KU LEUVEN

Biosafety in the laboratory

3rd, revised edition

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PREFACE

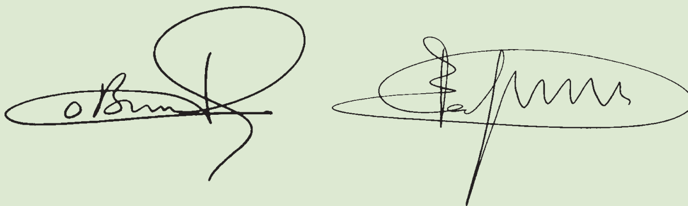
This is the third, revised edition of the booklet 'Biosafety in the laboratory'. In this booklet you will find all the information that was already present in the earlier editions. Only a few alterations have been made, especially in the text concerning decontamination and inactivation.

Modern biotechnology is a very important technology in the current biological and biomedical research. Thousands of researchers in the life sciences are using micro-organisms, plants or animals on a daily basis to answer relevant scientific questions. More and more use is made of genetically modified organisms.

The scientific community itself has been the first to stress the importance of working safely with GMOs. Nowadays many regulations and guidelines exist for GMOs. These are designed to protect human health and the environment.

We want to stress the importance of working safely and responsibly with biological and especially genetically modified material. It is the obligation of the researcher to apply all the necessary safety measures. It is also the obligation of the researcher to teach all inexperienced personnel the theoretical and practical aspects of biosafety.

We hope that this booklet, together with practical instructions and guidance, will prove to be a useful tool to welcome people in a safe manner into the fascinating world of biotechnological laboratory research.



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1. INTRODUCTION

Biological material is used intensively in biological and biomedical research. In this type of research the use of modern molecular biological techniques like recombinant-DNA technology is still on the increase. This booklet gives a brief overview of the basic principles that are important for the safe use of pathogenic and/or genetically modified organisms. Three types of safety measures can be distinguished:

a. measures to protect the worker,
b. measures to protect the experiment, and
c. measures to protect humans and the environment.

In this booklet, these different measures will be discussed for micro-organisms, as well as for plants and animals. Every researcher that makes use of these types of organisms in the laboratory should know the basic principles of biosafety by heart and act accordingly. Only in this way a situation can be created that is safe both for yourself, your fellow human beings, and the environment.

The safe use of biological material is also required by two types of legislation:

1. The legislation on the protection of workers of activities with biological agents;
2. The legislation on the environment with regard to working with genetically modified organisms.

For both types, there is European legislation which has been incorporated into national law.

Figure 1: The biohazard symbol. This symbol indicates that work is being done with biologically hazardous organisms (starting from risk class 2)

2. BIOSAFETY

What is biosafety?

The biosafety of laboratory work is the central focal point of this booklet. Biosafety is about the intrinsic hazards of living organisms and how to handle them safely. Genetic material as such ('naked' DNA) can be dangerous as well. Before starting to work with pathogens or genetically modified organisms (GMOs) in a laboratory one should stop and think about the possible hazards of these organisms and take proportionate measures to minimise any risks for human health and the environment. ....Safety first...!

What are the hazards?

Biological material and living organisms are neither intrinsically dangerous, nor intrinsically safe. Any danger will depend on the characteristics of the material or the organisms. Characteristics that represent a danger are the following:

- **Pathogenicity**
  The pathogenicity of an organism indicates whether an organism - for instance a bacterium, a virus, fungus or a parasite - is able to cause a disease in a plant, animal or human. Factors like infectious dose, virulence and the production of toxins by the pathogen play a role in the extent to which the organism is able to cause disease.

Infectious dose
Contamination by one pathogen does not automatically lead to infection. The infectious dose differs from one pathogen to another. Below, some examples are given of human pathogens' infectious dose when they are spread by their normal route of infection.

Pathogen	Infectious dose
Adenovirus	> 150 pfu (intranasaly)
Influenza virus	790 pfu (nasopharyncheal)
Tuberculosis bacteria	10 bacteria (inhalation)
Thyphoid bacteria	10^5 bacteria (ingestion)

Pfu= plaque forming units

Source: Material Safety Data Sheets, Health Canada

- **Toxicity**
  Toxicity means poisoning. Most substances are not poisonous when they are used under normal circumstances. The toxicity of a substance is mostly given as an LD50 for verte-

brates in weight units per kilogram body weigth. The LD50 (LD stands for: lethal dose) is the amount at which exposure to the substance leads to the death of 50% of the animals exposed. When the toxicity of living organisms (especially bacteria) is considered, toxicity often coincides with pathogenicity.

- **Allergenicity**
  Allergenicity is a non-toxic, immune system mediated, undesired reaction of the body to a substance or agent. Immune globuline E (IgE) and mast cells (immune system cells that, among other things, produce heparin) often play a role in the allergic reaction. An allergic reaction may lead to sneezing, skin irritation, asthma attacks, chronic lung disorders, and sometimes even to a lifethreatening shock.

> In order to prevent that laboratory staff be affected with lung and skin disorders, the possibility of an allergic reaction should be taken into account. Direct contact with allergens (inhalation, skin contact) should therefore be avoided.

- **Disturbance of ecological balances**
  The aspect of disturbance of ecological balances is especially relevant for activities involving GMOs. Disturbance of an ecological balance may happen when a GMO possessing a certain characteristic is accidentally spread to the environment, or when genetic material originating from that organism spreads to other organisms in the environment. The potential hazards of recombinant-DNA technology and the risk assessment of activities involving this technology will be discussed more in detail in the following chapters of this booklet.

- **Other harmful effects**
  Sometimes there are other unwanted effects that urge one to be even more cautious when handling biological material. It is not possible to give an exhaustive list of these effects. What matters is that one stops to think about the characteristics of biological material, before starting to work with it. One important class of genes that should be looked at carefully are genes that produce proteins with immune modulating properties, although not all immune modulations are harmful. For certainty about the possible level of harm the effects of the immune modulation should be thought through carefully and quite often consultation with experts will be necessary. One example is the handling of a vacciniavirus in which a gene responsible for immune suppression is cloned. Immune suppression may lead to the body not being able to fight an infection by the virus. In some exceptional cases, infections with vacciniaviruses may lead to fatal encephalitis.

Working with 'naked' DNA

In our environment we are confronted with loads of DNA every day. In the soil, on our skin, DNA is everywhere. In the laboratory DNA is mostly used in the form of plasmids, digested DNA, or primers. DNA as such is not hazardous. But on the other hand, working with DNA is not always without risk. This is especially the case when the DNA codes for a cellular oncogene. An oncogene is a gene that is dominantly transforming: one that, when incorporated into the genome, instantly leads to the transformation* of the cell. Transformation - the transition to a quickly and infinitely dividing cell - is the first and most important step in tumor formation. An accident with needles containing such material holds a risk. Personnel working with such material should be aware of that risk, wear gloves, and reduce the handling of sharps to an absolute minimum. Once this DNA is incorporated into a bacterial genome or the genome of a cell line, the resulting GMO no longer represents a hazard, unless the cell line is a packaging cell line resulting in the incorporation of the oncogene into a viral vector that is able to transmit the oncogene.

Oncogenes and viral vectors

The cloning of DNA coding for a dominant transforming cellular oncogene is especially hazardous when it is cloned into a viral vector that incorporates itself into the genome of the cell. As regards the hazard to the worker, a distinction should be made between ecotropic and amphotropic viral vectors, since the former are only able to infect mouse cells, whereas the latter can infect human cells as well, and therefore represent a greater risk.

*see clarification of terms

3. CLASSIFICATION AND RISK ASSESSMENT

Pathogenic organisms

Organisms are divided into four categories of risk. Organisms that are not able to cause disease belong to risk group 1. Pathogenic organisms belong to the risk groups 2, 3 or 4, depending on their degree of pathogenicity and the availability of effective treatment. To distinguish between the classification of natural non-modified pathogens and GMOs, the pathogen classification uses the term risk groups or sometimes also biological risk class, while for the GMO classification the term risk class is used. Below an overview is given of the definitions of the different risk groups.

Group 1	Very unlikely to cause disease in humans, animals or plants.	
Group 2	Human pathogens	(micro-)organisms that can cause disease in humans and pose a hazard to persons that are directly exposed to it. Their spread to the community is unlikely. Profylaxis or effective treatment is mostly available.
	Animal pathogens	(micro-)organisms that can cause disease in animals and that possess in different extend one of the following properties: limited geographical importance, transmission to other limited or non-existent species, absence of vectors or carriers. Limited economic and/or medical impact. Profylaxis and/or effective treatment is mostly available.
	Fytopathogens	(micro-)organisms that can cause disease in plants, but for which there is no higher risk of an epidemic when they are accidentally disseminated into the Belgian environment. Profylaxis or effective treatment is available. Non-indiginous or exotic fytopathogens that are not able to survive in Belgium because of the absence of target plants or because of unfavourable weather conditions, belong to this risk group.
Group 3	Human pathogens	(micro-)organisms that can cause serious disease in humans and pose a hazard to persons that are directly exposed to it. There is a risk of spread to the community. Profylaxis or effective treatment is mostly available.
	Animal pathogens	(micro-)organisms that can cause serious disease or epizotic in animals. Spread to other species is more than possible. Some of these pathogenic agents require specific sanitary measures. Profylaxis or effective treatment is mostly available.
	Fytopathogens	(micro-)organisms that can cause a disease in plants with important economic or environmental consequences and for which treatments are non-existent, difficult to apply or costly. Accidental spread may lead to local epidemics. Exotic strains of fytopathogens usually occuring in the Belgian environment and not in the list of the quarantine organisms also belong to this risk group.

Group 4	Human pathogens	(micro-)organisms that can cause serious disease in humans and pose a severe hazard to persons that are directly exposed to it. There is a high risk of spread to the community. Profylaxis or effective treatment is mostly not available.
	Animal pathogens	(micro-)organisms that can cause a very serious pandemic or epizotic in animals with high levels of mortality or dramatic economic consequences for the afflicted regions. Medical profylaxis is not available or one exclusive sanitary profylaxis is possible or obligatory.
Fytopathogenic quarantine organisms		Fytopathogenic organisms that are subject to additional quarantine regulations that are designed to combat relevant plant diseases or diseases that can affect plant products.

Belgian definitions of the different risk groups. Most definitions of risk groups are developed from the WHO classification of infective micro-organisms.

In addition to classifying a pathogen, it is very important to mention its host, since infectious diseases are an interaction between a pathogen and a host. Some pathogens have a broad host range, whereas others may only be able to infect one or a few hosts. Moreover, the risk group of a particular pathogen that can infect both humans and animals may differ from one host to another. For instance, the biological risk class of Herpes virus B is 3 for humans, while it is 2 for animals. There are published lists of the biological risk classes of different pathogens, which are a convenient help for risk assessment. In Belgium, the following lists are important:

- The pathogen classification list in Vlarem II, chapter 5.51 (http://www.biosafety.be), and
- annex 1 to the Royal Decree of August 4, 1996, concerning the protection of employees against the risks of exposure to biological agents at work (this annex applies to human pathogens only; Official Journal of Laws and Decrees in Belgium, October 1, 1996)

Annex III of this booklet lists the biological risk classes of a number of relevant pathogens. Opportunistic pathogens are organisms that are only able to cause disease in animals or humans which have an impaired immune system. These organisms belong to biological risk class 1.

There are no legal requirements for working with non-genetically modified risk class 1 organisms. It is, however, strongly recommended to apply the principles of *'Safe Microbiological Practices'* (SMP) when handling non-modified micro-organisms or cells (see chapter 5). Risk class 2, 3 and 4 agents may only be handled in laboratories that are specially designed for that purpose.

Genetically modified organisms (GMOs)

Recombinant-DNA technology has become so important that one can no longer imagine modern biological and biomedical laboratories without the technique. *Escherichia coli* K12 is the number one laboratory organism, which is used by almost every researcher as a means of cloning or expressing genes or sequences.

Definition of a GMO

GMOs are organisms whose genetic material has been altered in a way that is not possible by reproduction or natural recombination. Techniques that lead to the formation of a GMO include:

* recombinant-DNA- and RNA-techniques involving the use of host/vector systems
* techniques involving the direct introduction into a micro-organism of heritable material prepared outside the micro-organism, including micro-injection, macro-injection and micro-encapsulation
* cell fusion or hybridisation techniques where living cells containing new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally

The following techniques are not considered to result in genetic modification, on condition that they do not involve the use of recombinant-DNA molecules or genetically modified organisms

* *in-vitro* fertilisation
* natural processes such as: conjugation, transduction, viral infection, transformation
* polyploidy induction.

Exceptions to the guidelines

The following GMOs are excluded from the regulations and also from the scope of this booklet if they have been made using the techniques listed below, on the condition that they do not involve the use of recombinant-nucleic acid molecules or GMOs other than those produced by one or more of the techniques listed below:

* Mutagenesis.
* Cell fusion (including protoplast fusion) of prokaryotic species that exchange genetic material by known physiological processes.
* Cell fusion (including protoplast fusion) of cells of any eukaryotic species, including production of hybridomas and plant cell fusions.
* Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent) with or without prior enzymatic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting micro-organism is unlikely to cause disease to humans, animals or plants. Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular microorganisms.

Recombinant-DNA GMO's

Today a whole range of organisms can already be genetically modified, a.o bacteria, yeasts, fungi, insects (fruit fly), parasites, nematodes, plants, frogs, mammals (mice, rats, rabbits, goats, sheep, pigs, cattle). Genetic modification in general involves the following components:

* A host organism (the organism which is to be modified); note that the meaning of the term ‘host’ in this context differs from that in the context of pathogenic organisms - See clarification of terms..
* A donor sequence or insert, isolated from a certain organism (the donor organism). However, synthetically produced DNA sequences are also being used more and more often. These sequences can be identical to sequences present in living organisms, but they can also be completely new.
* And in many, but not all cases a (genetic) vector.
  In the case of transformation of bacteria, plasmids are mostly used as a vector. In other cases viruses or viral vectors may be used. Examples where no genetic vector is used are the micro-injection of DNA in the pronucleus of a fertilised egg, or the modification of plants by means of particle bombardment. Depending on the system used the vector will remain present in the final GMO or not.

Risk assessment

GMOs, like non-GMOs, are neither intrinsically hazardous, nor intrinsically safe. That is why risk assessment is performed on a case-by-case basis. The risk assessment procedure consists of of three subsequent steps:

1. Firstly, the characteristics of the host, vector and donorsequences that are potentially hazardous like pathogenicity, toxicity, the possibility of uncontrolled spreading of the organism or its genetic material, are identified. This leads to a preliminary identification of the risk level.
2. Secondly, the circumstances under which the organisms can be handled safely are determined, taking into account the following aspects:

* The characteristics of the environment that could be exposed to the GMOs
* The type and scale of the activity
* Any non-standard activities or actions

3. Finally, a risk class is determined, based on the results of the first two steps..

As for pathogens, four risk classes have been determined for GMOs:

Risk class 1	GMO activities holding no or a negligible risk	Activities for which level 1 containment is appropriate to protect human health as well as the environment
Risk class 2	GMO activities holding a low risk	Activities for which level 2 containment is appropriate to protect human health as well as the environment
Risk class 3	GMO activities holding a moderate risk	Activities for which level 3 containment is appropriate to protect human health as well as the environment
Risk class 4	GMO activities holding a high risk	Activities for which level 4 containment is appropriate to protect human health as well as the environment

Risk classes as defined by the European directive 98/81/EC concerning the contained use of genetically modified micro-organisms.

There is already quite some experience with the risk assessment of GMOs. In annex 2 of this booklet a set of guidelines is given for the risk categorisation of GMOs. These guidelines are not legally binding, but following these guidelines and the risk assessment principles set out above will facilitate a good implementation of the legal requirements. After determining a first classification, and depending on the type and scale of activity, it should also be determined whether or not specific extra measures will be necessary in addition to the basic requirements of that containment level, or whether some measures can be left out. The legal requirements for the four containment levels in laboratories, animal houses and greenhouses are listed in annex 1.

Below a comparison is given between the categorisation of pathogens and GMOs in their risk classes and the accompanying basic containment levels:

Class	Pathogens	GMOs	Basic containment level
Risk class 1	Non-pathogens	No or negligible risk	Level 1 for GMOs, SMP for non-modified micro-organisms or cells*
Risk class 2	Mild pathogens	Low risk	Level 2
Risk class 3	Moderate pathogens	Moderate risk	Level 3
Risk class 4	Strong pathogens	High risk	Level 4

* Note: level 1 gives requirements for both the infrastructure and the working practices, although the requirements for the infrastructure are very limited. SMP (Safe Microbiological Practices) only describes requirements for the working practices.

