

## **General position statement by the German Central Committee on Biological Safety on the risk assessment of**

### ***E. coli* K12 derivatives with a plasmid containing the (c)DNA of the genome of a replication-competent virus**

Since the development of various reverse genetics systems, the genomes of a large number of viruses can be easily modified *in vitro* and corresponding viral particles can be produced in cell cultures. For this purpose, the viral genomic DNA or a DNA copy (cDNA) of the viral genomic RNA is integrated into a suitable vector. The resulting plasmid or RNA, transcribed of it *in vitro*, can then be introduced into cell cultures or host organisms susceptible to the virus. Recombinant replication-competent virus particles are then formed and released by the cells. The additional presence of helper plasmids or viruses may be required.

The plasmids are typically amplified in derivatives of the K12 strain of *Escherichia coli* of **risk group 1**. According to § 5 (3) GenTSV, when the genome of a donor organism of risk groups 2 to 4 is transferred, the hazard potential of the donor organism must be fully taken into account in the risk assessment of the resulting genetically modified organism (GMO). The GMO must thus at least be assigned to the risk group of the donor organism. According to § 5 (5) GenTSV, a GMO can only be assigned to a lower risk group, if the vector-recipient system meets the requirements for biological safety measures according to § 6 (4) and (5) GenTSV.

The decisive factor in assessing the hazard potential of *E. coli* K12 derivatives transformed by plasmids containing a viral genome or its cDNA is the possibility that these GMOs could cause viral infections in humans, animals or plants. The formation of viral particles in *E. coli* can be ruled out, since they lack eukaryotic host factors that are essential for the expression and packaging of viral genomes. Therefore, a direct infection by virus-containing culture supernatants of *E. coli* can be excluded. However, plasmid transfer from *E. coli* to eukaryotic cells and the associated start of the viral replication cycle is conceivable. Accordingly, in the course of the risk assessment of works with the above referenced GMOs, the probability of such plasmid transfer must first be estimated. In a second step, the specific replication properties of the cloned viral genome, which are largely determined by its type and polarity, must then be included in the risk assessment.

In prokaryotes, the exchange of DNA between closely related species is a natural process enabled by specialised proteins of different secretion systems. By contrast, a specific DNA transfer from prokaryotes to eukaryotes is an extremely rare event in nature, which has only been described for a few prokaryotes, e. g. *Agrobacterium tumefaciens*, and which is mediated by special mobilisation factors. There is currently no evidence that DNA transfer from *E. coli* to eukaryotes can occur in nature [1]. Independently of it, *E. coli* safety strains are characterised by a defect in their secretion system. Furthermore, the plasmids used in this process must not contain any mobilisation factors or their recognition sequences if the

plasmids are to be recognised as part of a biological safety measure. The use of a vector-recipient system recognised as a biological safety measure is thus not expected to result in targeted plasmid transfer from *E. coli* to eukaryotic cells. However, *in vitro* studies have shown that the use of such safety strains can also lead to non-specific plasmid transfer to mammalian cell cultures, possibly mediated by phagocytosis or membrane fusion. If the plasmid contains the genomic DNA or cDNA of a virus, an infection could be initiated. However, such an event is extremely rare. In one study, for example, a 4-hour incubation with  $4 \times 10^9$  colony forming units (cfu) *E. coli* HB 101 containing a pBR322-derived plasmid with three copies of the linearised genome of the DNA virus *Simian virus 40* (SV40), cloned in direct sequence, led to a single infection event in  $10^7$  cells of the monkey cell line CV1 [2]. Likewise, ten infection events were observed after 24-hour incubation of  $2.5 \times 10^6$  cells of the monkey cell line BGM with  $5 \times 10^9$  cfu *E. coli* JW1106 containing a pBR322 derivative with the poliovirus cDNA. The cDNA of the positive-stranded RNA virus was not under the control of a promoter. Therefore, there must have been an initiation of the transcription of viral genomic RNA at a cryptic promoter within the vector backbone. If a eukaryotic promoter was cloned upstream of the cDNA, the frequency of infection increased by a factor of 10 [3]. No information is available on the possible transfer of *E. coli* plasmid DNA to plant cells.

