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DATE	18 februari 2021
REFERENCE	CGM/210218-01
SUBJECT	Advies 'Heroverweging inschaling werkzaamheden replicatiedeficiënte lenti- en gammaretrovirale vectordeeltjes'

Geachte mevrouw Van Nieuwenhuizen,

COGEM has reviewed its earlier advice on working with lentiviral vectors under contained use and notifies you of the following.

Summary
In 2009 COGEM issued generic advice on appropriate containment levels for laboratory activities involving lentiviral vectors derived from the *Human immunodeficiency virus 1*. This advice reviews the criteria used by COGEM to determine appropriate containment levels for laboratory activities. In the light of advances in understanding and the results of research commissioned by COGEM, the Commission has reviewed the criteria for activities involving replication-deficient lentiviral vectors. In its reassessment, COGEM also included consideration of activities involving replication-deficient gammaretroviral vectors derived from mouse gammaretroviruses.

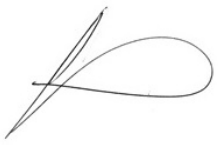
COGEM is of the opinion that the production of lentiviral vectors and their use in transduction and working with cells transduced with lentiviral vectors pose a negligible risk to the environment when these activities are carried out at the minimum containment level ML-I, on the condition that there is no chance of the presence or generation of replication-competent virus (RCV) and the cells to be used do not contain any wild-type lentiviruses. Because the generation of RCV cannot be ruled out when gammaretroviral vectors are produced, COGEM advises that work with gammaretroviral vectors should continue to be carried out at containment level ML-II. COGEM considers that transduction with gammaretroviral vectors and activities involving cells transduced with gammaretroviral vectors may be carried out at containment level ML-I as long as certain conditions are met: the gammaretroviral vector batch

must be free of RCV, the cell lines to be used must be of human origin and shown to be free of any replication-competent exogenous gammaretroviruses, and activities must not involve any cells which could contain gammaretroviruses.

COGEM points out that certain activities involve a risk of exposing laboratory workers to vector particles and that this exposure may lead to adverse effects in those workers. Exposure to vector particles can be prevented by carrying out the activities under working conditions and using safety equipment that are equivalent to the requirements for containment level II.

The attached report contains COGEM's advice and a discussion of the underlying reasoning.

Hoogachtend,



Prof. dr. ing. Sybe Schaap
Voorzitter COGEM

c.c.
- Dr J. Westra, Head of the GMO Office
- Ministry of Infrastructure and Water Management, Environmental Safety and Risks Directorate
  Directorate-General for the Environment and International Affairs

Review of containment levels for working with replication-deficient lentiviral and gammaretroviral vectors under contained use

COGEM Advisory Report CGM/210218-01

1. Introduction

In 2009 COGEM issued generic advice on appropriate containment levels for activities involving lentiviral vectors derived from the *human immunodeficiency virus 1* (HIV) under contained use.[1] That advice contained an overview of the criteria used by COGEM to determine appropriate containment levels for the production of lentiviral vectors, the transduction of mammalian cells using these vectors and the culture and analysis of the transduced mammalian cells. In the light of advances in understanding and the results of research commissioned by COGEM,[2] in this present advice the Commission has reconsidered the criteria for determining containment levels for in vitro activities, in particular regarding the risk of the presence of free lentiviral particles. At the same time, the containment levels for in vitro activities involving replication-deficient gammaretroviral vectors based on mouse gammaretroviruses and products derived from them were also reviewed.

2. Background information on lentiviruses and gammaretroviruses

Lentiviruses (to which HIV belongs) and gammaretroviruses belong to different genera within the *Retroviridae* family.[3] The viruses have a single-stranded RNA genome which is synthesised by a virus-specific polymerase, the reverse transcriptase, into a double-stranded complementary DNA which integrates into the genome of the infected cell.[4,5]

The RNA genome of lentiviral and gammaretroviral particles is enclosed within capsid proteins and surrounded by a lipid membrane, a structure called the envelope. The viral particles contain all the proteins needed to initiate the viral life cycle, including the reverse transcriptase, integrase and protease enzymes. These proteins are encoded in the viral genome by the (*pro/*)*pol* gene.[a] Embedded in the lipid membrane are the envelope proteins, which determine the host tropism of the virus. These proteins are encoded by the *env* gene.

The life cycles of lentiviruses and gammaretroviruses are similar. Infection of the host cell is induced by specific binding of the envelope proteins onto a cellular receptor, sometimes in combination with a co-receptor. During this process the viral particle is partially dismantled. In the cytoplasm the viral RNA is converted in a complex series of steps into a double-stranded DNA copy, which integrates into the cellular genome (proviral genome). Identical long terminal repeats (LTRs) are included at both the 5' and 3' ends of the proviral DNA. These sequences play a key regulatory role in the integration of the proviral DNA and the transcription of the viral genome.

The transcription machinery of the host cell synthesises the viral RNA from the integrated proviral DNA. After transport to the cytoplasm, two copies of the viral RNA are enclosed within the capsid protein

[a] In lentiviruses the *pro* gene is located in the *pol* open reading frame (ORF). In some retroviruses the *pro* gene can have its own ORF.

(encoded by the *gag* gen). Essential for the binding of the capsid protein with the viral RNA and the eventual formation of the viral particles is the packaging signal Ψ in the RNA. The infectious viral particles are formed during the budding on the plasma membrane, a process in which the enclosed viral genome is surrounded by a lipid membrane containing integrated envelope proteins.