4. THE SPREAD OF ORGANISMS IN THE LABORATORY

Natural routes of infection

Pathogens all have their own route of infection, by which they spread from one host-organism to another. The table below lists a number of important routes of infection:

Route of infection	example
Skin contact	fungi
Through air or aerosols	flu
Through pricking (insects or needles)	malaria Yellow fever
Blood-blood contact	HIV-virus Hepatitis B
Through wounds	staphylococci
Through faecal material	typhoid bacteria Poliovirus

All these routes of infection may, depending on the type of work that is being performed, occur in the laboratory. As regards organisms that are able to spread through the air, very small droplets play a role, but infection may also be the result of direct contact, for instance with hands, handkerchieves, or clothes.

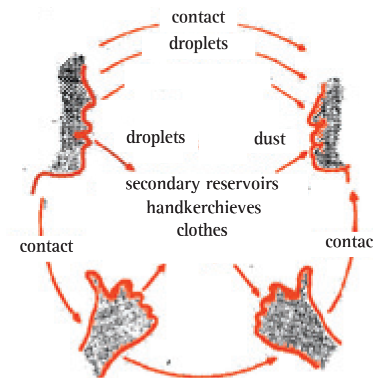


Figure 2: the spread of micro-organisms via air

Routes of contamination

Laboratory personnel may be exposed to organisms in different ways. Any open source of organisms (for instance an open petri dish) may lead to the spread of organisms. However, under normal circumstances, a container holding living pathogens of GMOs will only be opened in (semi) sterile surroundings, so as to prevent contamination of the container's content itself: for instance close to a Bunsen burner or in a safety cabinet. In practice, the cause of most laboratory infections is unknown. When the cause of the infection is known, it often concerns prick accidents, spilling, broken glassware, mouth pipetting, or biting or scratching by a laboratory animal.

Aerosols

One of the routes of infection that deserves special attention is infection through aerosols. Aerosols are very small droplets of fluid that can spread through the air. They are formed during activities such as opening bottles containing fluids and having a wet cap, vortexing, blending, emptying a pipette by blowing, or heating a wet inoculation needle in a flame. The formation of aerosols should be avoided as much as possible. When working with organisms that hold a certain risk (starting from risk class 2), one should perform aerosol producing activities in a safety cabinet.

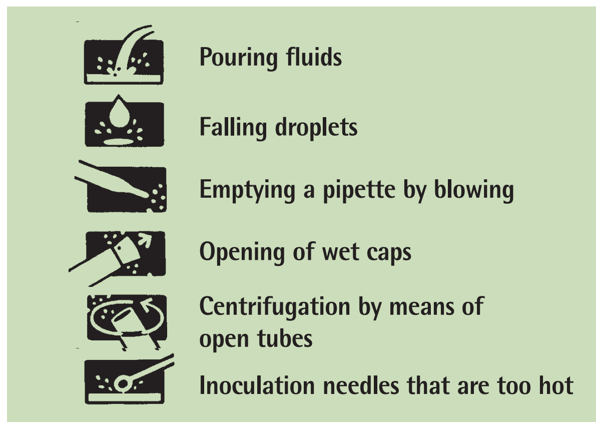


Figure 3: aerosolproducing procedures

Undesired spread of organisms or genetic material

It may have become clear that the spread of hazardous organisms represents a danger both to yourself and to your colleagues. When it is possible for organisms to spread to a colleague, they may spread to the environment as well. This dissemination of organisms or genetic material to the environment is often undesired, since it may involve the spread of

pathogens or toxins, or lead to the disruption of ecological balances. This is undoubtedly true with regard to organisms belonging to risk classes 2, 3 and 4. However, even the spread of organisms (and their genetic material) belonging to risk class 1, and thus presenting only a minor risk, should be limited.

Bacteria, yeasts and fungi

Bacteria are often capable of transferring genetic material. This is especially the case when vectors are used that are self-transmissible. In practice, to avoid genetic material from being easily transferred, vectors are usually used that are difficult to mobilise, or not mobilisable at all.

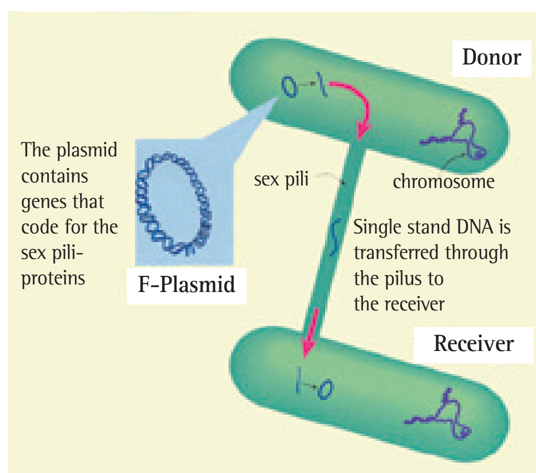


Fig. 4 Transmission of a self-transmissible plasmid through conjugation

Self-transmissibility

Natural plasmids in bacteria sometimes have the property of being able to transmit themselves to related bacteria. This means that, among other things, they carry the genes that are responsible for building the structures ('pilli') that are used by one bacterium to physically connect with another bacterium. The plasmid is then transmitted through this channel. The transmission frequency of self-transmissible plasmids is relatively high (10^{-3} to 10^{-5}).

Animal and human cells

Animal and human cells cannot spread to the environment just like that. In addition, non-contaminated cells are unable to spread genetic material to the environment by accident. Animal and human cells cannot survive in non-sterile surroundings. Cells that are specially designed to survive in non-sterile surroundings, such as fish or frog eggs, are an exception to this rule. As regards non-contaminated cells, the measures that are taken to prevent the cell culture from being contaminated are sufficient to prevent the cells from being spread to the environment. Genetic material of animal or human origin can only be spread to the environment when the cells involved are infected by biological agents, such as viruses, that are able to mobilize their genetic material. From a biosafety point of view, the question whether or not cells are infected by biological agents is very important. Any viruses present may represent a danger to the researcher or to the environment, and any safety measures should take account of this.

'Contaminated' animal or human cells

With regard to activities involving the use of animal or human cells, a distinction can be made between primary cells and established cell cultures. Primary cell cultures are created by growing cells directly from a biopsy. It is often not possible to determine beforehand whether or not these cells are contaminated by an infectious agent, such as a virus. For this reason, primary cell cultures should always be handled with care. Established cell lines are cultures that have been growing for longer periods of time and which are immortalised, so that they keep dividing infinitely. In most cases it is known whether they are free of contaminating biological agents. The immortalisation of such cells may have been achieved in different ways:

- spontaneously (as in NIH-3T3 cells)
- as a result of a natural viral infection (for instance HELA cells in which a few HPV virus genes, which cause immortalisation, are present)
- or by deliberate transfection of a factor that causes immortalisation.

When using virally immortalised cells in combination with vectors containing viral sequences, one should always check whether or not viral particles might be formed. If so, the cell line should be considered to belong to the same risk class as the virus concerned.

Viruses

A distinction can be made between wild type viruses and viral vectors (constructions derived from viruses). The use of viruses or viral vectors always implies the use of host cells. Without host cells no virus can be replicated. In practice, there are three types of activity: (1) the growing of cells to produce viral particles, (2) the handling of viral particle-containing supernatants (for quality controls, etc.), and (3) the transduction of a cell line, test animal or plant. Especially supernatants may contain very high levels of viral particles. These supernatants should be handled carefully. Once the cell, animal or plant has been infected, the danger depends on the virus' or viral particle's ability to replicate. In some

cases a replication-defective virus is used, which means that the virus can infect the cells, but is no longer able to replicate. The ability to spread or replicate may differ from one virus to another. Some viral particles are able to spread through the air or to survive for very long periods of time. Other viruses, such as HIV, are extremely vulnerable outside their host. Plant viruses sometimes need 'vectors' to be able to spread. These vectors are often insects that suck up the virus and spread it to other plants.

Transgenic plants

Transgenic plants are grown *in-vitro,* in growth chambers or greenhouses, and the plants are not able to disseminate just like that. Nevertheless, the undesired spread of transgenic plants deserves special attention. If no proper containment measures are taken, pollen may be disseminated to the environment through the air or aided by insects. Whether or not this presents a genuine risk, depends on how the plant reproduces: by self-pollination or by cross-pollination. The spread of pollen by strict self-pollinators has no effect whatsoever, but when a cross-pollinator is involved, it should be carefully checked whether any of its wild relatives, which it might successfully hybridize with, is growing in the vicinity. In addition to pollen, seeds originating from transgenic plants may sometimes easily be disseminated in the environment. Especially when they are very small or sticky, these seeds are very likely to be accidentally taken along by a researcher leaving the growth chamber or greenhouse.

It is not only pollen or seeds that may be responsible for the undesired spread of transgenic plants. Some plant parts may grow and turn into whole new plants themselves. These reproductive parts of plants should not be discarded without destroying them properly. For example, the branch of a willow can grow roots and leaves very easily, and the stembase of a cabbage can also grow roots. This is why laboratory staff handling transgenic plants or plant material should pay special attention to the possible spread of plant parts that are still able to reproduce. If there is a genuine possibility that a transgenic plant will be able to establish itself in the environment, or that it will hybridise with wild relatives, reproductive plant parts should be destroyed before they are discarded as waste.

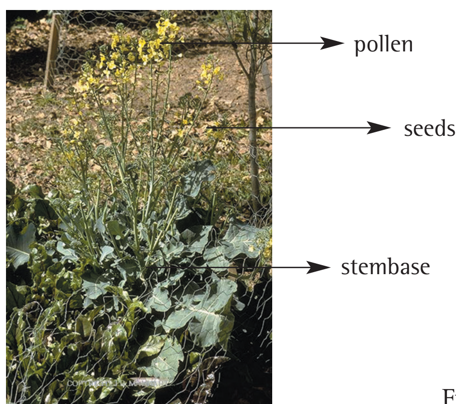


Figure 5: the reproductive parts of Brassica

The spread of transgenic plants or transgenic plant materials

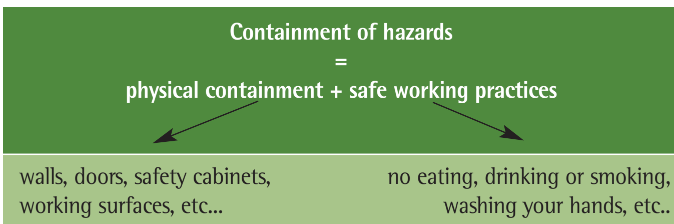
	Pollen	Seeds	Reproductive materials
Arabidopsis	In containment Arabidopsis is a self-pollinator. The spread of pollen is very unlikely to have an impact.	Seeds are very small; upon touch they can be catapulted into the air and spread very easily. Special 'aracons' or other comparable means of containment should be used.	No other than pollen and seeds.
Tobacco	Self-pollinator. The spread of pollen is very unlikely to have an impact.	No special remarks.	No other than pollen and seeds.
Rice	Self-pollinator. The spread of pollen is very unlikely to have an impact.	No special remarks.	No other than pollen and seeds.
Tomato	Insect / self-pollinator. Pollinating insects should be kept out.	No special remarks.	No other than pollen and seeds.
Potato	Self-pollinator. The spread of pollen is very unlikely to have an impact.	No special remarks.	The spread of tubers should be prevented.
Brassica	Insect / self-pollinator. Pollinating insects should be kept out.	No special remarks.	The spread of roots and stembases should also be prevented.
Maize	Wind-pollinator. Spread of pollen from the greenhouse is unlikely, especially when windows are fitted with insect nets.	No special remarks.	No other than pollen and seeds.
Wheat	Self-pollinator. The spread of pollen is very unlikely to have an impact.	No special remarks.	The spread of roots and stembases should also be prevented.

Transgenic animals

The unwanted spread of transgenic animals should be prevented. Depending on the animal, this can be very easy or rather difficult. Small rodents, like mice, should be kept in appropriate cages and the animal houses should be designed in such a way that it is impossible for the animals to escape. When a genetically modified micro-organism or a wild-type pathogen is administered to the animal, it should be determined on a case-by-case basis how to prevent the micro-organism from spreading. It may be necessary to keep the animals in individually ventilated cages, and to inactivate all materials that have been in contact with the animals (for instance the bedding material). When cells or other biological material are used in animals, it should be taken into account that viruses may be present in this material. Some cell lines are contaminated by viruses. If such viruses are present, the containment measures should be adapted if there is a risk that the virus might spread.

5. CONTAINMENT: A COMBINATION OF INFRASTRUCTURE AND WORKING PRACTICES

Four levels of containment appropriate to the four risk classes for pathogenic and/or genetically modified organisms have been defined. Containment is achieved through a combination of physical containment measures and safe working practices.



Each subsequent containment level (i.e. from level 1 to 2, 2 to 3, etc.) requires a number of additional containment measures and safe working practices. These containment levels have not only been formulated for laboratories, but also for greenhouses, animal houses and large-scale process installations. The annexes to this booklet contain an overview of the containment measures required in laboratories, animal houses and greenhouses. The requirements for large-scale process installations and hospital rooms can be requested from your biosafety coordinator or at www.biosafety.be.

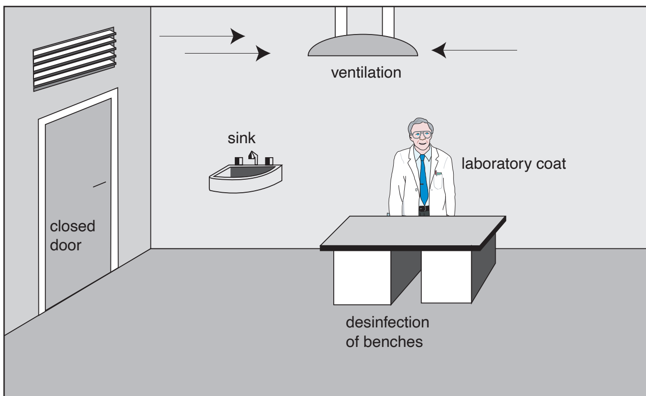


Figure 6: Containment in a laboratory

Containment at the source

As is the case for any other activity that may present a risk, one should always try to tackle the risk at its source. Among other things, this means that one should always opt for organisms, host organisms or vectors presenting less risk, but providing the same results as their alternatives that do pose a threat. In order to achieve containment at the source, researchers should, if possible:

- use a biologically contained or attenuated laboratory strain instead of a wild-type strain,
- use a non-mobilisable vector instead of a self-transmissible one,
- use a replication-defective virus or viral vector instead of a replication-competent one.

Physical containment

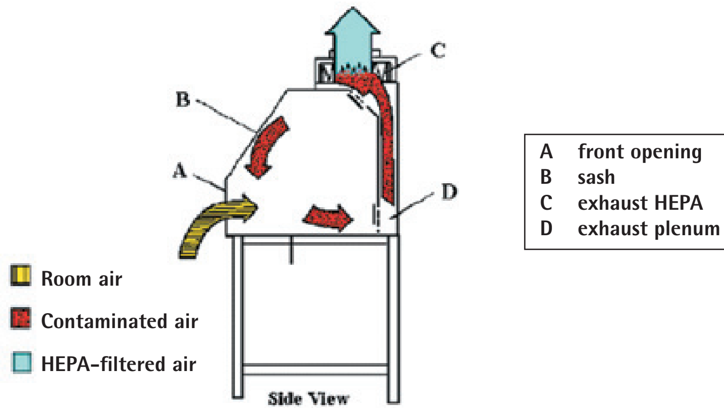
Physical containment comprises all physical measures that are taken to protect organisms from the surrounding environment and vice versa. This starts with the container holding the organism (for instance, a tube), but it also comprises the structure, floor, walls, ceiling, doors and windows of the laboratory, as well as benches that are suitable for working with pathogenic organisms or genetically modified micro-organisms. To allow the handling of micro-organisms, the benches should have a smooth surface and be readily decontaminable. A sink should be present to allow laboratory staff to wash their hands after an experiment. When a higher containment level is required (2,3 or 4), a number of additional physical containment measures will be taken (depending on the risk the activity represents), such as:

- class I, II or III safety cabinets
- negative air pressure in the laboratory
- an air-lock
- a shower
- HEPA filtration of the exhaust air
- special cups and other equipment to prevent the spread of aerosols
- gas-tightness of the laboratory to permit gas decontamination
- etc.

An overview of all legally required physical containment measures is listed in annex 1.