Since aerosols containing bacteria can be produced during work with *E. coli*, a plasmid transfer to cells of the experimenter after inhalation of corresponding aerosols cannot be excluded as an extremely rare event. Similarly, the possibility of uptake of *E. coli* and its plasmids by animals or their cells after the bacteria have been discharged into the environment cannot be completely excluded. However, a (mechanical) transfer of plasmid DNA from *E. coli* to plant cells seems unlikely. A permanent establishment of K12-derived *E. coli* safety strains in the environment is not to be existent, since *E. coli* K12 could no longer be detected in the environment or in the digestive tract of various animals after three weeks at the latest [4].

## Notes

The assessments listed below are based on the assumption that the *E. coli* K12 derivative of **risk group 1** used as the recipient organism as well as the vector fulfil the requirements of a biological safety measure.

If the intent is to use a recipient organism or a vector that does not meet the requirements for a biological safety measure set out in § 6 (4) or (5) GenTSV, such as an *E. coli* B strain or a binary vector based on the Ti plasmid of *Agrobacterium tumefaciens*, the GMO must generally be assigned to the same risk group as the replication-competent virus whose genome is transferred to the recipient organism.

## Note on virus genomes with point mutations, insertions or deletions

In *E. coli*, the viral genomic (c)DNA is amplified by bacterial DNA polymerases. The (c)DNA is thus subject to the bacterial DNA correction mechanisms. Consequently, the establishment of random mutations within the viral (c)DNA is considered very rare. DNA or RNA amplification by viral polymerases, which can lead to high mutation rates due to lack of nuclease activity, does not occur in *E. coli*. A reversion of point mutations, insertions or deletions, which may have been specifically introduced into the genomic (c)DNA of the virus, is thus unlikely in *E. coli*, provided that a DNA recombination event can be ruled out. Furthermore, it does not appear that any point mutations, insertions or minor deletions that

may have occurred in *E. coli* will lead to a growth advantage for *E. coli* or preferential amplification of mutated plasmids. Accumulation of randomly mutated plasmids is thus not to be expected. For the assessment of transformed *E. coli* described below, the risk group assignment of the intended viral mutants must thus be considered.

## Evaluation

### Plant viruses

There is currently no literature data suggesting that plasmid transfer from *E. coli* to plant cells is possible in nature or in the laboratory. This means that an infection of a host plant mediated by *E. coli* is not to be expected, regardless of the type and polarity of the viral genome encoded by the plasmid. Plant viruses with pathogenic potential for humans or animals have not been described. Hence, the transformed *E. coli* K12 derivatives do not possess any hazard potential for humans, animals or the environment. *E. coli* transformed by plasmids containing the genomic DNA or cDNA of a plant virus is assigned to **risk group 1** regardless of the risk group of the plant virus, provided that the vector-recipient system is a biological safety measure.

### Animal viruses with positive-stranded RNA genome

In viruses with a positive-stranded RNA genome (e. g. flavi-, picorna- and alphaviruses), the genomic RNA represents a mono- or bicistronic mRNA, of which at least the so-called non-structural proteins are translated in the form of a polyprotein. This polyprotein contains all enzyme activities and viral co-factors that are necessary for genome replication and, if necessary, transcription of further subgenomic mRNAs. Processing of the polyprotein into the individual protein units is performed by cellular proteases or by proteases that are also contained in the polyprotein. Thus, the genomic RNA of positive-stranded RNA viruses is infectious for susceptible cells even in the initial absence of viral proteins. Therefore, it cannot be excluded that transmission of a plasmid with the genomic cDNA of a positive-stranded RNA virus to the experimenter or a susceptible host animal could lead to infection due to the cloned or cryptic promoters present in the plasmid. The hazard potential of *E. coli* of **risk group 1** transformed by such plasmids depends on the risk group of the virus whose genomic cDNA is contained. If the plasmid contains the cDNA of a positive-stranded RNA virus of **risk group 1** or **2**, the transformed *E. coli* is assigned to **risk group 1** or **2** corresponding to the risk group of the virus. If the plasmid contains the cDNA of a positive-stranded RNA virus of **risk group 3** or **3\*\***, the transformed *E. coli* is assigned to **risk group 2**, provided that the vector-recipient system is a biological safety measure. This classification takes into account that plasmid transfer from *E. coli* to eukaryotic cells is an extremely rare event. The resulting low hazard potential of the genetic engineering operation is sufficiently covered by level 2 safety measures. The currently only known positive-stranded RNA virus of **risk group 4** is the *Foot-and-mouth disease virus* (FMDV), which occurs primarily as an animal pathogen in various ungulates. The classification in **risk group 4** takes into account the very high contagiousness of the virus and the great economic relevance of a possible resulting epizootic disease in these host animals. The low likelihood of *E. coli* spreading into the environment and subsequent transmission of plasmid DNA to a host animal can be adequately addressed by level 2 safety measures. *E. coli* transformed by plasmids containing the genomic FMDV cDNA thus is assigned to **risk group 2**, provided that the vector-recipient system is a biological safety measure. If additional positive-stranded RNA viruses are assigned to **risk group 4** in the future, the *E. coli* with plasmids containing the

