ZKBS
Zentrale Kommission für die Biologische Sicherheit

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General statement of the ZKBS on frequently performed genetic engineering work with the underlying criteria of comparability:

Gene transfer with Mastadenoviruses from primates

1 Definitions

- **Expression cassette**: Prokaryotic, eukaryotic or viral nucleic acid fragment containing a promoter, the transgene and a stop/polyadenylation signal. The expression cassette cannot replace the function of the adenoviral E1 region.
- **Recombination plasmid:** pBR-derived plasmid containing an expression cassette, flanked by adenoviral nucleic acid fragments that are usually homologous to the non-coding 5‘ and 3‘ regions of the AdV E1 region (see Fig. 2).
- **Adenoviral vector (AdV vector)**: Replication-defective adenoviral particles that can transduce a cell and transfer a nucleic acid fragment in the process.
- **Helper virus**: Adenovirus that provides proteins necessary for the replication of a replication-defective viral genome and its packaging. A helper virus can be replication-competent or replication-defective due to a deletion of the E1 region. Other deletions or modifications may be present in the genome of a replication-defective helper virus.
- **stuffer**: nucleic acid fragment without function

2 Adenovirus

This statement describes gene transfer with mastadenoviruses from primates (human adenoviruses, HAdV) and non-human primates (SAdV). For simplicity HAdV and SAdV are combined in the statement as **AdV**.

2.1 Taxonomy and occurrence

The *Adenoviridae* family is divided into six genera: *Aviadenovirus*, *Barthadenovirus*, *Ichtadenovirus*, *Mastadenovirus*, *Siadenovirus* and *Testadenovirus*.

HAdVs are assigned to different species of the genus *Mastadenovirus*. They were first isolated from adenoid tissue (hyperplastic tonsils) in 1953 [1]. The individual adenoviral (sero)types have been numbered in the order of their initial isolation. HAdVs are widespread, as evidenced by the presence of specific antibodies against HAdV types. In general, children have antibodies against at least one type of adenovirus after the first year of life. Adenovirus type 5 (AdV5) and other types (e.g. 1, 2 or 6) can persist in lymphoid tissue for long periods of time. HAdVs often cause mild respiratory infections in humans, but can also cause keratoconjunctivitis, meningitis or pneumonia [2, 3]. In rare cases, individual AdVs (such as AdV-F41), possibly in combination with other viruses, have been found to cause acute hepatitis in children [4].

Adenoviruses are able to cross the species barrier in some cases. This has been observed in particular for HAdV and SAdV, where close contact is assumed to be a prerequisite for successful transmission to another species. However, a number of factors determine whether a productive viral replication occurs in specific hosts or cell types, such as the presence of specific receptors, uncoating mechanisms or virus-host interactions during replication [5, 6]. Human cells are usually permissive for HAdVs, their infection is productive. In contrast, rodent cells are either non-permissive or semi-permissive for some HAdVs [2, 3, 7–9].

Today, assignment to the genus *Mastadenovirus* or to a species is mainly based on phylogeny and genome organisation data. According to these criteria, the HAdV serotypes are assigned to seven species of the genus *Mastadenovirus* [10]. Many of the AdVs isolated from non-human primates are also classified as HAdVs, e.g. AdVs from chimpanzees, gorillas and bonobos [10]. However, a clear distinction between the species isolated from humans and those

isolated from non-human primates is not always possible due to the high sequence identity of HAdV and SAdV. Chimeric AdVs have been observed in nature, arising from recombination events that occur in mixed infections [11–13]. In addition, the International Committee for the Taxonomy of Viruses, ICTV, lists nine species in which only SAdV are included [10].

2.2 Recommendation

According to § 5 para. 1 GenTSV in conjunction with the criteria in Annex 1 GenTSV, adenoviruses are assigned to the following risk groups as donor and recipient organisms for genetic engineering work:

- Adenoviruses in horses, sheep, pigs, birds and fish **Risk group 2**
- Adenoviruses in reptiles (Ref.: 6790-05-02-0057) **Risk group 2**

  Reason: Reptile adenoviruses exhibit a high degree of host specificity and can cause a potentially fatal disease in infected animals. The viruses are thought to be transmitted by droplet infection.

- Mastadenoviruses **Risk group 2**

  Mastadenovirus adami
  Mastadenovirus blackbeardi
  Mastadenovirus caesari, M. caesari vaccine strain ONRAB
  Mastadenovirus caviae
  Mastadenovirus dominans
  Mastadenovirus exoticum
  Mastadenovirus faecale
  Mastadenovirus russeli

- Recombinant replication-defective mastadenoviruses without risk potential: **Risk group 1**

  Mastadenovirus caesari rAd5-S-CoV2, vaccine strain „Sputnik V“ and *Mastadenovirus dominans* rAD26-S-CoV2 (Ref.: 45242.0193)

  Reason: The recombinant adenoviruses rAd5-S-CoV2 and rAd26-S-CoV2 are replication-defective viruses that carry a gene from SARS-CoV-2 without hazard potential and have been approved outside the EU as components of a combination vaccine against SARS-CoV-2. Both vaccine viruses were well tolerated in clinical trials and mostly only caused mild or moderate side effects.

  Mastadenovirus dominans Ad26.Mos2S.Env, Ad26.Mos1.Env, Ad26.Mos1.Gag-Pol and Ad26.Mos2.Gag-Pol, Ad26.RSV.preF (Ref.: 45242.0188)

  Reason: The recombinant adenoviruses Ad26.Mos2S.Env, Ad26.Mos1.Env, Ad26.Mos1.Gag-Pol and Ad26.Mos2.Gag-Pol as well as Ad26.RSV.preF are replication-defective viruses that carry nucleic acid fragments without the potential to cause HIV-1 or RSV infection. Recombinant viruses with the same vector backbone, which are used as vaccines against Zaire Ebola virus and SARS-CoV-2, have already been approved as medicinal products in the EU. These vaccines, as

well as Ad26.Mos4.HIV and Ad26.RSV.preF, were well tolerated in clinical trials and mostly caused only mild or moderate adverse events.

Mastadenovirus dominans Ad26.COVS1 (Ref.: 45242.0180)

Reason: The recombinant adenovirus Ad26.COVS1 is a replication-defective virus that carries a gene from SARS-CoV-2 without hazard potential and is to be tested as a vaccine against SARS-CoV-2. An Ad26-based vaccine against the Zaire Ebola virus was approved by the EU Commission in 2020. This and other Ad26-based vaccines were well tolerated in clinical trials, with mostly mild or moderate adverse events.

Mastadenovirus exoticum ChAdOx1 nCoV-19 (Ref.: 45242.0181)

Reason: The recombinant, replication-defective adenovirus ChAdOx1 nCoV-19, which is derived from ChAdY25, is a virus that carries a gene from SARS-CoV-2 without hazard potential and is to be tested as a vaccine against SARS-CoV-2. Vaccines based on the ChAdOx1 vector have already been well researched in preclinical studies. In addition, clinical data from ChAdOx1-based vaccines against FLUAV, *Mycobacterium tuberculosis* and MERS-CoV are available from 154 subjects to date. The ChAdOx1-based vaccines were well tolerated, with mostly mild or moderate adverse events.

2.3 Genome organisation

The genome of AdV from primates consists of a double-stranded linear DNA between 32 and 36 kb in length (Fig. 1a). Both ends of the DNA are characterised by inverse repetitive regions (inverted terminal repeats, ITR), with the 5' ITR containing the polymerase binding sites for initiation of DNA replication and the DNA packaging signal Ψ. The early (E) events in the productive infection cycle also include transcription of the E1a gene, with its product transactivating the expression of the other early viral genes E1b, E2, E3 and E4. Early transcription is followed by DNA replication, which initiates the late (L) phase of the productive infection cycle. In the late phase, the late genes are expressed, which mainly encode the structural proteins of the icosahedral capsid, and virus particles are produced. The productive cycle leads to the lysis of the infected host cell [2, 3].

In addition to transactivating early genes, E1a interacts with a variety of cellular proteins. For example, E1a can promote the entry of quiescent cells in the G_0 or G_1 phase into S phase [14]. While certain HAdV (e.g. AdV12) potentially induce neoplastic transformation in newborn rodents [15], AdV5 and AdV26 have been shown not to be associated with tumorigenesis [16]. For ChAdY25-based vectors, no causality in tumorigenesis has been described either. Therefore, a neoplastic transformation potential can be excluded for these AdV vectors [16].

