General position statement of the ZKBS

on frequently carried out genetic engineering operations based on the criteria of comparability:

Stable and transient gene expression using

γ-retroviral and lentiviral vectors

1. Description of the retroviral system

The following definitions are used:

- **retroviral vectors:** replication-defective virus-like particles derived from murine γ-retroviruses or lentiviruses that infect a target cell, thereby transferring a nucleic acid segment to them; the nucleic acid segment may subsequently be transiently present or integrate into the cell's genome

- **Transfer plasmid:** pBR-derived plasmid with one or more foreign genes which does not encode an envelope protein (potentially under the control of an eukaryotic or viral promoter) and non-coding nucleic acid segments of one or more murine γ-retrovirus or lentivirus; the non-coding nucleic acid segments include the 5' and 3' LTR, the packaging signal Ψ (potentially including an overlapping portion of *gag*), the PPT, and potentially lentiviral RRE (including an overlapping portion of *env*) and/or the lentiviral cPPT/cTS (including an overlapping portion of *pol*); potentially additional viral or cellular nucleic acid segments for expression regulation or enhancement (e.g. USE, IRES, 2A-peptide sequence, microRNA target sequences) are included; nucleic acid segments, encoding a complete retroviral protein, are not included

- **packaging plasmid:** pBR-derived plasmid with the gene *gag/pol* of murine γ-retroviruses or lentiviruses and/or one or more genes of any viral envelope protein; the gene of the envelope protein may be modified; optionally, the coding nucleic acid segments of the lentiviral proteins Tat, Rev and/or Vpr are included; a packaging signal is not included

- **packaging cell line:** stably transduced cell line of risk group 1, in whose genome the gene *gag/pol* of a murine γ-retrovirus and one or more genes of any viral envelope protein were stably integrated; the gene of the envelope protein may be modified; a packaging signal is not included

- **infection:** transfer of RNA that is contained within the retroviral vector into a target cell

- **transduction:** integration of DNA into the genome of a target cell after reverse transcription from RNA contained within the retroviral vector

1.1 General Introduction

Retroviruses (family: *Retroviridae*) are enveloped RNA viruses that have been subdivided by the *International Committee on Taxonomy of Viruses* (ICTV) into two subfamilies, the ortho-retroviruses and the spuma retrovirus, with six and five genera, respectively.

The genome of replication-competent retroviruses consists of two identical single-stranded RNA molecules with a length of 7 - 15 kb. During reverse transcription catalysed by the retroviral polymerase, a double-stranded DNA intermediate is formed, which then stably integrates into the genome of the infected cell (provirus). The three genes *gag, pol* and *env*, which encode the matrix, capsid and nucleocapsid protein, the protease, reverse transcriptase and integrase or the envelope proteins, are the basic components of each retroviral genome. Complex retroviruses also contain several other reading frames whose gene products carry out regulatory functions (Fig. 1). At both termini of the viral genome are the so-called long terminal repeats (LTR), which in the DNA provirus each consist of the sequence sections U3 (*unique 3‘*), R (*redundant*) and U5 (*unique 5‘*). Within these sections are the retroviral promoter and other *cis*-regulatory elements for enhanced gene expression (*enhancer*), polyadenylation and integration into the host genome. In contrast, the RNA genome of the virus has only the sections R and U5 at the 5' end and the sections U3 and R at the 3' end. The LTRs are completed during reverse transcription [1].
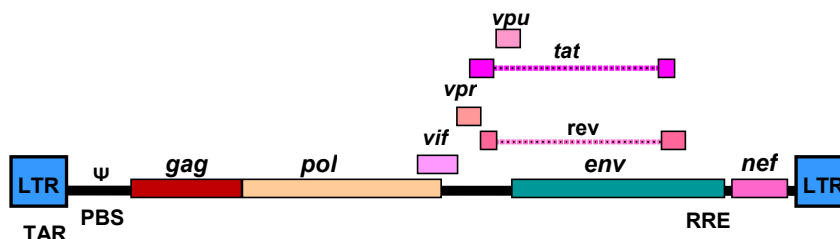
a)
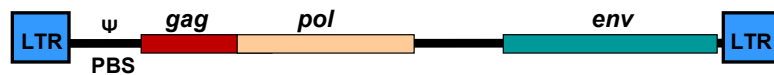
b)

Fig. 1: Gene maps of retroviral proviruses
a) Simple retrovirus using the example of *Murine leukemia virus* (MLV); PBS (primer binding site) denotes the binding site for the tRNA primer; Ψ marks the packaging signal; *gag* (group specific antigens, matrix, capsid and nucleocapsid proteins), *pol* (protease, reverse transcriptase and integrase) and *env* (envelope proteins) are coding regions; LTR (long terminal repeats) contain repetitive sequences that occur at both termini of the integrated DNA provirus. Figure modified according to [1].

b) Complex retrovirus using the example of *Human immunodeficiency virus* (HIV); In addition to the regulatory elements such as LTR, packaging signal Ψ, PBS, the trans-activation response element (TAR) and the rev response element (RRE) as well as the *gag, pol* and *env* genes, HIV has further reading frames encoding regulatory proteins. Figure modified according to http://hiv-web.lanl.gov/MAP/landmark.html.

1.2 Stable gene transfer using retroviral vectors

Recombinant retroviruses for stable gene transfer were originally developed on the basis of murine γ-retroviruses, especially the *Murine leukaemia virus* (MLV). They only transduce dividing cells, as the cytoplasmic reverse-transcribed DNA intermediate is unable to pass through the nuclear membrane. In non-dividing cells, therefore, there is no integration of the provirus into the host genome. Only when the nuclear membrane dissolves during cell division can the viral replication cycle be completed [2].

In order to stably transduce non-dividing cells or terminally differentiated cells, retroviral vectors derived from lentiviruses were also produced. While the early lentiviral vectors were derived from *Human immunodeficiency virus* (HIV) [3] and *Simian immunodeficiency virus* (SIV), vector systems based on *Feline immunodeficiency virus* (FIV) [4], *Equine infectious anemia virus* (EIAV) [5], *Caprine arthritis encephalitis virus* (CAEV) [6], *Bovine immunodeficiency virus* (BIV) [7] and *Visna-maedi virus* (VMV) [8] have also been developed.

