BIOSAFETY GUIDELINES

RISK ASSESSMENT OF GENETICALLY MODIFIED MICROORGANISMS

DEPARTMENT OF BIOSAFETY

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Any future regulations, guidelines and related documents will be posted to this website.

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RA=Risk assessment

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RA=Risk assessment

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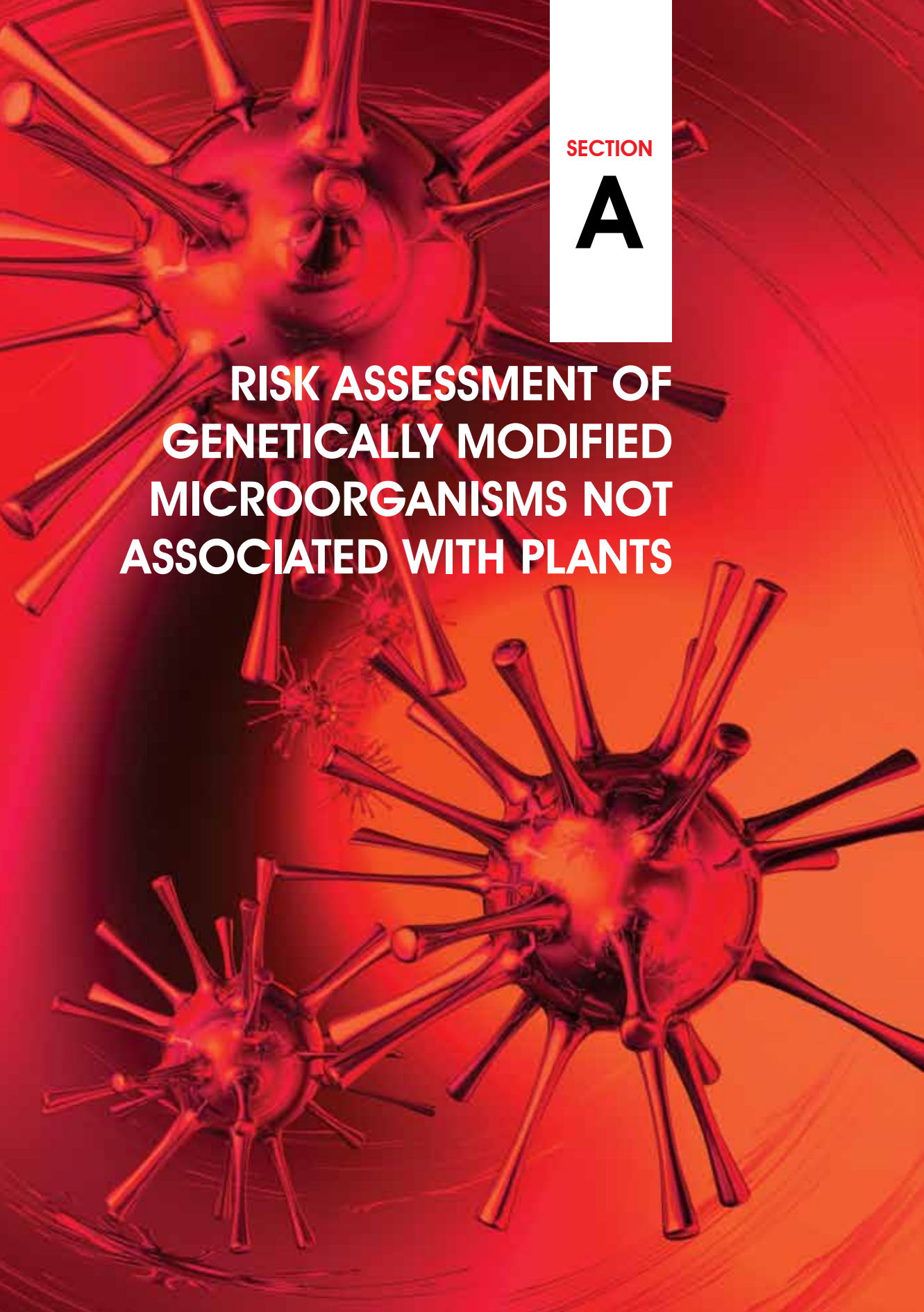
INTRODUCTION TO THE GUIDELINE

Living modified organisms (LMO) are created using recombinant DNA technology and involve the transfer of genetic material between unrelated organisms and species. Activities involving LMO (the importation, exportation, contained use and release) are regulated by the *Biosafety Act 2007* (the Act) and *Biosafety (Approval and Notification) Regulations 2010* to ensure safe application of modern biotechnology to protect human, plant and animal health, the environment and biological diversity. Under these legislations individuals and institutions that are carrying out contained use activity in the laboratory, that is, all research and development (R & D) activities involving modern biotechnology, import for contained use and export of LMOs, are required to inform or notify their activities to the National Biosafety Board (NBB). Approval from the NBB is required for the release and importation of LMOs and products of LMOs

It is important to appreciate that the genetic modification of a microorganism can affect its ability to cause harm to human health, animal health and the environment. Therefore, the Act requires risk assessment, risk management and emergency response plans, endorsed by the Institutional Biosafety Committee (IBC) before formal submission to the NBB from researchers and proposers of modern biotechnologies to ensure safe and responsible use of modern biotechnology. As referred to in *Part V, Section 36 (1)* of the Biosafety Act, "*an assessment of risk and adverse effect that such LMO and products of LMO will have or are likely to have on human, plant and animal health, the environment and biological diversity*", should be incorporated into the design, construction and operation of the release, import or contained use activities.

This Guideline on risk assessment of Genetically Modified Microorganisms (GMM) is applicable to all individuals involved in Research and Development (R & D) activities of modern biotechnology working in laboratories of government and non-governmental organisations. Adoption of this Guideline is essential for all public and private organisations, working on modern biotechnology, specifically involving GMM so as to conduct a proper risk assessment that will

enable safely handling and ensure protection of human, plant and animal health, the environment and biological diversity. This Guideline should be used in addition to relevant legislations and guidance documents that cover work with LMO as mentioned in Appendix 1.



SECTION

A

RISK ASSESSMENT OF GENETICALLY MODIFIED MICROORGANISMS NOT ASSOCIATED WITH PLANTS

CHAPTER 1

INTRODUCTION

1.1 SCOPE

Section 1 of this Guideline covers the risk assessment for human-health and environmental protection of work involving the genetic modification of all microorganisms, including bacteria, fungi, protists, cell-lines and viruses, which are human and animal pathogens. Section 2 of this Guideline covers the risk assessment for work with GMM associated with plants. These sections also cover guidance relating to the assignment and implementation of containment and control measures

Specific guidance giving more detailed information regarding aspects of genetic modification work with microorganisms and commonly used systems is also included in this Guideline. The relevant sections include:

- Hazards posed by inserted sequences
- Routine cloning work with *Escherichia coli*
- Bacterial gene-delivery systems
- Work with cell cultures
- Adeno-associated viruses
- Adenoviruses
- Baculoviruses
- Herpesviruses
- Poxviruses
- Retroviruses
- Viral reverse genetics

The term 'animal' is used here in the broadest sense and includes pathogens of both vertebrates and invertebrates. It also covers work with most types of cloned DNA, including prions, proviral DNA, oncogenes, growth factors, cytokines, non-coding elements, antisense constructs, siRNA and host range/virulence factors that are carried or

vectored by a microorganism. Many of the issues raised in this guidance are exemplified using cases of genetic modification work involving bacterial or viral systems.

1.2 DEFINITIONS

For the purposes of this Guideline, the definitions below apply.

1.2.1 Contained use

Any operation including R & D, production or manufacturing operation involving LMO or storage of LMO undertaken within a facility, installation or other physical structure such that it prevents the contact and impact of the LMO on the external environment.

1.2.2 Containment

The combination of buildings, engineering, equipment and work practices used to handle hazardous microorganisms safely.

1.2.3 Disabled strain

Strain of microorganism or virus that is genetically modified to minimise survival such that any inadvertent release is unlikely to initiate productive infection of the microorganism or virus outside of the experimental facility.

1.2.4 Donor organisms

The organism from which genetic material is obtained for transfer to the recipient organism for the intended genetic modification.

1.2.5 Hazard

A source that has a potential to cause harm. In case of microbiological hazards, this is associated with the microorganism itself (naturally occurring wild type or genetically modified).

1.2.6 Host organism

An organism that harbours a parasite or mutual commensal symbiont, typically providing nutrition and shelter. In the case of viruses, the host may be a cell culture that allows viral replication. A plant host is one that supplies food resources and substrate for certain insects, other fauna or microorganisms.

1.2.7 Institutional Biosafety Committee (IBC)

A formal expert committee appointed within an organisation undertaking modern biotechnology activities. The main scope of the IBC is to provide guidance on the safe use of modern biotechnology, monitor the said activities, establish and monitor the implementation of policies

and procedures for the purpose of the said activities and to determine the classes of Biosafety Levels for contained use activities.

1.2.8 Living modified organism (LMO)

Any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology.

1.2.9 Modern biotechnology

The application of *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of the nucleic acid into cells or organelles; or the fusion of cells beyond the taxonomic family that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection.

1.2.10 Pathogen

A microorganism capable of causing disease in a host.

1.2.11 Personal Protective Equipment (PPE)

Protective clothing, hair-nets, caps, masks, respirators, shoe covers, boots, gloves, goggles or other garments designed to protect the user's body or clothing from occupational exposure to infections from spills, contaminants and injury by sharps.

1.2.12 Provisional Containment Level

Initial containment level assigned to a GMM activity before a thorough risk assesssment is done. This containment level is based on the risk grouping and hazards to human health and environment. After a risk assessment is done, the final containment level for the gmm activity may change.

1.2.13 Recipient organism

The organism that receives genetic material from a donor organism for the intended genetic modification. The recipient organism is also known as parent organism.

1.2.14 Risk

The combination of likelihood and consequence of an undesirable event related to a specific hazard.

1.2.15 Risk assessment

Risk assessment is a scientific process of estimating the potential of a hazard to give rise to an adverse outcome. This estimation is based on a combination of the likelihood of the hazard occuring and the consequences if the hazard occurs.