An important difference between retroviruses and lentiviruses is that the viral DNA of lentiviruses is actively transported into the cell nucleus via the nuclear pore complex, whereas retroviruses must wait until the nuclear membrane has disassembled during mitosis. As a consequence, retroviruses and the vectors derived from them – unlike lentiviruses – can only infect dividing cells.

Another important difference between retroviruses and lentiviruses is that in addition to the previously mentioned genes *gag*, (*pro/*)*pol* and *env*, lentiviruses contain the four accessory genes *vif*, *vpr*, *vpu* and *nef* and the two regulatory genes *rev* and *tat*.

2.1 Endogenous retroviruses (ERVs)

The retroviral genome can remain present in the host cell as long as it survives by integrating into the genome of the host cell. If the retroviral genome integrates into the germ cells (the gametes or an embryo at an early stage of development), these integrated sequences can be passed on to subsequent generations; the integrated viral genome is then referred to as an endogenous retrovirus (ERV).[6] This is in contrast to transmissible infectious replication-competent retroviruses, which are referred to as exogenous viruses. Most ERVs disappear in time as a result of selection pressure in the host population. ERVs are found in the genome of modern vertebrates, but these are mostly 'remnants' of earlier infections with various retroviruses during the course of evolution of over millions of years.[7] Most of these ERVs have been inactivated (by mutations or epigenetic modifications) and do not code for functional proteins.[6] ERVs are also found in humans and have been classified into various classes: class I ERVs show sequence homology with the current gammaretroviruses and epsilonretroviruses; class II ERVs show sequence homology with the alpha-, beta- and deltaretroviruses; class III ERVs show sequence homology with the spumaviruses.[7]

In some animal species, including mice and rats, intact (or almost intact) ERVs are found which contain most or even all retroviral elements.[8] It has been stated that in rodents it is difficult to distinguish between the sequences of endogenous retroviruses and the proviral genome of exogenous retroviruses.[8] It has been shown that in mouse and rat cells containing intact or almost intact ERVs, it is possible to produce exogenous replication-competent retroviruses in vitro and under certain conditions, for example after exposure to mutagenic substances, after infection with a virus that can complement retroviral functions, or after repeated cell passages.[e.g.9,10] It is known that other mammals, such as cats and pigs, contain ERVs that can easily recombine with related exogenous viruses.[11]

Human ERVs (or HERVs) constitute about 8% of the genome and mainly originated from infections which occurred 30 to 40 million years ago with retroviruses that show a relationship with gammaretroviruses (class I) and betaretroviruses (class II).[6,12] However, the accumulation of inactivating mutations means that these sequences can no longer produce exogenous replication-competent retroviruses.[13,12,14,15] There is a group of HERVs, the HERV-K family, which can be transcriptionally active and able to generate 'virus

like particles', i.e. non-infectious virus particles, in tumour cells.[16,17] These HERVs, however, are not replication competent and multiple genetic alterations are needed to generate infectious exogenous retrovirus.[8,18] This family of HERVs belongs to class II.[6] ERVs showing significant homology with lentiviruses are not found in the human genome. Endogenous lenitiviruses are extremely rare and have so far only been found in the rabbit genome.[19]

3. Background information on replication-deficient lentiviral and gammaretroviral vectors

Vectors based on lentiviruses and gammaretroviruses can integrate into the genome of a cell and for this reason are frequently used in biomedical and genetic research. For reasons of biosafety the replication-deficient vectors mostly used are derived from the lentivirus HIV-1 and from a gammaretrovirus found in mice, Moloney murine leukemia virus (MoMLV; a virus within the species *Murine leukemia virus*).

3.1 Composition of replication-deficient lentiviral and gammaretroviral vectors

Replication-deficient lentiviral and gammaretroviral vectors are produced using split packaging systems.[4] The viral genes and sequences are split up and delivered in trans (mostly divided across multiple plasmids). The vector genome is delivered by a transfer plasmid which contains the viral 5' and 3' LTR sequences, the packaging signal Ψ and the transgene of interest. In the vector genome, all the genes essential for the life cycle of the virus particle are removed: the genes *gag*, (*pro/*)*pol* and *env*; in lentiviral vectors most of the accessory and regulatory genes *vif*, *vpr*, *tat*, *rev*, *vpu* and *nef* are also removed. Because the essential genes are not present in the vector genome, the vector is unable to replicate unaided.

The *gag/*(*pro/*)*pol* genes and the *env* gene (and in lentiviral vectors also the *rev* gene and possibly the *tat* gene (in translentiviral systems)) are delivered in trans, either on one or more packaging or helper plasmids or in a genetically modified (GM) cell line where these genes are stably integrated into the genome. As multiple recombinations are needed to obtain a replication-competent vector, the chance of the emergence of a replication-competent virus is reduced. The probability of this occurring is further reduced by minimising sequence agreement between the vector genome, helper plasmids and packaging cell lines.

For research purposes the vectors are generally pseudotyped with envelope proteins (glycoproteins) of other viruses, such as the vesicular stomatitis Indiana virus (VSIV, also called VSV) G protein, the rabies virus G protein, the gibbon ape leukemia virus envelope protein, the hemagglutinin/fusion glycoprotein of the *Measles morbillivirus* (Edmonston strain) and the Feline endogenous virus RD114 envelope protein. Various Murine leukemia virus envelope proteins (including the 10A1 and 4070A variants) can also be used.