In addition to these widely used vector systems, systems based on α-retroviruses (e.g. *Rous sarcoma virus*, RSV) [9], β-retroviruses (e.g. *Mouse mammary tumour virus*, MMTV) [10] or spuma retroviruses (e.g. *Eastern chimpanzee simian foamy virus*, SFVcpz) [11] have also been developed. However, because of their low distribution, they are not the subject of this position statement.

1.2.1 Design and production of retroviral vectors

Retroviral vectors are replication-defective virus-like particles that infect a target cell (meaning to transfer a nucleic acid into a target cell) and, as a rule, stably integrate a nucleic acid segment into its genome. Starting from the resulting provirus, one or more foreign genes can subsequently be permanently expressed in the transduced cell.

The preparation of retroviral vectors requires three components: a transfer plasmid similar to a retroviral provirus with the foreign gene to be transferred, (retro) viral structural proteins to form virus-like particles, and retroviral nonstructural proteins for integration of the foreign gene into the genome of a target cell (Fig. 2) [12].
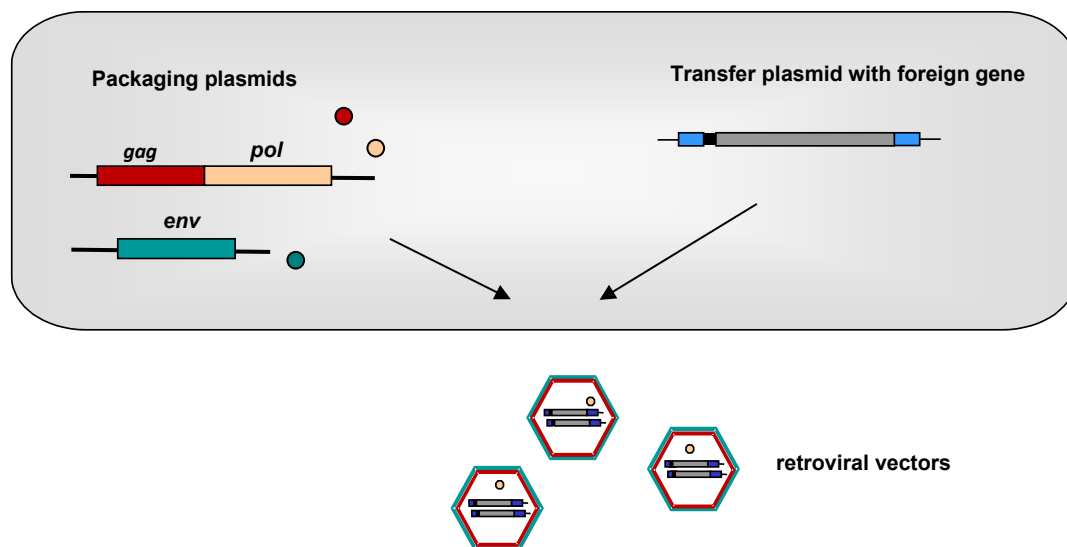

Fig. 2: Preparation of retroviral vectors
A transfer plasmid with the foreign gene to be transferred and all *cis*-regulatory sequences necessary for its integration, expression and packaging is transfected into a cell in which the essential (retro-)viral structural and nonstructural proteins are expressed. This is either achieved by transfection of appropriate packaging plasmids or by transduction of the cell line with the genes of these proteins. Starting from the transfer plasmid, an RNA similar to the retrovirus genome is subsequently transcribed. Together with the Pol polyprotein, which contains the proteins reverse transcriptase (RT) and integrase (IN) required for subsequent integration, it is eventually packaged by the viral structural proteins into virus-like particles, called retroviral vectors, and released by the cell. Figure modified according to [13].

In addition to the foreign gene, the transfer plasmid, which is typically derived from the vector pBR328, contains the packaging signal Ψ necessary for RNA packaging and the *cis*-regulatory elements necessary for the reverse transcription and integration. On the one hand, this is the

primer binding site (PBS), which enables the binding of a cellular tRNA and thus the initiation of negative-strand synthesis during reverse transcription. The subsequent initiation of the plus-strand synthesis also requires the polypurine tract (PPT). Also included are the complete 5' and 3' LTRs, which include the viral promoter, an enhancer, the polyadenylation signal as well as sequences that are important for the integration of the provirus. Moreover, lentiviral transfer plasmids in particular may contain various, sometimes heterologous, viral regulatory elements that improve the expression of the foreign gene, the transduction efficiency of the retroviral vector or its safety. Common heterologous elements include the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) for enhancement of mRNA stability, mRNA export and translation, upstream polyadenylation enhancer sequences (USEs), e.g. of *Simian virus 40* (SV40) for increasing foreign gene expression, as well as the human cytomegalovirus major immediate-early (HCMV MIE) promoter. The latter is often used alone or as a hybrid promoter in combination with the retroviral promoter within the 5' LTR to control expression of the foreign gene. The foreign gene may alternatively also be under the control of various other cellular or viral promoters. In addition to the use of inducible or cell-type-specific promoters, a binding site for a cellular microRNA can also be inserted to increase the accuracy of the foreign gene expression. This suppresses foreign gene expression in certain cell types or differentiation stages. If a bicistronic expression cassette is supposed to be expressed, an internal ribosome entry site (IRES) of a picornavirus is also usually inserted between the foreign genes [14].

The transfer plasmid, which does not code for retroviral proteins, is transcribed by cellular RNA polymerase II after transfection into an appropriate cell line. Mediated by the packaging signal, the RNA is then packaged and delivered into virus-like particles in the presence of viral structural (matrix, capsid, nucleocapsid and envelope proteins) and nonstructural proteins (at least protease, reverse transcriptase and integrase). In turn, the necessary (poly-)proteins are transiently or stably expressed in the cells, either after transfection of appropriate packaging plasmids, which themselves do not have a packaging signal, or after transduction of their respective genes.