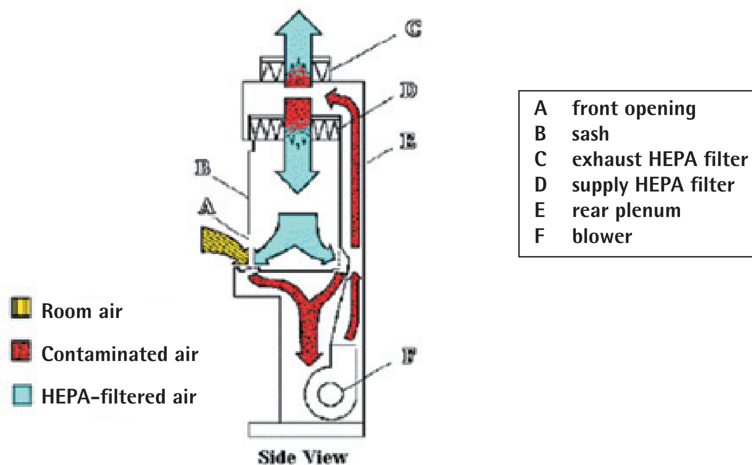
Safety cabinets

A safety cabinet is an important type of physical containment. In fact, a safety cabinet creates a safe working space within the laboratory. Safety cabinets are designed to protect the worker in the first place and the environment in the second place. A distinction is made between three types of safety cabinets: class I, II and III. A class I cabinet is a fume hood in which exhaust air passes through a HEPA filter. This type of cabinet provides worker protection, but no experiment protection.



Class II safety cabinets

A class II safety cabinet (a ‘downflow’ cabinet) provides worker, environmental and experiment protection. These cabinets have a downward laminar airflow. There are many different types of class II cabinets.



The proper use of a class II safety cabinet

A class II safety cabinet can only provide effective worker, experiment and environmental protection if it is properly used. For this reason, disruptions of the air flow inside the cabinet should be avoided as much as possible, which boils down to the following:

Guidelines for the proper use of a class II safety cabinet

1. Prepare an experiment thoroughly and collect all necessary materials before beginning work.

2. Allow the cabinet to run for 10 minutes before beginning work.

3. Decontaminate the work-surface and air intake grilles with a disinfectant such as 70 % ethanol.

4. Only place materials and equipment in the cabinet which are required for immediate work.

5. Never place objects over the front or rear intake grilles.

6. Remember to always work from a "clean" to a "dirty" side. On the dirty side you should place a small container for contaminated items such as pipette tips.

7. Move your arms slowly in a manner that will minimise the disruption of the airflow.

8. Perform all work well inside the cabinet, not half outside it.

9. Never use a Bunsen burner, since this may create turbulence in the airflow. Use disposable inoculation needles instead.

10. When your work is finished, decontaminate all materials (on the outside) before removing them from the cabinet.

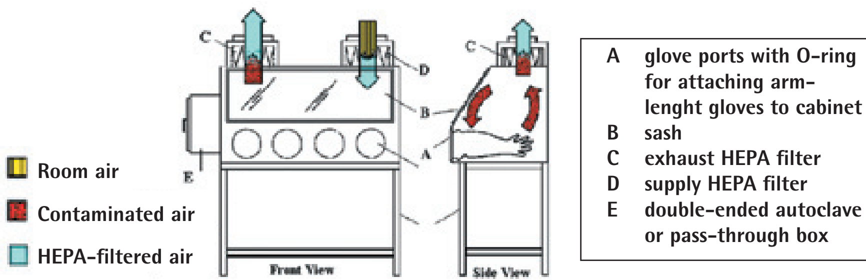
11. Decontaminate the working surface and the air intake grilles.

12. Allow the cabinet to run for another 5 minutes before turning it off.

13. Close the cabinet lid.

Class III safety cabinets

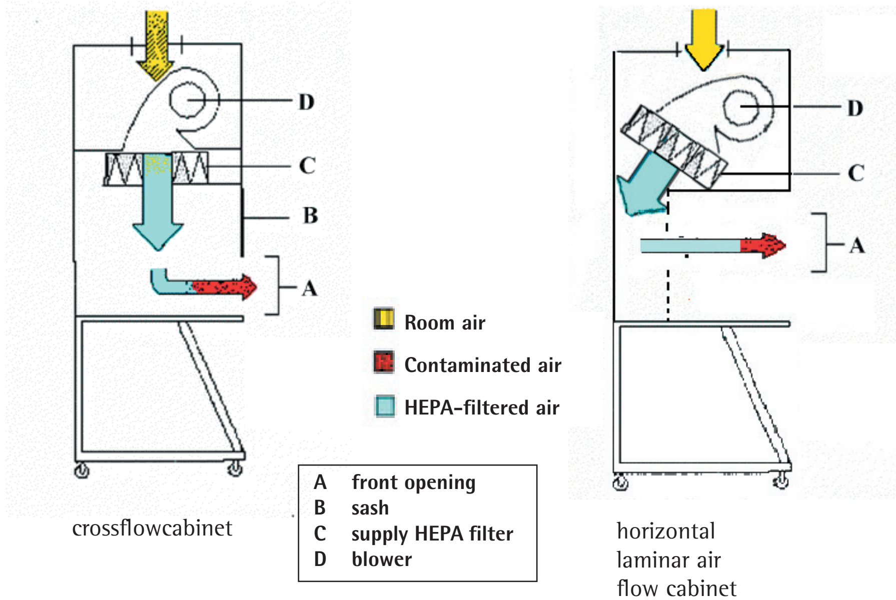
A class III safety cabinet provides maximum protection to the worker and the environment. The experiment, however, is less protected, because there is no downward air flow inside the cabinet. A class III cabinet is completely sealed, and arm-length rubber gloves are attached to ports in the cabinet in a gas-tight manner to allow for manipulation of the materials isolated inside. Materials are brought into and removed from the cabinet through a small air-lock or double-ended autoclave.



Safety cabinet	Protection		
	worker	environment	experiment
Class I	good	good	bad
Class II	good	good	good
Class III	excellent	excellent	good

Horizontal laminar air flow cabinets and cross flow cabinets

In laboratories where animal cells or in vitro plant materials are manipulated, use is sometimes made of a horizontal laminar air flow cabinet or a cross flow cabinet. These are cabinets in which sterile air is blown into the cabinet, whereas the contaminated air is blown into the laboratory space. This means that the laboratory worker is sitting in the contaminated air flow. In other words, these cabinets provide protection neither to the worker, nor to the environment. They are therefore not considered as real safety cabinets, and their use should be discouraged in most cases. These cabinets cannot be used for activities posing a risk to the worker or to the environment. The use of such cabinets may be permitted exceptionally, when no use is made of open containers holding GMOs, such as open petri dishes containing modified bacteria or opened Erlenmeyer flasks containing bacterial cultures.



crossflowcabinet

horizontal laminar air flow cabinet

HEPA filters
HEPA stands for 'High-Efficiency Particulate Air' filter. This filter contains a matrix which is folded many times; thus, a very large surface is reduced to a relatively small volume. HEPA-filters are highly efficient in capturing particles: only a maximum of 0.03 % of all particles at 0.3 micron in size can pass through them. Owing to its characteristics, a HEPA filter has a greater efficiency in capturing particles bigger and smaller in size than 0.3 micron.
Checking the functionality of HEPA filters
Once a year the HEPA filters in safety cabinets should be checked by specialists to see whether or not they are still effective. If not, the filters should be changed.

Safe working practices

Physical containment alone is not enough to create safe working conditions. It should be combined with safe working practices. Specific working practices have been defined for each containment level.

Basic working practices: Safe Microbiological Techniques

As far as level 1 containment is concerned, the working practices contribute most to creating safe working conditions. These basic working practices are called "Safe Microbiological Techniques" (SMT). Everyone that manipulates micro-organisms and cells in a laboratory should make it a daily routine to follow these procedures.

Safe Microbiological Techniques

1. Keep doors and windows closed when experiments are in progress.

2. Wear a laboratory coat.

3. Eating, drinking, smoking and storing food and drinks are not permitted in the laboratory.

4. Do not wear jewelry and/or a watch; keep your hands clean and your nails short at all times.

5. Decontaminate any spills of GMOs immediately:
   - remove fluids with tissues or paper towels and throw these into the waste basket for biologically contaminated waste
   - decontaminate the surface on which the material was spilled with 70% ethanol or another validated disinfectant
   - wash your hands afterwards

6. Minimise the creation and spread of aerosols by:
   - only using closed tubes to centrifugate
   - preventing caps from becoming wet
   - heating wet inoculation needles in the proper way, i.e.: first heat the shaft, then the eye
   - allowing inoculation needles to cool down before putting them back into the fluid
   - never using force to empty pipettes, but allowing gravity to do its work
   - pouring out fluids in a gentle manner and never from a great height

7. Mouth pipetting is forbidden:
   - always use a pipetting bulb or other mechanical pipette
   - use a mechanical micro pipette for small amounts

8. Decontaminate used materials before washing and reusing them:
   - this can be done by autoclaving them, or by immersing them in a validated disinfectant

9. Inactivate biological waste:
   - put the waste in a waste basket for biological waste. The waste should then be transported to the autoclave or to a waste incinerator that is capable of burning contaminated hospital waste.

10. Wash your hands after the experiment and before leaving the laboratory:
    - use disinfecting soap to do so

In addition to following these safe working practices, it is important to

1. keep a detailed laboratory notebook, containing a daily account of all biological materials used and all manipulations performed
2. routinely check the biological material you work with. If you receive material from a colleague, you should always check whether it is indeed the material you expected to receive (check the strain, the plasmid and its restriction pattern). It is equally important to check your own strains on a regular basis. Alternatively, you can start from a new, clean stock on a regular basis.

Any basic laboratory facility where the safe microbiological techniques are applied, meets the requirements of containment level 1. The laboratory itself does not need to be equipped with more than the basic facilities, such as a floor, wall, ceiling, smooth, readily cleanable work surfaces and a sink. Containment levels 2, 3 and 4 require a number of additional precautions, a.o.:

- gloves should be worn when experiments are being conducted
- access to the laboratory is restricted to authorised personnel only
- an insect and rodent control programme is in effect
- all liquid effluents released from sinks and showers should be inactivated
- additional measures to prevent the creation of aerosols should be taken, for instance, procedures with a potential for creating aerosols should be conducted within a safety cabinet
- clothing should be decontaminated before leaving the facility
- etc...

An overview of the requirements of containment levels 1 to 4 is given in annex 1.

The use of gloves

There are many misunderstandings regarding the use of gloves and in many cases gloves provide a false sense of safety. For level 1 activities gloves are not required. The application of the safe microbiological techniques is sufficient to work safely. Gloves are no basic requirement for level 2 either. But they should be worn when direct contact with the biological material poses a hazard, or prick or cutting injuries might occur, for instance when people are working with sharps and biologically hazardous material. Gloves are mostly used to prevent contamination of the experiment with micro-organisms, DNA or RNA present on your hands. One should not forget, however, that gloves can bear this material just as well, especially when they have been worn for a longer period of time and when they have been in contact with all kinds of materials. Thus, when using gloves, laboratory staff should keep the following guidelines in mind:

- Only wear gloves when strictly necessary, i.e. only when there is a genuine risk that you or the experiment might be contaminated.
- Only wear gloves when you are actually conducting the experiment. Do not wear gloves when handling all kinds of other materials like closed buckets, or when writing in your laboratory notebook.
- When using gloves, change them on a very regular basis.

Restricted access

From level 2 activities onwards the access to the laboratory is legally restricted. This means that only personnel that meets specific entry requirements is authorised to enter the laboratory. In practice, to prevent unauthorised personnel from gaining access, the laboratory should be kept locked when nobody is working inside. For level 3 and 4 activities this is an absolute requirement.

Authorisation to work in level 2,3 and 4 laboratories should only be given when the laboratory worker has proven that he has sufficient knowledge of safe working practices, that he has learnt all the specific safety procedures for this particular laboratory and knows how to apply them. There should be records of authorised personnel, which should be updated on a regular basis.

To prevent unauthorised access, level 2, and especially level 3 and level 4 laboratories should not be located near the entrance or exit of floors or buildings, or in areas where many people circulate.

6. CONTAMINATION, ACCIDENTS, DECONTAMINATION AND INACTIVATION

Minor contamination incidents can happen sooner than one might think. Some fluid may be spilled when it is being poured out, or some droplets may splash. It is important to deal with contaminations immediately. Other accidents, like the breaking of glassware, needle-prick or cutting accidents, may also happen before you know it. These different kinds of accidents always result in exposure to the organism. There is no immediate risk when a harmless class 1 organism is involved, but even in such cases it is necessary to clean and decontaminate. Not surprisingly, because if this is not done, micro-organisms or their remains may accumulate in the laboratory and thus become a potential source of contamination for the work of other laboratory staff. It goes without saying that accidents with organisms posing a genuine threat either to the laboratory worker and other humans or to the environment (risk class 2, 3 and 4 pathogens or GMOs) are possible as well. If such an accident occurs, it is an absolute must to clean and decontaminate immediately. Spills on damaged skin, or spills on intact skin of for instance human pathogenic viral particles, should never be disregarded.

Prevention

Since prevention is always better than cure, it is important to avoid accidents whenever possible. This can only be achieved by working carefully and thoughtfully. Experiments should be carefully planned, all necessary materials should have been collected beforehand, and afterwards everything should be put back where it belongs. Sloppiness is the major cause of accidents. Scalpels, for instance, should never be left unattended on one's bench. Put them in a safe container instead. As for needles, they should never be recapped after use. Place them in a special needle-waste container.

Notification

When a laboratory staff member has been exposed to dangerous human pathogens, this should always be reported, and not only when blood is pouring everywhere. An accident should be reported, even when there has been only a minor risk of contamination, or when there are doubts about the possibly hazardous characteristics of the material used (i.e. non-tested human blood). The people responsible for biosafety and/or workers protection should be notified, and if necessary, a doctor and insurance company (with a view to recovering the costs of expensive drugs) as well. Immediately after the accident the

appropriate measures should be taken. Antiviral drugs, for instance, should be administered to the victim as soon as possible after the accident.

Prick or cutting injuries

An accidental prick or cutting injury, or a splash in the eye or on the mucosae with material that is infectious to humans, should be dealt with as follows:

1. allow the wound to bleed freely
2. rinse the wound thoroughly
3. decontaminate the wound
4. provide further care (bandages, etc.) if necessary
5. make a note in the first aid notebook.

Depending on the type of agent the victim has been exposed to, further measures may be necessary. When the victim has been exposed to non-tested human blood, for instance, it should be determined whether he/she has been infected with Hepatitis B, C or HIV, and if so, appropriate therapy should be decided on.

How to decontaminate materials and work surfaces

There are several disinfectants available, all of which are characterised by their own specific mode of action. In general, there are two common modes of action:

1) by destroying the lipid membrane of a micro-organism which results in the leaking of cell material
2) by destroying proteins and enzymes necessary for the survival of the micro-organism

Ethanol, quaternary ammonium salts and surfactants (a.o. detergents and soap) exploit the first mode of action. Strong oxidising agents like chlorine of hydrogen peroxide exploit the second mode of action. Phenolics like lysol work by destroying both proteins and the lipid membrane.

Some more information on specific disinfectants:

- **Ethanol (alcohol)**
  Ethanol is highly effective against vegetative bacteria, fungi, and viruses surrounded by a lipid membrane, but not against spores. Its effectiveness against non-lipid viruses strongly varies. Ethanol should be used as a 70% solution. Mixtures of ethanol and other substances, such as ethanol mixed with 100 g formaldehyde per liter or 2 g chlorine per liter, are more effective than ethanol alone. Note that the degenerated alcohol used in laboratories is poisonous. Solutions of ethanol deteriorate over time, which is why they should be replaced at least once a month.

- **Sodium hypochlorite (bleach)**
  The chlorine released is a strong oxidising agent, which is effective against all types of micro-organisms. Sodium hypochlorite must be used as a 20 ml/l to 100 ml/l solution, depending on how “dirty” the circumstances are, for instance a 1 in 10 solution of domestic bleach. Solutions of chlorine deteriorate quickly. They should be replaced at least once a week to ensure their decontaminating effect.

- **Formaldehyde**
  Formaldehyde is a gas that is effective against all micro-organisms, provided that it is used at a temperature higher than 20°C and at a humidity of at least 70%. It is suited for the decontamination of fluids, safety cabinets and even whole laboratories, by means of atomisation. Note that formaldehyde is a suspected carcinogen. Moreover, it is an irritant and should not be inhaled. For this reason, formaldehyde is only used in exceptional cases, when a safety cabinet or the whole laboratory needs to be decontaminated. Examples are an airtight L3 laboratory that has been contaminated, or an airtight animal house, the floor and walls of which may be covered with dangerous micro-organisms. Decontamination by means of formaldehyde is a time-consuming and dangerous procedure that can only be performed safely by specialists. It is therefore recommended that the safety and prevention service be consulted before such procedures are carried out.