3.2 Risks of replication-deficient lentiviral and gammaretroviral vectors

Lentiviral and gammaretroviral vectors can integrate into the genome of the host cells; this process is called insertional mutagenesis.[b] Insertional mutagenesis may result in health risks if it affects a tumour suppressor gene (inactivates it) or activates a proto-oncogene, which can lead to cancer (oncogenesis).[20,21] It has been

[b] COGEM notes that the terms insertional mutagenesis and insertional oncogenesis are sometimes used interchangeably. If the integration of the vector genome into the cell genome leads to the development of cancer, COGEM uses the term insertional oncogenesis. If the integration does not lead to cancer, the term insertional mutagenesis is used.

reported that the gammaretroviral vectors derived from MoMLV prefer to integrate close to transcription start sites.[22,23,24] The promotor/enhancer activity of the LTRs at the ends of the integrated vector can lead to activation of proto-oncogenes present in the host genome.[20,21] In addition to insertional mutagenesis, the integration of the vector genome into the host genome can have adverse effects if the coded gene product is harmful to the cell or the host organism. As a possible additional adverse effect, infectious vector particles may cause an immune reaction.

3.3 Self-inactivating lentiviral and gammaretroviral vectors

Self-inactivating (SIN) vectors have been developed to improve the biosafety of lentiviral and gammaretroviral vectors.[25,26] These vectors lack the 3' LTR domain which contains the promotor and enhancer elements of the LTR. During reverse transcription to the proviral genome this deletion is transferred to the 5' LTR. As a consequence, the vector integrated into the DNA no longer has a functional LTR promotor/enhancer, which suppresses the initiation of transcription. In turn this reduces the chance of mobilisation of the vector from the host genome.[26] It is assumed that this also prevents activation of proto-oncogenes. These SIN vectors do have an internal promotor for the expression of the transgene, but this is not derived from a lentivirus or a gammaretrovirus and has a reduced transforming potential.[25,27,28]

In the literature, however, it is reported that transcription of the transgene from integrated SIN vectors may lead to read-through transcription in the cellular genome, which in turn could result in the generation of unintended viral-cellular fusion transcripts and possibly activation of downstream proto-oncogenes or inactivation of tumour suppressor genes.[29] Research is still being conducted into SIN vectors to further improve their safety and efficiency, and improvements are still being made to these vectors.[30]

4. Considerations

COGEM has assigned HIV-1 to pathogenicity class 3 and has assigned the gammaretroviruses (in mice) that it has classified, such as Murine leukemia virus (which includes MoMLV), to pathogenicity class 2.[31] Activities involving the GM viruses derived from them have been assigned to the corresponding containment levels (levels II and II respectively) in accordance with the Ministerial Regulation on Genetically Modified Organisms (GMO Regulation).[32]

In 2009 COGEM issued generic advice on the containment level for activities involving lentiviral vectors derived from HIV-1, setting out the criteria to be used to determine the appropriate containment level.[1] The containment level is determined partly by the packaging system used to produce the vector and whether or not the vector contains a SIN deletion. Other factors that are important for lowering the containment level for in vitro activities (production, transduction and activities with transduced cells) are the possible generation of a replication-competent virus based on the lentiviral vector (replication-competent lentivirus, RCL), the presence and number of free vector particles and the chance of complementation and mobilisation of the vector due to the presence of wild-type lentiviruses. In outline, the determination of the appropriate containment level proceeded as follows:

- If no conclusive assessment can be made of the presence of RCL or wild-type lentiviruses, given the pathogenicity class of HIV-1 (class 3) the activities must be carried out at containment level III.
- If the host cells are free of wild-type lentiviruses and no RCL can be generated (which is determined in part by the type/'generation' of the production system used to produce the vector) or is present in the vector batch, but 'free' (i.e. non-internalised) vector particles are present, the activities should be carried out at containment level II.
- If the host cells are free of wild-type lentiviruses and no RCL and no free vector particles are present, the activities may be carried out at the lowest containment level (level 1).

In the light of advances in understanding and the results of a research project commissioned by COGEM into the COGEM formula for calculating the presence of free vector particles,[2,33] in this present advice the environmental risk of the presence of free vector particles (i.e. non-internalised vector particles) is reassessed. This advice also proposes a containment level for activities involving gammaretroviral vectors based on mouse gammaretroviruses and products derived from them.

4.1 Environmental risk of exposure to free vector particles

Only laboratory workers can be exposed to free vector particles during contained use activities. If a laboratory worker is infected with free vector particles, they cannot pass on the infection to third parties, because the genetic information essential for replication is not present in these vectors (see §3.1). The free vector particles therefore pose no environmental risk.

4.1.1 Recombination and complementation in the laboratory worker

In a theoretical scenario, a laboratory worker infected with wild-type HIV-1[c] could be infected with a lentiviral particle while working with such vector particles. If co-infection of the same host cell takes place, complementation may occur resulting in mobilisation of the integrated vector genome. If the vector has a SIN deletion in the LTR, the chance of mobilisation following integration in the host cell genome will be considerably reduced (see §3.3). With a non-SIN vector mobilisation may occur and the vector could be co-packaged in virus particles along with the wildtype virus, but the lentiviral vector will not be able to replicate unaided because the genetic information essential for replication is absent. Co-infection with HIV could also lead to recombination with the vector, which in theory could result in the generation of a recombinant HIV. For both mobilisation (by complementation) and recombination, the lentiviral vector would have to infect the same cell as HIV-1. The chance of this occurring is small, however, because infection with HIV-1 is mainly restricted to CD4+ T cells. In addition, recombination will mostly result in a poorly replicating or even replication-deficient virus, given that uptake of vector sequences in HIV is very likely to disrupt the expression of viral genes and, because the genome will be larger, will be detrimental to the packaging of the viral genome in virus particles. COGEM points out that people infected with HIV