The envelope protein used in this process can originate from the same retrovirus or from any other virus. In the second case we speak of pseudotyping. It serves primarily to expand or alter the host range or cell tropism of the retroviral vectors for targeted infection of a particular host (typically human) and/or particular cell types. A variety of heterologously expressed viral envelope proteins have been successfully used for pseudotyping. The donors include, for example, arenaviruses [15], alphaviruses [16], hepadnaviruses [17], flaviviruses [18], filoviruses [19], paramyxoviruses [20] and rhabdoviruses [21]. In particular, the glycoprotein of the *Indiana vesiculovirus* (VSV-G) is widely used to provide retroviruses with a broad host range including human cells. Furthermore, some pseudotyped retroviral vectors, especially those with VSV-G, have a higher particle stability, facilitating concentration through ultracentrifugation [22]. In addition, a specific cell tropism can be achieved by fusing a binding partner of a cellular surface protein, such as the recognition domain of an antibody, with a viral envelope protein [23]. Since retroviral vectors with specific cell tropism are primarily intended for medical use in humans, they usually target epitopes of human cells. The viral fusion partner is often the haemagglutinin of measles virus (MeV-H) [20].

If the essential genes for packaging, reverse transcription and integration as well as potentially other regulatory genes are transfected, they are usually encoded on two to five plasmids. The key to maintain the safety of the retroviral vectors is that the *gag/pol* and *env* genes are present on two separate packaging plasmids. This reduces the probability of the formation of replication-competent retroviral particles significantly, since this would require two recombination events between three plasmids [12]. Similarly, the *gag/pol* and *env* genes are also introduced separately during the production of stably transduced packaging cell lines [24].

In addition to the risk of creating replication-competent retroviruses, the possibility of insertional mutagenesis represents the second safety risk of retroviral vectors. In this case, random integration of the provirus into the cellular genome can activate a cellular proto-oncogene or inactivate a cellular tumour suppressor gene. This can occur at different levels: (i) the provirus

affects adjacent enhancers or promoters of the cell and thereby the expression of the genes they control, (ii) the presence of the provirus leads to changes in the chromatin structure of regulatory domains and thereby to a change in gene expression, (iii) the promoter and enhancer of the provirus activate adjacent cellular proto-oncogenes, (iv) transcriptional read-through by the retroviral polyadenylation signal leads to the activation of neighbouring proto-oncogenes, (v) alternative splicing mediated by the retroviral splice donor and acceptor sequences results in the formation of deleterious fusion proteins; (vi) the integration of the provirus within open reading frames leads to the formation of protein fragments with dominant negative effect, (vii) the integration of the provirus within open reading frames results in the loss of function of a tumour suppressor gene [14; 25].

Retroviruses have different preferences regarding gene regions for their integration. While γ-retroviruses preferentially integrate into CpG islands, into regulatory elements and near transcriptional start sites in 5' flanking regions, lentiviral proviruses are more likely to be located within coding regions of a gene. Overall, the integration of a lentivirus appears to be less genotoxic than that of a γ-retrovirus [25; 26].

Various modifications of the transfer plasmid can be made to counteract the risk of insertional mutagenesis. For example, the previously mentioned USE and WPRE elements can significantly reduce the probability of read-through into neighbouring cellular genes. Similarly, inserting insulator elements can reduce activation of adjacent genes [14]. However, the most common safety measure is the insertion of a deletion within the U3 section at the 3' end. The resulting self-inactivating (SIN) vectors provide several advantages over their unmodified starting vectors. On the one hand, the deletion means that the provirus cannot be mobilized again after a single integration. This can be attributed to the fact that, due to the mechanism of reverse transcription, the incomplete U3 segment (ΔU3) is transcribed to the 5' end of the provirus. Since the deletion affects the retroviral promoter in addition to the enhancer, it thereby prevents the transcription of genomic RNA. Thus, uncontrolled, repeated integration of the recombinant nucleic acid segment into the cellular genome cannot occur – even in the presence of retroviral proteins (Fig. 3) [27]. On the other hand, the deletion of the retroviral promoter and enhancer at both ends of the provirus reduces the negative effects on the expression of adjacent cellular genes and on the elements controlling transcriptional activity within the provirus [14]. In order to further reduce the risk of insertional mutagenesis, approaches are now being pursued in which the integrase is modified with DNA-binding domains in a way to ensure that the integration takes place specifically into innocuous regions of the cellular genome [28; 29]. Furthermore, by specifically exchanging the lentiviral integrase for the enzyme of α-retroviruses, for example, new attempts try to achieve integration into regions of the genome in which there is a reduced potential for oncogenesis after insertional mutagenesis [14].

As an additional safety measure, the PBS can be mutated so that no cellular tRNA can bind to it. Reverse transcription of the transfer RNA is therefore dependent on the presence of an artificial tRNA provided in *trans*, either as a synthesized tRNA or as an RNA polymerase III-dependent gene on a co-transfected plasmid [30].

