown if access to basolateral surfaces was made possible by agents that open tight junctions [16, 17]. Moreover, VSV-G pseudotype retroviruses show greater stability, allowing particles to be concentrated by ultracentrifugation.

A specific cell tropism can be achieved when a ligand for a cellular surface protein, such as the recognition domain of an antibody, is inserted into the coat protein of murine retroviruses [18]. Since recombinant retroviruses with a specific cell tropism are primarily intended for use in somatic gene therapy, ligands for epitopes found on human cells are generally used.

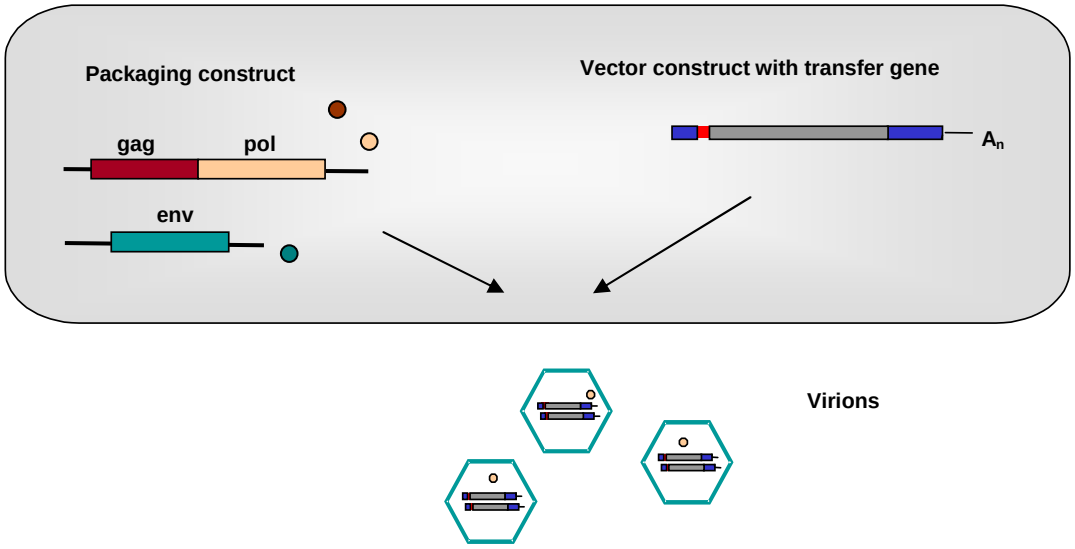

Figure 2:

Production of recombinant replication-defective retroviruses. The retroviral vector with the transgene intended for transfer is transfected into a packaging cell line constitutively expressing the retroviral structural proteins. Transcripts produced from the retroviral vector are not only translated but also packaged as genomic RNA, with the help of viral structural proteins, into new virions that are released from the packaging cell line. Figure modified from [19].

Using these recombinant retroviruses, which are generally replication-defective, cell lines can be infected where the transferred retroviral RNA is reverse transcribed into a provirus that stably integrates into the cell's genome. This results in expression of the transferred gene, as well as the selection marker if required.

So-called ping-pong amplification was developed to obtain higher virus titres. This involves a procedure where the recombinant retrovirus is amplified by alternating transfer to an amphotropic then ecotropic packaging cell line. The two packaging cell lines are co-cultivated to achieve this [20, 21, 22].

Lentiviral vectors

Lentiviruses have a complex genome (Fig. 1). With these vectors it is also possible to transduce non-dividing cells since the lentiviral pre-integration complex is able to pass through the intact nuclear membrane [3, 23]. This pre-integration complex comprises the enzyme integrase encoded by the *pol* gene, the *vpt* gene product, the matrix protein encoded by the *gag* gene, reverse transcriptase also encoded by the *pol* gene, as well as the viral RNAs. In addition to regulatory elements such as the LTRs, packaging signal ψ and structural genes *gag*, *pol* and *env* normally present on a retrovirus, lentiviruses possess additional elements and reading frames. The lentiviral transcriptional transactivator (tat) activates viral transcription by binding of the Tat protein to the tat activation region (tar), a cis-regulatory sequence located at the 5'-end of the viral RNA and DNA. Rev (regulator of expression of the virion) mediates the transport of simple spliced and unspliced viral RNA out of the nucleus through interaction with the rev response element, RRE, and allows transcription in the cytoplasm. The so-called accessory genes *vif*, *vpr*, *vpu* and *nef* are not essential for virus replication in cell culture. Vif (viral infectivity factor) increases the infectivity of the virion in primary T-cells; Vpr (virion associated protein) is involved in nuclear import of the pre-initiation complex. Nef and Vpu influence the infectiousness of the virions. Nef is also associated with reduced expression of CD4-receptors and MHC-I antigens on the infected cell's surface and Vpu degrades CD4-receptors in the ER, thus preventing complexes forming with the coat protein.