[c] As this concerns work with lentiviral and retroviral vectors derived from HIV-1 or mouse gammaretroviruses, only co-infection with HIV-1 has been considered as a relevant scenario. HIV-2 is not considered here because the sequence homology between HIV-2 and HIV-1 is moderate (48%) and therefore the chance of recombination and mobilisation between the lentiviral vector and HIV-2 will be smaller than with HIV-1. Sequence homology in other lentiviruses will be too limited for recombination. No evidence has yet been found that other gammaretroviruses can infect humans.

are treated with an antiretroviral therapy, which suppresses not only HIV-1 but also any infection with the lentiviral vector and will therefore further reduce the likelihood of mobilisation and recombination. Taking everything into consideration, COGEM considers that exposure of a laboratory worker infected with HIV-1 to free lentiviral vector particles poses a negligible environmental risk. The worker themself may be exposed to a risk (health and safety risk, see §4.2).

4.2 Health and safety risk of exposure to free vector particles

If a laboratory worker is exposed to free vector particles, the replication-deficient nature of the lentiviral and gammaretroviral vectors means that no further spread of the particles to the environment can occur. However, exposure to free vector particles does involve various potential health risks, such as the risk of oncogenesis from insertional mutagenesis (see §3.2), and potential adverse effects may occur depending on the transgene expression cassette that was used. In the Netherlands, the risks to third party employees, such as laboratory personnel, fall legally under the Working Conditions Act (*Arbeidsomstandighedenwet*)[34] and not under the environmental legislation.[d] The purpose of the Working Conditions Act is to ensure safe and healthy working conditions for all employees. Employers and employees are jointly responsible for implementing the Act at their own workplace. For this reason, during amendment of the Ministerial Regulation on Genetically Modified Organisms (GMO Regulation) and the proposed containment levels for activities, potential risks to laboratory workers cannot be taken into consideration within the current legal framework.

4.3 Presence of wild-type lentiviruses and exogenous gammaretroviruses

Two important aspects that must be taken into account when assigning containment levels to activities involving lentiviral and retroviral vectors concern the presence of wild-type lentiviruses or retroviruses in the cell lines to be used and the possibility of the generation of replication-competent virus (RCV). Lentiviruses and gammaretroviruses can be found in various animal species. COGEM considers it necessary to ensure that related wild-type viruses cannot be present during all activities involving lentiviral or gammaretroviral vectors in order to prevent recombination, complementation and mobilisation of these vectors.

No exogenous gammaretroviruses are known to infect humans. In the past, however, a human cell line was contaminated with mouse gammaretroviruses used as a xenograft in mice, leading to the acquisition of a new recombinant xenotropic gammaretrovirus.[35] Because it took some time before it became known that these cells produced xenotropic mouse retrovirus (about 10 years), cross-contamination with other cell lines may have occurred and so the presence of mouse gammaretroviruses in some human cell lines cannot be ruled out in advance.[36]

4.4 Risks of endogenous gammaretroviruses in activities involving gammaretroviral vectors

COGEM notes that it can be difficult to rule out the presence of gammaretroviruses in animal cell lines. Intact (or nearly intact) endogenous gammaretroviruses (ERVs) may be present in several vertebrates,

[d] *Lex specialis derogat legi generali*: the Working Conditions Act (Arbowet) has ‘precedence’ over the general environmental legislation.

including mice, birds, pigs, rats and cats. Often these endogenous gammaretroviruses are defective, but in some cases this is due to a single point mutation. In these cases there is a chance that the replication capacity of these cell lines may be restored after a number of passages or after treatment with UV radiation, X-rays or exposure to mutagenic substances, leading to the production of replication-competent gammaretrovirus. Since gammaretroviruses – in contrast to lentiviruses – cannot cause lytic infection, the production of gammaretroviruses from these endogenous gammaretroviral sequences is not always detected. As a result, gammaretroviruses may or may not be dormant in certain cell lines.

An example of this is rat cells. As far as we know, no exogenous gammaretroviruses infect rats.[e] However, the scientific literature contains a report of an ERV in rats, at the time referred to as rat leukemia virus (RaLV), which was shown to be related to the gammaretroviruses *Feline leukemia virus* and to a lesser extent *Murine leukemia virus*, and from which exogenous replication-competent RaLV can be spontaneously produced after repeated passages of embryonal rat cells.[10,37] In addition, it has been reported that recombination between exogenous RaLV and endogenous proto-oncogenes in tumour cells of rats can generate the Rasheed rat sarcoma virus (RaSV), a replication-deficient virus.[37,38]

Besides the fact that ERVs in cells of certain animal species (including mice, pigs, birds, rats and cats) can spontaneously produce exogenous gammaretrovirus under certain laboratory conditions, recombination can occur with a retroviral vector if there is a large sequence homology between the ERVs and the gammaretroviral sequences. If the endogenous gammaretroviral sequences contain the genetic information necessary to restore the replication capacity of the vector, a recombinant replication-competent gammaretrovirus may be generated. Complementation can also cause the retroviral vector to be mobilised in these cells. In the literature it has been reported that transfection with a defective MoMLV provirus in rat cells in which defective endogenous RaLV is present resulted in the production of a recombinant replication-competent retrovirus.[39] Although human cells also contain class I HERVs that exhibit sequence homology to current gammaretroviruses and epsilonretroviruses, these HERVs are inactivated to such an extent that recombination with a gammaretroviral vector is highly unlikely to result in recombinant replication-competent virus.