cDNA of the corresponding viruses must be evaluated by the German Central Committee on Biological Safety on a **case-by-case basis**.

### Animal retroviruses

The cDNA of retroviruses cloned into plasmids usually corresponds to the retroviral provirus. This virus contains a promoter sequence for cellular RNA polymerase II in its terminal non-coding regions. If plasmid DNA is transferred to a susceptible host cell, viral mRNA is transcribed and the viral replication cycle is initiated. The hazard potential of *E. coli* of **risk group 1** transformed by such plasmids depends on the risk group of the retrovirus whose genomic cDNA is contained. If the plasmid contains the cDNA of a retrovirus of **risk group 1** or **2**, the transformed *E. coli* is assigned to **risk group 1** or **2** corresponding to the risk group of the virus. If the plasmid contains the cDNA of a retrovirus of **risk group 3\*\***, the transformed *E. coli* is assigned to **risk group 2**, provided that the vector-recipient system is a biological safety measure. This classification takes into account that plasmid transfer from *E. coli* to eukaryotic cells is an extremely rare event. The resulting low hazard potential of the genetic engineering operation is sufficiently covered by level 2 safety measures.

### Animal viruses with negative-stranded RNA genome, except deltaviruses

In contrast to the situation with positive-stranded RNA viruses, proteins cannot be translated from the genome of negative-stranded RNA viruses (e. g. filo-, paramyxo- and rhabdoviruses) due to its opposite polarity to mRNA. At the beginning of the typically cytoplasmic replication cycle, mRNA must therefore first be transcribed. Except for viruses of the genus *Deltavirus* (see below), this step is catalysed by a viral RNA-dependent RNA polymerase and its (viral) co-factors. Cellular DNA-dependent RNA polymerases cannot perform this function due to their localisation in the cell nucleus and their specificity for DNA templates. Therefore, the genome of negative-stranded RNA viruses with a non-segmented genome (order *Mononegavirales*) is only infectious as a complex with the viral RNA polymerase (L), the viral nucleoprotein (N or NP) and the viral phosphoprotein (P or VP35) as well as possibly other viral or non-viral proteins. The L and N/NP proteins essential for transcription and viral genome replication are encoded at the opposite ends of the viral genome. In between, there is a variable number of additional open reading frames in which, among other things, the phosphoprotein and the envelope proteins are encoded. All viruses of the order *Mononegavirales* transcribe multiple subgenomic monocistronic mRNAs. Polyproteins with all enzyme activities and viral co-factors necessary for RNA synthesis are not encoded by negative-stranded RNA viruses. Complete antigenomic RNA (cRNA) is thus also not infectious in the absence of L, N/NP and P/VP35.