The gene products encoded by E1b inhibit the p53-dependent induction of apoptosis [2, 3]. The E2 region encodes three proteins that are required for viral DNA replication: the preterminal protein (PTP), DNA polymerase (Ad Pol) and a ssDNA binding protein (DBP) [3]. The gene products encoded by the E3 region are not essential for replication *in vitro* but are involved in immune evasion. E3 encodes several proteins that, among other functions, block major histocompatibility complex trafficking to the plasma membrane and inhibit lysis of adenovirus-infected cells by the tumor necrosis factor [2, 3]. The E4 region encodes several spliced mRNAs, and the expressed proteins are named after the open reading frames (ORF) of the E4 region (e.g. E4orf1 or E4orf2). They stimulate protein synthesis by activating the protein kinase

mTOR, protect the AdV genome ends by inhibiting the cell's own double-strand break repair mechanisms or stimulate transcription of the E2-ORFs [3].

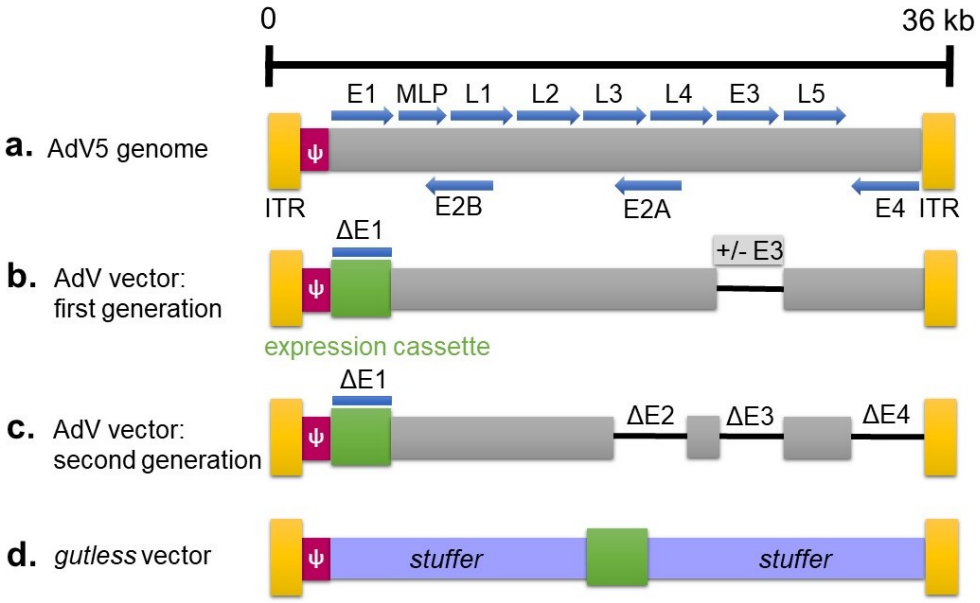

Fig. 1: Genome map of adenoviruses and derived vectors. a. The early transcription units E1, E2, E3, E4 are shown in the direction of transcription together with the major late promoter (MLP) and the region of late genes (L1 to L5). The ITR and the packaging signal ψ are indicated. **b**. Genome map of first-generation adenovirus-derived vectors with expression cassette. The E1 region is deleted and replaced by an expression cassette. The E3 region may also be deleted. **c.** Genome map of second-generation adenovirus-derived vectors with expression cassette. The E1 region is deleted and replaced by an expression cassette. The E2, E3 and E4 regions are also deleted. **d**. Genome map of adenovirus-derived gutless vectors with expression cassette. All viral coding nucleic acid fragments are deleted and replaced by an expression cassette and stuffer fragments. Figure modified from [17, 18].

3 Adenoviral vector systems

AdV-derived vectors are infectious, replication-defective particles with DNA portions of AdV that can transfer an expression cassette.

AdV vectors are versatile; they exhibit high transduction efficiency *in vitro* and *in vivo*, infect a wide range of cell types, including non-dividing cells, and can be cultured at high titers. AdV vector particles are used both *in vitro* for the transmission of heterologous genes and *in vivo* as vaccines or in somatic gene therapies [16, 19–24]. The adenovirus capsid can package DNA fragments of up to 105% of the length of the wild-type genome. With a genome length of about 36 kb, AdV5 therefore has a packaging capacity of about 1.8 to 2.0 kb of foreign DNA for 1st generation vectors [25]. When using 2nd generation adenoviral vectors or gutless vectors, correspondingly larger DNA fragments of up to 33 kb can be introduced into the AdV genome [26].

The adenoviral vectors used for gene transfer are currently categorised into three generations [18] (see Fig. 1):

- In first generation vectors, the E1 region is deleted, which makes the adenoviral vector particles replication-defective. This region serves as an insertion site for the expression

cassette. To increase their capacity, the E3 region has also been deleted in some of these vectors, resulting in further attenuation. However, they still contain the other early and late viral genes, which can be expressed in small amounts after infection [27, 28]. In clinical applications or in the event of accidental inoculation, the viral gene products can induce an immune response against the transduced cell, which can result in an inflammatory reaction and a shortened expression of the transgene. The observed low-level replication of viral DNA in the target cells can also be traced back to a weak expression of viral genes [29–31].

- In second generation vectors, the E2 and E4 regions are deleted in addition to the E1 and E3 regions. The late genes are still present. Compared to the first generation vectors, the additional deletions increase packaging capacity and reduce immunogenicity [18]. Due to the deletion of the E1 region, they are also replication-defective.

- gutless vectors (third generation) have the highest packaging capacity, because they only contain the flanking ITRs and the packaging signal Ψ as adenoviral sequences. In order to adapt the genome size to the viral capsid, the vectors usually contain a stuffer sequence. As a result of the deletion of all adenoviral genes, vectors are less immunogenic than second generation vectors [18] and they are also replication-defective.

The defective viral genome transferred by AdV vector particles to non-complementing target cells usually remains episomal, resulting in only transient expression of the expression cassette [32]. No new viral particles are produced.

In 1993, the first human gene therapy study in patients with cystic fibrosis using a recombinant AdV vector was conducted. In recent decades, the number of clinical trials based on AdV vectors has steadily increased. Today, about half of all gene therapy products in clinical trials are based on AdV vectors. They are used primarily as vaccines and in cancer therapy. However, AdV vectors are also used in regenerative medicine and stem cell research [16].

Cancer therapies based on defective AdV aim to transfer immunostimulatory, tumor suppressor or suicide genes into the tumor cell. For example, the introduction of a suicide gene into the tumor leads to the expression of an enzyme that converts an inactive pro-drug into cytotoxic metabolites that cause cell death [16].

The therapy with oncolytic AdVs is based on the replication competence of the viruses in tumor cells and the associated lysis. The released viruses then destroy further surrounding tumor cells in a cascade-like manner.

Recombinant replication-defective AdVs are approved as vaccine vectors and are used extensively. Already approved AdV vaccine vectors are based on AdV5, AdV26 or ChAdY25, are well tolerated and usually cause only mild or moderate vaccination reactions (see ZKBS recommendation in Chapter 2.2). For example, one approved Ebola vaccine is based on AdV26. Two vaccines for the prevention of SARS-CoV-2 infections are based on AdV5.

3.1 Production of recombinant AdV vector particles

Recombinant AdV vector particles can be produced by several methods. The expression cassette can be inserted into the AdV genome by direct ligation or homologous recombination.

3.2 Helper cell-dependent production by homologous recombination

The expression cassette is usually inserted by homologous recombination between the recombinant plasmid and the viral genome. For this purpose, very often a human cell line permissive for adenoviral replication is used.

An AdV genome of the first or second generation is transferred to human cells together with a pBR-derived recombination plasmid. The expression cassette is generally integrated into the deleted E1 region of the replication-defective AdV genome either present in a vector particle or integrated in a plasmid. The E1 gene products required for viral DNA replication are usually provided in trans via permissive recombinant cell lines that constitutively express the E1 gene. The recombinant viral genome is packaged by the viral structural proteins and released from the cell line as expression cassette containing AdV vector particles (Fig. 2) [33–35].