5. Proposed containment levels

COGEM considers the environmental risk of the presence of free vector particles to be negligible. Accordingly, COGEM has reconsidered which containment levels are appropriate for working with lentiviral vectors. This advice also proposes a containment level for activities involving gammaretroviral vectors based on mouse gammaretroviruses and products derived from them. The following sections review the appropriate containment levels for lentiviral vectors and then for gammaretroviral vectors.

5.1 Proposed new containment levels for activities involving lentiviral vectors

The following sections present a reconsideration of the appropriate containment levels for activities involving lentiviral vectors. For lowering the level of containment for activities involving lentiviral vectors,

[e] In this advice, the Kirsten murine sarcoma virus (Ki-MSV) and Harvey murine sarcoma virus (Ha-MSV) recognised by the ICTV are not considered to be natural rat viruses. These viruses were generated by passing mouse retroviruses (murine erythroblastosis virus and Moloney murine leukemia virus) in rats, whereby the viruses incorporated genetic elements (including the *ras* oncogene) from the rat genome by recombination.[36]

COGEM deems it necessary that no replication-competent lentivirus (RCL) can be generated and that the cells in the cell line to be used must be free of wild-type lentiviruses in order to rule out the possibility of recombination, complementation and mobilisation.

5.1.1 Production activities[f]

When using lentiviral vector production systems, the possibility of the generation of replication-competent lentivirus (RCL) depends on which vector production system is used (1st, 2nd or 3rd generation and whether or not they are combined with a SIN LTR).[1] When using lentiviral production systems in which the chance of the generation of RCL is known in advance to be negligible (i.e. 3rd generation SIN or translentiviral production systems), COGEM is of the opinion that production can be carried out at containment level ML-I. When using production systems in which the generation of RCL cannot be ruled out in advance, such as 2nd generation production system in combination with a non-SIN vector, the work must be carried out at containment level ML-III in accordance with the pathogenicity class of HIV-1.

5.1.2 Transduction activities

COGEM considers that when lentiviral vectors are used for the transduction of cells, the vector batch to be used must be free of RCL before the containment level for these activities can be lowered. If the lentiviral vectors to be used are produced by a production system with a negligible risk of generating RCL, COGEM is of the opinion that the environmental risks of transduction activities involving these lentiviral vectors carried out at containment level ML-I will be negligible. If the generation of RCL during production cannot be ruled out in advance, COGEM considers that the containment level can only be lowered to ML-I when the vector batch has tested negative for the presence of RCL before transduction.

5.1.3 Activities involving cells transduced with lentiviral vectors

Following lentiviral transduction, the GM cells are cultured for some time, after which they go through a number of inactivating washes to remove any remaining free vector particles. COGEM notes that these washing steps and biological decay will greatly reduce the number of free vector particles and therefore also the chance that laboratory workers will be exposed to them. COGEM is of the opinion that if the lentiviral vector batch used for transduction has been shown to be free of RCL, work with transduced cells can be carried out at containment level ML-I. Under this condition and the requirement that cells to be used must be free of lentiviruses, COGEM considers that the chance of the generation of RCL in the transduced cells and their transmission to humans and the environment are negligible.

5.1.4 Risks of contamination when working with lentiviral vectors at containment level I

As work with wild-type viruses (or replication-competent lentiviruses) is not permitted at containment level I, contamination from external sources during activities involving wild-type lentiviruses is not possible. It

[f] Recently, COGEM published an advice in response to a follow-up question of the GMO office concerning the risk of RCL generation when using different pseudotyping proteins. In her advice, the COGEM is of the opinion that the use of different pseudotyping vectors during production offers a negligible risk of RCL generation due to limited sequence homology and the amount of recombination steps required for RCL generation. However, when homologous proteins are used, i.e., derived from HIV, the possibility of RCL generation cannot be excluded due to the presence of homologous sequences. Therefore, COGEM is of the opinion that production activities which involve HIV envelope proteins should be carried out at containment level ML-III.

is possible, however, that activities involving lentiviral constructs derived from these wild-type viruses are carried out in the same ML-I space. When working with non-SIN lentiviral vectors, it is possible that the vector genome can be mobilised from the host genome if complementation occurs during or after the transduction of a host cell as a result of contamination with lentiviral constructs. COGEM points out that any vector particles that might subsequently be generated would be replication-deficient because they would lack the genetic information essential for replication. If a SIN vector is used the chance of mobilisation is negligible.

In the most serious case, it would be theoretically possible for a replication-competent virus to be generated as a consequence of recombination. COGEM notes that this is only possible if: a) one cell is simultaneously contaminated with different constructs, whereby b) the constructs together contain all the genetic information necessary for restoring replication capacity, c) these constructs contain overlapping nucleotide sequences that make recombination possible, and d) multiple recombinations occur simultaneously. The chance of all these conditions being met during a laboratory experiment with lentiviral vectors is considered to be negligible.

5.2 Proposed containment level for gammaretroviral vectors

The following sections review the containment levels for activities involving gammaretroviral vectors derived from mouse gammaretroviruses. In COGEM's opinion, the necessary conditions for lowering the containment level for activities involving these gammaretroviral vectors are that no replication-competent gammaretrovirus (RCR) can be generated and that the cells must be free of gammaretroviruses. An important aspect that also plays a role is the presence of intact (or nearly intact) endogenous gammaretroviruses in the cell lines used.

5.2.1 Production of gammaretroviral vectors

During the production of gammaretroviral vectors, replication-competent gammaretrovirus (RCR) may be generated by recombination between the various plasmids required for the production of the viral vector, or with endogenous gammaretrovirus sequences which may be present in the cell line used for production.[40] Although reconstruction of the whole gammaretroviral genome requires multiple recombination events to construct an infectious virus particle, COGEM is of the opinion that the chance of RCR being generated when using a gammaretroviral production system cannot be entirely ruled out.[40] To prevent the spread of RCR in the environment, COGEM therefore advises that production of gammaretroviral vectors should carried out at containment level ML-II, in accordance with the pathogenicity class of mouse gammaretroviruses.