- **Hydrogen peroxide**
  Like hypochlorite, this substance is effective against all types of micro-organisms, owing to its oxidising characteristics. It is used as a 6% solution. Hydrogen peroxide is not suited for use on aluminium, copper, sink or bronze.

- **Modern wide-spectrum disinfectants**
  There are many modern wide-spectrum disinfectants on the market. Mostly they combine different chemical substances with different modes of actions against micro-organisms. They can contain oxidising chemicals, quaternary ammonium salts, surfactants and other substances. These disinfectants can be used for decontamination as well, provided that they are validated for the types of micro-organisms you work with. Do not only check the user manual of these disinfectants, but also check what substances in what concentration are present and what modes of actions are combined in the disinfectant.

Decontamination, especially the decontamination of surfaces, can never be 100% effective. Decontamination can only result in a sharp reduction of the number of viable micro-organisms (a well-performed bacterial decontamination will lead to a 90% reduction, viral decontamination is often more effective). What is more, after the decontamination, the remains of micro-organisms often stay present. This is why it is important to use hot water and soap to clean the work surfaces, floors and door knobs during the weekly cleaning of the laboratory. You need to decontaminate twice in order to decontaminate successfully.

The effectiveness of different decontaminants

	Fungi	bacteria	mycobacteria	spores	lipid viruses	non-lipid viruses
Ethanol	-	+++	+++	-	+	V
Hypochlorite	+	+++	++	++	+	+
Formaldehyde	+++	+++	+++	+++*	+	+
Peroxide	+	+++	++	++	+	+

* above 40 °C V = variable

Inactivation

It is often quite difficult to destroy biological material. Bacterial spores, for instance, are resistant to temperatures of 100°C. Sterilisation - heating water under pressure at a temperature of 121°C for 20 minutes - is the recommended method in such cases. Sometimes the water is even heated at temperatures of 134°C. This is a very effective way to inactivate micro-organisms, provided that the steam can reach all areas in need of sterilisation. Air pockets are a well-known problem. Even animal cells are known to be able to survive in an air pocket during the sterilisation process. A sterilisation tank should be filled in a very careful manner, and all caps should be loosened. Sterilisation is the recommended method to kill bacteria, yeasts, and fungi. For other organisms, like plant and animal cells, and some viruses, simpler methods are available. Heating up to 80°C or exposing the cells to strong detergents is often sufficient to kill them. It is, however, highly important to check whether the method to be used is a validated one; in other words, whether the organisms will be effectively destroyed. Inactivation is much more effective in killing micro-organisms than superficial decontamination: if well-performed, inactivation can be 100 % effective.

When bacteria present in fluids need to be killed, sodium hypochlorite solutions are often used. It is important to make sure that the final level of chlorine in such solutions is high enough to kill the bacteria (20-100ml/l). It should be mentioned that sterilisation is still the most environmentally friendly method. In practice, the fluids containing chlorine are poured down the drain all too often. Principly, fluids containing substances that might affect the proper functioning of a water purification plant should not end up in the company's waste water. For a decontaminant to be effective, it is also very important that it is in contact with the micro-organisms long enough, i.e. for at least 15 to 30 minutes in general.

The appropriate decontamination and inactivation method for some relevant organisms

	Decontamination				Inactivation		
	1% NaOH	2% glutar-aldehyd	70% ethanol	Formal-dehyd	Wet heat 121 °C, 20'	Dry heat 160 °C, 60'	Heating 60 °C
E.coli	x	x	x	x	x	x	
Lactobacillus	x	x	x	x	x	x	
Salmonella	x	x	x	x	x	x	
Aspergillus	x	x		x	x		
Adenovirus	x	x					x
Influenzavirus	x	x	x	x			x, 30’
HIV	x	x	x				x, 30’
Vaccinia	x	x		x			x**

* only applies to small volumes of serum

** only for heat-labile antigen vaccinia

Biological waste

Biological waste must be disposed of. An important distinction should be made between biological waste that has been inactivated before disposal, and biological waste that has not been inactivated before disposal. The latter has to be treated as hazardous medical waste and should be transported to an incinerator that is suited for the incineration of hazardous medical waste.

Biological waste includes:

- all genetically modified and/or pathogenic biological material: cell cultures, cultures of micro-organisms, tissues, blood, etc.
- typical laboratory waste of organic origin: gels, etc.
- all kinds of biologically contaminated material: gloves, paper tissues, disposable culture flasks, pipettes, etc.
- materials that are not necessarily contaminated, but cannot be thrown into an ordinary waste disposal bag because they have sharp edges or look dirty (bones, blood, etc.).

Biological waste does not include:

- radioactive contaminated material. Such material should be dealt with separately.

How to manage biological waste

A distinction can be made between solid or pasty waste and fluid waste. Depending on whether the material is inactivated and/or chemically polluted, waste is categorised as follows:

Type of waste	Inactivated	Chemically polluted	Destination
Solid/pasty waste	+	-	=> residual waste
Solid/pasty waste	+	+	=> chemically polluted waste
Solid/pasty waste	-	+ or -	=> hazardous medical waste
Fluid waste	+	-	=> waste water
Fluid waste	+	+	=> chemically polluted waste
Fluid waste	-	+ or -	=> hazardous medical waste

Hazardous medical waste is collected in special containers that are suited for the transport of medically hazardous waste. Once closed, these containers cannot be opened anymore. In addition, they are resistant to leaking, so that falling from heights poses no problem. These special containers should be transported by an accredited transporter to an incinerator that is suited for the incineration of medical waste on a very regular basis. The following guidelines exist for the temporary stocking of the containers, and the transport to the incinerator:
- a maximum of 2 months, at a temperature of no more than 4°C;
- a maximum of 2 weeks, at a temperature of no more than 20°C;
- a maximum of 1 week, if the temperature of the stock room can become higher than 20°C

Biological waste from animal houses

Waste from animal houses belongs to the same categories mentioned above. Transgenic animals such as mice or rats should be killed and taken to an incinerator. The bedding material and faeces of these animals should not be regarded as biological waste. This material can be disposed of as residual waste without inactivating it beforehand. It is a different matter, however, when tests involving genetically modified and/or pathogenic micro-organisms are performed on animals. The micro-organisms used are a source of contamination. Animals containing these micro-organisms should therefore be killed and sterilised or incinerated (as hazardous medical waste) and all the bedding material and faeces should be collected and inactivated (either in-house or in an incinerator).

Waste from plants

Care should be taken in dealing with waste material from transgenic plants. The plants' reproductive parts should all be collected. Flowers (containing pollen), seeds, and - depending on the type of plant - other reproductive parts should be collected and inactivated (see chapter four of this booklet).

There are different ways to inactivate this material : sterilisation is the most rigorous method, but simpler methods may sometimes be enough, like chopping the material into small pieces. Also steaming of the plant material, or soil that has been into contact with the transgenic material is a very good means of inactivation. All non-reproductive parts of transgenic plants can be composted (dry or wet) and after being composted this material can be disposed of as residual waste, or garden waste.

When plants have been infected with genetically modified and/or pathogenic micro-organisms, they should be inactivated as a whole, as well as the soil or other possibly contaminated material.

7. WORKING WITH COMMONLY USED LABORATORY ORGANISMS

Working with genetically modified *Escherichia coli* and fages

Classification

Genetically modified *E.coli* K12, B and C strains and all fages can be manipulated in laboratories that meet the L1 criteria on condition that:

- the vectors used are not self-transmissible
- the genes inserted do not produce hazardous products (such as toxins) or have hazardous effects

These K12, B and C strains are attenuated (weakened) laboratory strains that have been biologically constrained and are no longer pathogenic. The table below lists some examples of classifications, which have been made on the basis of the guidelines in annex 2. Because it is assumed that the inserts used in these examples are not hazardous, article e or j of the guidelines are applicable (depending on whether or not the insert has already been characterised). It goes without saying that the nature of the insert is a strong determinant of the final classification. This means that, when hazardous inserts are used, for instance one that can produce a toxin, another article of the guidelines will be applicable and a higher level of containment will be required.

Strain	Vectors	Classification*	Classification rule**	Comments
Belonging to *E. coli* K12, B or C	Non-selftransmissible vectors *(tra⁻)*	L1	1.e or j	Most commercially available systems belong to this category
Belonging to *E. coli* K12, B or C	Selftransmissible vectors *(tra⁺)*	L2	2.e or j	An example is some mini Tn5 systems
Other *E. coli* strains	Non-selftransmissible vectors *(tra⁻)*	L2	3.e or j	Unless proven otherwise one should consider these hoststrains to be pathogenic

* on the assumption that non-hazardous inserts are used (hazardous inserts would require a higher risk class)

** as applied in the schemes of annex 2

Laboratory requirements

In many cases *E.coli* K12, B or C strains are used in combination with non-self-transmissible vectors. In such cases a basic L1 laboratory is sufficient. The work surfaces should of course be easy to decontaminate. Open handling of E. coli on a bench is permitted, provided that the creation of aerosols is minimised (see SMP guidelines). Inoculations can be performed near a flame. Instead of working with open sources on a bench, researchers can alternatively perform these operations in a safety cabinet. The requirements of Safe Microbiological Practices should be met.

Waste

All solide and pasty materials (culture mediums); all liquids containing modified coli bacteria and all materials that are possibly contaminated with coli bacteria should be inactivated or decontaminated. Living coli bacteria must never end up in the residual waste. Clearly marked waste bins for biological waste should therefore be present, and fluids should be sterilised before they are poured out in the sink. Alternatively, chlorine could be used to inactivate the materials, but this is a less environmentally friendly option. An exception to this rule is *E. coli* K12 strains carrying a non-mobilisable (mob-) and well-characterised vector, a fully characterised insert (with regard to its origin, size, gene product, function(s), etc.), and producing a fully known, non-hazardous gene product. Strains meeting these criteria do not need to be inactivated. However, these are very rare exceptions, which hardly ever occur in research settings. Moreover, an explicit and specific authorisation stating that inactivation is unnecessary is always required.

Note

It should be checked on a regular basis (twice a year) whether the used strains are still the proper ones. Alternatively, the strains can be replaced regularly by new ones from a clean stock.

Risk assessment example

As an illustration of how a risk class and the necessary containment measures are determined, and also how this booklet can be used, some practical examples of risk assessment are given below:

Practical example of how to determine classification

1. Cloning a chymosin gene into a pUC18 using *E.coli* JM109 as a host, and performing small-scale, standard laboratory procedures only.

Step 1: Is the combination of host and vector suited for risk class 1? To find this out, the *E. coli* strain should be looked at first: all *E. coli* K12, B and C strains are suited for risk class 1, and it is

therefore sufficient to check in available catalogues, with suppliers or in strain databases (ATCC, DSMZ, etc.) whether the JM109 strains belong to these classes of strains. *The answer in this case is:* **yes, JM109 belongs to the** *E. coli* **K12 class.**
Next, the vector's suitability for risk class I should be determined. PUC18 is a well-characterised vector that has no hazardous properties and is non-self-transmissible. **The vector is suited for risk class I.** (a detailed description of the requirements for vectors can be found in annex 2, chapter A to this booklet. A description of what is meant by "well-characterised" is also given there). **It can therefore be concluded that the combination of this specific host and vector is suited for risk class I.**

Step 2: Now the relevant containment category can be looked up in annex 2. **In this particular case, category 1 of the first direction of annex 2, chapter A, is applicable** (since the combination of host and vector is suited for risk class I) However, a final assessment cannot be given before the risk the insert itself poses has been determined.

Step 3: In this case a piece of bovine genetic material encoding for the production of chymosin, a cheese-clotting enzyme, is used. It is well-characterised, known to be no toxin, to have nothing to do with viruses or pathogenic organisms, and to pose no threat in any other way either. It can therefore be concluded that category j of the second direction of annex 2, chapter A is applicable here, and that the corresponding containment level is **L1.**

Step 4: Are there any special details with regard to (1) the environment that might be exposed to the materials, (2) the type and level of the activities, and (3) any non-standard procedures? In this particular case only small-scale, standard activities will be performed, such as transformation, small-scale culturing of bacteria in Erlenmeyer flasks, protein isolation, gel electrophoresis. An activity is only considered to be large-scale when large volumes are being produced in production facilities. However, even small fermenters require special attention as far as their leak tightness and the physical containment of the downstream processing are concerned. *In this case no special procedures, representing an additional risk or requiring a specific form of additional containment, are performed. The eventual containment level will therefore be* **standard L1-containment. This booklet provides the following information on this topic:**
• L1-containment requirements: annex 1 to this booklet
• Detailed SMP-procedures: chapter 5 of this booklet
• Decontamination and inactivation procedures: chapter 6 of this booklet.

A possible variation to this example:

Cloning the chymosin gene into a self-transmissible plasmid in *E.coli* JM109 and transferring the modified bacteria onto a plastic matrix, during which procedure the formation of aerosols cannot be avoided.
In this case, the content of step 2 changes: the vector does not meet the risk class I requirements. As a consequence, the second category of the 'first direction' of annex 2, chapter A, now becomes applicable instead of the first one. The gene product is still well-known and non-hazardous. As a result, category j of the 'second direction' remains applicable and the corresponding containment level is **L2**. What is more, a very specific aerosol-producing procedure is being performed, for which an additional containment measure is required, on top of the standard L2 measures: the aerosol-producing manipulations should be performed in a class II safety cabinet. **The eventual containment level is therefore L2, supplemented with the use of a class II safety cabinet. This booklet contains the following information on this topic:**
• L2 containment requirements: annex 1 to this booklet

- Description of how to use a class II safety cabinet: chapter 5 of this booklet
- Decontamination and inactivation procedures: chapter 6 of this booklet.

Are there any cases requiring special attention with regard to additional containment measures that might be necessary?

1. The formation and dissemination of aerosols cannot be minimised (cfr. variation above). Using a class II safety cabinet will be required in many cases.
2. GMOS or pathogens are handled for a longer period of time during a non-destructive measurement without any containment measures being taken. If the organisms pose a hazard it should be determined whether they might spread. If so, the laboratory worker and the laboratory itself often need extra protection (for instance negative air pressure and HEPA filtration of the exhaust air and/or gloves and face protection).
3. GMOs or pathogens are manipulated in fermenters and purified in a downstream process. Some fermenters are more leaktight than others, which is also true of downstream processing equipment. When risk class II, III or IV organisms are used, the fermenter's air exhaust system should contain a special hydrophobic absolute filter or a HEPA filter.
4. GMOs or pathogens are administered to a plant or animal (internally or externally). It should be determined on a case-by-case basis whether dissemination of the GMO or pathogen to the environment should be countered, and how. The measures to be taken strongly differ from one plant or animal to another. The method of administration is often decisive for the way in which a micro-organism is able to spread.

What should one do when there are doubts about the risk class and necessary containment measures?

In case the preceding step does not give you a decisive answer, you should subsequently

1. look up any missing information that might help you in assessing the materials
2. consult your superior
3. consult your group leader
4. consult internal biosafety experts (see annex 5 to this booklet), or external biosafety experts (www.biosafety.be)

Working with non-pathogenic genetically modified yeasts

Classification

Non-pathogenic genetically modified yeasts, such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Pichia pastoris* can all be handled under L1 containment conditions, provided that no hazardous genes (e.g. genes producing toxins) have been cloned into the organism. The yeast strains used in laboratories are all deficient in some way and need a special feeding medium to which a particular essential substance has been added. These deficiencies are often deficiencies in amino-acid.

Laboratory requirements

The standard L1 laboratory facilities as required for *E. coli* are sufficient here. However, it is recommended to perform procedures during which the yeasts are exposed to the envi-

ronment in a safety cabinet. Yeasts are airborne and able to survive in the air for long periods of time. In this way they can contaminate all kinds of other cultures. In addition, sensitised people might suffer from allergic reactions after having been exposed to yeast or its spores. For this reason, yeast is often manipulated in a separate laboratory.

Waste

All waste consisting of or containing genetically modified yeasts should be inactivated or decontaminated.

Note

As was the case for *E. coli,* the organism will move one level up in both risk class and the corresponding measures to be taken when a gene that produces a hazardous substance, such as a toxin, is cloned into the yeast.