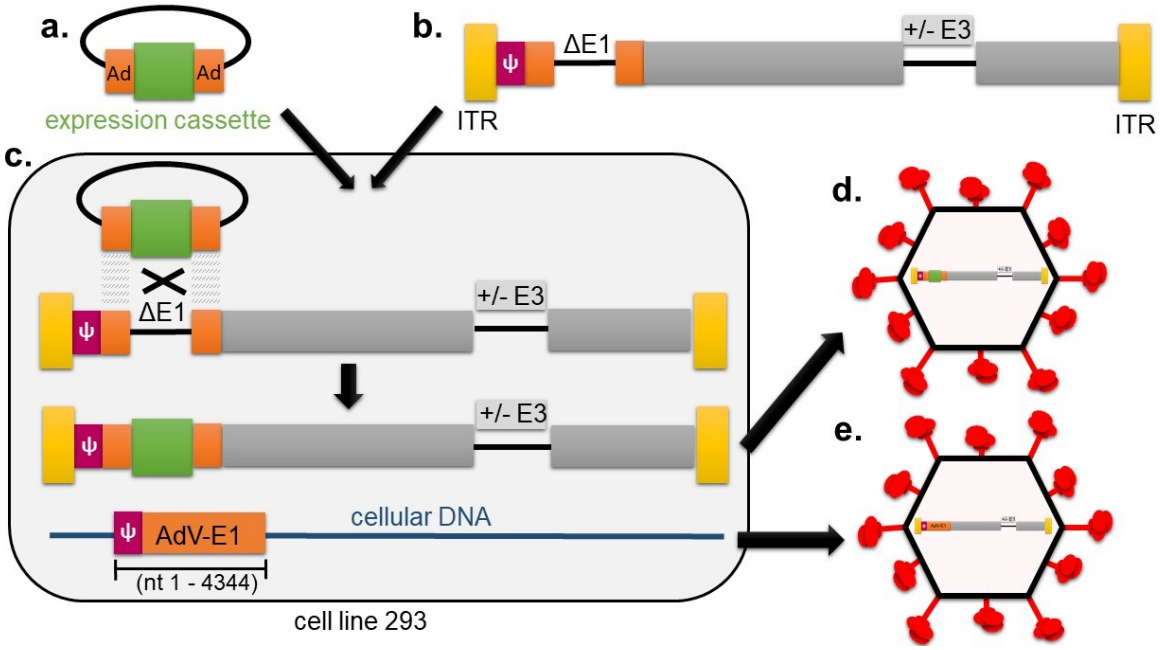

Fig. 2: Exemplary production of a replication-defective first-generation AdV vector particle with an expression cassette in a helper cell line. a. Recombination plasmid with expression cassette. **b**. AdV genome with deletion of the coding nucleic acid fragments of the early genes E1a, E1b and if applicable E3. **c**. The defective AdV genome is co-transfected with the recombination plasmid into a helper cell line. The genome of the helper cell line contains the 5' end of the AdV genome with the E1 region and expresses the E1 gene products, thereby complementing the replication defect of the co-transfected defective AdV genome. During viral DNA replication, recombination between the recombination plasmid and the defective AdV genome occurs via the DNA sequence homologies of the non-coding regions of E1. A replication-defective AdV genome is formed, in which the deleted E1 region contains the inserted expression cassette. **d**. The remaining late genes in the AdV genome are expressed. Their gene products package the AdV genomes, with and without expression cassettes, into infectious, replication-defective particles that are released by the helper cell line.**e**. Homologous recombination between the helper virus genome and the E1 gene integrated in the cell genome can result in the formation of replication-competent virus particles.

Transfer of the expression cassette into the AdV genome by homologous recombination can also be achieved using the efficient recombination machinery in *E. coli* K12 derivative BJ5183 (*recBC sbcBC*) [36]. In the AdEasy adenoviral vector system, DNA constructs with the AdV genome and the expression cassette flanked by nucleic acid fragments of the integration site in the AdV genome are co-transferred to *E. coli* BJ5183. In the AdV genome, the E1 region or and the E3 region is deleted.

The expression cassette is typically inserted in place of the E1 region, rendering the AdV vector particles replication-defective. The result is a recombinant plasmid containing an AdV genome with inserted expression cassette [37, 38]. The plasmid is isolated and transfected into a helper cell line providing the E1 proteins necessary for viral replication. Replication-defective AdV vector particles with an expression cassette are produced and released by the helper cell line. However, homologous recombination between the helper virus genome and the E1 gene integrated in the cell genome can result in the production of replication-competent virus particles (see Chapter 3.5 'Safety of helper cell lines').

3.3 Production of gutless vector particles using an AdV helper virus

In gutless or high capacity vectors, all viral genes have been deleted so that the immune response against the vector is reduced and the capacity for foreign DNA is increased [39–43]. In these vectors, only those viral nucleic acid fragments that act in cis and are essential for DNA replication and packaging of viral DNA are still present, such as the ITRs with the polymerase binding sites for the start of DNA replication and the DNA packaging signal ψ (see Fig. 1c and Fig. 3b).

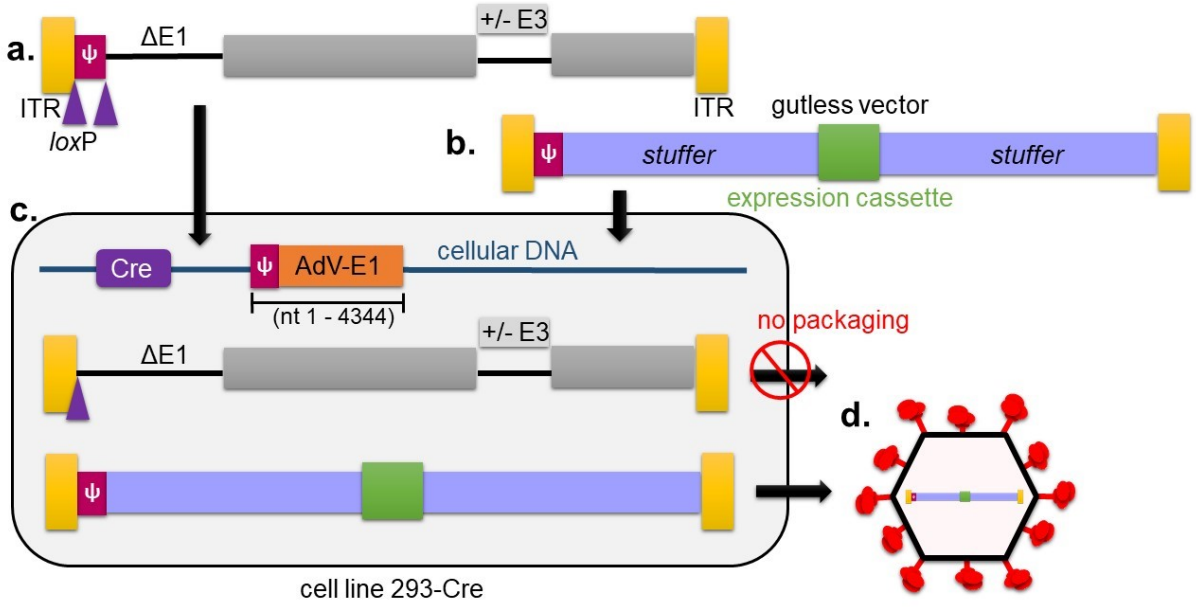

Fig. 3: Production of gutless vector particles utilizing the Cre recombinase system. a. The packaging signal ψ of an AdV helper virus genome (AdV genome) is flanked by two loxP sites that are recognized by the Cre recombinase. The E1 region and possibly also the E3 region are deleted, while all other adenoviral genes are still present. **b.** The expression cassette to be transferred was inserted into the gutless vector DNA and is located in between the stuffer sequences, the ITRs of AdV and the packaging signal ψ. **c.** The AdV helper genome **a.** and the gutless vector DNA **b.** are transferred together into the helper cell line 293-Cre **c.** The genome of this helper cell line contains the genes of the Cre recombinase (Cre) and an AdV genome fragment including the packaging signal ψ and the region coding for E1 (nucleotides 1 – 4344 [34]) that are constitutively expressed. AdV-E1 complements the deleted E1 in the helper virus genome. Thus, the expression of the remaining adenoviral genes of the helper virus genome is achieved, The helper virus genome and as well as the gutless vector DNA replicate. While gutless vectors can be package due to a packaging signal ψ, the Cre recombinase excises the packaging signal ψ from the helpter virus genome, rending it no longer packable. **d.** The gutless vector particles with expression cassette are released by the helper cell line. The figure has been modified from [41].

In principle, gutless vectors rely on a helper virus to provide the proteins necessary for replication of the viral genome and its packaging. If such a helper virus is replication-competent, its particles contaminate the vector particles produced and must be subsequently removed.

Another method for producing gutless vector particles is the Cre/loxP helper-dependent system (see Fig. 3), which is derived from bacteriophage P1. It has the advantage that a replication-defective helper virus can be used, which increases the system's safety [41, 42].