5.2.2 Transduction activities

As noted earlier, cell lines of diverse animal species (including mouse, bird, cat, pig and rat cells) may contain intact (or nearly intact) endogenous gammaretroviruses and gammaretroviruses may be produced undetected in these cell lines. During transduction of cells possibly containing such endogenous (undetected exogenous) gammaretroviruses with a gammaretroviral vector, there is a chance of homologous recombination occurring, which could result in a recombinant replication-competent gammaretrovirus or the mobilisation of the retroviral vector. COGEM therefore advises that transduction with gammaretroviral

vectors in combination with cell lines belonging to a species other than human cell lines, which by nature do not contain intact (or nearly intact) endogenous gammaretroviruses, should be carried out at containment level ML-II.

In human cell lines, no intact (or nearly intact) endogenous gammaretroviruses are present. Also, no known exogenous gammaretroviruses can infect humans. Transduction of cells of human origin containing residues of endogenous gammaretroviral sequences (class I HERVs) with a gammaretroviral vector has never been observed to result in the generation of RCR. An exception to this are human cell lines which in the past have been contaminated with a gammaretrovirus in an animal laboratory.[35,36] Although the chance of working with a human cell line contaminated in this way is small, it is important that researchers test the human cell line for the presence of gammaretroviruses.

All things considered, COGEM is of the opinion that when working with gammaretroviral vectors in human cell lines, which by nature do not contain any intact (or nearly intact) endogenous gammaretroviruses, the risk of RCR generation is negligible as long as the vector batch used is also free of RCR and the cells used have been tested for the presence of replication-competent gammaretroviruses. In addition, COGEM points to the importance of verifying the identity of the cell lines to be used. Under these conditions, COGEM advises that transduction with gammaretroviral vectors in combination with human cells should be performed at containment level I. COGEM advises imposing the following additional conditions:

- Activities involving cells transduced with gammaretroviral vectors and activities involving cells belonging to a species which is known to frequently contain endogenous gammaretroviruses (including mouse, bird, pig, cat and rat cells) should be separated in space and time to prevent any cross-contamination. Options for ensuring this separation include the use of distinct laboratory areas or the use of separate safety cabinets.
- For simultaneous work with other cells, it must first be demonstrated that they have tested negative for gammaretroviruses.

COGEM advises that transduction with cell lines that have not tested negative for the presence of gammaretroviruses should be carried out at containment level II.

5.2.3 Activities involving cells transduced with gammaretroviral vectors

COGEM is of the opinion that work with cells transduced with gammaretroviral vectors can be carried out at a lower containment level if it meets the same conditions and additional requirements for lowering the containment level for transduction with gammaretroviral vectors. The use of culture and washing steps greatly reduces the number of free vector particles, which limits the possible health risk to laboratory workers of exposure to free vector particles.

6. Conclusion and advice

Given that SIN and non-SIN replication-deficient lentiviral and gammaretroviral vectors cannot spread because the genetic information required for replication is absent, COGEM considers that the environmental risks of the presence of free vector particles during work with these types of vector are

negligible. In addition, COGEM notes that laboratory workers do face potential health risks associated with working with free vector particles.

In this advice COGEM has reviewed the containment levels for activities involving lentiviral vectors and has proposed an appropriate containment level for activities involving gammaretroviral vectors based on mouse gammaretroviruses and products derived from them.

COGEM advises that production or transduction with lentiviral vectors and activities involving cells transduced with lentiviral vectors can be carried out at containment level I, as long as the following conditions are met:

- The cells to be used are free of lentiviruses during all activities in order to rule out the generation of recombinant virus or mobilisation of the vector genome.
- It has been confirmed that no RCL can be generated during production, or in the case of transduction activities (or subsequent activities involving cells transduced with lentiviral vectors) the vector batches used or to be used are free of RCL.

COGEM is of the opinion that when working with gammaretroviral vectors, the generation of RCR during production of these vectors cannot be ruled out and advises that the production activities are carried out at containment level ML-II. COGEM advises that transduction with gammaretroviral vectors and activities involving gammaretroviral transduced cells that make use of non-human cell lines should be carried out at containment level ML-II because of the possible presence of intact (or nearly intact) ERVs. COGEM also advises that transduction with gammaretroviral vectors and activities involving cells transduced with gammaretroviral vectors should be carried out at containment level I only if the following conditions are met:

- The cell lines to be used are of human origin and have been shown to be free of replication-competent gammaretroviruses (demonstrated by a validated test) in order to rule out the generation of recombinant virus or mobilisation of the vector genome.
- The gammaretroviral vector batch to be used for transduction is free of RCR.
- Activities involving cells transduced with gammaretroviral vectors and activities involving cells belonging to a species which is known to frequently contain intact (or nearly intact) endogenous gammaretroviruses (including mouse, bird, pig, cat and rat cells) should be carried out in separate spaces and at different times to prevent any cross-contamination. Options for ensuring this separation include the use of distinct laboratory areas or the use of separate safety cabinets.
- For simultaneous work with other cells, it must first be demonstrated that they are negative for gammaretroviruses.

If these conditions are met when working with lentiviral and gammaretroviral vectors and the additional requirements (for gammaretroviral vectors) are applied, COGEM considers that the chance of RCR generation during the activities at containment level ML-I is negligible.