Working with genetically modified cell lines

Classification

Classifying animal or human cell lines is not that simple. The determining factor is the cells' ability to produce hazardous biological agents, notably viruses. First of all a distinction should be made between primary cells and "established cell lines". Primary cells should at least be classified as L2 organisms. When established cell lines are involved, it should be determined whether they might produce virus particles, taking into account any viral sequences that might be present in the vector being used in combination with the cell line. Some examples:

Cell line	Viral sequences in cell line	Vector (by way of example only)	Classification*	Classification rule**	Comments
Primary mouse cells	Not known	Not relevant	L2	-	Classification at a lower level only upon proof that no hazardous biological agents are produced
NIH-3T3	None	SV40ori vector	L1	4, e or j	Zero risk of virus particle formation
HELA	E6E7 genes from HPV	SV40ori vector	L1	4, e or j	Combination of viral sequences in cell line and vector will not result in the formation of virus particles

293	E1 and piece of E4 genes from adenovirus	E1 deleted adenoviral vector	L2	8, e or j	Recombination of sequences in the cell line and vector may result in the formation of replication-competent virus particles
EBV immortalised cells	Cells contain complete EBV virus	Not relevant	L2	-	Classification corresponds to that of the virus present in the cells

* on the assumption that non-hazardous inserts are used (hazardous inserts would require a higher risk class)
** as applied in the schemes of annex 2

Laboratory requirements

The laboratory requirements may vary depending on the classification. A basic L1 laboratory is sufficient for cells like NIH-3T3. In practice, cells like these are usually inoculated and grown in well-equipped cell culture facilities that meet more than the basic L1 requirements. A class II safety cabinet is usually present to protect the cells against infectious agents when they are handled in the open. Instead of class II safety cabinets, horizontal laminar flow cabinets are often used. The latter are suited for level 1 activities with cells that do not involve infectious agents. They are, however, not sufficient for manipulations with cells belonging to L2. In that case a class II safety cabinet is required to protect both the laboratory worker, the environment and the experiment. In comparison with those of L1, the L2 requirements are more stringent with regard to access to the laboratory and activities that might lead to the creation of aerosols.

Waste

All the biological waste originating from genetically modified cells needs to be inactivated. However, a distinction should be made between cells requiring L1 containment and those requiring L2 containment. As far as L1 cell cultures are concerned, it is sufficient to expose them to a strong detergent for a period of time and then pour it out in the sink. Contaminated materials can be dealt with in the same manner. L2 cell cultures require more rigorous measures, to ensure that any hazardous biological agents present in the material are killed as well. Sterilisation is the most appropriate method to this end.

Note

Never work with autologous cells. Especially when genes encoding for growth factors or oncogenes would be cloned into these cells, contamination could have detrimental effects. The reason for this is that the immune system would not recognise the cells as foreign.

Classification

Genetically modified viruses are generally classified in the same risk class as their biological counterparts (see the annexes to Vlarem II (www.biosafety.be)), unless the insert increases the risk. In the latter case the modified virus will be classified at a higher risk level than its biological counterpart. The use of viruses and viral vectors cannot be detached from the use of cell lines. This implies that here too the combination of viral sequences in the vector and in the cell line should be considered. To produce replication-defective virus particles, cell lines containing the missing viral sequences needed to create virus particles are used. Only when there is no risk that replication-competent virus particles might be formed, the virus can be classified at a lower level.

Packaging cell line	Viral sequences in the cell line	Vector (by way of example only)	Classification*	Classification rule**	Comments
293	E1 and piece of E4 gene from adenovirus	E1 deleted adenoviral vector	L2	8 e or j	Combination of viral sequences in cell line and vector may result in the formation of a replication-competent virus
PER.C6	E1 gene from adenovirus	E1 deleted adenoviral vector with no sequence overlap	L1	9 e or j	Zero risk of replication-competent virus particle formation
PA317	Gag-pol-env from amphotrophic mouse retrovirus	Retroviral vector lacking the gag-pol-env genes	L2	8 e or j	Small risk of RCR formation, though not unlikely. Sequence overlap occurs in many cases
psi-CRE	Gag-pol and env genes from ecotropic mouse retrovirus as two separate constructs	Retroviral vector lacking the gag-pol-env genes	L1	7 e or j	Risk of RCR formation is smaller than with PA317. Moreover, the virus is ecotropic and therefore unable to infect human cells.
Phoenix-ampho	E1/E4 from adenovirus, SV40 large T and episomal gag-pol and env genes from amphotrophic mouse retrovirus	Retroviral vector lacking the Gag-pol-env genes	L2	8 e or j	Risk of RCR formation is smaller than with PA317, but not zero, due to the episomal presence of gag-pol and env in multiple copies

* on the assumption that non-hazardous inserts are used (hazardous inserts would require a higher risk class)

** as applied in the schemes of annex 2

Laboratory requirements

For generating adenoviral or mouse retroviral particles, whether they are replication-defective or not, a L2 laboratory is recommended. In addition, a class II safety cabinet should be used, since a horizontal laminar flow cabinet does not provide sufficient worker and environment protection. Access to the laboratory is subject to strict rules.

Waste

All solid, fluid and contaminated materials should be inactivated or decontaminated before they are reused or discarded as residual waste. Sterilisation is the recommended method to this end. Alternatively, the material can be transported in a special container for infectious medical waste to an incinerator suited for the incineration of hospital waste.

Note

Activities involving different types of viral vectors should not be performed in the same laboratory at the same time, let alone in the same safety cabinet. After finishing work with a particular type of virus, the researcher should clean and decontaminate the work surface and the safety cabinet before conducting a new experiment, involving another type of virus.

In the above, it has been assumed that the inserts used were non-hazardous. When viral vectors are used, especially vectors that insert their genetic material in the cell's genome, extra care should be taken when, for instance, dominant cellular oncogenes are used. The insert will then be classified in a higher risk class. Genes encoding some immune-modulating proteins can also be regarded as hazardous. In some cases a risk class III classification will be necessary, for which a lot of effort is required due to the stringent containment measures and working practices that need to be applied.

The number of viral vector systems composed of components originating from different viruses, is growing rapidly. The most well-known example is the pseudo-typing of retroviral vectors using the VSV-G protein. Such combinations of viral components deserve special attention, since the host range may change, for instance from non-infectious for humans to infectious for humans The risk class should be adjusted to the actual risk.

Classification

Most researchers work with 'disarmed' *A. tumefaciens* strains, which means that the strains are no longer oncogenic. They no longer cause crown galls. These disarmed strains belong to risk class 1. This does not apply to wild-type strains or *A. rhizogenes*.

Strain	vectors	Classification*	Classification rule**	Comments
Disarmed A. *tumefaciens*	Binary and other plant transformation vectors	L1	1 e or j	The strains are biologically contained in comparison with wild-type strains
Not disarmed A. *tumefaciens*	Binary and other plant transformation vectors	L2	3 e or j	These strains are pathogenic
A. *rhizogenes*	Binary and other plant transformation vectors	L2	3 e or j	These strains are pathogenic

* on the assumption that non-hazardous inserts are used
** as applied in the schemes of annex 2

Laboratory requirements

Basic L1 containment measures are sufficient for disarmed *A. tumefaciens* strains. It is enough to follow the rules laid down for *E. coli* K12. As far as not-disarmed tumefaciens strains or *A. rhizogenes* are concerned, basic L2 containment measures will suffise. In cases when the creation and dissemination of aerosols containing the bacterium cannot be avoided, a class II safety cabinet should be used.

Waste

All materials, whether solid or fluid, should be decontaminated, as was the case for *E. coli*. Sterilisation is the recommended method to this end.

Note

Recently transformed, little plants are often transferred onto fresh culture mediums. This activity is often performed in a cross-flow cabinet, which is permitted as long as no open sources of *Agrobacterium* are involved.

Researchers working with transgenic plants should abide by the following rules of thumb:

1. When working in fytotrons or growth chambers:

- growth chambers should be closed and should not lead out into the open air
- plant material should be sterilised before it is discarded as residual waste (in case the material still contains genetically modified A. tumefaciens)
- the spread of seeds should be avoided and reproductive parts of plants should be inactivated before they are discarded as residual waste

2. When working in greenhouses

- when flowering insect pollinators are used, all openings should be covered with insect screens, and the greenhouse itself should not lead out into the open air (an airlock should be present). When non-flowering plants, or flowering self-pollinators, apomicts or wind-pollinators are used, insect screens are not required, and an airlock is not really necessary.
- In this case too, the spread of seeds should be avoided. Seeds should be carefully collected, and soil that might contain seeds should be inactivated before reusing it or discarding it as waste. Reproductive plant parts should be inactivated before they are disposed of as residual waste.

8. REFERENCES

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9. CLARIFICATION OF TERMS

Aerosol: very small droplets of fluid that can spread via air
Amphotropic: able to infect both mouse and human cells
Biological agent: in its legal definition this means organism that is able to cause disease
Donorsequence: a piece of genetic material that is transferred from one organism - the donor - to the receiving organism, also named insert. In many cases the insert nowadays is made synthetically.
Ecotropic: only able to infect mouse cells
Host:
(1) in the context of natural pathogens this means the organism that is naturally infected by the pathogen.
(2) in the context of genetic modification this is the organism receiving the genetic modification.
HEPA: High Efficiency Particulate Air (filter)
Pathogenicity: ability to cause disease
Reproductive: able to grow out to a autonomously functioning organism
Toxicity: poisoning
Transduction: equals viral infection. However, this terms is mostly used for infection of bacteria by fages and infection of cell by a viral vector.
Transformation:
(1) in the context of tumours this means the alteration of a normal cell into a infinitively dividing tumour cell,
(2) in genetic modification the term is generally used for the process of genetic modification (transformation = genetic modification)
Vector: literally this means 'carrier'.
(1) in the context of genetic modification this is the plasmid, cosmid, artificial chromosome or other genetic element that is used to carry the genetic modification into the host organism.
(2) in the context of viral infection of plants the term vector is used for the organisms that carries the virusinfection from one plant to the other. In some cases aphids are the vector.
Viral vector: a viral construction developed from a wildtype virus and that can carry a piece of genetic material and can behave autonomously, in the case it possesses all the sequences necessary to spread from one cell to the other, or defectively, if it lacks one or more sequences for its spreading.

ANNEX 1: CONTAINMENT REQUIREMENTS

In this annex the belgian containment requirements are given for laboratories, animalaria and greenhouses. The requirements for hospital rooms and large-scale processing equipment are not given here. These requirements can be found at www.biosafety.be. Also the requirements for so-called quarantine-organisms are not given here. If you want to work with one of the pathogenic organisms that are present on the lists of quarantine-organisms of the FAVV, one should ask them for the requirements to work with these organisms.

Belgian requirements for L1-L4 laboratories for work with genetically modified organisms and/or pathogens

1. Equipment and technical requirements

Specifications		Containment level			
		L1	L2	L3	L4
1	The laboratory is separated from other working zones in the same building or is located in a separate building	not required	not required	required	required
2	Entry to the laboratory via an airlock	not required	not required	equired or only entrance via L2	required
3	Doors can be locked	not required	required	required	required
4	Doors that close automatically	not required	optional	required	required
5	Windows that cannot be opened	not required	not required, but closed during experimentation	required	required (able to withstand breaking)
6	Airtight laboratory that enables decontanimation with a gas (fumigation)	not required	not required	required	required
7	Laboratory furniture that enables a programme to combat rodents and insects	not required	recommended	required	required
8	Observation window or equivalent system that enables one to see who is working in the laboratory	not required	optional	optional	required
9	Decontamination-facilities for the personnel	required (sinks)	required (sinks)	required (sinks in the airlock or near the exit)	required (sinks and shower, the latter fitted with a chemical sprinkler in case use is made of isolation suit fitted with positive airpressure)
10	Sinks that can be operated non-manually	not required	optional	required	required
11	Coathooks or changing room for the protective clothing	recommended	required	required	required
12	Tubing fitted with a system that prevents backward flow of fluids	not required	not required	recommended	required
13	Surfaces able to withstand acids, bases, organic solvents and decontaminants, water resistant and easy to clean	required (bench)	required (bench)	required (bench, floor)	required (bench, floor, walls, ceiling)

Specifications		Containment level			
		L1	L2	L3	L4
14	Autonomous electricity backup system	not required	not required	recommended	required
15	Fire alarm (not taking into account local fire regulations)	not required	not required	required	required
16	System that enables communication with the outside world	not required	not required	required	required (non-manual operation)
	VENTILATION				
17	Input airflowsystem separated from adjacent laboratories	not required	not required	recommended	required
18	Extract airflowsystem separated from adjacent laboratories	not required	not required	recommended	required
19	Input and extract airflowsystem interconnected to prevent accidental overpressure	not required	not required	required	required
20	Input and extract airflowsystem can be closed using valves	not required	not required	required	required
21	Negative airpressure in the controlled zone when compared to adjacant zones	not required	not required	required (control and alarm-systems)	required (control and alarm-systems)
22	HEPA-filtration of the air[1]	not required	not required	required (on the extract air)	required (on the input airflow and double filtration on the extracted air)
23	System that allows changing of the filters without contamination of the environment	-	-	required	required
24	HEPA-filtered air may be recirculated	-	-	optional	no
25	Specific measures to ventilate to such an extent that contamination of the air is minimised	optional	optional	required[2]	required

[1] In case of use of virus not retained by HEPA filtration, special appropriate measures must be taken with regard to the extract air

[2] Measures to be specified in the notification and the competent authorities should determine the requirements in the authorisation.

2. Safety equipment

Specifications		Containment level			
		L1	L2	L3	L4
26	Microbiological safety cabinet	not required	optional (class I or II)	required (class I or II)	required (class III; in case of class II an isolation suit should be used fitted with positive airpressure)
27	Autoclave	if autoclave, then on site	in the building	in the laboratory or in an adjacent room[3]	In the laboratory
28	Double-ended autoclave	not required	not required	optional	required
29	Centrifuge in the containment zone	not required	required; not required, if leakfree tubes are used	required	required
30	Vacuumgenerator fitted with a HEPA filter	not required	not required	recommended	required

[3] Transfer of material to an autoclave situated outside the laboratory should occur according to a validated method that guarantees an equivalent protection level.

3. Working practices and waste management

	Specifications	Containment level			
		L1	L2	L3	L4
31	Restricted access	recommended	required	required (and control)	required (and control)
32	Notification on the door: a: "Biohazard" symbol b: co-ordinates of the person in charge c: containment level d: type of biolocal risk e: list of persons who have access f: criteria for admittance	recommended (b, c)	required (a, b, c)	required (a, b, c, d, e, f)	required (a, b, c, d, e, f)
33	Laboratory to contain its own equipment	not required	not required	required	required
34	Protective clothing	required	required	required (specific for the containment zone)[4]	required (specific for the containment zone)[5]
35	Decontamination of the protective clothing before it leaves the containment zone	not required	not required	recommended	required
36	Gloves	not required	optional	required	required
37	Mouth mask	not required	not required	optional	required
38	Face protection (eyes, mucosa)	not required	optional	required	required
39	Fysical containment of live micro-organisms (closed systems)	recommended	required	required	required
40	Generation of aerosols	minimize	minimize	prevent	prevent
41	Specific measures to prevent the spread of aerosols (including appropriate equipment)	not required	recommended	required	required
42	Mechanical pipetting	required	required	required	required
43	No drinking, eating or smoking, no use of cosmetics, no manipulation of contacts, or the stocking of consumables	required	required	required	required
44	Appropriate laboratory registers	required	required	required	required
45	Verification of control measures and protective equipment	required	required	required	required
46	Notice on the use of decontaminants	required	required	required	required
47	Decontaminants in the siphons	not required	not required	recommended	required
48	Instruction of the personnel	required	required	required	required
49	Written instructions on the biosafety procedures	required	required	required	required
50	Efficient vector control (e.g. for rodents and insects)	not required	recommended	required	required
51	Circulation of animals	forbidden	forbidden	forbidden	forbidden
52	In case of manipulation of zoopathogens, period during which contact of the personnel with the host animals should be avoided	not required	not required	recommended, period to be specified in the authorisation	required, period to be specified in the authorisation
	WASTE MANAGEMENT				
53	Inactivation of biological waste and/or biological residues using an appropriate validated method before dumping	required	required	required	required
54	Inactivation of contaminated material (glassware, etc.) using an appropriate and validated method before reuse or destroying	required	required	required	required
55	Inactivation of the effluents of sinks and showers using an appropriate and validated method before dumping	not required	not required	optional	required

[4] Suitable footwear (optional)
[5] Full change of clothing and shoes before entrance and leaving