If a plasmid with the genomic cDNA of a negative-stranded RNA virus is transferred, it would only be infectious if the genomic RNA or cRNA and all mRNAs coding for proteins of the replication complex were simultaneously transcribed. Although this is technically possible, at least in individual cases [5], by using several promoters, such an approach is not common in practice. The typical reverse genetics systems for negative-stranded RNA viruses use plasmids in which the transcription of genomic RNA or cRNA is controlled by a phage or eukaryotic promoter. The mRNAs of the proteins of the replication complex are provided by separate expression plasmids. The accidental presence of several cryptic promoters within the viral genome sequence (at least *upstream* of P and L and possibly N) is unlikely. If plasmids containing only one promoter are used, it cannot be excluded that an infection is initiated by plasmid transfer from *E. coli* to cells that are susceptible to the virus. Hence, the *E. coli* do not possess any hazard potential for humans, animals or the environment. *E. coli* transformed by plasmids containing the genomic cDNA of a negative-stranded RNA virus

under the control of a single promoter is assigned to **risk group 1** regardless of the risk group of the virus with the exception listed below, provided that the vector-recipient system is a biological safety measure.

If plasmids are used that contain several promoters for the simultaneous transcription of genomic RNA or cRNA and subgenomic mRNAs, the hazard potential of transformed *E. coli* of **risk group 1** is determined by the risk group of the negative-stranded RNA virus whose genomic cDNA is contained. If such a plasmid contains the cDNA of a negative-stranded RNA virus of **risk group 1** or **2**, the transformed *E. coli* is assigned to **risk group 1** or **2** corresponding to the risk group of the virus. If such a plasmid contains the cDNA of a negative-stranded RNA virus of **risk group 3** or **3\*\***, the transformed *E. coli* is assigned to **risk group 2**, provided that the vector-recipient system is a biological safety measure. This classification takes into account that plasmid transfer from *E. coli* to eukaryotic cells is an extremely rare event. The resulting low hazard potential of the genetic engineering operation is sufficiently covered by level 2 safety measures. If such a plasmid contains the cDNA of a negative-stranded RNA virus of **risk group 4**, the transformed *E. coli* must be evaluated by the German Central Committee on Biological Safety on a **case-by-case basis**.

### Deltaviruses

The genus *Deltavirus* currently only includes the species *Hepatitis delta virus* (HDV). Other HDV-like viruses have recently been identified. HDV has a circular single-stranded RNA genome with negative polarity. However, a rod-shaped RNA double strand is formed due to extensive complementarity. The HDV genome does not contain a gene for its own RNA polymerase. Transcription and genome replication take place in the cell nucleus and are catalysed by cellular DNA-dependent RNA polymerase II, possibly also involving DNA-dependent RNA polymerase I and/or III. A plasmid containing three consecutive copies of the HDV genome led to transcription and genome replication in human cell culture. In addition, the genomic RNA was processed and circularised. The eukaryotic promoter in the plasmid controlled the transcription of the genomic negative-strand [6]. It cannot be ruled out that the transmission of a plasmid with the cDNA of the possibly extended genome of HDV or HDV-like viruses of **risk group 2** to the experimenter or a susceptible host animal with the aid of the cloned or cryptic promoters in the plasmid may initiate transcription and genome replication. This means that *E. coli* of **risk group 1** that were transformed by such plasmids have a low hazard potential and are assigned to **risk group 2**.

### Animal viruses with DNA genome, except polyoma-, papilloma-, asfar- and poxviruses

For the transcription of their (early) mRNAs, DNA viruses with single-stranded (e. g. parvoviruses), double-stranded (e. g. herpes- and adenoviruses) or partially double-stranded (hepadnaviruses) genomes use cellular RNA polymerase II or, in exceptional cases, cellular RNA polymerase III, which are located in the cell nucleus. The only exceptions are viruses of the families *Asfarviridae* and *Poxviridae* (see below), which encode their own transcription enzymes, including an RNA polymerase, capping enzymes and a poly(A) polymerase. The genomes of the other viruses contain promoter sequences to which the corresponding cellular polymerases can bind. If plasmid DNA is transferred to a susceptible host cell, this leads to the transcription of viral mRNAs and the initiation of the viral replication cycle (with restrictions for viruses of the *Polyomaviridae* and *Papillomaviridae*, see below). The hazard potential of *E. coli* of **risk group 1** transformed by plasmids with the DNA of the possibly extended genome of a DNA virus depends on the risk group of the respective virus, with the exceptions listed below. If the plasmid contains the DNA of a DNA virus of **risk group 1** or **2**, the transformed *E. coli* is assigned to **risk group 1** or **2** corresponding to the risk group of