The Cre/loxP helper-dependent system consists of three components: an AdV helper virus, a gutless vector DNA and a helper cell line. The AdV helper virus is an E1- and possibly E3-deleted AdV in which the packaging signal ψ is flanked by loxP sites. In most cases, a marker gene additionally to the ψ packaging signal is present between the loxP sites. The loxP sites are recognition sites for Cre recombinase, an enzyme of bacteriophage P1. The 293-Cre cell line, which stably expresses the Cre recombinase, is used as the helper cell line. After infection of the helper cell line, the packaging signal ψ of the helper virus DNA and, if applicable the marker gene, are excised.

The E1 gene of the helper cell line mediates transcription of all viral genes still present in the helper virus genome and replication of the helper virus genome.

A gutless vector DNA is transferred into the helper cell line containing the expression cassette and the stuffer sequence (see Figs 1 and 3) as well as the ITRs and the packaging signal ψ of an AdV. The gutless vector DNA is replicated by the viral proteins provided by the helper virus. Since the gutless vector DNA still contains the packaging signal ψ, only this DNA is packaged by the viral capsid proteins provided by the helper virus. Packaging of the helper virus DNA is disrupted due to excision of its packaging signal ψ by the Cre recombinase (see Fig. 3). In addition to vector particles, helper virus particles may be present in the cell culture supernatant. Furthermore, homologous recombination between the E1 gene integrated into the cell genome and the helper virus genome can lead to the formation of replication-competent virus particles (see Chapter 3.5 "Safety of helper cell lines"). The gutless vector particles are purified by CsCl gradient centrifugation. Due to differences in density, helper virus particles and gutless vector particles sediment in different sections of the gradient [44]. This enables an extensive but not complete removal of the remaining helper viruses from the gutless vector particles [44].

3.4 Production of gutless vector particles using a helper plasmid

The production of gutless vector particles using the AdV helper virus genome was further refined by replacing the helper virus with a helper plasmid depleted of essential genes for particle production [45, 18]. The helper plasmid does not contain the E1 gene, the flanking ITRs or the ψ packaging signal (see Fig. 4). In addition, the E3 gene has been removed.

This system still allows homologous recombination between the integrated adenovirus nucleic acid fragment and the helper plasmid (nucleotide 3034 – 5015 = 1428 bp, pIX-AdV-genome fragment). However, the formation of AdV particles is very unlikely, since several illegitimate recombination events would be required to generate a packageable genome from the helper plasmid. Two recombination events with the adenovirus nucleic acid fragment present in the cell are required to integrate the left ITR, ψ and E1 genes (illegitimate at the 5' end, homologous at the 3' end of the fragment). Additionally, two more illegitimate recombination events with the gutless vector DNA would be required to integrate the right ITR. Thus, replication-competent particles are not expected to be generated by homologous recombination. Measures to separate replication-defective gutless vector particles are therefore not necessary.

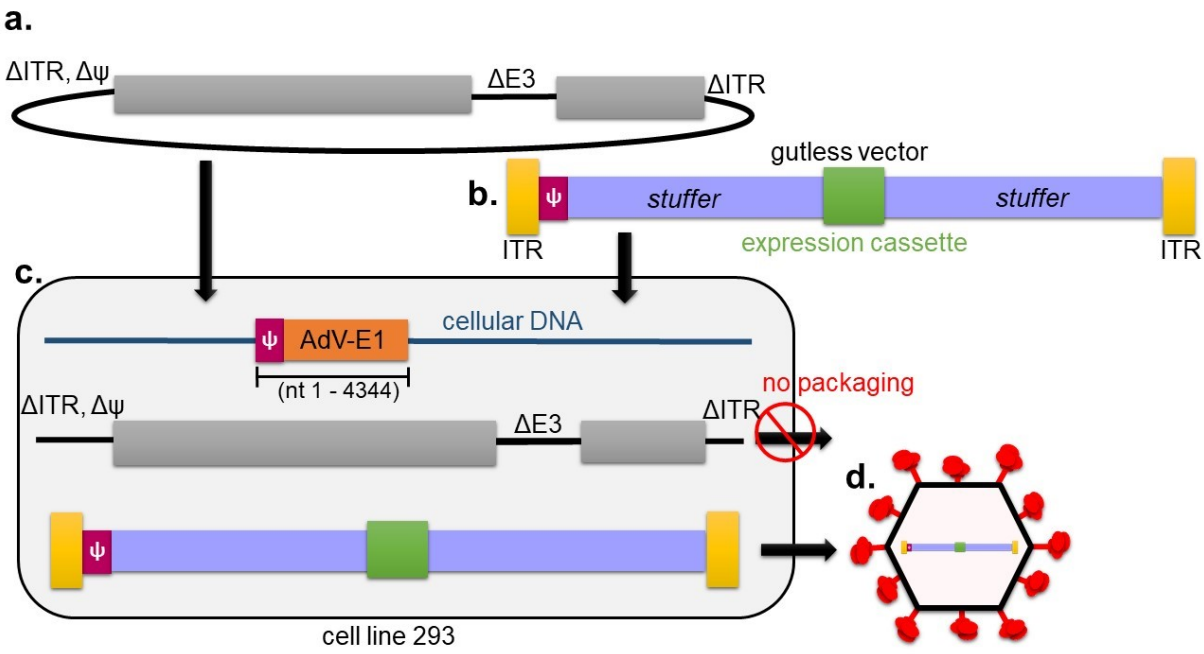

Fig. 4: Production of gutless vector particles using a helper plasmid system. In order to generate an AdV helper plasmid **a.**, the flanking ITR, the packaging signal Ψ and the E3 gene were removed from the AdV helper virus genome containing loxP sites (see Fig. 3a) . The expression cassette to be transferred is inserted into the gutless vector DNA **b.**, which also contains the ITR and the AdV packaging signal ψ. To produce adenoviral vectors, the gutless vector DNA is co-transfected with the AdV helper plasmid into a helper cell line (here 293) **c.**, which then releases the viral vector **d.**.

3.5 Safety of helper cell lines

Replication-defective adenoviral vector particles and replication-defective helper viruses lack the E1 region. For their production and propagation, permissive cells are required to provide the gene functions of the E1 region.

A frequently used helper cell line is the human cell line 293, which was generated by transfecting human embryonic kidney cells with fragmented AdV5 DNA [33]. Their genome contains a copy of the left end (nucleotides 1 to 4344) of the AdV5 genome, including the ITR, the packaging signal ψ, the late pIX gene, and the E1a and E1b genes, which are constitutively expressed [33, 34].

Many first-generation AdV vectors, as well as helper viruses, contain nucleic acid fragments homologous to the integrated copy of the left end of the AdV genome (see Fig. 5a).

It has been shown that homologous recombination events can lead to the reintegration of the E1 gene into the vector or helper virus genomes, leaving them replication-competent. Replication-competent AdV vector particles are still rare in the first passages, but when large amounts of virus are produced over many passages, contamination rates can be high [46–48]. Another helper cell line with a similar E1 integration pattern is 911, a cell line established from human embryonic retinoblasts [35].