1. Equipment and technical requirements

	Specifications	Containment level			
		A1	A2	A3	A4
1	The animalarium is separated from other working zones in the same building or is located in a separate building	not required	required	required	required
2	Entry via an airlock	not required	recommended	required	required (with three compartments)
3	Doors that can be locked	required	required	required	required
4	Doors that close automatically	not required	required	required	required
5	Windows that cannot be opened	not required	not required, buth windows should be closed during experimentation	required	required (able to withstand breaking)
6	Airtight laboratory that enables decontamination with a gas	not required	optional	required	required
7	Building designed in such a way that accidental escape of animals is avoided	recommended	required	required	required
8	Observation window or equivalent system that enables one to see who is working in the laboratory	recommended	recommended	required	required
9	Decontamination facilities for the personnel	required (sinks)	required (sinks)	required (sinks near the exit or in the airlock) shower: recommended	required (sinks and shower, the latter fitted with a chemical sprinkler in case use is made of isolation suit fitted with positive air pressure)
10	Sinks that can be operated non-manually	not required	recommended	required	required
11	Coathooks or changing room for the protective clothing	required	required	required	required
12	Tubing fitted with a system that prevents backward flow of fluids	not required	not required	recommended	required
13	Separate room for the stocking of clean cages, feed and caging material	recommended	required	required	required
14	Surfaces resistant to decontaminants, impermeable and easy to clean	required (cages, benches)	required (cages, benches, floor)	required (cages, benches, floor, walls, ceiling)	required (cages, benches, floor, walls, ceiling)
15	Facility for the washing of cages	required	required	required	required
16	Automous electricity backup system	not required	not required	recommended	required
17	Fire alarm (not taking into account local fire regulations)	not required	not required	required	required
18	System that enables communication with the outside world	not required	not required	required	required (non-manual operation)
	VENTILATION				
19	Input airflowsystem separated from adjacent laboratories	not required	not required	recommended	required
20	Extract airflowsystem separated from adjacent laboratories	not required	optional	recommended	required
21	Input and extract airflowsystem interconnected to prevent accidental overpressure	not required	optional	required	required
22	Input and extract airflowsystem can be closed using valves	not required	optional	required	required

Specifications		Containment level			
		A1	A2	A3	A4
23	Negative airpressure in the controlled zone when compared to adjacent zones	not required	optional	required (control and alarm systems)	required (control and alarm systems)
24	HEPA-filtration of the air[6]	not required	optional	required (on the extracted air	required (on the inward airflow and double filtration on the extraced air)
25	System that allows changing of the filters without contamination of the environment	-	-	required	required
26	HEPA-filtered air may be recirculated	-	-	optional	forbidden
27	Specific measures to ventilate to such an extent that contamination of the air is minimized	optional	optional	required[7]	required

[6] In case of use of virus not retained by HEPA filtration, special appropriate measures must be taken with regard to the extract air

[7] Measures to be specified by the notifier and by the competent authorities

2. Safety equipment

Specifications		Containment level			
		A1	A2	A3	A4
28	Microbiological safety cabinet	not required	optional (class I or II)	optional (class I or II)	optional (class III, or class II with the use of an isolation suit fitted with overpressure)
29	Animals in cages or equivalent isolation installations	optional	optional	optional	optional
30	Cages fitted with a HEPA filter	not required	optional	required	required
31	Autoclave	on site	in the building	in the animalarium or an adjacent room[8]	in the animalarium
32	Double-ended autoclave	not required	not required	recommended	required
33	Fumigationsystem or decontamination immersion bath	not required	recommended	required	required

[8] Transfer of material to an autoclave situated outside the laboratory should occur according to a validated method that guarantees an equivalent protection level.

3. Working practices and waste management

	Specifications	Containment level			
		A1	A2	A3	A4
34	Restricted access	required	required	required (and control)	required (and control)
35	Notification on the door: "Biohazard" symbol, coordinates of person in charge, containment level, type of biological risk, list of persons who have access, criteria for admittance	required, except the "Biohazard" symbol	required	required	required
36	Animal housing to contain its own equipment	not required	recommended	required	required
37	Protective clothing that is specific for the containment zone	required	required	required, + optionally appropriate shoe protection	required, complete change of clothing and footwear when entering or leaving
38	Decontamination of the protective clothing before it leaves the containment zone	not required	not required	required	required
39	Gloves	optional	recommended	required	required
40	Mouth mask	not required	optional	optional	required
41	Face protection (eyes, mucosa)	not required	optional	optional	required
42	Generation of aerosols	minimise	minimise	prevent	prevent
43	Specific measures to prevent the spread of aerosols (including appropriate equipment)	not required	recommended	required	required
44	Mechanical pipetting	required	required	required	required
45	No drinking, eating or smoking, no use of cosmetics, no manipulation of contacts, or the stocking of consumables	required	required	required	required
46	Register in which all activities are notified (transport of laboratory animals, inoculation of GMM's etc....)	required	required	required	required
47	Verification of control measures and protective equipment	required	required	required	required
48	Notice on the use of decontaminants	required	required	required	required
49	Decontaminants in the siphons	not required	recommended	required	required
50	Instruction of the personnel	required	required	required	required
51	Written instructions on the biosafety procedures	required	required	required	required
52	Efficient vector control (eg. for rodents and insects)	recommended	required	required	required
53	Isolation of laboratory animals used for experimentation	required	required (separate room)	required (separate room)	required (separate room)
54	In case of manipulation of zoopathogens, period during which contact of the personnel with the host animals should be avoided	not required	not required	recommended, period to be specified in the authorisation	required, period to be specified in the authorisation
55	**WASTE MANAGEMENT** Inactivation of biological waste and/or biological residues (contaminated cadavers, faeces[9]) using an appropriate and validated method before dumping	required	required	required	required
56	Inactivation of contaminated material (glassware, cages, etc...) using an appropriate and validated method before reuse or destroying	required	required	required	required
57	Inactivation of the effluents of sinks and showers using an appropriate and validated method before dumping	not required	not required	recommended	required

[9] Faeces from transgenic animals does not have to be inactivated

Requirements for G4 greenhouses are not given in the Belgian legislation.

1. Equipment and technical requirements

	Specifications	Containment levels		
		G1	G2	G3
1	The greenhouse is a permanent structure	not required	required	required
2	A 1.5m free zone in concrete or free of plant growth surrounding the growth chamber or greenhouse	not required	required	required
3	A secured fence	not required	not required	required
4	Aisles	stabilised soil	solid material	solid material
5	Entry via a separated room with two interlocking doors	not required	optional	required
6	Doors that can be locked	not required	required	required
7	Structure[10] resistant to shocks	not required	recommended	required
8	Structure[11] waterimpermeable and easy to clean	not required	recommended	required
9	Windows that cannot be opened	not required	not required	required
10	Airtight room that permits decontamination with a gas	not required	not required	required
11	Decontamination facilities for the personnel	required (sinks)	required (sinks)	required (sinks in the airlock or near the exit), shower: optional
12	Sinks that can be operated non-manually	not required	not required	required
13	Tubing fitted with a system that prevents backward flow of fluids	not required	not required	recommended
14	Surfaces resistant to acids, alkalines, organic solvents and desinfectants	not required	recommended	required
15	Waterimpermeable floor	not required	recommended	required
16	Management of flow of contaminated water	optional	minimise flow[12]	prevent flow
17	Autonomous electricity backup system	not required	not required	required
18	Fire alarm system (not taking into account local fire regulations)	not required	optional	required
19	System to communicate to the outside world	not required	optional	required
20	**VENTILATION** Input and extract airflowsystem interconnected to prevent accidental overpressure	not required	optional	required
21	Input and extract airflowsystem can be closed using valves	not required	optional	required
22	Negative airpressure in the controlled zone when compard to adjacent zones	not required	not required	optional
23	HEPA-filtration of the air[13]	not required	not required	required (on inward and extracted air)
24	System that allows changing of the filters without contamination of the environment	–	–	required

[10] With structure the walls, roof and floor are meant.

[11] The greenhouse should be a permanent construction with a seamless water tight covering, situated on a premises such that it is impossible for surface water to come in, and with doors that close automatically and can be locked.

[12] If transmission via soil is possible.

[13] In case of use of virus not retained by HEPA filtration, special appropriate measures must be taken with regard to the extract air.

2. Safety equipment

	Specifications	Containment levels		
		G1	G2	G3
25	Autoclave	on site	in the building	in the greenhouse
26	Double-ended autoclave	not required	not required	optional
27	Fumigationroom or immersion bath for the transfer of living materials	not required	not required	optional

3. Working practices and waste management

	Specifications	Containment levels		
		G1	G2	G3
28	Restricted access	required	required	required (and control)
29	Notification of the biological risk	not required	required	required
30	Specific equipment	not required	not required	required
31	Protective clothing	required	required	required (and specific for the containment zone
32	Decontamination of the protective clothing before it leaves the containment zone	not required	not required	required
33	Gloves	not required	optional	optional
34	Shoe-coverings or decontamination bath for the shoes	not required	optional	optional
35	Generation of aerosols	minimise	minimise	prevent
36	Specific measures to prevent the spread of aerosols	not required	recommended	required
37	Mechanical pipetting	required	required	required
38	No drinking, eating or smoking, no use of cosmetics, no manipulation of contacts, or the stocking of consumables	required	required	required
39	Register in which all activities are notified (transport of plant material, inoculation of GMM's etc....)	required	required	required
40	Verification of control measures and protective equipment	required	required	required
41	Notice for the use of decontaminants	required	required	required
42	Instruction of the personnel	required	required	required
43	Written instructions on the biosafety procedures	required	required	required
44	Circulation of animals	forbidden	forbidden	forbidden
45	Measures to control undesired species such as insects, rodents, arthropods	required	required	required
46	Selfspreading organisms: - transport in the installation between containment zones - note in the register - decontamination of containers before transport	 container: optional not required not required	 container recommended required	 double container required required
47	Control of contaminated run-off water	optional	minimise run-off[14]	prevent run-off
48	**WASTE MANAGEMENT** Inactivation of biological waste and/or biological residues using an appropriate and validated method before dumping	required	required	required

[14] If transmission via soil is possible.

Specifications		Containment levels		
		G1	G2	G3
41	Inactivation of contaminated material (glassware, etc) using an appropriate and validated method before reuse or destroying	required	required	required
42	Inactivation of the effluents of sinks and showers using an appropriate and validated method before dumping	not required	not required	optional

ANNEX 2: GUIDELINES FOR THE CLASSIFICATION OF GMO-ACTIVITIES

Introduction

These guidelines are meant to offer help in the determination of the appropriate containment measures for activities with genetically modified organisms. In this annex a distinction is made between activities involving micro-organisms and cells (chapter A), activities with plants (chapter B) and activities with animals (chapter C). The transformation of plants using *Agrobacterium tumefaciens* is classified as an activity with the micro-organism.

The use of these guidelines

These guidelines offer especially help for determining the necessary containment measures for activities with micro-organisms and cells (chapter A of these guidelines). In chapter A there is a description of how to use the classification schemes. Also, the necessary definitions of T1, T2 and T3 toxins are given as well as the requirements for risk class 1 hostorganisms and vectors. For activities with transgenic plants, chapter B gives some guidance. Activities with transgenic animals and especially the combination of the use of genetically modified micro-organisms or cells with animals are difficult to classify. Chapter C gives some guidance for these activities.
Of course there are cases that are not described in these guidelines. In such cases it is best to contact internal or external biosafety experts as given in annex 5 in this booklet.

Sources

These guidelines have been developed from the guidelines from the dutch genetic modification advisory committee (COGEM), as far as applicable in Belgium, and from the classification guidelines in the annexes of section 51 of Vlarem II.

CHAPTER A. GUIDELINES FOR THE CLASSIFICATION OF ACTIVITIES INVOLVING GENETICALLY MODIFIED MICRO-ORGANISMS AND CELLS

Classification rules

On the following pages classification schemes are presented that can be used to determine a first indication of the necessary containment level. As already explained in chapter 3 and in the example in chapter 7 of this booklet, this is only a first, but important step. To come to a final conclusion one still has to consider: (1) the environment to which the GMO would be exposed, (2) the type and the scale of the activity, and (3) possible non-standard manipulations.

The classification scheme is divided into four parts:

- Part 1: activities with host/vectorsystems that do or do not fulfill the criteria for categorisation in risk class 1 and activities with non-viral pathogenic hostorganisms
- Part 2: activities involving animal cells without viral vectors and with baculoviruses
- Part 3: activities involving animal cells in combination with viruses or viral vectors
- Part 4: activities in plant cells

The schemes should be used as follows: In a first step the four schemes are screened in the ‘first direction’ and is determined what type of host and vector is applicable. In a second step it is determined in the ‘second direction’ whether it concerns activities with non-characterised or characterised genetic material. In a third step it is determined within the applicable type of genetic material (characterised or non-characterised) what type of insert is applicable. In the case of non-characterised genetic material it is a choice between categories a to e, and in the case of characterised genetic material it is a choice between categories f to j. From the combination of applicable categories of host, vector and insert the applicable classification can be read.

Definition of classes of toxins

a. A T-3 toxin is a toxin with an LD50 for vertebrates of less than 100 nanograms per kg bodyweight.
b. A T-2 toxin is a toxin with an LD50 for vertebrates between 100 nanograms and 1 microgram per kg bodyweight.
c. A T-1 toxin is a toxin with an LD50 for vertebrates between 1 microgram to 100 micrograms per kg bodyweight.

Requirements for host/vectorsystems to be classified in risk class 1

Requirements for smallscale laboratory activities (= < 100 liter effective culture volume)

1. The host is not pathogenic.
2. The host is free of known biological agents that are potentially hazardous.
3. The vector is well-characterised, this is: the type of vector should be defined (plasmid, cosmid, mini-chromosome, etc.), the size should be known, function and origin of structural genes and markergenes should be known, as well as restrictionsites and replicon. Commercially available vectors are mostly well-characterised.
4. The vector may not have hazardous consequences (may not transfer virulence or toxindetermining elements).
5. The vector is limited to only necessary elements as far as possible.
6. The vector may not advance the stability of the GMO in the environment.
7. The vector should be difficult to mobilise (Tra-).
8. The vector is not allowed to pass on resistance genes to micro-organisms that do not posses these genes naturally, if the uptake of such a resistance gene by this organism would disable the use of antibiotics in healthcare.

Risk groups (biological risk classes)

With organisms of risk groups 4, 3 and 2 is meant the organisms that have been classified as pathogens in the risk groups 4, 3 and 2 as described in the annexes of Vlarem II.

1. Activities with host/vectorsystems that do or do not fulfill the criteria for categorisation in risk class 1 and activities with non-viral pathogenic hostorganisms

Second direction ↓	First direction →		1. Activities in a host/vectorsystem that fulfills the criteria for categorisation in risk class 1	2. Activities in a host/vectorsystem that does not fulfill the criteria for categorisation in risk class 1	3. Activities in pathogenic hosts, excluding viral pathogens. The host is a pathogen of respectively risk group 4, 3, or 2:
Activities in which genetic material is or has been added that has not been characterised	a. The donor produces a toxin of respectively class:	T3	L4	L4	Resp. L4, L4, L4
		T2	L3	L4	Resp. L4, L4, L4
		T1	L2	L3	Resp. L4, L3, L3
	b. The donor is a for eucaryotic cells infectious virus of respectively:	Risk group 4	L3	L4	Resp. L4, L4, L4
		Risk group 3	L2	L3	Resp. L4, L3, L3
		Risk group 2	L1	L2	Resp. L4, L3, L2
	c. The donor is a defective for eucaryotic cells infectious virus of respectively:	Risk group 4	L2	L2	Resp. L4, L3, L3
		Risk group 3	L1	L2	Resp. L4, L3, L2
		Risk group 2	L1	L2	Resp. L4, L3, L2
	d. The donor is a non-viral pathogen of respectively	Risk group 4	L3	L3	Resp. L4, L4, L4
		Risk group 3	L2	L2	Resp. L4, L3, L3
		Risk group 2	L1	L2	Resp. L4, L3, L2
	e. The donor is an organism of risk group 1, or a plant or an animal		L1	L2	Resp. L4, L3, L2
Activities in which characterised genetic material is or has been added	f. The sequence contains genetic information that codes for a toxin of respectively class	T3	L4	L4	Resp. L4, L4, L4
		T2	L3	L4	Resp. L4, L4, L4
		T1	L2	L3	Resp. L4, L3, L3
	g. The sequence contains genetic information for the formation of a for eucaryotic cells infectious virus of respectively:	Risk group 4	L3	L4	Resp. L4, L4, L4
		Risk group 3	L2	L3	Resp. L4, L3, L3
		Risk group 2	L1	L2	Resp. L4, L3, L2
	h. The sequence contains genetic information for the formation of a defective, for eucaryotic cells infectous virus, of respectively:	Risk group 4	L2	L2	Resp. L4, L3, L3
		Risk group 3	L1	L2	Resp. L4, L3, L2
		Risk group 2	L1	L2	Resp. L4, L3, L2
	i. The sequence contains genetic information that codes for a hazardous gene product, other than in f.*		L1 or L2*	L2 or L3*	Resp. L4, L3, L2*
	j. The sequence does not contain genetic information that codes for a hazardous gene product		L1	L2	Resp. L4, L3, L2

* What constitutes a hazardous gene product is the most difficult risk classification question. Virulence gene products might be an example of a hazardous gene product. Final risk classification depends on the suspected effect of the gene product in the used hostorganism.