The development of lentiviral vectors is particularly relevant for gene therapy. However, due to the pathogenicity of lentiviruses there are concerns with respect to the safety of these vectors. A particular problem is the possible creation of replication-competent lentiviruses during vector production. However, possible recombination creating replication-competent lentiviruses can be minimized by using a three or four plasmid system comprising a vector plasmid, a packaging plasmid and one or two additional plasmids (coat protein gene and Rev protein gene). A minimal vector plasmid contains all the *cis*-active sequences essential for packaging, reverse transcription and integration. These are limited to the LTRs, the packaging signal □, the primer binding site (PBS) and the polypurine tract (PPT), as well as the desired transgene, if required, with a heterologous promoter (see Fig. 3a). Current constructs are also based on inducible systems, e.g. using tetracycline or ecdyson [24,25], or tissue specific promoters [26]. If the rev-RRE system from HIV is used to export the mRNA molecule to the cytoplasm, then the vector must also carry the RRE sequence. The proteins required for infection of a cell are supplied *in trans*. To increase the efficiency of gene transfer, additional regulatory sequences can be added to the vector. For example, the central polypurine tract (cPPT) supports translocation of the pre-integration complex into the cell nucleus [27]. A further example is the posttranscriptional regulatory element of wombat hepatitis virus (WPPE), which increases the RNA stability, thus mediating a higher expression rate of the transgene [28,29].

Since lentiviruses show limited cell tropism, the vectors derived from them are generally pseudotyped with coat proteins that extent the cell tropism. Often this involves using the *env* gene of amphotropic MLV, but mostly VSV-G is used [30]. Moreover, a range of other heterologously expressed viral coat proteins have already been successfully used for pseudotyping (e.g. lyssaviruses [31], baculoviruses [32], filoviruses [33] and alphaviruses [34]).

Due to the cytotoxic properties of HIV Gag/Pol proteins, establishing packaging cell lines for lentiviral vector systems is only possible in inducible or attenuated systems [35]. Usually three or four plasmids carrying the genes and regulatory sequences required for producing the vector divided among them are co-transfected into a cell line (see Fig. 3). There, replication-defective lentiviral vectors are packaged and released from the cell line.

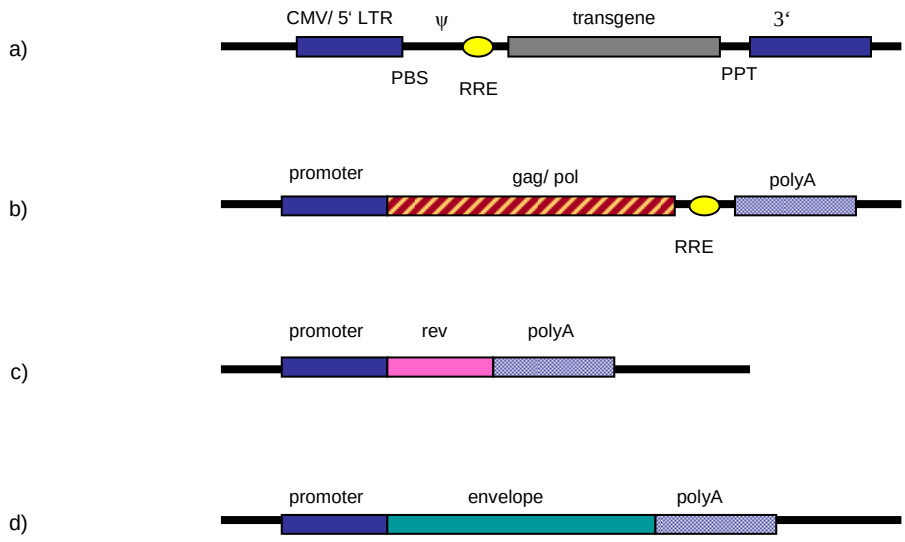

Figure 3: Components for producing HIV-based lentiviral vectors. Four plasmids are used for their production:

a) The minimal HIV vector comprises the CMV/5'-LTR hybrid promoter that allows tat-independent transcription, the 3'-LTR, the packaging signal ψ, the Rev binding element RRE for cytoplasmic export of the RNA, the PBS and PPT sequences required for reverse transcription, and the transgene expression cassette, which can also contain internal promoters and more than one gene to be transferred. All enzymatic or structural HIV proteins have been removed.

b) The HIV packaging plasmid contains the genes from HIV required for packaging retroviral RNA and provides these proteins *in trans*: 5' and 3'-LTRs are replaced by a heterologous promoter and a heterologous polyadenylation signal.

c) This plasmid carries the rev gene. The gene is under the control of a heterologous promoter; the polyadenylation site is also heterologous.

d) This plasmid carries a coat protein gene. The promoter of the gene and the polyadenylation site are also heterologous.

The figure was modified from [36].

A further safety problem that not only applies to lentiviral vectors but also MLV-derived vectors is the possibility of activating cellular oncogenes by random integration of the vector provirus into the host genome. This activation can happen at three levels: 1) the vector influences neighbouring cellular enhancers or promoter elements and thus gene expression; 2) promoters and enhancers in the integrated provirus activate neighbouring cellular oncogenes; 3) the presence of the vector DNA can lead to changes in the chromatin structure of regulatory domains and thus influence gene expression. With the so-called self-inactivating (SIN) vectors the U3-region of the 3'-LTR is deleted, which removes the promoter and enhancer properties contained in this region [3, 27, 37]. This not only limits potential activation of neighbouring cellular genes, but also during a viral replication cycle the deletion ends up at the 5'-LTR, thus shutting off vector expression and also preventing mobilization of the integrated vector provirus (see Fig. 4).

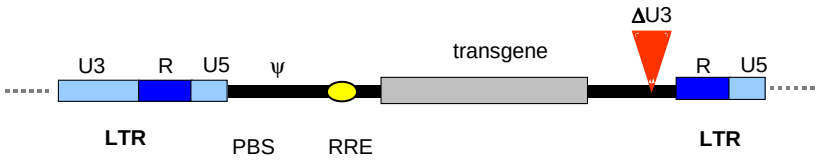

Figure 4: A self-inactivating (SIN) vector

As an additional safety measure, the PBS in the vector can be mutated so that no cellular tRNA can bind to it. To produce the vector it is then necessary to provide the appropriate mutated tRNA *in trans*, either as a synthesized tRNA or as a pol III gene on a co-transfected plasmid [38].

Introducing chromosomal insulators into the vector construct and the use of cell and tissue specific promoters represent further strategies for preventing activation of cellular oncogenes by unspecific integration. More recently, the development of vectors is towards trying to target integration into harmless regions of the human genome. Experiments are focusing on modifying the integrase, e.g. by inserting a specific DNA binding domain [39, 40, 41].

Adenovirus/retrovirus hybrid vectors

Chimeric vectors based on adenoviral and retroviral vectors were developed to achieve efficient gene transfer and long-term gene expression [42, 43]. Following co-infection, three replication-defective adenoviral vectors individually introduce the retroviral vector, the retroviral packaging functions (*gag/pol* region) and an *env* gene into a cell, which then becomes a retroviral-producing cell. The released retroviral vectors can then stably transduce other cells. Assessing the risk for producing adenoviral vectors follows the general position statement of the ZKBS on frequently carried out genetic engineering operations based on the criteria of comparability: gene transfer using adenovirus type 5, Ref. No. 6790-10-28, 2nd revised version from November 2001. Risk assessment of cells following co-infection of all three adenoviral vectors – as for a packaging cell line transfected by a retroviral plasmid – depends on the release of retroviral vectors.