1.2.2 Murine γ-retroviral vectors

Murine γ-retroviral vectors can be prepared either by co-transfecting the transfer plasmid and typically two packaging plasmids, or by transfecting the transfer plasmid into a packaging cell line. Depending on the nature of the envelope proteins expressed here, a distinction is made between ecotropic, amphotropic and xenotropic murine γ-retroviral vectors. The host range of the ecotropic vectors is restricted to cells of mice and rats. By contrast, amphotropic vectors have a broader host range, including both murine and non-murine cells, including human cells. Similarly, xenotropic vectors also have a broad host range, including humans. However, they cannot infect most mouse laboratory strains [31]. In the development of retroviral vectors, the envelope proteins of the murine γ-retroviruses are increasingly being modified or exchanged to achieve a broader host range, tropism targeted to a particular cell type, or higher vector stability [32].
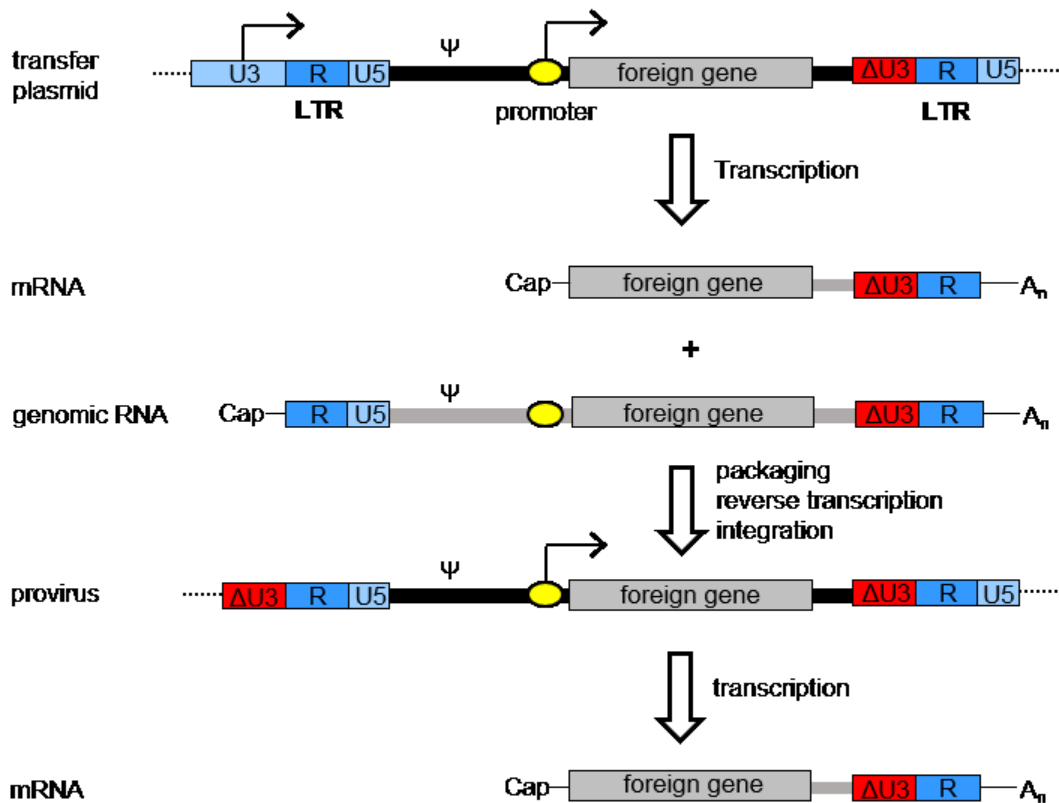
Fig. 3: Integration of a self-inactivating (SIN) vector
A deletion that inactivates the viral promoter contained in this section is inserted into the U3 section (ΔU3) at the 3' end of the retroviral cDNA. After transcription of the transfer plasmid by the cellular RNA polymerase II, the retroviral genomic RNA is formed starting from the promoter in the intact 5'-U3 section. The retroviral genomic RNA is packaged in the presence of viral structural and nonstructural proteins. The resulting vectors can then be used to infect other cells. Due to the mechanism of reverse transcription, the ΔU3 portion of the 3' end of the genomic RNA in these cells is transferred to the 5' end of the plus-strand of the resulting cDNA. The provirus resulting from the integration into the host genome thus does not have a promoter at its 5' end that could be used to transcribe genomic RNA again. The provirus is therefore no longer mobilizable. Accordingly, the corresponding vectors are self-inactivating after one-time integration. Figure modified according to [31].

1.2.3 Lentiviral vectors

Since established cell lines generally do not readily tolerate the expression of the lentiviral *gag/pol* gene, the establishment of packaging cell lines for lentiviral vector systems is only possible in inducible or attenuated systems [33]. Therefore, lentiviral vectors are typically produced by co-transfecting all necessary plasmids. Depending on the production system, this includes three to six plasmids. Like the γ-retroviral vectors, they encode the gag-pol polyprotein as well as a heterologous envelope protein to expand the narrow lentiviral host range and cell tropism. However, additional regulatory proteins and their recognition sequences are needed for efficient vector production and transduction. One of these is the protein Rev, which, depending on the rev response element (RRE), mediates the transport of singly spliced and unspliced viral RNA from the nucleus and facilitates their translation in the cytoplasm. Furthermore, RNA molecules are packaged up to 70 times more efficiently in viral capsids with RRE [14]. Moreover, the lentiviral transcriptional transactivator Tat may be encoded on one of the plasmids. This protein is essential for the recognition of the natural lentiviral promoter and binds there to the trans-activation response element (TAR), a *cis*-regulatory sequence located at the 5' end of the viral RNA and DNA. In more current systems, however, this protein is no longer necessary since hybrid promoters consisting of the lentiviral promoter and that of HCMV

or of the α-retrovirus RSV are increasingly being used. In the latest lentiviral production systems, the protein Vpr is also used to ensure transport of the Pol polyprotein into the particles (TaKaRa Lenti-X packaging system). Another, exclusively nucleic acid-based, regulatory element is the combination of the central polypurine tract (cPPT), a copy of the 3' end PPT, and the central termination sequence (cTS). Together, these elements result in increased integration and thus increased transduction efficiency of the vectors. This may be due to an improved nuclear import of the pre-integration complex [34].

In contrast to the 1st generation production systems, which encode all lentiviral proteins on two packaging plasmids, the production systems of the 2nd, 3rd or more recent generations are distinguished by the fact that the coding nucleic acid segments were reduced to a necessary minimum. Accordingly, these systems offer significantly higher safety than systems of the 1st generation. However, because of the compact genome structure of lentiviruses, packaging plasmids have homologous regions to the transfer plasmid. Thus, the packaging signal Ψ overlaps with the *gag* gene at up to 400 bp, with an overlap of 40 bp being essential. Likewise, the RRE, which can be up to 850 bp long, contains portions of the *env* gene. Finally, the cPPT also overlaps with the *pol* gene by 130 bp [12; 14]. However, recombination between the at least three plasmids of a lentiviral production system has never been described and is not considered to be probable [14].