1.3 FORMAT FOR RISK ASSESSMENT

1.3.1 The first stage of the risk assessment process for a GMM is to identify the potential harmful properties of the GMM to determine an initial classification for the GMM.

This is achieved by the identification of hazards associated with the recipient, donor organism, vector and insert where appropriate. There are some types of activities where particular caution must be exercised. These are generally cases where the pathogenicity or host range of a pathogen has been enhanced or altered and include:

i. Activities with genetically modified (GM) pathogen that carry genetic inserts that may confer potentially harmful biological activity. Examples are a known virulence factor, a toxin or a determinant of immune evasion.

ii. Activities with GM pathogen that have been modified to alter host range (*e.g.* viral attachment and entry determinants; bacterial host range factors), may require a higher containment level compared to the recipient organism or vector construct.

1.3.2 The following procedures represent a recommended model for risk assessments of GMM, although it is not intended to be prescriptive:

i. **Overall nature of the intended GMM**. Assessment of the risks it may pose to human health and the environment.

ii. **Risk assessment for human health**. Identification of potential mechanisms by which the GMM might pose a hazard to human health; Assessment of the potential severity, likelihood of occurrence and considerations of uncertainty. Establishment of a BSL that is sufficient to safeguard human health.

iii. **Risk assessment for the environment**. Assessment of the risks it may pose to the environment (includes plant, animal health and biological diversity), consideration of the potential severity and likelihood of occurrence. Establishment of a BSL that is necessary to protect the environment.

iv. **Review of procedures and control measures**. Implementation of any additional control measures necessary to safeguard both human health and the environment.

v. **Assignment of Biosafety Levels (GM-BSL) of containment LMO**

The biosafety principles and practices described in the World Healh Organization (WHO), *Laboratory Biosafety Manual, 3rd edition*

(WHO, 2004) provide the fundamental guidelines for laboratories working with pathogenic organisms. Four levels of biosafety based on international approaches are described that are arranged in order of increasing stringency to reflect the level of risks involved. They are: Biosafety Level 1 (BSL1), Biosafety Level 2 (BSL2), Biosafety Level 3 (BSL3) and Biosafety Level 4 (BSL4). Under the *Biosafety (Approval and Notification) Regulations 2010*, four classes of Biosafety Levels (BSL) are specified in the *Second Schedule* for any activity involving modern biotechnology. This classification is consistent with internationally recognised standards. The IBC plays a fundamental role in determining the classes of BSL for contained use for the purpose of modern biotechnology research and development undertaken within the facility where the IBC has been set up. Five categories of containment facility for genetic modification activities are described in the "*Biosafety guidelines for Contained use activity of LMO, 2010*":

- Genetic modification of microorganisms (GM-BSL)
- Genetic modification of plants (GP-BSL)
- Genetic modification of animals (GA-BSL)
- Genetic modification of arthropods (GI-BSL)
- Genetic modification of aquatic organisms (GF-BSL)

Genetic modification work involving microorganisms that fall into the lowest class of activity i.e. GM-BSL1 will require minimal assessment. It is a legislative requirement to assess the risks and employ measures to minimise the chances of exposure. The level of detail required will vary from case to case and will depend upon the nature of the hazards and the degree of uncertainty. Where a potential for harm is identified, a more detailed consideration of the risks associated with the activity should be undertaken. Less detail will be required for less hazardous work, such as routine cloning work in disabled *E. coli* or the generation of E1/E3-deleted adenovirus vectors carrying harmless inserts.

1.3.3 The final risk assessment must contain enough background and detail to ensure that a reviewer with a limited understanding of the precise nature of the work will not require further information to comprehend the nature of any hazards.

Supplementary information can take the form of references to scientific literature and reports, which can be used to justify statements made. All potential hazards should be acknowledged and information should be based upon established scientific knowledge wherever possible. The lack of scientific evidence for a particular hazard being legitimate

should not be automatically taken to mean that it does not exist. Any uncertainty should be taken into consideration in the risk assessment.

1.3.4 All genetic modification risk assessments should be reviewed regularly and be updated in the light of new scientific knowledge or where there has been a change in the nature of the activity (including a change in scale or any new procedures and containment measures).

Documentation is important for genetic modification work. All data should be recorded and used to supplement the risk assessment where appropriate. Risk assessments should be kept for 6 years after the work has ceased (storage of materials is also considered to be active work in this case).

1.3.5 The risk assessment should also consider the purpose of the work.

For example, if the GMM is ultimately intended to be a therapeutic product then the assessment will require updating as the product moves between basic laboratory research, upstream development, preclinical and clinical phases.

1.3.6 Containment and control measures must be assigned on the basis of both human health and environmental aspects of the risk assessment.

In the majority of cases where human pathogens are modified, the containment and control measures appropriate for the protection of human health will also be sufficient to protect the environment. In other cases, the measures needed to protect human health may be minimal whereas much more stringent measures will be required to protect against harm to the environment. This is particularly true for work with animal pathogens or where the recipient organism is modified such that it poses a risk to animal health or plants. The nature of the intended GMM will identify whether human health or environmental concerns take priority, as explained below.

1.4 OVERALL NATURE OF THE HAZARDS POSED BY THE INTENDED GMM

A risk assessment for human, plant and animal health and a risk assessment for environmental and biological diversity protection are required in all cases under the *Biosafety Act 2007*. However, the

balance of the significance given to each section will vary depending on the nature of the organism. For example, GMM based upon human pathogens will require careful assessment of the risks to human health and the activity class will ultimately reflect the measures needed to prevent infection of staff in these cases. Conversely, GMM based upon animal pathogens or plant pathogens will probably require more detailed and careful assessment of the risks to the environment. The final activity class will ultimately reflect the measures needed to prevent release and the potential consequences. Any change in the nature of the intended GMM must also be considered, as the balance of risks to human health and the environment respectively may well differ from those of the recipient organisms, e.g. if the host range of the pathogen has been altered. While humans in the community are considered to be a part of the environment, it is logical to consider risks to human health in one section as detailed below.

1.4.1 A decision can be made from the outset as to which part of the genetic modification risk assessment is the more applicable and should take precedence

- For GMM that are primarily a potential risk to human health, a detailed **risk assessment for human health** can be carried out first and a provisional containment level set based upon human health protection.
- For GMM that are primarily an environmental concern, a detailed **risk assessment for the environment** can be carried out first and a provisional containment level to prevent harm to the environment set.

1.4.2 This recommended approach to the risk assessment of GMM is illustrated in Figure 1.

1.5 RISK ASSESSMENT FOR HUMAN HEALTH

The objective is to identify all potential hazards to human health and then to assess the likelihood and potential severity of the consequences, should the hazards be realised. Risk assessment for human health consideration should take into account the following details:

- expected toxic or allergenic effects of the GMM and/or its metabolic products;
- comparison of the GMM to the recipient or (where appropriate) parental organism;
- pathogenicity;
- expected capacity for colonisation;

- if the GMM is pathogenic to humans who are immunocompetent;
- diseases caused and mechanism of transmission including invasiveness and virulence;
- infective dose;
- possible alteration of route of infection or tissue specificity;
- possibility of survival outside of human host;
- biological stability;
- antibiotic-resistance patterns;
- allergenicity;
- toxigenicity; and
- availability of appropriate therapies and prophylactic measures.

1.5.1 Risks associated with the recipient strain

i. Particular care must be given to the assessment of GMM that have the potential to enter human cells or establish an infection in human hosts. All biological agents (any organism that may cause infection, allergy, toxicity or any other hazards to human health) are classified into one of four Risk Groups (RG) based on *WHO Biosafety Manual, 3rd edition, 2004*. Further guidance can be found in Appendix 2 of tthis Guideline. Specific guidance on certain commonly used GM bacterial and viral vector systems is given in Appendix 3 of this Guideline.

ii. The degree of pathogenicity of the recipient strain and the severity of the consequences of exposure should be estimated. Where the recipient is a known human pathogen, the organism will be assigned to Risk Group (RG) classification based on the Seventh Schedule [Subregulation 6(1)] of the *Prevention and Control of Infectious Diseases Act 1988; Prevention and Control of Infectious Diseases (Importation & Exportation of Human Remains, Human Tissues and Pathogenic Organisms and Substances) Regulations, 2006*. The classification of microorganism as in Appendix 2 does not determine the containment levels. To determine the containment levels required for GMM, the risk assessment should be done.

    Properties of recipient strain that should be considered where relevant are:

    - nature of pathogenicity and virulence, infectivity, allergenicity, toxicity and vectors of disease transmission;
    - nature of indigenous vectors and adventitious agents, where they could mobilise the inserted genetic material, and the frequency of mobilisation;
    - nature and stability of disabling mutations, if any;

- any prior genetic modifications;
- host range (if relevant);
- any significant physiological traits which may be altered in the final GMM and if relevant their stability;
- natural habitat and geographic distribution;
- interaction with, and effects on, other organisms in the environment (including likely competitive or pathogenic properties); and
- ability to form survival structures (such as spores).

iii. Attenuated derivatives of pathogens may be assigned to a lower RG than indicated in the approved list, if it can be verified that the strain is adequately disabled. This can be described as *biological containment* and represents engineered genetic control measures that will permit the safe handling of otherwise pathogenic species.

iv. In most cases, the origin and nature of attenuating lesions should be well understood and will form an important part of the risk assessment. In some instances, however, the nature of the attenuation may not be well understood but a history of safe use may