2. Summary of relevant criteria for assigning safety and containment levels for genetic engineering operations involving gene transfer using retroviral vectors

The hazard potential in handling recombinant retroviruses derived from murine retroviruses is evaluated as low, even if it involves human pathogens. In the course of an infection the provirus genome integrates randomly into the genome of the host cell, and in individual cases can induce activation of cellular oncogenes or alter the transcription activity of other regulatory genes by insertion mutagenesis. The risk of these events is defined by the replication proficiency in the host, the potential target cells and natural defence mechanisms of the infected organism. Naturally occurring infections of humans with murine retroviruses are not known. In addition, these retroviruses have a low physical stability. Transmission via the respiratory tract has not been described. The major criterion for individually allocating the safety level for genetic engineering operations with recombinant murine retroviruses is the host spectrum range of the packaged recombinant retroviruses.

Ecotropic murine retroviruses are generally not considered a potential hazard for humans or animals (see ZKBS position statement on the risk assessment of ecotropic murine C-type retroviruses). It cannot be excluded that amphotropic murine retroviruses have a low hazard potential for humans, since primate cells can be infected with amphotropic retroviruses *in vitro* and under certain conditions *in vivo* [1, 44]. However, humans and old world monkeys have protection against infection with murine retroviruses. The glycosylation system of the mouse cell generates the so-called Gal epitope on both cellular and retroviral glycoproteins that form components of the virus coat. Interaction with anti-α-Gal antibodies circulating in human blood activates the complement system, which results in lysis of the retroviral particles [45].

Replication proficient recombinant retroviruses can arise if in the packaging cell line recombination events between homologous sequences in the helper genome and the vector genome create replication-competent retroviruses. Such an event depends on the vector/packaging cell line system used. Safety can be ensured by having little sequence homology between helper and vector genomes and introducing stop codons, mutations and deletions that in the case of recombination lead to a replication defect. An example here is the amphotropic packaging cell line PA317. While the appearance of replication-competent retroviruses has been described following transfection of the cell line with the N2 vector [46], LN-derived vectors are constructed in such a way that only replication-defective retroviruses are formed in the case of recombination [47]. Furthermore, the probability of a recombination event is reduced by using packaging cell lines where the genes for *gag/pol* and the coat protein *env* are separated from each other. This is mainly the case when producing recombinant retroviruses with altered coat proteins. Any appearance of replication-competent amphotropic retroviruses can be tested by the S^+L^-assay [48]. This involves adding the cell supernatant to be tested to PG-4 cells, which contain a defective Molony Mouse Sarcoma virus (M-MSV). If replication-competent retroviruses are present in the supernatant, they can compliment the defect in M-MSV and foci are formed.

The use of ecotropic packaging cell lines that can create replication-competent retroviruses represents no hazard potential for humans and animals.

Retroviruses with altered coat proteins usually infect human and other cells. The hazard potential of these retroviruses depends on the amphotropic murine retroviruses. When using the coat protein of the cat virus RD114 or monkey virus SSAV, the corresponding pseudotype is not efficiently inactivated by the complement system [49, 50], but efficient inactivation is observed for pseudotypes with the VSV-G protein [51]. Experimental operations with pseudotypes should take into account that the vector can also use different transmission paths from the wild type virus [10].

Replication-competence is relevant for safety in the production of lentiviral vectors. The possibility of homologous recombination between overlapping retroviral DNA sequences in the vector and helper constructs can lead to the formation of replication-proficient lentiviruses that are pathogenic, and like wild type viruses are assigned to risk group 3**. The use of codon optimized packaging plasmids reduces the degree of homology and length of homologous regions between the packaging plasmid and vector and can therefore contribute to reducing the risk of creating replication-proficient lentiviruses [52]. Only the hazard potential of replication-defective lentiviral vectors corresponds to the hazard potential of amphotropic murine retroviral vectors.

3. Criteria for comparability of genetic engineering operations involving gene transfer using retroviral vectors

The following points summarize the general criteria for comparability of genetic engineering operations involving gene transfer using retroviral vectors.