1.2.4 Adenovirus/retrovirus hybrid vectors

To achieve efficient gene transfer and gene expression, chimeric vectors based on adenoviral and retroviral vectors have been developed [35; 36]. With this hybrid system, three replication-defective adenoviral vectors transfer upon co-infection the transfer construct with the foreign gene, the retroviral packaging functions (*gag/pol* gene) and an *env* gene to a cell, which thus becomes the producer cell of retroviral vectors. The delivered retroviral vectors can then stably transduce additional cells. The risk assessment of the production of the necessary adenoviral vectors is carried out in accordance with 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.: 6790-10-28, 3. Revised version from November 2011. As with a packaging cell line transfected with a retroviral plasmid the risk assessment of cells after co-infection with the three adenoviral vectors is based on the type of the released retroviral vectors.

1.3 Transient gene expression using retroviral vectors

Considering the potential for insertional mutagenesis using integrative retroviral vectors, specific retroviral vectors for transient gene expression have recently been developed. Targeted proteins or regulatory nucleic acid segments that are essential for reverse transcription or subsequent integration of the transfer construct are mutated in these approaches. The production of corresponding retroviral vectors is performed as described for the traditional retroviral vectors.

1.3.1 Integrase-defective vectors

Integration of the retroviral cDNA is catalysed by the viral integrase. This protein, which is part of the Pol polyprotein, exhibits endonuclease as well as ligase activity. Therefore, to avoid integration, this protein and its recognition sequences are obvious targets for mutagenesis.

Mutations that inhibit integrase activity are divided into two classes. Class I mutations only affect integration and have no effect on other stages of retroviral replication. In contrast, class II mutations cause pleiotropic effects that also affect other processes of virus replication. For example, class II mutations may also negatively impact reverse transcription and particle formation. Thus, they are unsuitable for the generation of retroviral vectors [26; 37]. Among the class I mutations, mutations of the amino acids conserved in all retroviruses and

retrotransposons of the so-called catalytic triad Asp-Asp-Glu are of particular significance. For the HIV integrase (HIV-IN), these are the amino acids Asp64, Asp116 and Glu152; for the MLV integrase, the amino acids Asp125, Asp184 and Glu220. Integrase-defective retroviral vectors typically contain a mutation of these amino acids, with the mutation Asp64Val being the most frequently introduced mutation in HIV-based vectors [31]. Other possible mutations involve amino acids that mediate DNA binding of the enzyme, as in the case of the HIV protein Asn120, Gln148, Trp235, Arg262, Arg263, Lys264, Lys266, and Lys273. Mutations that prevent the required multimerisation of the enzyme, such as His12 (HIV-IN), are also conceivable [26]. Finally, the recognition sequences of the integrase, the so-called attachment sites (att), within the U3 and U5 section can be inactivated through mutation [37].

If integration of the reverse-transcribed DNA intermediate is prevented, the cellular DNA-repair enzyme activity leads to the enrichment of circular, double-stranded DNA forms. Mediated by the promoter present in the retroviral LTR or additional promoters, these by-products, which also occur during natural replication of a retrovirus, can be transcribed with slightly lower efficiency than the integrated provirus. In contrast, replication of the episomal forms does not take place. Due to their high stability, however, they are usually removed from the cells only through dilution as cell division progresses [38; 39]. Integrase-defective retroviral vectors can therefore serve for transient gene expression. If additional regulatory elements are inserted into the transfer construct that enable replication, stable expression of the episomes can also be achieved [12].

Although the integrase activity of these vectors has been inhibited, studies show that integration of the transfer construct can still occur. This is due to the cellular repair mechanisms, which lead to an integrase-independent insertion of the transfer construct into existing double-strand breaks [38]. The frequency of these nonspecific insertions varies significantly. Depending on the system studied, the integration frequency was reduced by 10 to 10,000 times compared to an integration-competent vector [26]. Taking into account the high variability and the sometimes small reduction of integration events, integrase-defective retroviral vectors cannot be expected to sufficiently counteract the risk of insertional mutagenesis. The possibility of insertional mutagenesis therefore continues to be fully factored into the risk assessment of such vectors.

1.3.2 mRNA transfer using retroviral vectors

Another approach to transient gene expression is the so-called retrovirus particle-mediated mRNA transfer (RMT). It makes use of the fact that the RNA molecules packed in a retroviral particle possess both a 5' cap and a 3' polyadenylation. Therefore, they represent functional mRNAs and are translated by the cellular machinery unless reverse transcription of the RNA occurs [40]. Generally, reverse transcription can be inhibited in two different ways. First, the reverse transcriptase can be inactivated. Second, the PBS necessary for the initiation of cDNA minus-strand synthesis can be mutated so that it is not recognized by any cellular tRNA. The latter approach has already been developed for safety reasons and is now used in the absence of an artificial tRNA for transient gene expression [31].

Since this approach does not lead to the formation of double-stranded DNA, insertional mutagenesis can definitely be ruled out. Vectors containing a transfer RNA with a mutated PBS therefore have no hazard potential, provided they were prepared in the absence of the complementary artificial tRNA.

2. Summary of relevant criteria for the biosafety classification of genetic engineering operations with retroviral vectors

In the production of HIV-derived lentiviral vectors, the likelihood of replication-competent HIV particles being formed is the key aspect of the risk assessment, since HIV particles are pathogenic to humans and belong to risk group 3**. This particular aspect is of less importance for the risk assessment of murine γ-retroviral vectors since replication competent and defect

murine γ-retroviral vectors will be assigned to the same risk group according to their host spectrum.