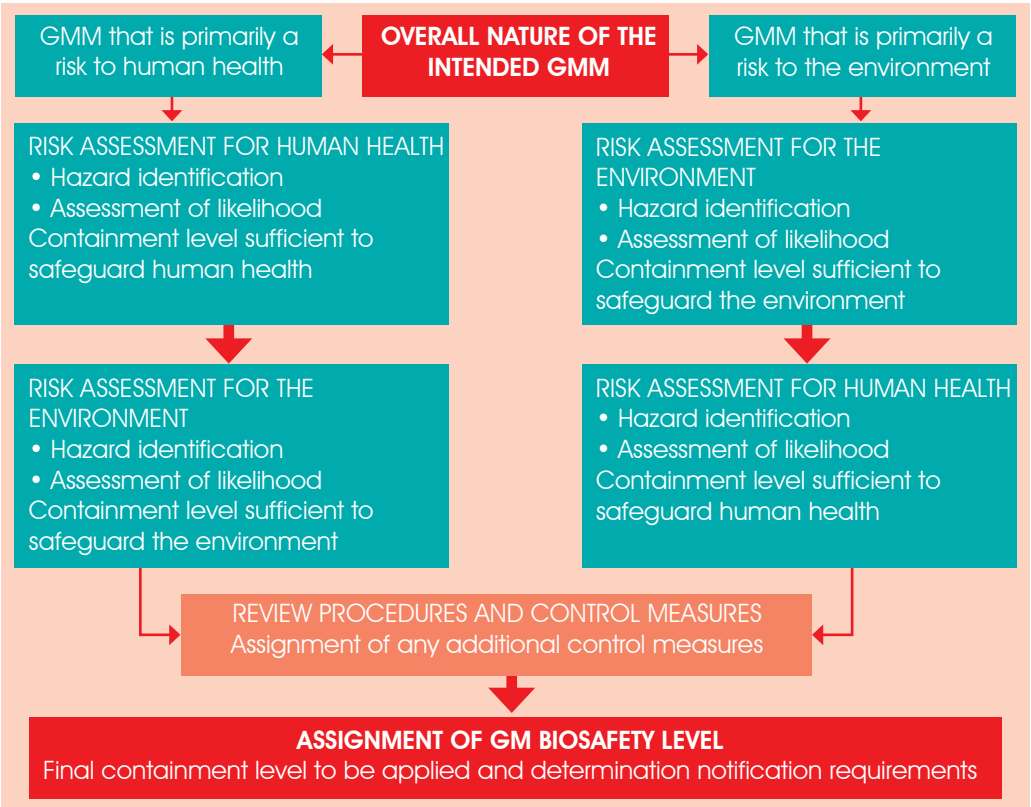


Figure 1. *Illustration of a recommended approach to the risk assessment of GMM*

permit the assignment of a lower RG. Some examples of attenuated strains in use are given below:

- Wild type, pathogenic *E. coli* strains are classified as RG 2 and as such, should be handled at BSL2 containment. However, many derivatives of the *E. coli* K-12 strain have been demonstrated to be avirulent, have a long history of safe use and the genetic lesions are well understood. Many of these strains can be handled safely at BSL1.
- Wild type Adenoviruses are RG2 pathogens and should be handled at BSL2. Many adenoviral vector strains have been constructed that are deleted for E1, encoding key genes required for viral growth. These strains are disabled and incapable of establishing a productive, transmissible infection in humans. These vector strains can be considered to be avirulent and may be handled safely at BSL1.
- Some vectors derived from herpes simplex virus, (HSV), a RG2 virus have targeted gene deletions with attenuating effects that are indeterminable outside of a human host. Many of these vectors have been tested extensively in humans and these strains have demonstrated a good safety profile. Examples are Human HSV-1716, a replication restricted Human Herpes simplex type 1 virus and Disabled Infectious Single Cycle (DISC) HSV Vector, DISC, that can be handled at BSL1.
- The highly attenuated strain of vaccinia virus, Modified Vaccinia Ankara (MVA) strain, while being poorly understood in terms of the nature of attenuating lesions, also has a long history of safe use as a vaccine. MVA has been administered to numerous animal species including monkeys, mice, swine, sheep, cattle, horses, and elephants, with no local or systemic adverse effects. This strain can be handled at BSL1.

v. The reclassification applies only to disabled recipient strains. Any harmful properties associated with the insert or the final GMM may present an increased risk and warrant additional control measures. Strains for which evidence of attenuation is not available must be carefully considered on a case-by-case basis. In assessing whether a strain is adequately disabled, the possibility of reversion or complementation should be considered and it should be confirmed that the GMM remains disabled. The likelihood of reversion will depend on the mechanism of attenuation, i.e. deletion mutants are less likely to revert to wild type than point mutations or conditional-lethal mutants. The decision on reclassification and containment level of the GMM should be made by consultation with the Institutional Biosafety Committee.

vi. A consequence of a reversion event in an attenuated or disabled recipient could be the generation of a pathogenic strain that expresses the inserted gene. One approach that can be used to minimise the likelihood of such an event is to place the insert at the site of an attenuating mutation. Thus, any recombination event that restores previously deleted sequences will result in the deletion of the inserted gene. It is recognised that this technique will not be appropriate in all systems. However, this method should be used whenever practicable, especially when working with harmful genes. In particular, where it is proposed to insert a harmful gene into a virus other than the site of a disabling mutation, full justification should be given in the risk assessment.

1.5.2 Risks associated with genetic inserts

i. This primarily applies to inserted genes encoding products with potentially harmful biological activity, for example toxins, cytokines, growth factors, allergens, hormones or oncogenes. Consideration should be given to the the following characteristics:

- specific identity and function of the insert (genes);
- kinetics and level of expression of inserted genetic material;
- source of the genetic material, identity of the donor organism(s) and characteristics where appropriate, history of prior genetic modifications if appropriate;
- location of inserted genetic material (possibility of insertional activation/deactivation of host genes); and
- and the possible consequences of exposure to the GMM carrying the gene.

In cases where the insert is not being expressed, or where the expressed product is produced in an inactive form (such as in an insoluble inclusion body) it is unlikely that the gene product will give rise to harm. This is often the case when human genes are expressed in *E. coli* or other prokaryotic host systems, since proteins lack the required post-translational modifications and may not be biologically active. However, this is not always the case; for example, many non-glycosylated cytokines are both soluble and biologically active when expressed in *E.coli*. Likewise, expression of potentially harmful genes would not be predicted in prokaryotic systems if they were under the control of eukaryotic promoters. The sequence should be carefully scrutinised to ensure that no cryptic prokaryotic promoters have been generated during the cloning steps or due to sequence optimization of the control regions.

ii. Careful consideration should be given to potentially harmful prokaryotic genes expressed in prokaryotic systems (*e.g.* a

bacterial toxin) and products active in eukaryotic cells carried by viral vectors, particularly genes encoding regulators of cell growth and differentiation, for example signalling molecules, apoptosis regulators, differentiation mediators and oncogenes.

iii. Almost any gene that encodes a product involved in cell-to-cell or intracellular signalling, interaction with the environment, cell cycle control, differentiation or apoptosis could be regarded as potentially oncogenic in some circumstances (*e.g.* perhaps if expressed constitutively at high levels). While development of a cancer is acknowledged to be a multi-step process requiring a number of genetic lesions to generate a malignant tumourigenic cell, expression of some genes (*e.g.* those encoding growth factors) can allow proliferation or confer an extended life span upon otherwise quiescent cells. This may predispose a cell to accumulating oncogenic lesions and is particularly relevant if the gene is stably introduced into a cell. That cell and its progeny might be one step nearer to forming a cancer and such a potentially serious outcome should not be dismissed lightly.

Further specific guidance on the hazards posed by genetic inserts, including oncogenes, can be found in Chapter 3.

1.5.2.1 Hazards arising from the alteration of existing pathogenic traits

Many modifications will not involve genes with products that have activities that will be directly harmful, but adverse effects may nevertheless arise as the result of exacerbation or alteration of existing pathogenic traits. There are many different ways in which the pathogenicity or virulence of the host organism can be affected and the following potential mechanisms should be considered. However, the list is not exhaustive and all modifications should be carefully assessed in the light of scientific knowledge:

- **The inserted gene encodes a pathogenicity or virulence determinant**

  For example, in bacterial systems this could be a toxin, invasin, or surface determinants such as pili, lipopolysaccharide (LPS) and capsule that may affect the infectivity and virulence of a bacterial host organism.

- **The modification affects the infectivity or virulence of the host organism**

  There are many possible mechanisms by which the inherent pathogenicity of the host organism can be affected. Unforeseen effects may also be observed while making seemingly innocuous alterations to the genes of the organism. This is particularly relevant to complex systems such as bacteria where genes are often part of a cluster

or encode a component of a regulatory network. The modification or deletion of one gene may have ramifications beyond the loss or alteration of the known functions of the encoded products. The expression of other genes may be affected and biosynthetic or signaling pathways may be disrupted, resulting in altered pathogenic traits.

- **The modification alters susceptibility to the immune system**

  The ability to evade the immune system is an important determinant of pathogenesis for many microorganisms. Immune evasion determinants are frequently dispensable for growth *in vitro* and their deletion can be viewed as innocuous or attenuating. It can be argued that loss of immune evasion functions (*e.g.* deletion of E3 from Adenovirus or the IL-18 binding protein from Poxviruses) might result in more effective clearing of the organism during an infection. Similarly, insertion of genes encoding immunomodulatory functions that are not natural to the recipient organism might affect pathogenesis. For example, Vaccinia and Mousepox viruses modified to express Interleukin 4 are more pathogenic because the appropriate immune response for the effective clearance of viral infection is inhibited.

- **The modification alters tissue tropism or host range**

  There are many factors that might change the natural tropism of a microorganism. Modification or substitution of viral cellular entry determinants can give rise to viruses with altered cellular tropism. Some viruses (*e.g.* vaccinia virus) have a number of host range determining genes that confer the ability to replicate within certain cell types. Modification of viral entry determinants (*e.g.* viral surface glycoproteins) might permit the entry of the virus into normally refractory cell types and expression of the insert sequences might occur, even if replication is impossible. Pathogenic bacteria may also have determinants that affect host range or the ability to colonise certain sites. During the risk assessment, careful consideration should be given to the possible effects on tissues and sites not normally infected or colonised by the recipient organism and whether the normal route of transmission of the organism has been altered. In the case of replication-competent viruses with extended/altered tropism, it should be assumed that they would require a higher level of containment as compared to the recipient strain until the properties of the GMM are better understood.