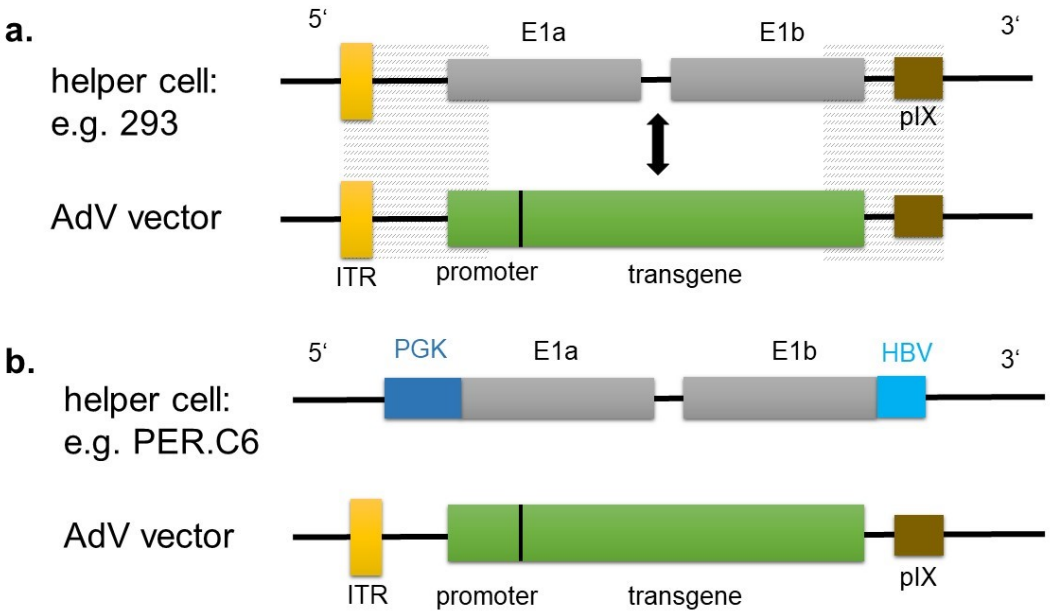

Fig. 5: AdV DNA in helper cell lines and in the vector with expression cassette. a. Helper cell lines such as 293 typically contain 5' regions including ITR, packaging signal ψ, and the E1a, E1b, and pIX genes of the AdV5 genome. The AdV vector DNA contains the expression cassette consisting of promoter and transgene in place of the E1a and E1b coding regions, but flanked by E1 nucleic acid fragments homologous to the integrated AdV genome region of the helper cell line (hatched areas), allowing for homologous recombination events. **b.** In the new helper cell lines, such as PER.C6, the E1 gene is reduced to the E1a and E1b reading frames and is under the control of a heterologous promoter, such as the promoter of the human phosphoglycerate kinase (PGK) gene and the polyadenylation signal of the hepatitis B virus (HBV) surface protein gene. The figure has been modified from [49].

PER.C6 is also a cell line established from human embryonic retinoblasts. The genome of this cell line contains a truncated E1 region (nucleotides 459 to 3510) under the control of a heterologous phosphoglycerate kinase (PGK) promoter and a heterologous polyadenylation signal (see Fig. 5) [49]. There are no homologies between the PER.C6 genome and that of AdV vectors [49, 50]. Cell lines such as PER.C6 used in combination with AdV vectors lacking the E1a and E1b nucleic acid fragments, prevents the generation of replication-competent adenovirus particles.

3.6 Safety of AdV vectors of other *Mastadenovirus* species

The generation of replication-competent AdV particles can also be prevented by using AdV vectors from other Mastadenovirus species, such as the ChAdY25 subtype, which is assigned to the species *Mastadenovirus exoticum* [51]. If there is low or no sequence homology between the AdV region present in the helper cell line and the nucleic acid fragments of the AdV vector, homologous recombination and release of replication-competent AdV particles are not expected [51]. This is the case for cell lines derived from 293 and ChAdY25.

3.7 Biodistribution, stability and shedding of virus particles

Biodistribution, shedding and duration of shedding of adenoviral vectors depend on vector serotype, the dose administered and the mode of administration [52]. Adenoviral particles are known to persist at the injection site for several days to weeks after administration [53]. During

biodistribution, AdV5 particles mainly enter the liver and spleen. AdV5 vector particles are much less frequently detected in other tissues such as heart, bone marrow, lung, kidney, intestine or gonads [54–56]. Three month after administration of 10^{11} AdV35 particles, a low number of particles could be detected in iliac lymph nodes [55]. Balb/c mice that had received the chimpanzee adenovirus-derived vaccine ChAdOx1-HBV at a dose of 2.4×10^{10} vector particles intramuscularly retained vector genome copies in all organs examined (brain, heart, kidney, liver, lung, lymph node, ovaries, skeletal muscle, spleen and testes). On day 56, the number of vector genome-positive samples was significantly reduced (9 out of 56 organ samples) [57].

The tropism of AdV depends in part on the cellular receptor binding specificity of the serotype. While the coxsackievirus and adenovirus receptor (CAR) has affinity for most human serotypes, bovine, porcine and ovine AdV enter host cells independently of CAR [58]. Tissues such as liver, stomach and gallbladder as well as some tumor tissues (lung, ovary and uterus) show high expression of CAR. In contrast, in kidney, prostate and parotid gland tumors CAR expression is downregulated [59]. Some species, such as mouse, rat, dog and pig, have receptors with a high degree of homology to the human CAR. Almost all HAdVs contain the Arg-Gly-Asp (RGD) motif in the fiber knot, which might interact with integrins as co-receptors. Other known receptors of different AdV types are CD46, CD80/86, sialic acid, proteoglycans, major histocompatibility complex (MHC)-I and vascular cell adhesion molecule (VCAM)-1 [58]. Adenoviruses are generally unable to cross the blood-brain barrier. Using the melanotransferrin (MTf)/P97 transcytosis pathway, it was shown that transcytosis of AdV5 across the blood-brain barrier is possible using the generated fusion protein CAR-MTf [60].

Shedding of virus particles from the treated patient or laboratory animal is possible via urine, faeces, sweat, saliva, nasopharyngeal fluid, tears, or seminal fluid. In general, the determination of shed virus particles is based on the detection of their genome using non-quantitative or quantitative PCR (qPCR) or, less commonly, on the detection of protein using enzyme-linked immunosorbent assays (ELISA) [61]. Biological assays are used to determine the infectivity of vector particles. Most commonly, shedding data is collected using the xenograft mouse model. The authors of a mouse study argue that laboratory personnel are not at risk of exposure to infectious particles on the fourth day after intravenous administration of an AdV5, because three days after administration, no or little plaque formation was detected in oral mucosa, urine, faeces, or skin samples taken from the area overlying the established tumors. Therefore, a downgrade from biosafety level 2 to biosafety level 1 could be considered in the Biosafety Guidelines of the NIH in the USA [62].

Few data is available on virus shedding after oral or subcutaneous administration. However, viral DNA was occasionally detected by qPCR in the faeces and saliva of raccoons (3 days), foxes (4 days) and dogs (1 day) during the first few days after oral administration of high doses of the rabies vaccine AdRG1. 3-ONRAB®, based on replication-competent AdV5 particles [56].

A comprehensive analysis of clinical trial results in 2007 showed that 29 out of 50 publications reported shedding of vector DNA or infectious particles in secretions after different routes of administration. Long-term shedding in sputum for more than 90 days was observed in lung cancer patients after intratumoral administration, among others [63]. In some studies, blood, faeces or throat swabs from healthcare workers in close contact with patients were also analysed. No vector genomes or virus particles were found [61, 64].

In summary, no general statement can be made on biodistribution or the time point at which treated laboratory animals cease to shed infectious AdV particles and are no longer considered to be GMO carriers. Therefore, when disposing of litter and carcasses or handling tissues from laboratory animals inoculated with AdV vector particles, the instructions in Chapter 6, numbers 3 and 4 should be followed.

4 Criteria for the safety classification of genetic engineering work with AdV vectors

According to the current state of science and technology, the safety assessment of genetic engineering work with E1-deleted AdV genomes in *E. coli* K12 and its derivatives does not pose a risk to human health or the environment, provided that no other nucleic acid fragments with hazard potential are introduced. If, however, the complete genome or a replication-competent genome (e.g. only E3 deleted) of an AdV is amplified, even if it is interrupted by nucleic acid fragments of the plasmid, the hazard potential of the AdV must be fully considered in the risk assessment (general statement of the ZKBS on the risk assessment of *E. coli* K12- derivatives with a plasmid with the (c)DNA of the genome of a replication-competent virus, Ref. 6790-10-89).

When eukaryotic cells are transfected with amplified recombinant plasmids, it is relevant whether AdV vector particles can be generated and their hazard potential is crucial for the safety assessment. The risk potential for humans is considered low, provided that no heterologous nucleic acid sequences are inserted into the AdV genome that increase the risk potential of AdV. AdV vector particles are considered to pose a low risk when handled because they efficiently infect human cells and because it cannot be excluded that the viral genome – as a rare event – integrates into the host genome. The possibility of such integration should be considered, particularly as the adenoviral vector DNA may have low-level replication due to the presence of viral gene products. On the basis of clinical data, AdV5, AdV26 or ChAdY25 are not considered to pose a hazard even if they are integrated (see Chapter 2.2).

Depending on the generation of the AdV vectors used (see Fig. 1) replication-competent AdVs can be formed and released after co-infection of a cell with an AdV and an AdV vector.

In the case of first-generation AdV vectors, in which the E1 region in the AdV genome has usually been deleted, it is possible that, as described in Chapter 3.2 homologous recombination may lead to the formation of replication-competent wild-type adenoviral particles whose hazard potential is equivalent to that of the original AdV.

For subsequent generations of AdV vectors (see Fig. 1) it cannot be assumed that replication-competent AdV will be formed *in vivo*. Either several essential regions of the viral genome have been deleted, so that the replication defect can only be resolved/reversed by several recombination events, or in the helper cell line there are no nucleic acid fragments homologous to the AdV genome. To date, no cases of recombination between AdV and AdV vectors have been reported in clinical trials [65].