A **replication competence of nominally replication-defective retroviral vectors** is provided when replication-competent retroviruses are formed by recombination events between homologous sequences of the packaging plasmids or the genome of the packaging cell line and the transfer plasmid. The probability of such an event depends on the number of packaging plasmids that encode the necessary genes for the production of retroviral vectors. For instance, the probability of recombination is significantly reduced if *gag/pol* and the gene encoding the envelope protein are present on separate plasmids. This is the case for all lentiviral production systems of the second or later generations. When using such systems for the production of lentiviral vectors, it can be assumed that no recombination generating replication competent lentiviruses occurs. Additionally, the safety of retroviral vectors may be further increased by reducing sequence homologies between transfer and packaging plasmids or packaging cell line, e.g. through codon optimisation and introducing additional stop codons, deletions and mutations that result in a replication defect in case of recombination.

Even if a replication defect exists, retroviral vectors can pose a hazard potential to humans. This is due to the fact that in the course of infection, the proviral genome can integrate non-directionally into the genome of the host cell, and thus in individual cases may cause the change of the transcriptional activity of regulatory genes, the activation of cellular proto-oncogenes or the deactivation of cellular tumour suppressor genes through **insertional mutagenesis**. The risk of this event is defined by the potential target cells as well as the natural defence mechanisms of the infected organism and may be reduced by different modifications of the transfer plasmid.

Only the **host range achieved by the vectors** is relevant for the biosafety classification of genetic engineering operations with murine γ-retroviral vectors. Due to the restriction of the cell tropism, ecotropic murine γ-retroviral vectors are generally not expected to cause a hazard potential for humans or animals (see position statement of the ZKBS on the risk assessment of ecotropic mouse C-type retroviruses, ref_ 6790-10-47, April 1996). Even in the event of a contamination of these vectors with replication-competent ecotropic murine γ-retroviruses there is no hazard potential for humans or animals. A low hazard potential for humans cannot be ruled out for amphotropic or xenotropic murine γ-retroviral vectors, since primate cells can be infected both *in vitro* and under certain conditions *in vivo* with these vectors [1; 31; 41]. Murine γ-retroviral vectors with altered envelope proteins can usually infect human and other cells. Accordingly, their hazard potential is equivalent to that of amphotropic and xenotropic vectors. Furthermore, while handling these pseudotypes experimentally, it should be taken into account that the vector could also use other transmission pathways than the wild-type virus [32].

3. Application of retroviral vectors on animals

Retroviral vectors are often used to transduce animal cells *ex vivo* and then introduce them into a laboratory animal. Alternatively, a direct injection of retroviral vectors into laboratory animals for *in situ* transduction may be performed. Since the virus-like particles used for this purpose and the transduced cells are genetically modified organisms (GMOs), the corresponding genetic engineering operation must be carried out in a genetic engineering facility in accordance with § 8 (1) GenTG.

However, by transducing or introducing transduced somatic cells, the laboratory animal itself does not become a GMO within the meaning of § 3 (3) GenTG. This can be justified as follows: Transduction only affects some somatic cells but not germline cells. The animal cannot pass on the genetic modification introduced by transduction. Correspondingly, there is no change in the genome of the animal. In contrast, if cells of the germline of a laboratory animal are genetically modified by means of transduction, the animal and its offspring must be regarded as GMOs due to the possibility of inheriting the genetic modification.

According to § 3 (1a) GenTG, an independent evaluation of cells is intended only for eukaryotic cells in cell culture and thus in the context of *in vitro* cultivation. Accordingly, the targeted removal of transduced cells from the animal and their further cultivation are subject to the regulations of the GenTG. The transduced cells contained in the animal, however, must not be regarded as stand-alone GMOs while part of the biological unit 'animal'. This also applies to cells of a different species. The presence of transduced cells does not make the animal a carrier of GMOs.

However, the animals treated with retroviral vectors should be considered as carriers of GMOs for as long as the virus-like particles can be detected in these animals. The question of whether and, possibly until when, the corresponding laboratory animals are carriers of GMOs, is therefore dependent on the stability of the infectious retroviral vectors. There are several studies on this subject matter. For unmodified retroviral vectors, half-lives of 2 – 8 h in cell culture medium at 37 °C are shown depending on the production cell line and the associated composition of the cell membrane [42]. In contrast, a lentiviral vector, pseudotyped with the VSV-G protein, showed a half-life of 24 h in cell culture medium at 37 °C. After intravenous injection of the same vector into rats, however, starting with 10^7 *transduction units* (TU), a reduction of infectious particles in the plasma by 4 log levels was already observed after the first hour. After 24 h, no infectious particles were detected (detection limit 50 TU/animal) [43]. In another study with a VSV-pseudotyped lentiviral vector, no vector RNA was detected in blood (detection limit 10 IU/ml), urine or faeces after intravenous injection of 10^9 *infectious units* (IU) into mice after one day. Moreover, at no point in the experiment were infectious particles found on the surfaces inside the cage, although control experiments showed that the retroviral vectors remain infective on plastic surfaces at room temperature for up to 24 h and on moist litter for up to 72 h. Infectious particles could be isolated from the injection site for up to 24 h after injection [44]. Based on these data, it can be assumed that in animals that were transduced with replication-defective retroviral vectors, no recombinant infectious particles are present one day after the injection. Accordingly, a release of these particles is therefore no longer expected after this point in time, at the latest. If the injection site has been disinfected and the cage was changed, the handling of these animals is no longer subject to the regulations of the GenTG after this point in time.

In contrast to *in situ* transduced animals, animals that received *ex vivo*-transduced cells are initially carriers of GMOs only if infectious retroviral vectors adhere to these cells. To ensure this is not the case, the transduced cells should be washed several times. For example, in a study with lentivirally transduced 293 cells, washing twice with physiological saline solution resulted in a 100-fold reduction of the free particles. After two passages ultimately no vector RNA was detected (detection limit 260 copies/ml) [45].

If, due to the production system of the retroviral vectors, a contamination with replication-competent retroviruses has to be assumed and the experimental animals are potentially permissive for them, a release of genetically modified viruses cannot be ruled out. Animals that were infected with the described vector solutions or onto which cells transduced with these solutions were transferred must therefore be considered permanent carriers of GMOs. In addition, they may also be able to release GMOs. The handling of such animals is therefore permanently subject to the regulations of the GenTG.