- **The modification alters the susceptibility of the organism to prophylaxis**

  In the event of exposure to humans, the availability of effective prophylaxis may be an important supplementary safety measure. Therefore, careful consideration should be given as to whether the modification will result in reduced susceptibility of the GMM

to the prophylactic treatment that is effective against the recipient organism. For example, this could be additional antibiotic resistance conferred upon bacteria during the modification process or the conferring of drug resistance to a virus (e.g. deletion of poxvirus or herpesvirus thymidine kinase functions results in resistance to nucleoside analogue-based antivirals). Furthermore, some modifications might result in a GMM that is immunogenically novel and staff that are normally immune to the recipient organism might be susceptible to the GMM. Moreover, in such cases, a vaccine that may protect against the infection by the recipient organism may not be effective against the GMM.

1.5.2.2 Transfer of harmful sequences between organisms

i. There are many mechanisms by which sequences may be transferred between microorganisms and the factors that affect the frequency of such events and the likelihood of a harmful consequence are complex. Such issues must be carefully considered in the risk assessment. During the risk assessment process, it is important to consider the potentially harmful consequences of sequences inserted into a GMM being transferred to other organisms, or that the GMM itself may acquire sequences that increase its pathogenicity.

ii. With the notable exception of some viruses (where recombination events between virus genomes and viral sequences present in infected cells are an important consideration), the transfer of genetic information present on the genomes of microorganisms is much less likely than if they are present on an episomal form, such as a plasmid, cosmid or artificial chromosome. The frequencies of successful horizontal gene transfer in the environment are low, even for genes located on plasmids, although there is a finite possibility that any gene may be transferred, even if the mechanism is just a passive one involving release of DNA from senescing (biological aging) cells, and this should not be discounted.

- **Sequence mobilisation in bacteria**

i. Whether or not a prokaryotic GMM will be able to survive in the environment in the event of a breach of containment is a key consideration. The longer the organism can survive, the greater the likelihood that a transfer event will be successful in generating an organism that poses a threat to human health. For example, some disabled *E.coli* K-12 strains will survive for up to several days in the gut and for similar lengths of time in the environment. Genes carried on plasmids require particular consideration as transformation and conjugation events could result in the transfer of harmful sequences between bacteria. Sequences present on bacterial chromosomes are less likely to be transferred. However, phage-mediated mobilisation of inserted sequences should be considered as a possibility.

ii. If the sequence is plasmid-borne, then the mobilisation status of the plasmid backbone should be considered. As a general rule, non-mobilisable plasmids should be used. If mobilisable plasmids are to be used, this should be fully justified by the risk assessment and suitable controls implemented. It is also important to consider whether there is any selection pressure in the local or wider environment that might contribute to its persistence. It may be that the 'harmful' sequence (*e.g.* a drug-resistance marker) is naturally occurring, and therefore the impact of transfer will be diminished. However, the possible consequences of the transfer of novel constructions should be assessed; whether the sequence gives an advantage to naturally occurring pathogens or if the sequence gives an advantage to naturally occurring pathogens.

- **Recombination between related viruses**

  While the phenotype of the GM virus that is under construction is the primary consideration, some thought must also be given to the possibility that harmful sequences may be transferred as the result of a recombination event. Scenarios that need to be considered at this stage include the possibility that a disabled vector might recombine with the recipient or wild type virus or with viral sequences present in the infected cell and revert to a replication-competent derivative of the GMM. One way in which this might arise is as the result of an accidental cross contamination in a laboratory handling both disabled and wild type virus. If a recombination event could give rise to a harmful derivative of a GM virus by restoring previously deleted or mutated genes, then great care should be taken to prevent cross-contamination in the laboratory. It is reasonable to assume, however, that genetic inserts that are positioned at the site of the disabling mutation would be lost in the event of a recombination event that restores competency. Inserted sequences should be so positioned wherever possible and any decision to place genetic inserts at any other site should be fully justified by the risk assessment.

- **Reassortment between segmented RNA viruses**

  Some RNA viruses have segmented genomes (*e.g.* Influenza virus) and can achieve genetic variability in nature by 'swapping segments' with related viruses. Reverse genetics approaches permit rational genetic modification of these viruses and it is important to consider that cross- contamination or accidental inoculation of a worker who is already carrying an infection with a wild type virus could result in the generation of novel strains that could be regarded as harmful. If such an event is a possibility then great care should be taken to prevent cross-contamination in the laboratory, or exposure of staff that may be harbouring an infection with a wild type virus.

1.5.3 Likelihood that the GMM will be a risk to human health

i. The initial stages in the risk assessment process so far involve identifying those features of the GMM that have the potential to cause harm and the mechanisms by which these hazards could be realised. While it may be possible to draw up theoretical scenarios whereby the GMM may be hazardous to human health, the chances of them being realised should be evaluated and understood.

ii. It is therefore important to consider the likelihood that the identified hazards will be manifested. Factors that come into play are (i) judgements as to the overall fitness of the GMM and (ii) the probability that rare events may occur (*e.g*. the likelihood of gene transfer). Estimating the likelihood of a harmful consequence being realised will be difficult where there is no firm data on which to base a judgement. In general, the weight given to information used in these considerations should reflect the quality of the supporting data. Where the likelihood of harm is poorly understood, a precautionary approach should be adopted until evidence to the contrary has been obtained.

- **Consideration of the ability of GMM to become established in the host**

i. An assessment should be made as to the ability of the GMM to establish an infection, how efficient that infection would be and its ability to spread within a host or through a community. This represents an evaluation of the 'fitness' of a GMM and should be based upon available scientific knowledge. Any uncertainty should be taken into consideration in the risk assessment and a precautionary approach taken.

ii. It is important to remember that fitness and pathogenicity are not interdependent. Some modifications, while theoretically making the GMM more pathogenic, may also render the GMM less fit. For example, overexpression of a toxin in a bacterium may make the GMM more pathogenic than the recipient strain, although the overexpression of that toxin might be deleterious to the metabolism of the GMM. This would mean that the GMM is less fit compared to the recipient organism, even though the expressed product itself is hazardous. Another example would be insertion of a foreign gene into the E3 locus of an Adenovirus. The modified virus will be less likely to establish an infection and spread in the community as the loss of E3 makes the virus more susceptible to immune surveillance. Therefore, the virus is arguably less fit. In this case, the pathogenicity of the virus is increased, since there would be a more severe inflammatory response than would be the case with wild type virus, particularly in an immunocompromised individual.

- **Consideration of the probability that rare events will occur**

i. It is often possible to assign a frequency to a given event. Often, this can take the form of a precise numerical frequency obtained in-house or through published data. For example, published data exists that compares the frequency of transfer of mobilisable, mobilisation defective and non-transferable plasmids. Similarly, the rates of mutation and frequencies of recombination during microbial replication are open to quantitative analysis and some are known and published.

ii. In many cases this will not be possible and an approximate, semi-quantitative or descriptive assessment of the frequency, based upon experience with similar GMM or techniques can be used. For example, the likelihood of an attenuated or disabled GMM reverting to wild type status can be assessed on the basis of the number of discrete events that would need to take place, i.e. the more events that are needed, the less likely it is that reversion will occur.

iii. However, it should not be assumed that failure to observe an event is evidence that it does not occur. As part of such considerations, it should be recognised that microorganisms often have extremely short generation times and therefore adapt to specific environments and selective pressures rapidly. This is particularly true for viruses and during the course of evolution, they have proven to be particularly adept at responding to selective pressures by infecting new cell types or host organisms. This is a consequence of the high level of genetic variability, particularly in RNA viruses that replicate using an error-prone mechanism.

iv. Mutant genomes are continually being generated and the effects of selection pressures should be assessed. For example, although variants will often be maintained at low frequencies by negative selection, in a situation where a microorganism can replicate in an environment that differs from that in which it is normally found, the probability of one of the genetic variants becoming dominant will be increased. When undertaking risk assessments of GMM, it is important to have some awareness of this genetic variability. Even if the GMM that is initially constructed is not well adapted to growth in a particular environment or host, there is a possibility that it will adapt as new variants arise. Therefore, it is necessary to proceed with caution and use recipient strains that are sufficiently defective wherever possible. This will virtually eliminate problems arising from genetic variability.

1.6 RISK ASSESSMENT FOR THE ENVIRONMENT

There is a requirement under both the *Biosafety Act, 2007*, to consider risks to the environment and biological diversity. The environment likely to be exposed, in most cases, will be limited to the workplace

environment and the area immediately surrounding the facility. However, depending on the specific characteristics of the contained use and the facility, a wider environment may need to be considered. The extent of the environmental exposure may be influenced by the nature and scale of the activity, but consideration should also be given to all possible modes of transmission into the wider environment. These can include physical modes (such as local drains, water-courses, waste disposal, air movement) and biological vectors (such as movement of infected animals and insects).

i. The objective of the risk assessment for environmental (including biological diversity) protection is to determine the likelihood and the possible consequences of an unintended release of a GMM from containment into the environment. In a properly maintained and managed facility with the correct containment measures in place, the likelihood of such a release will be low. However, it is important to identify all possible hazards and consider any routes by which the GMM could be released (including waste disposal, equipment failure and human spread).

ii. Clearly, the concern is for GMM that could feasibly cause harm to the environment and biological diversity. Therefore, GMM with the potential to infect or colonise animals and plants are of primary concern. Particular attention should be paid to GMM derived from pathogens that can infect vertebrate and invertebrate animals, especially domestic farm animals of economic importance. However, if the GMM in question is incapable of infecting or impacting upon any species other than humans, then this should be stated and supported in the risk assessment. GMM that could impact upon any environmental ecosystem (including microbial, animal and plant populations) should also be carefully assessed and any possible adverse effects on microbial ecosystems accounted for.

iii. The risk assessment should consider the local environment surrounding the containment facility as well as the wider environment, especially if there is a possibility that the GMM could survive and disseminate. For example, an arthropod-borne protozoan pathogen and its intermediate vector may be present in adjacent laboratories. Such instances might necessitate the implementation of additional controls.

iv. The procedure for environmental risk assessment is similar to that of the risk assessment for human health - to identify risks to the environment and then to assess the likelihood and potential severity of the consequences, should the risks be realised. This procedure will be illustrated throughout the following sections using a hypothetical model case study of GMM that could impact upon the environment in the event of release from containment.