Note:

a. The evaluated retroviral vectors are murine retroviral or lentiviral vectors derived from pBR328 and contain the 5'- and 3'-LTR from retroviruses and the packaging signal ψ, which can be extended with additional nucleotide sequences of the *gag* region, the cloning site for the transferred nucleic acid fragment and if required a selection marker. The selection marker or nucleic acid fragment intended for transfer may possibly be under the control of an additional promoter. No coat protein is present. Lentivirus vectors may also have the regulatory sequence RRE.

b. The packaging cell lines are established cell lines of risk group 1 that contain the *gag/pol* genes of murine leukaemia virus (MLV) (without packaging signal ψ) and as required the genes for unaltered coat proteins from ecotropic MLV, amphotropic MLV, other viruses such as GaLV or VSV, or for recombinant coat proteins with ligands for cellular surface proteins. The genes for recombinant coat proteins are present either as individual coat proteins or together with the unaltered coat protein of MLV.

c. The genes essential for packaging lentiviral vectors (*gag/pol* and *env*) and other regulatory reading frames are separated from each other on two to three different pBR328 plasmid derivatives, which are transfected together with the lentiviral vector into the cell. The plasmids carry no packaging signal so that the transcripts are not packaged into particles. Recombination between the constructs to produce replication-competent lentiviruses is not anticipated.

Criteria:

Introduction of retroviral including lentiviral vectors into *E. coli*:

3.1. When subgenomic viral or cellular nucleic acid fragments are introduced into *E. coli* K12 derivatives using the murine retroviral or lentiviral vectors discussed above, then the genetically modified organisms are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 1**.

Producing ecotropic retroviruses in a cell line:

3.2. If subgenomic viral or cellular nucleic acid fragments are introduced into an ecotropic packaging cell line belonging to risk group 1 using the murine retroviral vectors discussed above, then the genetically modified organisms are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 1**.

3.3. Recombinant replication-defective ecotropic retroviruses released from packaging cell lines described in 3.2. are assigned to **risk group 1**, also when possibly contaminated with replication-competent ecotropic retroviruses. Genetic engineering operations with genetically modified organisms that fulfil these criteria, including the infection of further

cells in **risk group 1** as well as inoculation of animals, are comparable to each other and should be assigned to **containment level 1**.

3.4. Cells from risk group 1 infected with recombinant replication-defective ecotropic retroviruses described in 3.3. are assigned to **risk group 1**, as long as the infected cells release no retroviruses with a broader host range. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 1**.

Producing amphotropic retroviruses in a cell line:

3.5. If amphotropic packaging cell lines belonging to risk group 1 are infected with ecotropic retroviral vectors described in 3.3., then the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 2**.

3.6. If subgenomic viral or cellular nucleic acid fragments are introduced into amphotropic packaging cell lines belonging to risk group 1 using the retroviral vectors discussed above, then the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 2**.

3.7. If subgenomic viral or cellular nucleic acid fragments are introduced into a co-culture of ecotropic and amphotropic packaging cell lines belonging to risk group 1 using the retroviral vectors discussed above, then the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 2**.

3.8. Recombinant replication-defective murine retroviruses released from the packaging cell lines described in 3.5., 3.6., or 3.7. are assigned to **risk group 2**, also when possibly contaminated with replication-competent amphotropic retroviruses. Genetic engineering operations with genetically modified organisms that fulfil these criteria, including the infection of further cells in risk group 1, as well as inoculation of animals, are comparable to each other and should be assigned to **containment level 2**.

Producing lentiviruses in a cell line:

3.9. If lentiviral vectors described in 3.1. are introduced into cell lines of risk group 1 together with additional pBR328-derived vectors carrying subgenomic nucleic acid fragments for lentiviral packaging, then the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 2**.

3.10. Recombinant replication-defective lentiviruses released from the cell lines described in 3.9. are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria, including the infection of further cells in risk group 1, as well as inoculation of animals, are comparable to each other and should be assigned to **containment level 2**.

Infection of cell lines with recombinant retroviruses including lentiviruses

3.11. Cells of risk group 1 infected with recombinant replication-defective retroviruses described in 3.8., 3.10., 3.17. or 3.19., where contamination with replication-competent retroviruses is not expected, are assigned to **risk group 1**, as long as the cells cannot complement the replication defect. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 1**.