4. Criteria of comparability of genetic engineering operations with retroviral vectors

General comparability criteria in genetic engineering operations with retroviral vectors are summarized below.

Introduction of a transfer or packaging plasmid into *E. coli*:

4.1   When subgenomic viral or cellular nucleic acid segments are introduced into an *E. coli* K12 derivative using a transfer or packaging plasmid, the genetically modified organisms are assigned to **risk group 1**. Genetic engineering operations with

genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

Generation of ecotropic murine γ-retroviral vectors:

4.2.   When a transfer plasmid and one or more packaging plasmids based on a murine γ-retrovirus are transfected into a cell line of **risk group 1** and the encoded envelope proteins are exclusively ecotropic, the genetically modified organisms are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

4.3.   When a transfer plasmid is introduced into a packaging cell line with genes of a murine γ-retrovirus and the encoded envelope proteins are exclusively ecotropic, the genetically modified organisms are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

4.4.   Ecotropic murine γ-retroviral vectors released by the cell lines described in 4.2. and 4.3. belong to **risk group 1**, even if a contamination with replication-competent ecotropic murine γ-retroviruses has to be assumed. Genetic engineering operations with these vectors, including the transduction of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 1**.

Generation of amphotropic or xenotropic murine γ-retroviral vectors:

4.5.   When a transfer plasmid and one or more packaging plasmids based on a murine γ-retrovirus are transfected into a cell line of **risk group 1** and amphotropic or xenotropic envelope proteins are encoded, the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.6.   When a transfer plasmid is introduced into a packaging cell line with genes of a murine γ-retrovirus in which amphotropic or xenotropic envelope proteins are encoded, the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.7.   When a transfer plasmid is introduced into a co-culture of two packaging cell lines with genes of a murine γ-retrovirus in which ecotropic and amphotropic or xenotropic envelope proteins are encoded, the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.8.   When amphotropic packaging cell lines of **risk group 1** are transduced by the ecotropic murine γ-retroviral vectors described in 4.4., the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.9.   Amphotropic and xenotropic murine γ-retroviral vectors released from the cell lines described in 4.5., 4.6., 4.7. and 4.8. are assigned to **risk group 2**, even if a contamination with replication-competent amphotropic or xenotropic murine γ-retroviruses has to be assumed. Genetic engineering operations with these vectors, including the transduction of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 2**.

4.10. If the integrase activity in the amphotropic and xenotropic murine γ-retroviral vectors generated as described in 4.5., 4.6. or 4.7. is inactivated through mutation, the mutant vectors are assigned to **risk group 2** due to their described tendency to non-specific integration. Genetic engineering operations with these vectors, including the infection of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 2**.

4.11. Cell lines of **risk group 1** that release the mutated amphotropic or xenotropic murine γ-retroviral vectors described in 4.10. are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.12. If the reverse transcription in the amphotropic and xenotropic murine γ-retroviral vectors generated as described in 4.5., 4.6. or 4.7. is inhibited through mutation of the PBS, the mutated vectors are assigned to **risk group 1**. Genetic engineering operations with these vectors, including the infection of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 1**.

4.13. Cell lines of **risk group 1** that release the mutated amphotropic or xenotropic murine γ-retroviral vectors described in 4.12. are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

Generation of lentiviral vectors:

4.14. When a transfer plasmid and at least two packaging plasmids of the 2nd, 3rd or newer generation, which are based on a lentivirus, are transfected into a cell line of **risk group 1**, the genetically modified organisms are assigned to **risk group 2**. The gene of the envelope protein and the *gag/pol* gene must be present on separate packaging plasmids. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.15. Lentiviral vectors released from the cell lines described in 4.14. are assigned to **risk group 2**. Recombination between the plasmids resulting in replication-competent lentiviruses has not be assumed when using packaging systems of the 2nd, 3rd or more recent generation. Genetic engineering operations with these vectors, including the transduction of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 2**.

4.16. If the integrase activity in the lentiviral vectors generated as described in 4.14. is inactivated through mutation, the mutant vectors are assigned to **risk group 2** due to their described tendency to non-specific integration. Genetic engineering operations with these vectors, including the transduction of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 2**.

4.17. Cell lines of **risk group 1** that release the mutant lentiviral vectors described in 4.16. are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.18. If reverse transcription in the lentiviral vectors generated as described in 4.14. is inhibited by mutation of the PBS, the mutated vectors are assigned to **risk group 1**. Genetic engineering operations with these vectors, including the infection of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 1**.

4.19. Cell lines of **risk group 1** that release the lentiviral vectors described in 4.18. are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

Note: Due to its higher safety, the use of a SIN transfer plasmid is recommended for the generation of HIV-derived lentiviral vectors.

Retroviral vectors with modified envelopes:

4.20. If the lentiviral *env* gene is exchanged for the gene of an envelope protein of an ecotropic murine γ-retrovirus during the generation of the lentiviral vectors described in 4.14., 4.16. or 4.18., the genetically modified organisms are assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

4.21. Pseudotyped lentiviral vectors with exclusively ecotropic envelope proteins that are released from the cell lines described in 4.20. are assigned to **risk group 1**. Genetic engineering operations with these vectors, including the transduction or infection of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 1**.

4.22. If, during the generation of the retroviral vectors described in 4.5., 4.6., 4.10., 4.14. or 4.16., the retroviral *env* gene is exchanged for the potentially modified gene of an envelope protein of any virus (except unmodified proteins of an ecotropic murine γ-retrovirus) or if this gene is additionally expressed, the genetically modified organisms are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.23. Pseudotyped retroviral vectors with potentially modified envelope proteins of foreign viruses that are released from the cell lines described in 4.22. are assigned to **risk group 2**, provided the envelope proteins are not exclusively unmodified proteins of ecotropic murine γ-retroviruses. Genetic engineering operations with these vectors, including the transduction of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 2**.