Some environmental factors for consideration include:

- ecosystems to which the microorganism could be unintentionally released from the contained use
- expected survivability, multiplication and extent of dissemination of the GMM in the identified ecosystems
- anticipated result of interaction between the GMM and the organisms or microorganisms which might be exposed in case of unintentional release into the environment
- known or predicted effects on plants and animals such as pathogenicity, toxicity, allergenicity, vector for a pathogen,altered antibiotic-resistance patterns, altered tropism or host specificity, colonisation
- known or predicted involvement in biogeochemical processes

1.6.1 Risks associated with the recipient strain

i. The characteristics of the recipient strain that will be of relevance to the final GMM include pathogenicity, infectivity, toxicity, virulence, allergenicity, colonisation, parasitism, symbiosis and competition. If the recipient organism is invasive or pathogenic, then the GMM may also exhibit the same features, albeit exacerbated or attenuated by the modification. In the same way that the RG and containment requirements are important preliminary issues for genetic modification work with human pathogens, it is also important to consider the classification of animal pathogens (which are pathogens of domestic farm animals and poultry) These GMM pathogens may require licenses/permits from the Department of Agriculture or Malaysian Quarantine and Inspection Services (MAQIS) to handle or import them. The containment conditions specified within those licences must be strictly adhered to.

ii. Survivability of the GMM will be a key attribute. If aGMM is not capable of surviving for significant periods in the environment, as may be the case for many of the disabled organisms used in containment (e.g. *E. coli* K-12 and many viral vectors), none of the other hazard areas are likely to come into play. In many cases, a disabled GMM can probably be considered safe from an environmental standpoint as they are biologically, if not physically, contained. Conversely, if a GMM can survive and perhaps disseminate in the environment, then other possible hazards should be considered. For example, vaccinia virus is highly stable, resistant to dehydration and capable of infecting multiple species. Therefore, there is the possibility that an inadvertently released GM derivative of vaccinia virus could survive and become disseminated. This means that alterations in pathogenicity, possible adverse effects of any inserted gene products and the consequences of recombination with wild type vaccinia virus will also need to be considered.

iii. When assessing whether a GMM might survive in the environment, it should include all types of association with living organisms, as well as the possibility of persisting in soil, water or other sites, whether or not in a vegetative state, or undergoing active replication.

1.6.2 Risks associated with genetic inserts

i. GMM might pose a risk to the environment by virtue of the properties inherent to the genetic insert, even if the recipient microorganism poses no specific risk. For instance, the products of the inserted sequences may have the desired effect in the intended experimental system but nevertheless kill (or be detrimental for) natural flora and fauna (*e.g.* expression of a recombinant pesticidal protein in a prokaryotic system).

ii. Furthermore, promoters and control sequences may not show the same expression characteristics or tissue restrictions in other species as they would in the intended experimental system. The level and kinetics of expression, as well as the activity of the product, will therefore be important considerations in these cases.

Further guidance on the possible hazards associated with inserted genes can be found in Chapter 3.

1.6.3 Risks arising from the alteration of existing pathogenic traits

The recipient strain may not have any inherent properties that pose a risk to species in the environment or to ecosystems but the genetic modification may confer characteristics upon the GMM that alter its capacity to cause harm to the environment. There are many different ways in which the properties of the host organism can be affected and the following possible mechanisms should be considered. Although the list is not exhaustive, all modifications should be carefully assessed.

1.6.3.1 The modification alters stability or survivability

As already discussed, the ability of a GMM to survive in the environment is a key determinant of its potential to cause harm. Therefore, any modification that alters the survivability of the GMM should be carefully assessed, i.e. genetic modifications that enhance the ability of a GMM to resist oxidative stress, ultraviolet (UV) irradiation, temperature fluctuations or dehydration. For viruses in particular, it is important to consider the possible effects of alterations to the virus surface or envelope constituents as this may affect viral survivability in the environment. For example, retroviruses are generally highly unstable and sensitive to UV light, temperature and dehydration. Pseudotyping a retroviral vector with the surface glycoprotein of vesicular stomatitis virus (VSV-G) is known to increase their resistance to certain environmental stresses and may, therefore, increase their ability to survive.

1.6.3.2 The modification alters pathogenicity or infectivity

i. It should be considered whether the modification results in increased pathogenicity or infectivity for species present in the environment. This could result from the alteration of known virulence determinants or be as a result of modifications that affect the susceptibility of the GMM to host immune systems.

ii. As an example, Rinderpest is a morbillivirus that is primarily a pathogen of cattle. However, it also has the ability to infect rabbits. The P-gene of morbilliviruses is thought to be a major pathogenicity determinant and changes in this gene can determine the efficiency of infection in cattle and rabbits. Thus, modifications to the P-gene that resemble rabbit-adapted Rinderpest, or incorporation of the P-gene from a rabbit-adapted strain into other related morbilliviruses, might result in a GMM that is of increased risk to the rabbit population.

iii. Another example would be a GM derivative of *Mycobacterium bovis* (BCG) that is modified to express a bovine cytokine. *M. bovis* (BCG) is attenuated for humans and has a long history of safe use as a vaccine. A GM derivative expressing a bovine cytokine may remain attenuated for humans and the expressed gene product (intentionally selected due to its reduced efficacy in humans) may improve the strain's utility as a vaccine. This GMM may be relatively safe for humans, but it might be potentially hazardous for cattle, the natural host.

1.6.3.3 The modification alters tissue tropism or host range

i. Particular attention must be given to the generation of a GMM that is pathogenic for an animal species derived from a recipient strain that is normally non-infectious to that host. The nature of this kind of experiment means that they could give rise to novel animal pathogens and thus it is vitally important that the environmental risks are carefully assessed.

ii. Altered host range may result from the modification of cellular entry or invasion determinants. Retargeting and/or extending the host range of viral vectors are a common practice and a desirable goal for the development of therapeutic viral GMM. Other microorganisms have host range determining factors that affect the ability to colonise, replicate or establish infections in certain host species or cell types. For example, vaccinia virus can enter most mammalian cell types, but its ability to replicate is determined to some extent by the presence and expression of a number of 'host range genes'. It is vitally important that the ramifications of modifications to determinants such as these are carefully considered from an environmental perspective.

iii. For example, *Neisseria meningitidis* is a commensal bacterium that is occasionally pathogenic for humans. This pathogenicity is partially determined by the expression of transferrin binding-proteins (TBPs) that are required by the bacteria to scavenge iron from human hosts. Replacement of the genes encoding TBPs in *N. meningitidis* with equivalent genes from the unrelated pig pathogen *Actinobacillus pleuropneumoniae* could result in a GM *N. meningitidis* derivative that is pathogenic for pigs (see the example risk assessment on the development of an animal model for *N. meningitides* disease in Example 3 of Appendix 4 of this Guideline).

1.6.4 Transfer of harmful sequences between organisms

It is important to consider the potentially harmful consequences should sequences inserted into a GMM be transferred to other organisms in the environment, or that the GMM itself may acquire sequences from the environment that might increase its pathogenicity. Sufficient consideration should also be given to the possibility that an attenuated or disabled GMM could revert to wild type status or become competent and be able to survive and spread. Sequence mobilisation in bacteria will be the major mechanism by which sequences could be transferred in the environment, although there are many mechanisms by which sequences may be transferred between microorganisms and such factors must be carefully considered in the risk assessment.

1.6.4.1 Sequence mobilisation in bacteria

i. If the sequence is plasmid-borne, then the mobilisation status of the plasmid backbone should be considered. As a general rule, non-mobilisible plasmids should be used. If mobilisable plasmids are to be used, this should be fully justified by the risk assessment and suitable controls implemented. The frequencies of successful horizontal gene transfer in the environment are low, even for genes located on plasmids. However, the possibility remains that any gene may be transferred and this necessitates the need to focus on the nature of the gene itself, any likely selective advantage it might confer and whether it is a novel construction or already abundant in the environment.

ii. Once again, the survivability of the GMM is a key determinant. It is important to remember than a GMM that has a limited capacity to persist in the environment will be under extreme selection pressure to acquire the capability. For example, it is known that *E. coli* K-12 can survive for several days in the gut and for similar lengths of time in the environment. Under conditions of stress, plasmid transfer may be more likely, so it should not be assumed that gene transfer would not occur in the environment because a disabled host is being used.

1.6.5 Phenotypic and genetic stability

The stability of the genetic modification should also be considered, particularly where there is the possibility that a GMM attenuated or disabled for growth might revert to wild type or pathogenic phenotype and become an environmental hazard. Therefore, the genetic stability of the modification may be linked to phenotypic stability, especially where the modification restricts the GMM's ability to survive and to spread The loss of an inserted gene from a GMM is unlikely to constitute a hazard. However, inherent genetic instability leading to incorporation of genes elsewhere in the genome of the same GMM could be hazardous. A GMM with a restricted capacity to survive will be under stress in the environment and there will be a strong selection pressure for the reversion of attenuating and disabling genetic lesions. The possibility that a GMM will be genetically unstable outside of the conditions in which it was intended to exist should be taken into account and consideration given to any detrimental effects this might cause.

1.6.6 Likelihood that the GMM will be a risk to the environment

i. The initial stages of the environmental risk assessment process thus far has involved identifying those features of the GMM that have the potential to cause harm to the environment and the mechanisms by which these hazards could be realised. A GMM may well have characteristics that make it a potential environmental hazard. However, the chances of the hazards being realised should be evaluated and understood.

ii. It is therefore important to consider the risk of the identified hazards being manifested by (i) assessing the likelihood that the GMM will be a hazard and (ii) making a judgement as to the possible consequences should the hazard be realised. Estimating the likelihood of a harmful consequence being realised will be difficult where there is no firm data on which to base a judgement. In general, the weight given to information used in these considerations should reflect the quality of the supporting data. Where the likelihood of harm is poorly understood, a precautionary approach should be adopted until evidence to the contrary has been obtained.

iii. A determination of the risk of harm posed by a GMM can be estimated using a risk estimation matrix (see Table 1). Risk can be expressed as 'high', 'medium', 'low' or 'negligible' and requires an assessment of likelihood and an assessment of the possible consequences that the hazard will be realised. However, this matrix is not definitive, and all potential environmental hazards should be acknowledged and carefully assessed.