6.1 Observations from a health and safety perspective

Free vector particles are a potential risk to laboratory workers, but further spread of these vector particles is not possible. The safety of employees falls under the working conditions legislation and not under the GMO or environmental legislation. COGEM notes that from a health and safety perspective, measures are required to prevent exposure to free vector particles and possible infection. To limit the risks to workers, COGEM considers that when the risk of exposure to free vector particles is higher, i.e. during the production of the vector particles or the use of transductions (preparing GM cells) which involves handling large amounts of vector particles, it is necessary to carry out open handling in a class II safety cabinet (VK-II cabinet) and to wear gloves.

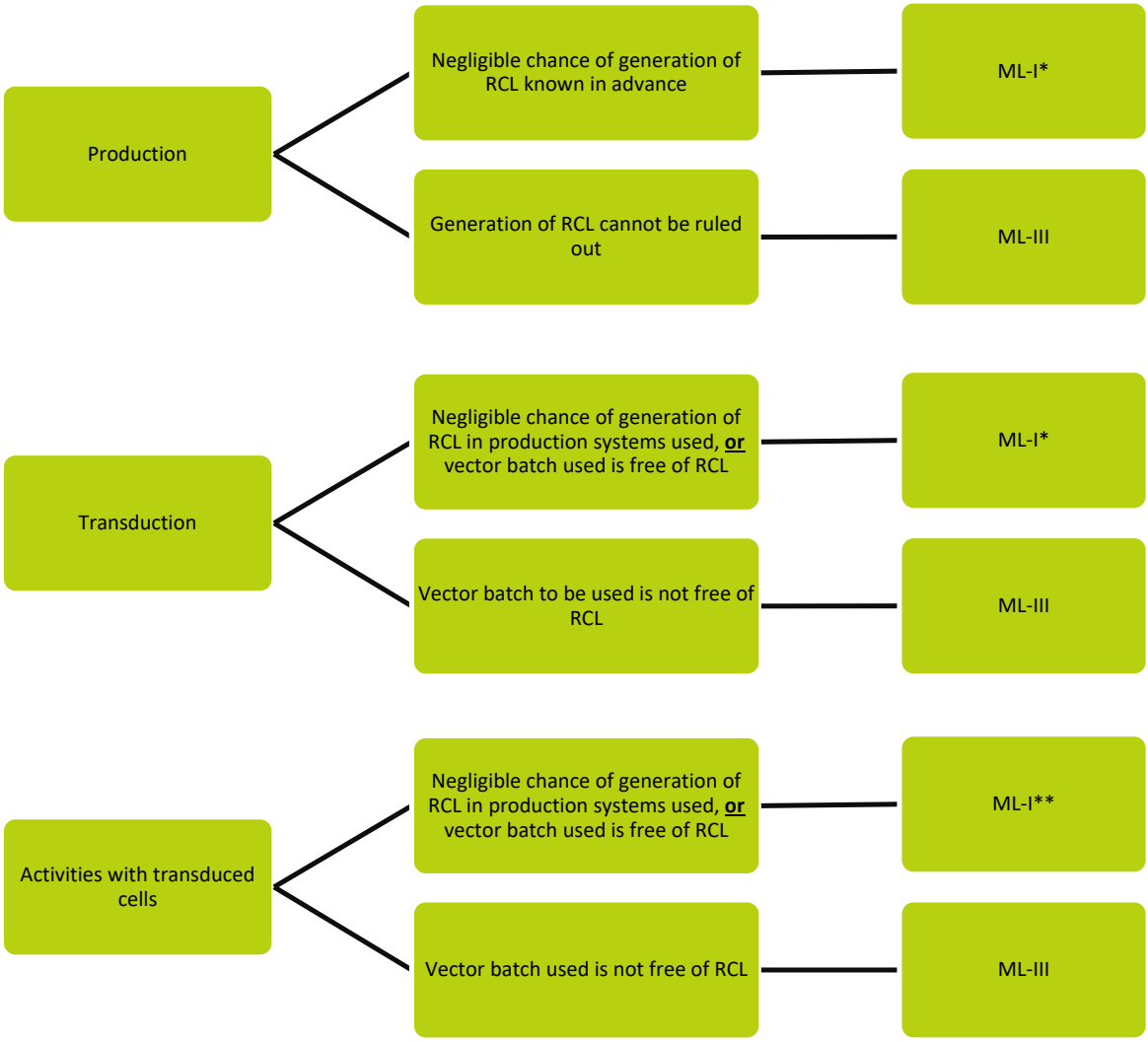
After a transduction experiment the GM cells are cultured for a certain time, after which they go through a number of washes (in some cases inactivating washes) to remove any remaining free vector particles. The washing steps and biological decay will in time greatly reduce the number of free vector particles during activities with transduced cells. COGEM points out that the amount of residual free vector particles can be determined experimentally. When using lentiviral vectors, an alternative method for determining the amount of residual vector particles is the calculation formula developed by COGEM.[1,33] This formula is not suitable for calculating free gammaretroviral vector particles because there are still uncertainties about the half-lives of these vectors. The COGEM formula is used to estimate the reduction in the number of free infectious vector particles during the transduction and washing of the GM cells and relates this to the number of vector particles added during transduction. This is expressed as a reduction ratio (Rr). The number of particles can then be calculated from the reciprocal value of the reduction ratio (1/Rr). A research project commissioned by COGEM into the experimental validation of the COGEM formula has shown that most of the values of the parameters in the formula are variable and sometimes also method-dependent.[2] The published research report therefore contains a revised formula, which can only be used for cultures with adherent cells and the values obtained are only valid for lentiviral vectors.