3.12. Cells of risk group 1 infected with recombinant replication-defective amphotropic retroviruses described in 3.8., are assigned to **risk group 2**, when contamination with replication-competent retroviruses is expected, or if the cells can complement the replication defect. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 2**.

3.13. Primary human cells in risk group 2 infected with recombinant retroviruses described in 3.3., 3.8., 3.10., 3.17. or 3.19., are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 2**.

<u>Retroviruses including lentiviruses with modified coats:</u>

3.14. If subgenomic viral or cellular nucleic acid fragments are introduced into cell lines belonging to risk group 1 using the lentiviral vectors discussed above, and replication-defective lentivirus particles are formed with coat proteins of ecotropic MLV, then the genetically modified organisms are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 1**.

3.15. Recombinant replication-defective lentiviruses released by the cell lines described in above 3.14. are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil these criteria, including infection of further cells in risk group 1, as well as inoculation of animals, are comparable to each other and should be assigned to **containment level 1**.

3.16. If murine retroviral vectors discussed above are used to introduce subgenomic viral or cellular nucleic acid fragments into packaging cell lines belonging to risk group 1 that express gag/pol from MLV and the coat proteins from other viruses, e.g. GaLV, VSV or recombinant MLV coat proteins, replication-defective, pseudotype retrovirus particles are formed. The genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 2**.

3.17. Recombinant replication-defective retroviruses released by the packaging cell lines described in 3.16. are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria, including infection of further cells in risk group 1, as well as inoculation of animals, are comparable to each other and should be assigned to **containment level 2**.

3.18. If subgenomic viral or cellular nucleic acid fragments are introduced into cell lines belonging to risk group 1 using the lentiviral vectors discussed above, and replication-defective lentiviral particles are formed with coat proteins from other viruses such as GaLV, VSV, RD114, or recombinant MLV coat proteins containing ligands for a cellular

surface protein, then the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria are comparable to each other and should be assigned to **containment level 2**.

3.19. Recombinant replication-defective lentiviruses released by the cell lines described in 3.18. are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil these criteria, including infection of further cells in risk group 1, as well as inoculation of animals, are comparable to each other and should be assigned to **containment level 2**.

Note:

If nucleic acid fragments with oncogenic potential are transferred, then handling of such GMOs should adhere to the same safety measures for personal protection as the ZKBS published in its “Recommendations for safety measures in handling nucleic acids with oncogenic potential” from September 1991.

If genetic engineering operations involve transfer of nucleic acid fragments with oncogenic potential using lentiviral vectors that have increased particle stability or a host range for human epithelial cells due to pseudotyping, or are not recognized by the human complement system due to an altered glycosylation pattern, then in addition to the safety measures of containment level 2, it is recommended to wear mouth and nose protection to avoid contact infections. The criteria for nucleic acids with oncogenic potential are defined in the general position statement of the ZKBS on adenoviral vectors with cell cycle regulating genes (May 2004).

Genetic engineering operations with retroviruses including lentiviruses in animals:

1. Animals infected by recombinant retroviruses including lentiviruses, where the animals are considered to be already contaminated with such replication-competent retroviruses, are carriers of these GMOs without the animals themselves becoming new genetically modified organisms. The safety measures for animal housing are based on the risk group of the recombinant retrovirus.
2. The transfer of replication-defective recombinant viruses does not generate transgenic animals. For an undetermined time, the animals are carriers of genetically modified cells of **risk group1**, which probably cannot be localized in the animal. It is however assumed that the genetically modified cells remain within the animal. The animals are to be kept in the genetic engineering facilities where the genetic engineering operations are carried out (§11 Para. 1 if the Genetic Engineering Act (GenTG)).
3. Animals receiving cells described in 3.11. are not GMOs and are not able to release GMOs. They are carriers of genetically modified cells of **risk group1** for an undetermined period of time.
4. Animals receiving cells described in 3.12. and 3.13. are not GMOs and are not able to release GMOs. They are carriers of genetically modified cells of **risk group 2** for an undetermined period of time.

Note:

Since it is assumed that the animals listed in sections 2., 3. and 4. have individual somatic cells containing the transferred recombinant nucleic acids for an undetermined period of time, it is recommended that the animals are disposed of in such a way that none of their remains can reach the food chain.

4. References

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