1.6.7 Assessment of likelihood

i. Assessment of likelihood is a key factor in whether or not the hazard will be realised is the environment into which the GMM would be released. It is therefore important to consider the nature of the GMM in relation to the receiving environment. There may be characteristics of the local environment that will contribute to the likelihood of the hazard being manifested (*e.g.* climatic, geographical or soil conditions and the types of potential host species present). For the purposes of using the risk estimation matrix, likelihood can be expressed as 'highly likely', 'likely', 'unlikely' or 'highly unlikely'.

ii. Even if the GMM could conceivably survive and disseminate in the environment, it may be that the environment itself would not be able to support it. For example, GMM derived from animal pathogens of non-Malaysian hosts may have limited capacity to become disseminated within Malaysia even if it could survive for extended periods. Similarly, the transmission of some pathogens requires an intermediate vector that might not be present in Malaysia. The possibility of unknown hosts or intermediate vectors should be accounted for, as should the longer-term possibility that such hosts and vectors will become native to Malaysia, for example, as a result of climate change or introduction by migrant workers. However, in general, the risk that such GMM could pose a risk to the environment will be low.

iii. When estimating the probability and frequency of events, consideration should also be given to the number of viable organisms as opposed to the actual volume that might be involved in the incident. This will depend on the nature of the experiment. However the probability that a risk will be realised will often depend on the number of GMM that are being handled and, consequently, the number that could escape.

1.6.8 Assessment of the consequences

i After the likelihood of all the hazards has been assessed, the consequences of each hazard should be estimated. Evaluation of the magnitude of potential consequence is difficult since a degree of judgement is involved, although a qualitative appraisal of the impact on other species or ecosystems should be possible. For the purposes of using the risk determination matrix, consequences could be described as being 'major', 'intermediate', 'minor', or 'marginal'.

ii. It should be borne in mind that even if the consequences of a hazard are deemed 'major', if the likelihood of the hazard being manifested at all was 'highly unlikely' then there is a 'negligible' risk of harm. Likewise if the consequence of a hazard were 'marginal' or 'minor',

then even if the likelihood of its manifestation were 'highly likely', the risk of harm would still be 'low' (See Table 1).

iii. However, a precautionary approach to risk estimation is advised. In situations where the likelihood of the hazard being manifested is 'highly unlikely', should there be a 'major' consequence to the identified hazard, then it is unlikely that BSL1 would be appropriate, even though there is a 'negligible' risk of harm. A balanced view of the risks is therefore required.

iv. The risk estimation matrix is a tool and should not be seen as definitive. It is important that uncertainty is taken into consideration in the risk assessment and the use of assumptions is made clear when drawing conclusions with respect to the level of risk. This is particularly pertinent in situations where the consequences of the hazard are major. The basis of any assumption should be explained and the robustness of the argument supporting it should be acknowledged.

Table 1. Risk estimation matrix

		RISK ESTIMATE			
Likelihood of Hazard	4 Highly likely	Low	Moderate	High	High
	3 Likely	Low	Low	Moderate	High
	2 Unlikely	Negligible	Low	Moderate	Moderate
	1 Highly unlikely	Negligible	Negligible	Low	Moderate
		1 Marginal	2 Minor	3 Intermediate	4 Major
		Consequence of Hazard			

Key to Risk estimation matrix

Likelihood	Likelihood assessment definitions
Highly unlikely	May occur only in very rare circumstances
Unlikely	Could occur in some circumstances
Likely	Could occur in many circumstances
Highly likely	Is expected to occur in most circumstances

Consequences	Consequence assessment definitions relating to the health of people and the environment
Marginal	Minimal adverse health effects
	Minimal or no damage to the environment or disruption to biological communities
Minor	Adverse health effects that are reversible
	Damage to the environment or disruption to biological communities that is reversible and limited in time and space or numbers affected
Intermediate	Adverse health effects that are irreversible
	Damage to the environment or disruption to biological communities that is widespread but reversible or of limited severity
Major	Adverse health effects that are severe, widespread and irreversible
	Extensive damage to the environment or extensive biological and physical disruption of whole ecosystems, communities or an entire species that persists over time or is not readily reversible

Risk estimate	Risk estimate definitions
Negligible	Risk is insubstantial and there is no present need to invoke actions for mitigation
Low	Risk is minimal, but may invoke actions for mitigation beyond normal practices
Moderate	Risk is of marked concern that will necessitate actions for mitigation that need to be demonstrated as effective
High	Risk is unacceptable unless actions for mitigation are highly feasible and effective

v. It may be necessary to evaluate whether any specific control measures are required to adequately protect the environment. Containment measures should be applied until the risk of harm is 'negligible'. Further guidance on containment measures to protect both the environment and human health can be found in Chapter 2.

vi. Some examples risk assessment are given in Appendix 4 of this Guideline.

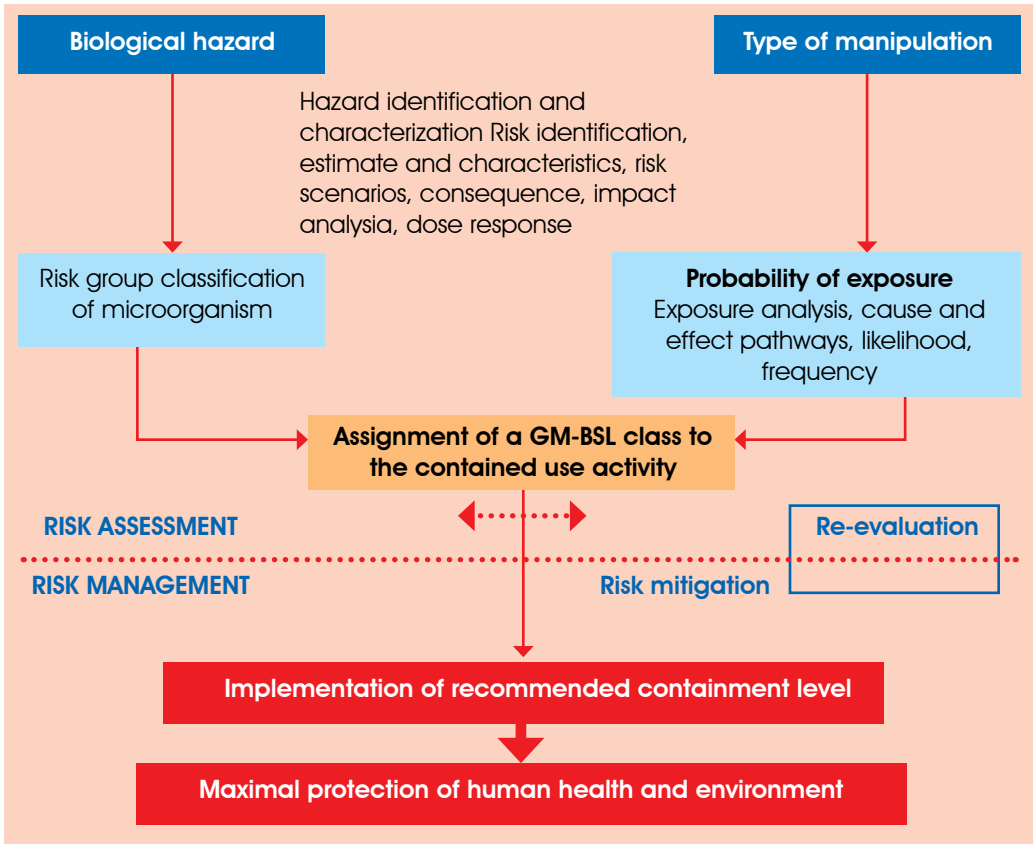


Figure 2. *Identification of biological hazards and determination of the Risk Groups and biosafety level (GM-BSL) of the GMM*

CHAPTER 2

CONTAINMENT AND CONTROL OF ACTIVITIES WITH GENETICALLY MODIFIED MICROORGANISMS

2.1 CONTAINMENT LEVELS FOR GM MICROORGANISMS

i. It is recommended that the appropriate BSL (GM-BSL 1, 2, 3 or 4) that is necessary to protect human health and environment be set. This is based upon:

- RG and/or BSL (GM-BSL) appropriate to the parent or recipient microorganism.
- Any identified hazards arising as a consequence of the genetic modification.
- The severity of any harmful consequences and the likelihood that they might occur.
- Containment measures required by *Animal Act 1953 (Revision of Laws (Rectification of Animals Act 1953) Order 2006*.

Other regulatory requirements should also be followed in addition to the requirements of the Biosafety Act 2007 such as:

- Any license needed by *Malaysian Quarantine and Inspection Services* (MAQIS) Act 2011, for importation.
- Any license needed by the *Plant Quarantine Act 1976* for the importation and activity on scheduled plant species.
- Any license needed by the Prevention & Control of Infectious Diseases Act 1988 and Prevention & Control of Infectious Diseases (Importation & Exportation of Human Remains, Human Tissues and Pathogenic Organisms & Substances) Regulations 2006.

Therefore, a judgement can then be made about whether the GMM will be more hazardous, less hazardous or equivalent to the parent strain. Comparing the predicted properties of the GMM to the recipient strain can be used to estimate

the provisional containment level. In many cases this will correspond to the containment level that is appropriate for the recipient strain. However, it may be clear in some cases that the GMM will be less hazardous than the recipient strain (for example the genetic modification results in significant attenuation or disablement of the host strain). In that event, it may be that a lower containment level than that appropriate for the recipient strain will be sufficient to protect human health. Equally it may be that the GMM will be considerably more hazardous than the recipient strain (*e.g.* where a pathogenicity determinant has been cloned into a recipient that is only partially disabled). In that event, it may be appropriate to assign the GMM to a higher provisional containment level than that appropriate for the recipient strain.

ii. Users should judge whether the measures required for the recipient strain as described in the *Biosafety guidelines for Contained use activity of LMO 2010* are also appropriate for the GMM. If some measures are no longer needed or any extra measures are required, then the BSL should be adjusted accordingly to afford sufficient protection for human health and environment.

iii. In some instances, the GMM will be based on an organism which is harmful to animals, but which is not a human pathogen. In such cases an initial classification based solely upon human health considerations might legitimately yield the conclusion that BSL1 is sufficient to protect human health. However, this may well be inadequate for environmental protection. The potential for environmental harm can be considered separately as set out in sections on environmental risk assessment. In cases where the major hazards are posed to the environment rather than human health, priority can be given to environmental risk assessment from the outset and a provisional containment level set on the basis of environmental protection.

iv. A judgement should be also be made about whether the GMM will be a risk to the environment. If all risks are deemed to be 'low' or 'negligible', then no specific measures will be required. However, if any risk exceeds this level, then control measures should be implemented such that the risk of harm to the environment is reduced to 'low' or 'negligible'.

v. The requirements of the final BSL must be sufficient to control all the potential harmful properties of the GMM and offer sufficient protection for both human health and the environment. The minimum BSL set for both human health and environmental protection risk assessments only broadly define the containment measures needed as a function of the properties of the GMM.