4.24. If, during the generation of the retroviral vectors described in 4.12. or 4.18., the retroviral *env* gene is exchanged for the potentially modified gene of an envelope protein of any virus, or if this gene is additionally expressed, the genetically modified organisms continue to be assigned to **risk group 1**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

4.25. Pseudotyped retroviral vectors with potentially modified envelope proteins of foreign viruses that are released from the cell lines described in 4.24., are assigned to **risk group 1**, provided that their reverse transcription was inhibited by mutation of the PBS. Genetic engineering operations with these vectors, including the infection of additional cells of **risk group 1** and inoculation of animals, are comparable to one another and are assigned to **safety level 1**.

Infection of cells with retroviral vectors

4.26. Cells of **risk group 1** transduced by the ecotropic retroviral vectors described in 4.4. or 4.21. are assigned to **risk group 1**, unless the transduced cells release retroviral vectors with expanded host range. Genetic engineering operations with genetically

modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

4.27. Cells of **risk group 1** transduced by or infected with the retroviral vectors described in 4.9., 4.10., 4.12., 4.15., 4.16., 4.18., 4.23. or 4.25. and in which no contamination with replication-competent retroviruses has to be assumed are assigned to **risk group 1**, provided that the cells do not complement the replication defect and no infectious retroviral vectors adhere to the cells. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 1**.

4.28. Cells of **risk group 1** transduced by the amphotropic or xenotropic murine γ-retroviral vectors described in 4.9. are assigned to **risk group 2**, if a contamination with replication-competent retroviruses has to be assumed or if the cells complement the replication defect. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

4.29. Primary cells of **risk group 2** transduced by or infected with the retroviral vectors described in 4.4., 4.9., 4.10., 4.12., 4.15., 4.16., 4.18., 4.21., 4.23. or 4.25. are assigned to **risk group 2**. Genetic engineering operations with genetically modified organisms that fulfil the mentioned criteria are comparable to one another and are assigned to **safety level 2**.

Notes on genetic engineering operations with retroviral vectors on animals:

1. When transduced germline cells or retroviral vectors intended to transduce germline cells are transferred to animals, these animals and their offspring are to be regarded as GMOs.

2. When transferring retroviral vectors to animals, no transgenic animals are produced if only somatic cells are transduced. Unless the animals complement the replication defect of the retroviral vectors, they are also unable to release GMO. Due to the low stability of retroviral vectors, the animals infected with them are no longer regarded as carriers of GMOs after one day and proper disinfection of the injection site. After this period and a cage change, keeping these animals is therefore no longer subject to the provisions of the GenTG.

   Retroviral vectors transfer subgenomic retroviral nucleic acid segments and a heterologous nucleic acid segment. The heterologous nucleic acid segment does not complement the replication defect. If the animal does not complement the replication defect, an abortive infection occurs. The viral nucleic acid is not mobilized and therefore not transferred to other cells. No new retroviral particles are generated. The nucleic acid is transferred to somatic cells of the animal. It only leads to the transient presence of the transgene in the animal. The transfer and integration of the nucleic acid into the germline cells has not to be assumed.

3. Animals, into which the transduced cells described in 4.26. or 4.27. have been transferred, are not GMOs, if the cells are somatic cells. They are also unable to release GMOs. If it is ensured that no infectious retroviral vectors adhere to these cells, the animals are also not regarded as carriers of GMOs. This also applies if the transduced cells are of a different species. Accordingly, the keeping of these animals is not subject to the regulations of the GenTG.

   The cells do not complement the viral replication defect. The viral nucleic acid is not mobilized and therefore not transferred to other cells. No new retroviral particles are generated.

4. Animals, into which the cells described in 4.28. and 4.29. have been transferred, are not GMOs, if the cells are somatic cells. The assessment is based on the hazard potential

of the replication-competent viruses introduced by the transduced cells. On the one hand, this could be ecotropic, amphotropic or xenotropic replication-competent murine γ-retroviruses as contamination in the production of corresponding murine γ-retroviral vectors (GMOs of risk group 1 or 2). On the other hand, this could be non-recombinant, replication-competent viruses that had already infected the primary cells before the transduction. Accordingly, the safety measures of the animal facilities are based on the hazard potential of the replication-competent viruses (S1 or S2).

5. When transduced cells or tissues containing them are deliberately removed from animals and cultured *in vitro*, these cells and tissues are to be regarded as GMOs.

Notes on genetic engineering operations with retroviral vectors that transfer nucleic acid segments with oncogenic potential:

If nucleic acid segments with oncogenic potential are used, the precautionary measures for personal protection as required by the 'Position statement of the ZKBS: precautionary measures for handling of nucleic acids with neoplastic transforming potential', ref_ 6790-10-01, updated version from December 2016, must be adhered to during handling.

If retroviral vectors which exhibit an enhanced particle stability due to pseudotyping are used in genetic engineering operations for the transfer of nucleic acid segments with oncogenic potential, precautionary measures for personal protection may be required that exceed those named in the "Position statement of the ZKBS: assessment of genetically modified organisms in which nucleic acid segments with neoplastic transforming potential have been integrated" (ref_6790-10-36, updated version from December 2014). Wearing a surgical mask is recommended to prevent smear or droplet infections if such pseudotyped vectors are able to transduce human epithelial cells of the mucosa of the nose, mouth or throat due to the receptor specificity of the used envelope protein. Wearing a respirator mask with class 3 retention capacity is recommended to prevent infection through aerosols if such pseudotyped vectors are able to transduce human lung epithelial cells due to the receptor specificity of the envelope protein used. VSV-G-pseudotyped vectors are exempted from this recommendation since those vectors are known to transduce human lung epithelial cells only with low efficiency from the apical side. Due to the broad cell tropism of VSV-G-pseudotyped retroviral vectors wearing a surgical mask is recommended.

The criteria for evaluation of a nucleic acid with regard to its oncogenic potential are set out in the aforementioned position statement with ref_6790-10-01.

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