2. Activities with animal cells without use of viral vectors and with the use of baculoviruses

Second direction ↓	First direction →		4. Activities in animal cells without the use of a viral vector	5. The viral vector is, or has been developed from a baculovirus that is biologically contained, or that becomes biologically contained as a result of the construction	6. The viral vector is, or has been developed from a biologically notcontained baculovirus and that also will not become biologically contained as a result of the construction
Activities in which genetic material is or has been added that has not been characterised	a. The donor produces a toxin of respectively class	T3	L1	L2	L3
		T2	L1	L1	L2
		T1	L1	L1	L2
	b. The donor is, or has been developed from, a defect for eucaryotic cells infectious virus of respectively risk group 4, 3 or 2, and the viral sequences that have been brought into the host can give rise to the formation of autonomously replicating virusparticles	Risk group 4	L4	L4	L4
		Risk group 3	L3	L3	L3
		Risk group 2	L2	L2	L2
	c. The donor is, or has been developed from, a defect for eucaryotic cells infectious virus ofrespectively risk group 4, 3 or 2, and the viral sequences that have been brought into the host cannot give rise to the formation of autonomously replicating virusparticles	Risk group 4	L3	L2	L3
		Risk group 3	L2	L2	L2
		Risk group 2	L1	L1	L2
	d. The donor is a non-viral pathogen of respectively	Risk group 4	L3	L2	L3
		Risk group 3	L2	L2	L2
		Risk group 2	L1	L1	L2
	e. The donor is an organism of biological risk group 1, or a plant or an animal		L1	L1	L2
Activities in which characterised genetic material is or has been added	f. The sequence contains genetic information that codes for the production of a toxin of respectively risk group	T3	L1	L2	L3
		T2	L1	L1	L2
		T1	L1	L1	L2
	g. The sequence contains genetic information for the formation of a for eucaryotic cells infectious virus, of respectively risk group 4, 3 or 2 and the viral sequences that have been brought into the host can give rise to the formation of autonomously replicating virusparticles	Risk group 4	L4	L4	L4
		Risk group 3	L3	L3	L3
		Risk group 2	L2	L2	L2
	h. The sequence contains genetic information for the formation of a defect for eucaryotic cells infectious virus, of respectively risk group 4, 3 or 2 and the viral sequences that have been brought into the host cannot give rise to the formation of autonomously replicating virusparticles:	Risk group 4	L3	L2	L3
		Risk group 3	L2	L2	L2
		Risk group 2	L1	L1	L2
	i. The sequence contains genetic information that codes for a hazardous gene product, other than in f.*		L1	L1	L2
	j. The sequence does not contain genetic information that codes for a hazardous gene product		L1	L1	L2

* What constitutes a hazardous gene product is the most difficult risk classification question. Virulence gene products might be an example of a hazardous gene product. Final risk classification depends on the suspected effect of the gene product in the used hostorganism.

3. Activities with animal cells in combination with viruses or viral vectors

Second direction ↓	First direction →		7. The viral vector is a complete or defective ecotropic mouse retrovirus	8. The viral vector is a human or animal pathogenic autonomously replicating infectious virus of respectively risk group 4, 3 or 2 or a defective virus that has been developed from it and the possibility that autonomously replicating virusparticles emerge does exist	9. The viral vector is, or has been developed from a satelite virus or is a defective virus developed from a human or animal pathogen of respectively risk group 4, 3 or 2; the possibility that autonomously replicating virusparticles emerge does not exist
Activities in which genetic material is or has been added that has not been characterised	a. The donor produces a toxin of respectively class	T3	L2	Resp. L4, L4, L4	Resp. L3, L3, L3
		T2	L2	Resp. L4, L4, L3	Resp. L3, L2, L2
		T1	L2	Resp. L4, L4, L3	Resp. L3, L2, L2
	b. The donor is, or has been developed from, a for eucaryotic cells infectious virus of respectively risk group 4, 3 or 2, and the viral sequences that have been brought into the host can give rise to the formation of autonomously replicating virusparticles*	Risk group 4	L4	Resp. L4, L4, L4	Resp. L4, L4, L4
		Risk group 3	L3	Resp. L4, L3, L3	Resp. L3, L3, L3
		Risk group 2	L2	Resp. L4, L3, L2	Resp. L3, L2, L2
	c. The donor is, or has been developed from, a defect for eucaryotic cells infectious virus of respectively risk group 4, 3 or 2, and the viral sequences that have been brought into the host cannot give rise to the formation of autonomously replicating virusparticles*	Risk group 4	L3	Resp. L4, L3, L3	Resp. L3, L3, L3
		Risk group 3	L2	Resp. L4, L3, L2	Resp. L3, L2, L2
		Risk group 2	L1	Resp. L4, L3, L2	Resp. L3, L2, L1
	d. The donor is a non-viral pathogen of respectively	Risk group 4	L3	Resp. L4, L3, L3	Resp. L3, L3, L3
		Risk group 3	L2	Resp. L4, L3, L2	Resp. L3, L2, L2
		Risk group 2	L1	Resp. L4, L3, L2	Resp. L3, L2, L1
	e. The donor is an organism of biological risk risk group 1, or a plant or an animal		L1	Resp. L4, L3, L2	Resp. L3, L2, L1
Activities in which characterised genetic material is or has been added	f. The sequence contains genetic information that codes for the production of a toxin of respectively class	T3	L2	Resp. L4, L4, L4	Resp. L3, L3, L3
		T2	L2	Resp. L4, L4, L3	Resp. L3, L2, L2
		T1	L2	Resp. L4, L4, L3	Resp. L3, L2, L2
	g. The sequence contains genetic information for the formation of a for eucaryotic cells infectious virus, of respectively risk group 4, 3 or 2 and the viral sequences that have been brought into the host can give rise to the formation of autonomously replicating virusparticles*	Risk group 4	L4	Resp. L4, L4, L4	Resp. L4, L4, L4
		Risk group 3	L3	Resp. L4, L3, L3	Resp. L3, L3, L3
		Risk group 2	L2	Resp. L4, L3, L2	Resp. L3, L2, L2
	h. The sequence contains genetic information for the formation of a defect for eucaryotic cells infectious virus, of respectively risk group 4, 3 or 2 and the viral sequences that have been brought into the host cannot give rise to the formation of autonomously replicating virusparticles*	Risk group 4	L3	Resp. L4, L3, L3	Resp. L3, L3, L3
		Risk group 3	L2	Resp. L4, L3, L2	Resp. L3, L2, L2
		Risk group 2	L1	Resp. L4, L3, L2	Resp. L3, L2, L1
	i. The sequence contains genetic information that codes for a hazardous gene product, other than in f.**		L2	Resp. L4, L3, L2	Resp. L3, L2, L2
	j. The sequence does not contain genetic information that codes for a hazardous gene product		L1	Resp. L4, L3, L2	Resp. L3, L2, L1

* The risk classification may in some cases have to be higher than given above in the case of the formation of an infectious virusparticle with an increased host(cell)range or an increased virulence or pathogenicity.

** What constitutes a hazardous gene product is the most difficult risk classification question. Virulence gene products might be an example of a hazardous gene product. Final risk classification depends on the suspected effect of the gene product in the used hostorganism.

4. Activities using plant cells

Second direction ↓	First direction →		10. Activities in which characterised genetic material is or has been added	11. Activities in which genetic material is or has been added that has not been characterised
Activities in which genetic material is or has been added that has not been characterised	a. The donor produces a toxin of respectively class	T3	L2	L3
		T2	L1	L2
		T1	L1	L2
	b. The donor is, or has been developed from, a for eucaryotic cells infectious virus of respectively risk group 4, 3 or 2, and the viral sequences that have been brought into the host can give rise to the formation of autonomously replicating virusparticles	Risk group 4	L4	L4
		Risk group 3	L3	L3
		Risk group 2	L2	L2
	c. The donor is, or has been developed from, a defect for eucaryotic cells infectious virus of respectively risk group 4, 3 or 2, and the viral sequences that have been brought into the host cannot give rise to the formation of autonomously replicating virusparticles	Risk group 4	L1	L2
		Risk group 3	L1	L2
		Risk group 2	L1	L2
	d. The donor is a non-viral pathogen of respectively	Risk group 4	L1	L2
		Risk group 3	L1	L2
		Risk group 2	L1	L2
	e. The donor is an organism of biological risk group 1, or a plant or an animal		L1	L2
Activities in which characterised genetic material is or has been added	f. The sequence contains genetic information that codes for the production of a toxin of respectively class	T3	L2	L3
		T2	L1	L2
		T1	L1	L2
	g. The sequence contains genetic information for the formation of a for eucaryotic cells infectious virus, of respectively risk group 4, 3 or 2 and the viral sequences that have been brought into the host can give rise to the formation of autonomously replicating virusparticles	Risk group 4	L4	L4
		Risk group 3	L3	L3
		Risk group 2	L2	L2
	h. The sequence contains genetic information for the formation of a defect for eucaryotic cells infectious virus, of respectively risk group 4, 3 or 2 and the viral sequences that havebeen brought into the host cannot give rise to the formation of autonomously replicating virusparticles:	Risk group 4	L1	L2
		Risk group 3	L1	L2
		Risk group 2	L1	L2
	i. The sequence contains genetic information that codes for a hazardous gene product, other than in f.		L1	L2
	j. The sequence does not contain genetic information that codes for a hazardous gene product		L1	L2

CHAPTER B. GUIDELINES FOR THE CLASSIFICATION OF ACTIVITIES IN GROWTH CHAMBER AND GREENHOUSES INVOLVING TRANSGENIC PLANTS OR PLANTS THAT CARRY GENETICALLY MODIFIED MICRO-ORGANISMS

Containment level G1:

- Sterile or sterilised plants.
- Strict autogamous plants (strict selfpollinators).
- The plants that are not able to survive in the ecosystem (for instance banana).
- The plants that have no relatives in the ecosystem with which they could hybridise successfully (for instance banana).
- The plants that have been infected by a virus of biological risk class 2, by a genetically modified virus of risk class 1 or 2, by a viral vector of biological risk class 1 or 2 or that carry a viral genome of biological risk class 1 or 2.
- The plants that carry a non-selftransmissible fytopathogen of biological risk class 1 or 2.

Containment level G2:

- The crosspollinating, selfpollinating, windpollinating and insectpollinating plants.
- The plants whose full life cycle can only take place in the ecosystem and whose seeds can survive for long periods of time (depending on the case and the experience).
- The plants that have been infected by a virus of biological risk class 3, by a viral vector of biological risk class 3, or that carry a viral genome of biological risk class 3.
- The plants that carry a selftransmissible fytopathogen or a GMO of (biological) risk class 2 or a fytopathogen or a GMO of (biological) risk class 3.

Containment level G3:

- The plants that have similar properties as described in level G2, but that carry a hazardous transgene, or a virus of biological risk class 4, or a viral genome of biological risk class 4.
- The plants that carry genes that are the subject of first tests of gene flow, and that originate from a pathogen that is hazardous for humans or the environment, and of which the risks are not yet known.
- The plants that carry a fytopathogen or GMO of (biological) risk class 4.

Containment level G4:

- The plants that carry a virus of biological risk class 4 that poses an enormous threat to the environment (or for which there is a zero-tolerance), of that carry a gene that is responsible for the production of a substance that is very hazardous for humans or animals.
- The plants that carry a fytopathogen or a GMO of (biological) risk class 4, that poses an enormous threat to the environment (or for which there is a zero tolerance).
- The plants that carry transgenes, originating from a for humans, animals or the environment very dangerous pathogenic organism and of which the risks are not known.

CHAPTER C. GUIDELINES FOR THE CLASSIFICATION OF ACTIVITIES IN ANIMAL HOUSES INVOLVING TRANSGENIC ANIMALS OR ANIMALS THAT CARRY GENETICALLY MODIFIED MICRO-ORGANISMS OR CELLS

Containment level A1:

- The animals that cannot spread easily themselves (pigs, sheep, cattle) and that have taken up a transgene in their genome without the help of a viral vector.
- The animals that cannot spread easily themselves and that carry a risk class 1 GMO.

Containment level A2:

- The animals that are able to spread easily themselves (small rodents, rabbits, insects, fish) and that have taken up a transgene in their genome without the help of a viral vector.
- The animals that are able to spread themselves and that carry a risk class 1 or 2 GMO.

Notes

The classification of animals that carry genetically modified micro-organisms or cells is more complex than the classification of the genetically modified micro-organisms or cells as such. This is because with such activities not only the risk class of the modified micro-organism is important, but also the combination animal/micro-organism, the way the micro-organism is administered, and the possibilities of the micro-organism to spread from the animal to the environment. In a normal laboratory the micro-organism is kept in a closed container, and if this container is opened and there is a risk of spread to the environment, then in many cases the container will only be opened in a safety cabinet (class 2 and higher). The laboratory animal cannot be considered a closed container. This is why for every specific combination of laboratory animal/micro-organism it has to be determined whether there is a need for additional measures to prevent escape of the micro-organism to the environment. In annex 1, under containment levels A1 to A4 these additional measures have been described under: "recommended" or "optional".

An example: a risk class 2 genetically modified adenovirus should be handled in a laboratory using L2 containment measures. When such a virus is vaporised as an aerosol into the lungs of a mouse, basic A2 containment measures will not be enough to prevent the unwanted spread of the virus. The mouse will have to be kept in special cages fitted with a filtertop, and all the waste (faeces, urine, bedding material, etc.) will have to be inactivated. If the same experiment would be done in a pig, then again additional measures would be necessary, but measures that are different than in the case of the mouse. It will not be possible to keep the pig in a filtertop cage. The stable as a whole will have to provide the proper containment. This means that there will have to be an airlock, negative airpressure, fumigation will have to be possible, personnel will have to wear special protective clothing that will remain in the airlock when leaving the stable, and there will have to be special equipment for the collection and inactivation of faeces and urine (a closed collection system connected to a inactivation tank).

ANNEX 3: THE RISK GROUPS OF SOME RELEVANT PATHOGENS

In this annex the risk groups are given of a number of relevant pathogens. This list is a shortened version of the lists given in the annexes of Vlarem II. If a particular pathogen is not present on this shortlist, the complete Vlarem list should be consulted. Is the pathogen also not present on the Vlarem list, then you should contact your internal or external biosafety expert, or the Section Biosafety and Biotechnology of the WIV (www.biosafety.be).

H = risk group for humans
A = risk group for animals
P = risk group for plants
OP = opportunistic-pathogen
(*) = have a limited risk of infection, because they are not able to spread via air
+ = virus of which the biological risk depends on the hostanimal
Human and animal pathogens: bacteria and related organisms

H	A	
3	3	*Bacillus anthracis*
OP	OP	*Bacillus cereus*
	2	*Bacillus lentimorbus*
	2	*Bacillus popiliae*
	2	*Bacillus sphaericus*
	2	*Bacillus thuringiensis*
	2	*Bordetella avium*
2	3	*Bordetella bronchiseptica*
2		*Bordetella parapertussis*
2		*Bordetella pertussis*
2	2	*Campylobacter coli*
2	2	*Campylobacter fetus* subsp. *fetus*
	3	*Campylobacter fetus* subsp. *venerealis*
2	2	*Campylobacter jejuni*
2	2	*Campylobacter* spp.
2		*Chlamydia pneumoniae*
2	2	*Clostridium botulinum*
2	2	*Clostridium tetani*
2		*Enterobacter* spp.
2		*Enterococcus faecalis*
2	2	*Escherichia coli* (excluding non-pathogenic strains)
	2	*Helicobacter hepaticus*
2		*Helicobacter pylori*
2		*Klebsiella mobilis (Enterobacter aerogenes)*
2		*Klebsiella oxytoca*
2	2	*Klebsiella pneumoniae*
2	2	*Klebsiella* spp.
2	2	*Listeria ivanovii*
2	2	*Listeria monocytogenes*
2	3	*Mycobacterium avium* subsp. *paratuberculosis*
3	3	*Mycobacterium bovis* (except the BCG strain)

H	A	
3		*Mycobacterium leprae*
3	3	*Mycobacterium tuberculosis*
2		*Neisseira gonorrhoeae*
2		*Neisseira meningitidis*
2	2	*Neisseira* spp.
2		*Pseudomonas aeruginosa*
2	3	*Salmonella Abortusequi*
2	3	*Salmonella Abortusovis*
2	2	*Salmonella choleraesuis (enterica)* subsp. *arizonae*
2	3	*Salmonella Dublin*
2	2	*Salmonella Enteritidis*
2	3	*Salmonella Gallinarum*
2		*Salmonella Paratyphi A,B,C*
2	3	*Salmonella Pullorum*
3 (*)		*Salmonella Typhi*
2	2	*Salmonella Typhimurium*
2		*Shigella boydii*
3 (*)		*Shigella dysenteriae* (Type 1)
2		*Shigella flexneri*
2		*Shigella sonnei*
2	2	*Staphylococcus aureus*
	2	*Staphylococcus epidermidis*
2	2	*Streptobacillus moniliformis*
2	2	*Streptococcus agalactiae*
	2	*Streptococcus dysgalactiae*
	3	*Streptococcus equi*
2		*Streptococcus pneumoniae*
2		*Streptococcus pyogenes*
2	2	*Streptococcus* spp.
2	2	*Streptococcus suis*
	2	*Streptococcus uberis*
2		*Vibrio cholerae* (including El Tor)
3	3	*Yersinia pestis*

Note: This is a shortened list of pathogens. If your organism is not on this list, first consult the lists at www.biosafety.be before concluding that your organism is not pathogenic.