If helper cell lines are used that cannot exclude the possibility of the emergence of replication-competent AdV particles through homologous recombination (see Chapter 3.5 ‘Safety of helper cell lines’), it must be assumed that such viruses will be contaminating the cell culture.

In the newly developed helper cell lines, in which the helper genes of the E1 region are reduced to the coding nucleic acid fragments and in which homologous recombination with the replication-defective AdV genome is not assumed, only illegitimate recombination would be conceivable. However, this is an extremely rare event and does not need to be considered in the risk assessment of such work.

After infection of cell cultures or animals, only the replication competence of the AdV vector particles shall be assessed:

- After infection with replication-competent AdV vector particles including an expression cassette or after corresponding transfection, new recombinant, replication-competent AdV

vector particles are generated, which are relevant for the safety assessment of the genetic engineering work.

- Following abortive infection with replication-defective AdV vector particles including the expression cassette, or following appropriate transfection, no new AdV vector particles are produced unless the infected cells or infected animal reverse the replication defect of AdV. The viral nucleic acid is not mobilised and transferred to other cells. When handling these cells or animals, it should be noted that they may release the administered vector particles if they are not taken up by a cell (see Chapter 1 Section 3 and 4).

  When experimental animals are infected with AdV vectors, homologous recombination between AdV and AdV vectors and the formation of replication-competent AdV can be excluded if the experimental animals are kept in a specific pathogen-free environment (SPF) according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) [66].

5 Criteria for the comparability of genetic engineering work with AdV vectors

General criteria for the comparability of genetic engineering work with adenoviral vector particles are summarised below. Genetic engineering work involving genetically modified organisms (GMOs) that meet the above criteria can be compared with each other and assigned to the safety level corresponding to the risk group of the GMO.

Transfer of genes for prion proteins or toxins by using adenoviral vectors, requires a case-by-case assessment by the ZKBS.

5.1 Transfer of plasmids containing adenoviral DNA to *E. coli* K12-derivatives

1. *E. coli* K12 derivatives including a recombination plasmid with subgenomic nucleic acid fragments of AdV are GMOs of **risk group 1**.
2. *E. coli* K12 derivatives including a recombination plasmid and, if applicable, a helper plasmid with subgenomic adenoviral nucleic acid fragments are GMOs of **risk group 1**.
3. *E. coli* K12 derivatives including a plasmid with an E1-deleted (replication-defective) adenoviral genome are **risk group 1** GMOs.
4. *E. coli* K12 derivatives including a plasmid with the complete genomic DNA of an AdV are GMOs of **risk group 2**.

5.2 Production of adenoviral vector particles in eukaryotic cells

1. Eukaryotic cells of **risk group 1** to which a recombination plasmid or subgenomic nucleic acid fragments of AdV have been transferred are GMOs of **risk group 1**.
2. Eukaryotic cells, including helper cell lines, of **risk group 1**, to which the complete genomic DNA of an AdV has been transferred, are GMOs of **risk group 2**. Replication-competent virus particles of **risk group 2** are formed and released.
3. Eukaryotic helper cell lines of **risk group 1**, to which E1-deleted AdV5, AdV26 or ChAdY25 genomes and a transgene without risk potential have been transferred, are GMOs of **risk group 1**, provided that it can be ruled out that replication-competent adenoviral virus particles are formed by homologous recombination. Replication-defective ad-

enoviral vector particles of **risk group 1** are released. If it cannot be ruled out that replication competent adenoviral vector particles are formed by homologous recombination, these must be included in the risk assessment. If complete separation of replication-competent AdV particles is not possible, the mixture of particles must be assigned to **risk group 2**.

4. Eukaryotic helper cell lines of **risk group 1**, to which E1-deleted AdV genomes (except AdV5, AdV26 or ChAdY25) and a transgene without risk potential have been transferred, are GMOs of **risk group 2**. If it cannot be ruled out that replication-competent adenoviral vector particles are formed by homologous recombination, these must be included in the risk assessment. Replication defective adenoviral vector particles and, if applicable, replication competent adenoviral virus particles of **risk group 2** are released.
5. Eukaryotic helper cell lines of **risk group 1**, which have been infected with a replication-competent helper virus and to which additionally non-coding subgenomic nucleic acid fragments of an AdV and a transgene without hazard potential have been transferred, are GMOs of **risk group 2**. Replication-competent virus particles of **risk group 2** and replication-defective adenoviral gutless vector particles (possibly with transgene) of **risk group 1** are formed and released. If the replication-competent helper viruses cannot be separated from the replication-defective adenoviral gutless *vector particles*, the particle mixture must be assigned to **risk group 2**.
6. Eukaryotic helper cell lines of **risk group 1**, which have been infected with a replication-defective helper virus or transfected with a helper plasmid and to which additionally non-coding subgenomic nucleic acid fragments of an AdV and a transgene without risk potential have been transferred, are GMOs of **risk group 1**, provided that (i) the replication capacity of the helper virus cannot be assumed to be restored by homologous recombination. If the proteins of the adenoviral E1 region are expressed in the cells, this leads to the formation and release of replication-defective adenoviral gutless vector particles of **risk group 1**.
7. Eukaryotic helper cell lines of **risk group 1** to which E1-deleted AdV genomes, including a replication-defective helper virus or a helper plasmid in combination with non-coding subgenomic nucleic acid fragments of an AdV, as well as a transgene with neoplastic transforming potential, have been transferred are GMOs of **risk group 2**. Recombinant, replication-defective AdV particles or replication-defective adenoviral gutless vector particles that can transfer a gene with a hazard potential are released. Additional safety measures must be observed when handling these GMOs (see chapter 1).

5.3 Transduction of eukaryotic cells with adenoviral vector particles

1. Eukaryotic cells of **risk group 1** to which DNA has been transferred using replication-defective adenoviral adenoviral gutless vector particles of **risk group 1** are GMOs of **risk group 1**. No replication-defective adenoviral vector particles or replication-competent virus particles are formed and released.
2. Eukaryotic cells of **risk group 1** to which nucleic acid fragments with neoplastic-transforming potential have been transferred using replication-defective adenoviral gutless vector particles of **risk group 2** are GMOs of **risk group 1** after completion of transduction, unless the cells are infected with a helper virus. No replication-defective adenoviral vector particles or replication-competent virus particles are formed and released.
3. Eukaryotic cells of **risk group 1** to which a transgene without hazard potential or with neoplastic transforming potential has been transferred using replication-defective adeno-

viral vector particles of **risk group 1** or **2**, in which contamination with replication-competent virus particles of **risk group 2** is not to be assumed, are GMOs of **risk group 1** after completion of transduction, provided it can be ruled out that (i) homologous recombination with any adenoviral nucleic acid fragments present in the cell cancels the replication defect and (ii) the adenoviral E1 region is present in the genome of the cells. adenoviral nucleic acid fragments present in the cell cancels the replication defect and (ii) the adenoviral E1 region is present in the genome of the cells. No replication-defective adenoviral vector particles or replication-competent virus particles are formed and released.

4. Eukaryotic cells of **risk group 1**, to which DNA has been transferred using replication-defective adenoviral vector particles of **risk group 1**, in which contamination with replication-competent virus particles of **risk group 2** cannot be excluded, are also GMOs of **risk group 2** after completion of transduction. Replication-competent virus particles of **risk group 2** may be formed and released.
5. Eukaryotic cells of **risk group 2**, to which a transgene without hazard potential or with neoplastic-transforming potential has been transferred using adenoviral vector particles, are also **risk group 2** GMOs after completion of transduction, as the hazard potential of the recipient organisms is fully taken into account in the risk assessment.