Therefore, it is important to take into account the nature of the work or any non-standard operations that might increase the risk of exposure or likelihood of release. It may be necessary to implement additional containment and control measures, which may have an impact on the final GM-BSL.

2.2 CONSIDERATION OF THE NATURE OF SAFE WORK PROCEDURES TO BE USED

The possibility of humans or the environment being exposed to a GMM depends upon what operations are being carried out (*e.g.* the scale of the operations) and the containment measures appropriate to the initial classification applied to the work.

The characteristics of the operation could affect the risk assessment and so should be taken into account as appropriate. These include the actual activities to be undertaken, work practices, scale and containment measures applied. The nature and scale of the activity need to be considered in order to estimate the possibility of exposure of humans and the environment and will also affect the choice of appropriate risk management procedures.

The assessment should especially take into account the question of disposal of waste and effluents. Where appropriate, the necessary safety measures should be implemented in order to protect human health and the environment.

2.3 NATURE OF ACTIVITIES TO BE UNDERTAKEN

i. In practice, for laboratory scale work where the effect of standard laboratory procedures on exposure are well known, detailed risk assessment of each individual procedure would be unlikely to be required unless a highly hazardous organism was being used.

ii. More detailed consideration however may be necessary for non-routine procedures or procedures which might have a significant effect on the degree of risk (*e.g.* procedures which generate aerosols).

iii. In particular, any non-standard operations that are not accounted for in the general requirements for a given BSL should be considered as increased risks might arise from certain procedures. For example:

- Inoculating animals with the GMM. Furthermore, the chances of recombination or reversion may be enhanced when work *in vivo* is undertaken, as compared to work *in vitro*.
- The use of sharps for administration of viable GMM or post-mortem analysis increases the likelihood of an exposure that

might lead to infection. In particular, the use of sharps should be minimised when working with retroviruses and oncogenic material.

If it is decided that any such non-standard operations are likely to generate risks that are not accounted for in the minimum BSL assigned in human health or environmental risk assessments, then additional control measures should be applied.

2.4 CONCENTRATION AND SCALE

i. The density of a culture can lead to a risk of exposure to high concentrations of the GMM, particularly in downstream processing operations. The effect of concentration on the possibility of a harmful event occurring must be considered.

ii. Scale is a factor that should be taken into account in the risk assessment, especially in the pilot manufacture of a GMM or GMM-derived products. Scale may be in terms of the absolute volume of a single operation or the frequent repetition of a process, because both could give rise to an increased possibility of exposure if the containment and control measures fail and thus affect the possibility of a harmful event occurring. While large scale does not necessarily mean high risk, increased scale may lead to an increased possibility of exposure both in terms of the number of humans and the amount of environmental exposure that might occur in the event of containment failure. Scale will also influence the most appropriate containment and control measures to be considered.

2.5 CULTURE CONDITIONS

i. In many contained use activities, the culture conditions are rigorously contained to protect the work. However, the nature and design of the growth vessels or other culture equipment will also influence the degree of risk to human health and the environment. Highly engineered and sealed fermentation vessels can significantly reduce exposure and hence risk from a GMM. Consideration of reliability and possible failure rates for such equipment is important where failure could lead to high levels of exposure to harmful GMM. Where such loss is reasonably foreseeable, additional containment measures may be required. The standard operating procedures of individuals undertaking work with cultured GMM such as centrifugation or sonication will have a significant impact on the effectiveness of any containment measures employed.

ii. In combination with physical culture conditions that act as containment measures, both biological and chemical measures that are employed to protect the work can also contribute significantly

to the containment measures that may be required. Examples of biological containment could well be auxotrophic mutants that require specific growth factors to be supplied to grow. Examples of chemical containment measures could be disinfectant solutions maintained in drainage systems.

2.6 ASSIGNMENT OF ADDITIONAL MEASURES TO MINIMISE RISKS

The "*Biosafety guidelines for Contained Use activity of LMO, 2010*" describe the underlying principles of containment and biosafety practices for all LMOs including GMM. These include the principles of Good Microbiological Practice and Good Occupational Safety and Hygiene. A summary of containment measures showing facility design and work practices for activity involving GMM is shown in Table 2 and Table 3. Additional measures may be needed to ensure safety, especially where the GMM is pathogenic for humans or able to infect human cells.

2.6.1 Prevention of cross contamination

Measures should be taken to prevent cross contamination during laboratory work in order to minimise the possibility of adverse consequences resulting from genetic transfer or complementation. If genetic transfer could give rise to a pathogenic species, then handling them in the same laboratory should be avoided, if possible. Where this is not practicable, measures should be taken to separate the work either spatially, temporally or both. Where a pathogen could be generated, then measures appropriate for the containment and control of that pathogen will be necessary.

2.6.2 Containment and management of aerosols

When handling a GMM that is spread via the airborne route, activities that may generate aerosols should ideally take place within a biological safety cabinet or a negative pressure isolator. Laminar flow cabinets and so-called clean-air systems are not sufficient to protect staff or prevent the dissemination of aerosols. If it is not possible or reasonably practicable for the work to take place in a cabinet (*e.g.* when working with large animals or bulky equipment) then other measures should be implemented to prevent aerosol dissemination and staff exposure. This may include mechanical air handling, High Efficiency Particulate Air (HEPA) filtration and the use of personal and respiratory protective equipment.

2.6.3 Monitoring of GMM stability

i. Where a risk assessment relies heavily on the premise that the GMM is disabled or biologically contained, it may be necessary to check for revertant strains that have lost disabling mutations. Such

an approach is taken when working with disabled retroviruses and Adenoviruses but this is unlikely to be necessary for disabled bacterial strains such as *E. coli* K-12. Molecular detection methods such as the Polymerase Chain Reaction (PCR) and Southern hybridization can be used to detect the presence of sequences deleted from the GMM.

ii. In certain circumstances, it may be possible to monitor the presence of a GMM outside of primary containment (*e.g.* the use of nutrient plates to monitor bacterial and fungal contamination). Such an approach could be used when using enteric pathogens with a low infectious dose. This could be used to assess potential GMM contaminations and the efficacy of working practices or decontamination procedures. Furthermore, it may also alert users to the potential escape of GMM from the containment facility.

2.6.4 Risk management issues

i. Organisations intending to carry out LMO activity should establish and maintain a risk management system to control or minimise risk to acceptable levels in relation to personnel as well as the environment. A useful reference is the *WHO Biorisk Management: Laboratory Biosecurity Guidance (WHO, 2006)* which provides guidance on procedures necessary to control risks associated with the handling or storage and disposal of biological agents.

ii. The management should be satisfied that the laboratory local rules give effective guidance on working practices and procedures. All workers should be trained in good laboratory techniques before commencing work and should be fully aware of the potential hazards of the work and confident that the measures in place are sufficient to protect them. In particular, they should have a working knowledge of the nature and importance of any disabling mutations. There should be a programme of internal safety inspections and active monitoring by the IBC or other competent person to ensure that the local rules are satisfactorily implemented.

iii. The maintenance schedule for protective apparatus such as isolators, biosafety cabinets and ventilation systems should be strictly adhered to. It is also important that any mobile equipment (biological safety cabinets and isolators) is validated for the conditions in which they are used – i.e. cabinets that are transferred to a new location will need to be retested and validated for use in that new location. It should be noted that such local exhaust ventilation systems (LEV) must be regularly maintained, examined and tested.

2.6.5 Preventing release into the environment

i. As previously discussed, it may be necessary to adjust the containment level to ensure that the possibility of release into

the environment is prevented. It is therefore important that all possible routes of release are known and controlled. One of the major release routes will be via contaminated waste and it is therefore important that GMM that pose an environmental hazard are adequately inactivated and appropriately disposed of.

ii. The route of release might affect the survivability of an organism. For example, a GMM may not survive for a significant time in an aerosol but might survive for protracted periods within an infected animal carcass. Furthermore, laboratory staff may inadvertently carry the GMM out of containment on contaminated equipment or clothing.

2.7 GMM ACTIVITY CLASSIFICATION (GM-BSL 1, 2, 3 OR 4)

i. A GMM activity class must be assigned in relation to the control measures needed to protect both human health and the environment (see Figure 1). The measures that are indicated as necessary by the risk assessment must be applied. The importance of the final activity classification is that it determines the minimum containment and control measures that must be applied.

ii. For class 1 activities, GM-BSL1 containment measures must be applied as a minimum. For class 2 activities, GM-BSL2 containment measures and so on. However, a risk assessment must be done to show that the BSL is deemed sufficient for the activity.

iii. To decide on the final classification, users should compare the measures warranted by the risk assessment with the appropriate containment measures described in the "*Biosafety guidelines for Contained use activity of LMO, 2010*". The risk assessment must take precedence in these cases and all measures identified as necessary must be applied. Furthermore, there is a general requirement for the exposure of humans and the environment to GMM to be as low as reasonably practicable and the principles of Good Microbiological Practice and of Good Occupational Safety and Hygiene should also be applied.

iv. Class 1 activities are described in the "*Biosafety guidelines for Contained Use activity of LMO, 2010*" as being of minimum or 'negligible risk'. It is unlikely that any non-disabled human or animal pathogen could be of negligible risk' (except where the host species is absent from the receiving environment). Such work should be assigned to BSL3 or higher. Since work with pathogens will almost invariably require some procedures and control measures (e.g. an autoclave in the building; restriction of access). It would not normally be possible to assign the activity to BSL1 A GMM that is a potential human or animal pathogen should not be assigned to GM-BSL1.