Human and animal pathogenic viruses

H	A	
		Adenoviridae
	2	Animal adenoviruses
2		Human adenoviruses
	4	African swine fever virus
		Arenaviridae
3		Flexal virus
4	+	Junin virus
4	+	Lassa virus
4	+	Machupo virus
	3	Equine arteritis
	3	Simian haemorrhagic fever virus
		Astroviridae
2	2	Astroviruses
		Baculoviridae
	2	Invertebrate baculoviruses
		Birnaviridae
	2	Drosophila X virus
	3	Infectious pancreatic necrosis virus
		Bunyaviridae
3	2	California encephalitis virus
3		Hantaan virus (Korean haemorrhagic fever)
		Filoviridae
4	4	Ebola virus
4	4	Marburg virus
		Flaviviridae
3		Dengue virus 1-4
3	+	Japanese encephalitis virus
3	+	Yellow fever virus
3		Hepatitis C virus
	3	Border disease virus
	3	Bovine diarrhoea virus
	4	Hog cholera virus
		Herpesviridae
	3	Avian herpesvirus 1 (ILT)
	3	Marek's disease
	3	Bovine herpesvirus 1
	2	Bovine herpesvirus 2
	2	Bovine herpesvirus 3
	2	Bovine herpesvirus 4
	2	Chimpanzee herpesvirus (pongine herpesvirus 1)
2		Cytomegalovirus (Human herpesvirus 5)
	2	Cytomegaloviruses of mouse, guinea pig and rat
2		Epstein-Bar virus (EBV, Human herpesvirus 4)
3	2	Herpes virus B
		Orthomyxoviridae
2	3	Avian influenza virus A-Fowl plague
2	2	Equine influenza virus 1 (H7N7) and 2 (H3N8)
2	3	Influenza viruses (Types A, B & C)
		Papovaviridae
	2	Animal papillomaviruses
2		Human papillomaviruses (HPV)
	2	Bovine polyomavirus (BPoV)
	2	Monkey (SV40, SA-12, STMV, LPV)
		Paramyxoviridae
2		Measles virus
2		Mumps virus
2	2	Parainfluenza viruses types 1-4
		Parvoviridae
	2	Adeno-associated viruses AAV
	2	Canine parvovirus (CPV)
2		Human parvovirus (B 19)
		Picornaviridae
2		Coxsackieviruses
2		Polioviruses
2	3	Swine vesicular disease virus
	2	Bovine rhinoviruses (types 1-3)
2		Human rhinoviruses
		Poxviridae
	2	Entomopoxviruses
	3	Fowlpox virus
	2	Other avipoxviruses
	3	Camelpox virus
2	2	Cowpox virus
2	3	Horsepox virus
3	3	Monkeypox virus
2	3	Rabbitpox virus-variant of vaccinia
2	2	Vaccinia virus
4		Variola (major & minor)virus
4	+	White pox (Variola virus)
	2	Swinepox virus
		Reoviridae
2	2	(ortho)reoviruses
2	2	Human rotaviruses
	2	Mouse rotaviruses (EDIM, epizootic diarrhoea of infant mice)
2	2	Rat rotavirus
		Retroviridae
	3	Avian leucosis viruses (ALV)
	3	Avian sarcoma viruses (Rous sarcoma virus, RSV)
	2	Bovine foamy virus
	3	Bovine immunodeficiency virus (BIV)
	2	Equine infectious anemia virus
	3	Feline immunodeficiency virus (FIV)
	3	Feline sarcoma virus (FeSV)
3		Human immunodeficiency viruses (HIV) types 1 & 2
3		Human T-cell lymphotropic viruses (HTLV) types 1 & 2
	3	Leukomogenic murine oncovirus (Murine lymphosarcoma virus: MuLV)
	3	Lymphosarcoma viruses of nonhuman primates
	3	Monkey mammary tumor viruses (MPTV)
	3	Murine mammary tumor viruses (MMTV)
	3	Murine sarcoma viruses (MuSV)
	3	Porcine sarcoma virus
	3	Rat lymphosarcoma virus (Rat LSA)
	2	Reticuloendotheliosis viruses (REV)
	2	Simian foamy virus
	3	Simian immunodeficiency viruses (SIV)
	3	Simian sarcoma viruses (SSV)

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H	A		H	A	
		Rhabdoviridae			**Unrelated agents connected with**
3	3	Rabies virus		3	Bovine spongiform encephalopathy (BSE)
2	3	Vesicular stomatitis virus (VSV)		3	Chronic wasting disease of deer
		Togaviridae	3		Creutzfeldt–Jakob disease
2	+	Semliki Forest virus	3		Gerstmann-Sträussler-Scheinker syndrome
2	2	Sindbis virus	3		Kuru
2		Rubella virus		3	Mink encephalopathy
		Not classified		3	Scrapie
3		Blood-borne hepatitis viruses not identified yet			
	3	Borna diseases virus			

Human and animal pathogenic fungi

H	A		H	A	
2	2	*Aspergillus flavus*	2	2	*Cryptococcus neoformans*
2	2	*Aspergillus fumigatus*		2	*Fusarium coccophilum*
OP	OP	*Aspergillus nidulans*	3	2	*Penicillium marneffei*
	2	*Aspergillus parasiticus*	OP	OP	*Pneumocystis carinii*
OP	OP	*Aspergillus terreus*	OP	2	*Rhizomucor pusillus*
OP	OP	*Aspergillus versicolor*		2	*Rhizopus cohnii*
2	2	*Candida albicans*		2	*Rhizopus microspous*

Human and animal pathogenic parasites

H	A		H	A	
	3	*Eimeria acervulina*		3	*Theileria hirei*
	3	*Eimeria burnetti*		2	*Theileria mutans*
	3	*Eimeria maxima*		2	*Theileria ovis*
	3	*Eimeria necratix*		3	*Theileria parva*
	3	*Eimeria* spp.		2	*Theileria taurotragi*
3	3	*Leishmania brasiliensis*	2	3	*Toxoplasma gondii*
3	3	*Leishmania donovani*		3	*Trichomonas foetus*
2		*Leishmania ethiopica*	2		*Trichomonas vaginalis*
3		*Leishmania major*		3	*Trypanosoma brucei brucei*
2	3	*Leishmania mexicana*	3		*Trypanosoma brucei gambiense*
2		*Leishmania peruviana*	3	3	*Trypanosoma brucei rhodesiense*
2		*Leishmania* spp.		3	*Trypanosoma congolense*
3	3	*Leishmania tropica*	3		*Trypanosoma cruzi*
3		*Plasmodium falciparum*		3	*Trypanosoma equiperdum*
2		*Plasmodium* spp. (with humans and apes)		3	*Trypanosoma evansi*
2		*Pneumocystis carinii*		2	*Trypanosoma vivax*
	3	*Theileria annulata*			

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Fytopathogens

On the following page the risk group of some relevant fytopathogens is given. Some fytopathogens however are also subject to additional quarantine regulations that are designed to combat relevant plant diseases or diseases that can affect plant products.

Fytopathogenic viruses

P	
2	Alfalfa mosaic virus
2	Apple chlorotic leaf spot virus
2	Apple mosaic virus
2	Apple stem grooving virus
2	Barley yellow mosaic virus
2	Beet western yellows virus
2	Carnation ringspot virus
2	Cucumber mosaic virus
3	Hop american latent virus
2	Hop mosaic virus
3	Lettuce mosaic virus
2	Maize dwarf mosaic virus
2	Melon necrotic spot virus
2	Papaya ringspot virus
2	Pea early-browning virus
2	Potato leafroll virus
2	Potato virus A
2	Potato virus M
2	Potato virus S
2	Potato virus X
2	Potato virus Y
2	Tobacco mosaic virus
2	Tobacco necrosis virus
2	Tobacco rattle virus
3	Tobacco streak virus
2	Tobacco stunt virus
3	Tomato bushy stunt virus
2	Tomato mosaic virus
3	Tomato yellow leaf curf virus
2	Tulip breaking virus
2	Turnip crinkle virus
2	Turnip mosaic virus
3	Wheat dwarf virus
3	Wheat spindle steak mosaic virus

Fytopathogenic bacteria and related organisms

P	
2	*Agrobacterium rhizogenes*
2	*Agrobacterium rubi*
2	*Agrobacterium tumefaciens*
2	*Erwinia carotovora* subsp. *betavasculorum*
2	*Erwinia chrysanthemi pv. chrysanthemi*
3	*Erwinia salicis*
3	*Erwinia tracheiphila*
2	*Pseudomonas cichorii*
2	*Pseudomonas fluorescens*
3	*Pseudomonas syringae pv. phaseolicola*
3	*Pseudomonas syringae pv. pisi*
2	*Pseudomonas syringae* subsp. *syringae*
2	*Rhodococcus fascians*
3	*Xanthomonas campestris pv. aberrans*
2	*Xanthomonas campestris pv. alfalfae*
3	*Xanthomonas populi*

Fytopathogenic fungi

P	
2	*Alternaria dauci*
3	*Alternaria solani*
2	*Botrytis allii*
2	*Botrytis elliptica*
3	*Botrytis fabae*
2	*Botrytis hyacynthi*
2	*Botrytis tulipae*
2	*Cladosporium phlei*
2	*Cladosporium variabile*
3	*Claviceps gigantea*
2	*Claviceps purpurea*
2	*Fusarium arthrosporioides*
3	*Fusarium coeruleum*
2	*Fusarium culmorum*
2	*Fusarium graminum*
2	*Fusarium oxysporum* f. sp. *betae*
3	*Fusarium oxysporum* f. sp. *lycopersici*
2	*Fusarium oxysporum* f. sp. *pisi*
3	*Fusarium oxysporum* f. sp. *trifolii*
3	*Fusarium solani* f. sp. *cucurbitae*
3	*Fusarium solani* f. sp. *phaseoli*
3	*Fusarium solani* f. sp. *pisi*
2	*Glomerella cingulata* (anamorph *Colletotrichum gloeosporioides)*
2	*Glomerella graminicola* (anamorph *Colletotrichum graminicola)*
2	*Glomerella tucamanensis* (anamorph *Colletotrichum falcatum)*
3	*Mucor circinelloides*
3	*Mucor piriformis*
3	*Mucor racemosus*
3	*Mucor strictus*
2	*Penicillium corymbiferum*
2	*Penicillium cyclopium*
2	*Penicillium digitatum*
2	*Penicillium expansum*
2	*Penicillium italicum*
2	*Phytophthora infestans*
2	*Phytophthora megasperma*
2	*Rhizoctonia carotae*
2	*Rhizoctonia fragariae*
2	*Rhizoctonia tuliparum*
2	*Rhizopus arrhizus*
2	*Rhizopus stolonifer*
2	*Sclerophthora macrospora*
2	*Sclerospora graminicola*
2	*Sclerotinia minor*
2	*Sclerotinia trifoliorum*
3	*Septoria apiicola*
2	*Septoria azaleae*
3	*Septoria chrysanthemella*
2	*Septoria lactucae*
3	*Septoria lycopersici* var. *lycopersici*

Fytopathogenic parasites

P	
3	*Heterodera glycines*

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ANNEX 4: AKNOWLEDGEMENT

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ANNEX 5: RESPONSIBLE PERSONS AND SOURCES

Names and coordinates of responsible persons

The list below is meant to serve as an overview of the persons responsible for the safety in biotechnological laboratories. These persons differ from one department to another. Fill out the list below and make sure that you have it within reach in your laboratory.

	Name	Telephone	Other
Emergence number			
Laboratory responsible person			
First aid responsible person			
Location of first aid kit			
Biosafety coordinator			
Workers protection contact			
Company doctor			
Waste contact person			
Regulatory affairs manager, VIB	René Custers	09 244 6611	rene.custers@vib.be

If you have general questions about classifications or necessary containment measures, you should first contact your superior. And in case this does not give you a decisive answer, you should subsequently contact your groupleader, internal biosafety expert or external biosafety expert.

ANNEX 6: SELFTEST

Question 1: What is the definition of a genetically modified organism?

Question 2: How many biological risk classes or risk groups are there to classify the ability of organisms to cause disease?

Question 3: Mention three types of hazards that are potentially associated with living and/or modified organisms.

Question 4: To what risk group (or biological risk class) belongs wild type *E.coli?*

Question 5: To what risk group (or biological risk class) belong *E.coli* K12 strains?

Question 6: Describe the different steps of the risk assessment process for genetically modified organisms

Question 7: What is an aerosol?

Question 8: In what way can the combination of an immortalised cell line with a viral vector give rise to the unwanted spread of genetically modified material?

Question 9: Mention four important physical containment measures.

Question 10: True or false: the culture fluid containing genetically modified micro-organisms of risk class 1 can be thrown down the sink after the experiment.

Question 11: Which of the following decontaminants does not work against spores? (1) chlorine, (2) formaldehyde, or (3) ethanol.

Question 12: True or false: contaminated materials that will be reused only have to be washed. They do not have to be decontaminated.

Question 13: What should one do after genetically modified bacteria have been spilled on the laboratory bench?

Question 14: Mention five important routines for working in a class II safety cabinet.

SELFTEST ANSWERS

Question 1: *A genetically modified organism is an organism in which the genetic material has been altered in a way that is not possible by means of reproduction or natural recombination.*

Question 2: *There are four risk groups (1 to 4). Group 1 organisms are not able to cause disease. Group 2, 3 and 4 are organisms that can cause disease.*

Question 3: *For instance pathogenicity, toxicity or disturbance of ecological balances.*

Question 4: *Wild type E.coli is risk group 2. It is therefore able to cause disease.*

Question 5: *E.coli K12 strains are attenuated and no longer able to cause disease. They belong to risk group 1.*

Question 6: *(1) List all the potential hazardous properties of host, insert and vector. (2) This enables you to identify a first idea of the appropriate risk class. (3) Starting from this risk class, identify the necessary containment measures to be able to handle the organism safely. Take into account the properties of the environment that could be exposed to the organism, the type and scale of the activity, and if applicable any non-standard operations. (4) Determine the final risk class.*

Question 7: *An aerosol is made up of very small droplets of fluid that are able to spread via the air. These droplets can contain viable micro-organisms and therefore provide a means for these organisms to spread to the environment.*

Question 8: *An immortalised cell may contain viral sequences (for instance HELA cells contain the E6E7 genes of HPV) that together with viral sequences present in the vector may give rise to the formation of infectious virus particles through recombination.*

Question 9: *Examples are: closed doors and windows, smooth benches that can be contaminated easily, a class II safety cabinet, negative air pressure, an airlock, HEPA-filtered outward airflow, etc.*

Question 10: *False: also risk class 1 GM micro-organisms should be inactivated before they are discarded as waste. There are only few exceptions to this rule for which you need an explicit authorisation of the competent authorities.*

Question 11: *Ethanol is not effective against bacterial spores. So if you for instance use Lactococci you should use another decontaminant.*

Question 12: *False: Potentially contaminated materials that will be reused need to be decontaminated (by sterilisation or another effective means) before they are washed.*

Question 13: *The spilled bacteria should be removed with paper towels or tissues and thrown into the waste basket for biologically contaminated waste. And the bench should be decontaminated thoroughly with tissues soaked in a decontaminant, for instance ethanol or chlorine.*

Question 14: *Important routines are: (1) close the door of the L2 laboratory behind you (2) turn the cabinet on 10 minutes before starting work (3) decontaminate the bench in the cabinet and the airslids before starting work (4) only put the materials in the cabinet that are strictly necessary, (5) do not put any material on the front and back airslids, (6) work deep enough in the cabinet, (7) move gently to minimise disturbance of the airflow, (8) decontaminate the bench and the airslids after the experiment (9) leave the cabinet working for another 5 minutes, (10) fill out the safety cabinet log (name, date, used biological material, sign for decontamination)*