6 Notes

1. If AdV vector particles are contaminated with helper viruses due to the system used to produce them, the hazard potential of the helper viruses is fully included in the risk assessment.
2. For the handling of adenoviral vector particles with nucleic acid fragments with neoplastic transforming potential, reference is made to the following general statements of the ZKBS:
   - Statement of the ZKBS: Precautionary measures when handling nucleic acids with neoplastic transforming potential (Ref. 6790-10-01, updated December 2016)
   - Recommendation of the ZKBS on adenoviral and AAV-derived replication-defective particles that transfer a nucleic acid fragment with neoplastic transforming potential (Ref. 6790-10-83, updated April 2020)
   - Statement of the ZKBS – Evaluation of genetically modified organisms into which nucleic acid fragments with neoplastic transforming potential have been introduced (Ref. 6790-10-36, updated February 2025)
3. **Experimental animals** from an SPF husbandry to which the transduced cells described in Chapter 5.3 Section 1 and 2 have been transferred, whereby contamination of these cells with recombinant AdV can be excluded, are not GMOs and are also not capable of releasing GMOs. The animals are also not to be regarded as carriers of GMOs.
4. **Experimental animals** inoculated with replication-defective AdV particles are not GMOs. However, the animals are to be regarded as carriers of GMOs as long as the vector particles are present. Transduced somatic cells in animals are also not GMOs, as animal cells in the organism are not microorganisms according to § 3 No. 1 of the Genetic Engineering Act as long as they are not propagated in cell culture. The period during which infectious vector particles remain in the animal depends on several factors (e.g. dose, route of application, serotype used, immunocompetence of the test animal). In addition, the animals may shed the administered vector particles again. In this case, litter and any surfaces that may have come into contact with the excretions must also be regarded as GMO-contaminated. The risk group of the recombinant AdV vector particles present determines the necessary safety measures.

If data, e.g. obtained like in [51, 65, 66] or suitable literature for the individual case or comparable experimental approaches, prove that the treated animal no longer releases AdV vector particles after a certain period of time, the state authority responsible for the enforcement of genetic engineering law can set deadlines based on this data, after which the release of GMOs can no longer be assumed. Reference is also made to § 24 para. 1 sentence 3 b GenTSV, according to which liquid and solid waste (e.g. litter) from plants of safety level 1 can also be disposed of without pre-treatment if it is so slightly contaminated that harmful effects on the protection goals of the GenTG are not to be expected.

5. **Tissues and cells** from laboratory animals that have been inoculated with AdV vector particles of **risk group 1 or 2** may contain vector particles of **risk group 1 or 2**, even beyond proof that the laboratory animals no longer release particles. **Safety level 1 or 2** measures are required for handling removed tissue or cells. If data obtained like in [51, 65, 66], or suitable literature for the individual case or comparable systems prove the absence of virus particles in the tissue concerned, the competent state authority can set deadlines after which contamination with AdV vector particles is not to be assumed and, if necessary, the genetic engineering work can be downgraded.
6. **Carcasses of laboratory animals** that have been inoculated with AdV vector particles of **risk group 1** can be disposed of in accordance with § 24 para. 1 No. 3 GenTSV without prior autoclaving. It can be assumed that the protection of legal interests in accordance with § 1 No. 1 of the Genetic Engineering Act is also guaranteed in this case, as the animals do not have an active metabolism and any remaining particles do not pose a potential risk.
7. In general, seven days after the inoculation of test animals with AdV vector particles, a significant depletion of the particles can be assumed [51, 65, 66]. Carcasses of laboratory animals that have been inoculated with AdV vector particles of **risk group 2** can therefore also be disposed of as laboratory animal waste from this point on in accordance with § 2 para. 2 sentence 2 GenTSV, without having to be autoclaved first. Working with the cadavers of these animals does not pose any potential hazard beyond that of Safety Level 1, so that the protection of legal interests in accordance with § 1 no. 1 of the Genetic Engineering Act is also guaranteed. It must be ensured that the cadavers mentioned in this note do not enter the food or feed chain.

7 Literature

1. **Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG** (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* **84**(3):570–3.
2. **Cooper M, Goebel W, Hofschneider PH, Koprowski H, Melchers F, Oldstone M, Rott R, Schweiger HG, Vogt PK, Zinkernagel R, Doerfler W** (1983). The Molecular Biology of Adenoviruses 1. Current topics in Microbiology and Immunology, vol. 109, Springer, Berlin, Heidelberg.
3. **Knipe DM** (2015). Fields Virology, 6. Auflage, Wolters Kluwer, Philadelphia.
4. **Jong YP de, Herzog RW** (2022). AAV and hepatitis: Cause or coincidence? *Mol Ther* **30**(9):2875–6.
5. **Lucher LA** (1995). Abortive adenovirus infection and host range determinants. *Curr Top Microbiol Immunol* **199**(1):119–52.
6. **Doerfler W** (1996). Medical Microbiology. Adenoviruses, 4th Ed, Springer, Galveston (TX).
7. **Braithwaite Aw** (1986). Semipermissive replication of adenovirus 5 in rat brain cells and evidence for an induction of cellular DNA replication in vivo. *J Gen Virol* **67**(2):391–6.
8. **Eggerding FA, Pierce WC** (1986). Molecular biology of adenovirus type 2 semipermissive infections. I. Viral growth and expression of viral replicative functions during restricted adenovirus infection. *Virology* **148**(1):97–113.
9. **Murray JD, Bellett AJ, Braithwaite Aw, Waldron LK, Taylor IW** (1982). Altered cell cycle progression and aberrant mitosis in adenovirus-infected rodent cells. *J Cell Physiol* **111**(1):89–96.
10. **International Committee on Taxonomy of Viruses**. ICTV - Taxonomy. https://ictv.global/. Accessed 03-07-2024.
11. **Dehghan S, Seto J, Liu EB, Ismail AM, Madupu R, Heim A, Jones MS, Dyer DW, Chodosh J, Seto D** (2019). A Zoonotic Adenoviral Human Pathogen Emerged through Genomic Recombination among Human and Nonhuman Simian Hosts. *J Virol* **93**(18):e00564-19.
12. **Pinski AN, Gan T, Lin S-C, Droit L, Diamond M, Barouch DH, Wang D** (2024). Isolation of a recombinant simian adenovirus encoding the human adenovirus G52 hexon suggests a simian origin for human adenovirus G52. *J Virol* **98**(4):e0004324.
13. **Kosoltanapiwat N, van der Hoek L, Kinsella CM, Tongshoob J, Prasittichai L, Klein M, Jebbink MF, Deijs M, Reamtong O, Boonnak K, Khongsiri W, Phadungsombat J, Tongthainan D, Tulayakul P, Yindee M** (2023). A Novel Simian Adenovirus Associating with Human Adenovirus Species G Isolated from Long-Tailed Macaque Feces. *Viruses* **15**(6):1371.
14. **Stacey DW, Dobrowolski SF, Piotrkowski A, Harter ML** (1994). The adenovirus E1A protein overrides the requirement for cellular ras in initiating DNA synthesis. *EMBO J* **13**(24):6107–14.
15. **Ogawa K** (1989). Embryonal neuroepithelial tumors induced by human adenovirus type 12 in rodents. 2. Tumor induction in the central nervous system. *Acta Neuropathol* **78**(3):232–44.
16. **Trivedi PD, Byrne BJ, Corti M** (2023). Evolving Horizons: Adenovirus Vectors' Timeless Influence on Cancer, Gene Therapy and Vaccines. *Viruses* **15**(12):2378.
17. **Robbins PD, Ghivizzani SC** (1998). Viral vectors for gene therapy. *Pharmacol Ther* **80**(1):35–47.
18. **Liu J, Seol D-W** (2020). Helper virus-free gutless adenovirus (HF-GLAd): a new platform for gene therapy. *BMB Rep* **53**(11):565–75.
19. **Chavda VP, Bezbaruah R, Valu D, Patel B, Kumar A, Prasad S, Kakoti BB, Kaushik A, Jesawadawala M** (2023). Adenoviral Vector-Based Vaccine Platform for COVID-19: Current Status. *Vaccines (Basel)* **11**(2):432.
20. **Xiang ZQ, Yang Y, Wilson JM, Ertl HC** (1996). A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* **219**(1):220–7.

21. **Ghosh-Choudhury G, Haj-Ahmad Y, Brinkley P, Rudy J, Graham FL** (1986). Human adenovirus cloning vectors based on infectious bacterial plasmids. *Gene* **50**(1-3):161–71.

22. **Benihoud K, Yeh P, Perricaudet M** (1999). Adenovirus vectors for gene delivery. *Curr Opin Biotechnol* **10**(5):440–7.

23. **Vile RG, Russell SJ, Lemoine NR** (2000). Cancer gene therapy: hard lessons and new courses. *Gene Ther* **7**(1):2–8.

24. **Gluzman Y, Reichl H, Solnick D** (1982). Helper-free adenovirus type-5 vectors. *In* Gluzman Y (Ed), Eukaryotic viral vectors, p. 187–92. Cold Spring Harbor Lab, Cold Spring Harbor, N.Y..

25. **Bett AJ, Prevec L, Graham FL** (1993). Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* **67**(10):5911–21.