the virus. If the plasmid contains the DNA of a DNA virus of **risk group 3** or **3\*\***, the transformed *E. coli* is assigned to **risk group 2**, provided that the vector-recipient system is a biological safety measure. This classification takes into account that plasmid transfer from *E. coli* to eukaryotic cells is an extremely rare event. The resulting low hazard potential of the genetic engineering operation is sufficiently covered by level 2 safety measures.

#### Polyoma- and papillomaviruses

Polyoma- and papillomaviruses have a circular double-stranded DNA genome. Consequently, if the DNA of these viruses is cloned into a plasmid, the genome must be artificially interrupted. The linear form of the viral genome in the plasmid is not infectious. Hence, to infect cells *in vitro*, a restriction digestion is first carried out to separate the linearised viral genome from the other plasmid sequences. This is followed by an *in vitro* ligation. The circular viral DNA without foreign sequences is infectious when it is subsequently transfected into susceptible cells. If plasmid DNA is accidentally transferred from *E. coli* to eukaryotic cells, it is not likely that a double-strand break occurs at both ends of the viral DNA and no essential viral DNA sections are lost. Subsequent circularisation of the linear viral genome is also not expected. Hence, the *E. coli* do not possess any hazard potential for humans, animals or the environment. *E. coli* transformed by plasmids containing a single copy of the genomic DNA of a polyoma- or papillomavirus is assigned to **risk group 1** regardless of the risk group of the virus, provided that the vector-recipient system is a biological safety measure.

#### Asfar- and poxviruses

Asfar- and poxviruses replicate in the cytoplasm of infected host cells. Accordingly, RNA and DNA polymerases as well as the necessary co-factors are encoded in their genome. In the absence of viral proteins, especially the viral RNA polymerase, which consists of several subunits, the viral genomic DNA is not infectious. Transcription by cellular RNA polymerases does not occur. Coincidental presence of several cryptic promoters for cellular RNA polymerases within the viral DNA, which enable the expression of all viral proteins necessary for the initiation of an infection, is unlikely. *E. coli* transformed by plasmids containing the genomic DNA of a poxvirus of **risk group 1, 2** or **3** or of the *African swine fever virus* (ASFV) of **risk group 4** thus is assigned to **risk group 1**, provided that the vector-recipient system is a biological safety measure. The World Health Organization recommends that genetic engineering operations using the complete or large parts of the genomic DNA of *Variola virus* (VARV) of **risk group 4** should not be performed. This recommendation applies in particular to operations aimed at producing replication-competent viral particles [7]. *E. coli* transformed by plasmids containing the genomic VARV DNA thus is assigned to **risk group 4**.

#### **Final notes**

The handling of *E. coli* transformed by plasmids containing the DNA of an oncogenic virus requires that additional safety measures are observed as outlined in the 'Position Statement of the German Central Committee on Biological Safety: assessment of genetically modified organisms into which nucleic acid sections with neoplastic transforming potential have been introduced' (Ref\_6790-10-36, updated December 2014).

This position statement replaces the following general recommendations of the ZKBS:

- General position statement by the ZKBS on the risk assessment of *E. coli* K12 containing the cDNA of a full-length retroviral genome (Az. 6790-10-89, December 2007)
- General position statement by the ZKBS on the risk assessment of *E. coli* K12 containing genomic DNA of papilloma viruses (Az.6790-10-102, April 2011)
- General position statement by the ZKBS on the risk assessment of *E. coli* K12 containing the cDNA of the full-length genome of SARS-Coronavirus (Az. 6790-10-97, updated July 2017)

## Literature

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7. **World Health Organization** (2016). WHO Recommendations concerning the distribution, handling and synthesis of variola virus DNA. Revised 13 January 2016. <https://www.who.int/csr/disease/smallpox/variola-virus-dna/en/>.