If it is not possible to use the COGEM formula, for example due to a lack of data on the half-life of the vector, a seven-day safety period may be observed. At the end of this period natural decay will have further reduced the number of free particles to a negligible amount.

7. Summary and diagrams

The flow charts on the following pages summarise the above advice on containment levels. The containment levels given apply on the condition that for all activities the cells to be used must be demonstrated to be free of lentiviruses or gammaretroviruses.

7.1 Lentiviral vectors

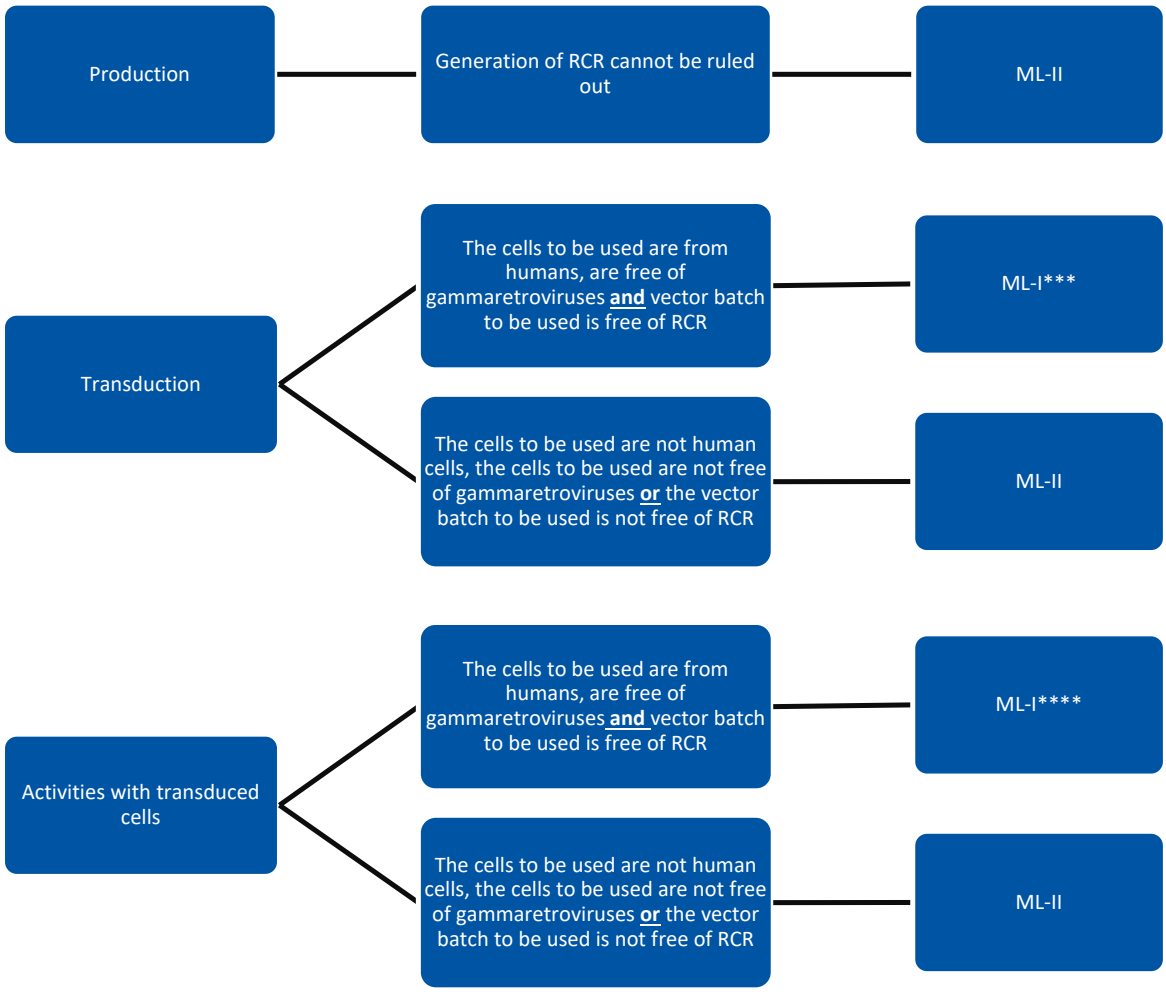


* *COGEM stresses the importance of taking additional measures for health and safety reasons, such as carrying out open handling in a category II biological safety cabinet and wearing gloves.*

** *COGEM stresses the importance of taking additional measures for health and safety reasons, such as carrying out open handling in a category II biological safety cabinet and wearing gloves in situations were free vector particles are present.*

RCL: replication-competent lentivirus.

7.2 Gammaretroviral vectors



*** *Subject to the requirement to keep the activities separated from cells belonging to a species which is known to contain intact (or nearly intact) endogenous gammaretroviruses (including mouse, bird, pig, cat and rat cells) or other cells which have tested negative for the presence of gammaretroviruses. COGEM also stresses the importance of taking additional measures for health and safety reasons, such as carrying out open handling in a category II biological safety cabinet and wearing gloves.*

**** *Subject to the requirement to keep the activities separated from cells belonging to a species which is known to contain intact (or nearly intact) endogenous gammaretroviruses (including mouse, bird, pig, cat and rat cells) or other cells which have tested negative for the presence of gammaretroviruses. COGEM also stresses the importance of taking additional measures for health and safety reasons, such as carrying out open handling in a category II biological safety cabinet and wearing gloves in situations were free vector particles are present.*

RCR: Replication-competent gammaretrovirus

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