26. **Parks RJ, Graham FL** (1997). A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J Virol* **71**(4):3293–8.

27. **Engelhardt JF, Simon RH, Yang Y, Zepeda M, Weber-Pendleton S, Doranz B, Grossman M, Wilson JM** (1993). Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: biological efficacy study. *Hum Gene Ther* **4**(6):759–69.

28. **Yang Y, Nunes FA, Berencsi K, Furth EE, Gönczöl E, Wilson JM** (1994). Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A* **91**(10):4407–11.

29. **Nelson JE, Kay MA** (1997). Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. *J Virol* **71**(11):8902–7.

30. **Goldsmith KT, Dion LD, Curiel DT, Garver RI** (1998). trans E1 component requirements for maximal replication of E1-defective recombinant adenovirus. *Virology* **248**(2):406–19.

31. **Steinwaerder DS, Carlson CA, Lieber A** (2000). DNA replication of first-generation adenovirus vectors in tumor cells. *Hum Gene Ther* **11**(13):1933–48.

32. **Ehrhardt A, Xu H, Kay MA** (2003). Episomal persistence of recombinant adenoviral vector genomes during the cell cycle in vivo. *J Virol* **77**(13):7689–95.

33. **Graham FL, Smiley J, Russell WC, Nairn R** (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**(1):59–74.

34. **Louis N, Evelegh C, Graham FL** (1997). Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. *Virology* **233**(2):423–9.

35. **Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, van Ormondt H, Hoeben RC, van der Eb AJ** (1996). Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther* **7**(2):215–22.

36. **Hanahan D** (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**(4):557–80.

37. **Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M** (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J Virol* **70**(7):4805–10.

38. **He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B** (1998). A simplified system for generating recombinant adenoviruses. *PNAS* **95**(5):2509–14.

39. **Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, Caskey CT** (1996). A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc Natl Acad Sci U S A* **93**(12):5731–6.

40. **Fisher KJ, Choi H, Burda J, Chen SJ, Wilson JM** (1996). Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. *Virology* **217**(1):11–22.

41. **Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL** (1996). A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* **93**(24):13565–70.

42. **Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S** (1998). Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat Genet* **18**(2):180–3.

43. **Kochanek S** (1999). High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum Gene Ther* **10**(15):2451–9.

44. **Jager L, Hausl MA, Rauschhuber C, Wolf NM, Kay MA, Ehrhardt A** (2009). A rapid protocol for construction and production of high-capacity adenoviral vectors. *Nat Protoc* **4**(4):547–64.

45. **Lee D, Liu J, Junn HJ, Lee E-J, Jeong K-S, Seol D-W** (2019). No more helper adenovirus: production of gutless adenovirus (GLAd) free of adenovirus and replication-competent adenovirus (RCA) contaminants. *Exp Mol Med* **51**(10):1–18.

46. **Lochmüller H, Jani A, Huard J, Prescott S, Simoneau M, Massie B, Karpati G, Acsadi G** (1994). Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. *Hum Gene Ther* **5**(12):1485–91.

47. **Zhang WW, Koch PE, Roth JA** (1995). Detection of wild-type contamination in a recombinant adenoviral preparation by PCR. *Biotechniques* **18**(3):444–7.

48. **Hehir KM, Armentano D, Cardoza LM, Choquette TL, Berthelette PB, White GA, Couture LA, Everton MB, Keegan J, Martin JM, Pratt DA, Smith MP, Smith AE, Wadsworth SC** (1996). Molecular characterization of replication-competent variants of adenovirus vectors and genome modifications to prevent their occurrence. *J Virol* **70**(12):8459–67.

49. **Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ, Valerio D, Hoeben RC** (1998). New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* **9**(13):1909–17.

50. **Genzel Y** (2015). Designing cell lines for viral vaccine production: Where do we stand? *Biotechnol J* **10**(5):728–40.

51. **Almuqrin A, Davidson AD, Williamson MK, Lewis PA, Heesom KJ, Morris S, Gilbert SC, Matthews DA** (2021). SARS-CoV-2 vaccine ChAdOx1 nCoV-19 infection of human cell lines reveals low levels of viral backbone gene transcription alongside very high levels of SARS-CoV-2 S glycoprotein gene transcription. *Genome Med* **13**(1):43.

52. **Baldo A, Leunda A, Willemarck N, Pauwels K** (2021). Environmental Risk Assessment of Recombinant Viral Vector Vaccines against SARS-Cov-2. *Vaccines (Basel)* **9**(5):453.

53. **Shimada M, Wang H, Ichino M, Ura T, Mizuki N, Okuda K** (2022). Biodistribution and immunity of adenovirus 5/35 and modified vaccinia Ankara vector vaccines against human immunodeficiency virus 1 clade C. *Gene Ther* **29**(10-11):636–42.

54. **Veneziale RW, Bral CM, Sinha DP, Watkins RW, Cartwright ME, Rosenblum IY, Treinen KA, Kishnani NS, Nelson J, Chen Z, Faha B, Maneval D, Munger RJ, Cai X-Y, Cullen C, Arezzo JC** (2007). SCH 412499: biodistribution and safety of an adenovirus containing P21(WAF-1/CIP-1) following subconjunctival injection in Cynomolgus monkeys. *Cutan Ocul Toxicol* **26**(2):83–105.

55. **Sheets RL, Stein J, Bailer RT, Koup RA, Andrews C, Nason M, He B, Koo E, Trotter H, Duffy C, Manetz TS, Gomez P** (2008). Biodistribution and toxicological safety of adenovirus type 5 and type 35 vectored vaccines against human immunodeficiency virus-1 (HIV-1), Ebola, or Marburg are similar despite differing adenovirus serotype vector, manufacturer's construct, or gene inserts. *J Immunotoxicol* **5**(3):315–35.

56. **Knowles MK, Nadin-Davis SA, Sheen M, Rosatte R, Mueller R, Beresford A** (2009). Safety studies on an adenovirus recombinant vaccine for rabies (AdRG1.3-ONRAB) in target and non-target species. *Vaccine* **27**(47):6619–26.

57. **Australian Government Department of Health, Therapeutic Goods Administration**. Nonclinical Evaluation Report. ChAdOx1 S COVID-19 Vaccine [COVID-19 Vaccine AstraZeneca]. https://www.tga.gov.au/sites/default/files/auspar-chadox1-s-covid-19-vaccine-astrazeneca-210215.pdf. Accessed 05-02-2025.

58. **Sharma A, Li X, Bangari DS, Mittal SK** (2009). Adenovirus receptors and their implications in gene delivery. *Virus Res* **143**(2):184–94.

59. **Hensen LCM, Hoeben RC, Bots STF** (2020). Adenovirus Receptor Expression in Cancer and Its Multifaceted Role in Oncolytic Adenovirus Therapy. *Int J Mol Sci* **21**(18):6828.

60. **Tang Y, Han T, Everts M, Zhu ZB, Gillespie GY, Curiel DT, Wu H** (2007). Directing adenovirus across the blood-brain barrier via melanotransferrin (P97) transcytosis pathway in an in vitro model. *Gene Ther* **14**(6):523–32.

61. **Schenk-Braat EAM, van Mierlo MMKB, Wagemaker G, Bangma CH, Kaptein LCM** (2007). An inventory of shedding data from clinical gene therapy trials. *J Gene Med* **9**(10):910–21.

62. **LaRocca CJ, Jacobsen KL, Inoko K, Zakharkin SO, Yamamoto M, Davydova J** (2023). Viral Shedding in Mice following Intravenous Adenovirus Injection: Impact on Biosafety Classification. *Viruses* **15**(7):1495.

63. **Griscelli F, Opolon P, Saulnier P, Mami-Chouaib F, Gautier E, Echchakir H, Angevin E, Le Chevalier T, Bataille V, Squiban P, Tursz T, Escudier B** (2003). Recombinant adenovirus shedding after intratumoral gene transfer in lung cancer patients. *Gene Ther* **10**(5):386–95.

64. **Tursz T, Cesne AL, Baldeyrou P, Gautier E, Opolon P, Schatz C, Pavirani A, Courtney M, Lamy D, Ragot T, Saulnier P, Andremont A, Monier R, Perricaudet M, Le Chevalier T** (1996). Phase I study of a recombinant adenovirus-mediated gene transfer in lung cancer patients. *J Natl Cancer Inst* **88**(24):1857–63.

65. **Wold WSM, Toth K** (2013). Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. *Curr Gene Ther* **13**(6):421–33.

66. **Guillen J** (2012). FELASA guidelines and recommendations. *J Am Assoc Lab Anim Sci* **51**(3):311–21.