Table 2. Containment measures showing Facility design and equipment for activities involving GMM in laboratories

Containment measures		Containment measures			
		GM-BSL1	GM-BSL2	GM-BSL3	GM-BSL4
Facility design					
1	Laboratory suite isolation	Not required	Not required	Required	Required
2	Laboratory sealable for fumigation	Not required	Not required	Required	Required
3	Entry to laboratory via airlock	Not required	Required	Required to extent the RA shows it is required	Required
4	Negative pressure relative to the pressure of the immediate surroundings	Not required	Required to extent the RA shows it is required	Required	Required
5	Input air and exhaust air from the laboratory should be HEPA filtered	Not required	Not required	HEPA filters required for exhaust air	HEPA filters required for input and exhaust air
6	An observation window or alternative is to be present so that occupants can be seen	Required if the RA shows it is required	Required if the RA shows it is required	Required	Required
7	Surfaces impervious to water, resistant to acids, alkalis, solvents, disinfectants and decontamination agents and easy to clean.	Required for bench	Required for bench	Required for bench and floor	Required for bench, floor, ceiling and walls

8	Shower	Not required	Not required	Required to extent the RA shows it is required	Required
9	Appropriate biohazard signages	Required	Required	Required	Required
Equipment					
10	Biological safety cabinets / enclosures	Not required	Class 2 BSC required to extent the RA shows it is required	Class 2 or Class 3 BSC required depending on RA	Class 3 glove box required
11	Autoclave	Required on site	Required in the building	Required in the laboratory suite	Double ended autoclave required in the laboratory

Note: RA – Risk assessment

Table 3. Containment measures showing work practices and waste management for activities involving GMM in laboratories

Containment measures		Containment measures			
		GM-BSL1	GM-BSL2	GM-BSL3	GM-BSL4
Waste management					
1	Inactivation of effluent from handwashing sinks and showers	Not required	Not required	Required if the RA shows it is required	Required
2	Inactivation of GMM in waste effluent and contaminated material	Required by validated means	Required by validated means	Required by validated means	Required by validated means
Work practices					
3	Access restricted to authorised personnel only	Not required	Required	Required	Required via airlock / card key

4	Specific measures to control aerosol dissemination	Not required	Required so as to minimise	Required so as to prevent	Required so as to prevent
5	Protective clothing	Suitable protective clothing required	Suitable protective clothing required	Suitable protective clothing required; footwear required where the RA shows it is required	Complete change of clothing and footwear required before entry and exit
6	Gloves	Required	Required	Required	Required
7	Effective control of disease vectors such as insects, rodents, arthropods which could disseminate the GMM	Required	Required	Required	Required
8	Safe storage of GMM	Required if the RA shows it is required	Required	Required	Required
9	Specified disinfection procedures in place	Required if the RA shows it is required	Required	Required	Required
10	Inventory / databases of GMM and all GM events for IBC inspection	Required if the RA shows it is required	Required	Required	Required
11	Written records of staff training	Required if the RA shows it is required	Required where and to extent the RA shows it is required	Required	Required

Note: RA – Risk assessment

GUIDANCE ON HAZARDS POSED BY INSERTED SEQUENCES

CHAPTER 3

3.1 BACKGROUND

i. The following section concerns exogenous inserted sequences that may have harmful biological activity (*e.g.* toxins), sequences which may be involved in the control of expression of such inserts (for example promoters and control regions) and other products that may have no inherently harmful activity but might have other adverse effects (such as allergens and antigenic proteins).

ii. It is therefore important to consider the potential biological activities of the product encoded by the insert and any adverse effects that might result following inadvertent release or exposure. For example, genes that may alter the growth status of cells (such as oncogenes, cytokines and growth factors) or have cytotoxic effects (such as toxins) will represent a much greater risk of harm than genes such as those encoding Green Fluorescent Protein (GFP) or Luciferase that have no direct effect on cellular processes. Other products may have ecological impact due to adverse effects on natural flora and fauna or microbial ecosystems.

iii. The fate of the expressed product should also be considered. The consequences of exposure to a GMM that secretes expressed biologically active molecules may be different to those arising from a GMM expressing the same molecule that does not. Biologically active molecules that are secreted may have wide-ranging, and possibly systemic, effects. Similarly, the biological activity of the product may be dependent upon the environment in which it is expressed. For example, if a product is biologically active at the cell surface but will only be expressed by an intracellular GMM, the consequences might be less severe. However, the possibility that lysis of the cell might allow dissemination of the molecule in the extracellular environment should be considered in this case.

iv. The elements that control gene expression in GMM should be understood as well as possible before a GMM is generated, particularly if that GMM is able to enter or infect the cells of humans

or animals. Furthermore, promoters and other control sequences differ in the cell types in which they can function. Therefore, it is important to consider the potential activity, properties and effects of an expressed product in any individual cell or tissue types that might be affected by the vector GMM or permissive for the sequences that control its expression. Most commonly used expression systems are well understood. However, novel constructs and artificial promoters will require greater scrutiny and testing prior to their use in an infectious GMM.

v. In cases where inserted genes encode products that may have adverse effects either on human health or to the environment, it may be necessary to assign specific control measures for the safe handling of the vectoring organism.

3.2 ONCOGENES

i. Oncogenesis is the process leading up to a cell losing the ability to effectively regulate its own growth and becoming tumourigenic or transformed. It is a multi-step process requiring mutations; the cell becoming more tumourigenic as the changes accumulate. Mutations often result in the derestricted or deregulated expression of a cellular mitogenic factor and/or loss of pro-apoptotic or cell cycle inhibitor (i.e. tumour suppressor). A single event, such as the overexpression of one gene, is unlikely to result in oncogenic transformation. Stable expression of a gene with potentially oncogenic properties might result in a cell and its progeny being one step closer to a tumourigenic phenotype. Such a potentially severe consequence of accidental exposure should be carefully considered.

ii. It is recognised that there is no precise definition of an oncogene. There are some genes that encode mitogenic factors with demonstrable oncogenic properties (*e.g. c-myc*), which, when expressed, result in deregulated growth. A transformed phenotype usually requires expression of an oncogene in conjunction with another gene (*e.g. c-myc* and *c-ras* co-expression can result in stable transformation of cells) or in cells with impaired tumour-suppressor function or apoptotic pathways (*e.g.* mutant *p53* or *bcl- 2*). Oncogenes could be any genes that are likely to contribute to cellular transformation. Many known oncogenes are involved in mitosis and intracellular signalling pathways and any genes involved in cell-cycle control, differentiation, apoptosis, intracellular signaling or extra-cellular interactions could be potentially oncogenic. In addition, any gene that confers a phenotype upon the cell that is associated with transformation (*e.g.* deregulated growth, loss of contact inhibition, density or anchorage-dependent growth) could have oncogenic properties.

iii. Particular attention should be paid to any modification work where there is a possibility that oncogenic sequences could be transferred and expressed in human cells. For routine cloning of eukaryotic DNA that could be oncogenic, non-mobilisible plasmid vectors should be employed. Where possible, the constructs should be devoid of functional eukaryotic promoter sequences to prevent expression in the event that they are inadvertently transferred to human cells.

iv. It is also prudent to avoid the use of recipient strains that are able to infect or colonise human hosts. For example, staff should not conduct genetic modification work with their own cells, or those of other laboratory staff. Furthermore, viruses and viral vectors with a human host range carrying potentially oncogenic sequences may pose risks to human health and safety over and above those hazards associated with the recipient strain itself. In particular, the use of virus vector strains that are capable of modifying host chromatin (*e.g.* retroviruses and lentiviruses) represent an immediate hazard to human health and appropriate controls will be required.

3.3 TOXINS AND CYTOTOXIC GENES

i. The development of GMM with cytotoxic properties is now an established technique for the therapeutic destruction of cells, for example tumour cells. Microorganisms able to infect or invade eukaryotic cells (*e.g.* viruses or invasive bacteria) can be modified to target cells specifically for destruction or be used to kill all cells in proximity to the point of inoculation.

ii. There are different approaches to generating cytotoxic GMM. One is the use of an inserted gene with a product that is inherently toxic, for example a plant toxin (*e.g.* Ricin) or a bacterial toxin (*e.g.* Diphtheria Toxin or Shiga Toxin). Bacterial toxins are primary determinants of pathogenicity in bacteria and therefore great care should be exercised when modification work involves the insertion of bacterial toxin sequences into prokaryotic hosts, even as part of routine cloning procedures. Non-mobilisible plasmid vectors should be employed and the constructs should be devoid of functional promoter sequences where possible. Since breakthrough expression might occur precautions must be taken to avoid exposure.

iii. Clearly, any GMM carrying genes of this type may be inherently harmful and may increase the hazards posed as compared to the recipient strain, even if it is adequately disabled or restricted to affecting a particular cell type. Another approach is to use a gene encoding an enzymatic protein that can convert a 'harmless' prodrug molecule into a cytotoxic compound. For example,

Herpes simplex virus thymidine kinase can be used to convert the antiviral Ganciclovir into a toxic guanidine analogue and bacterial nitroreductase can be used to convert the non-toxic compound CB1954 into a toxic alkylating agent. In most cases the GMM should only be of greater risk than the recipient strain in the presence of the prodrug and, arguably, such systems are generally much safer.

iv. It is important when dealing with cytotoxic products, whether encoded directly by the inserted gene or generated as a result of the encoded product's biological activity, to consider any potential effects upon cells other than those normally infected i.e. a *bystander effect*. In the past, the development of therapeutic GMM carrying cytotoxic products has been hampered due to the inability to destroy all the cells that are targeted. A bystander effect can, in some instances, be deemed a desirable attribute of the system. While this may be beneficial to the potential efficacy of a therapeutic GMM, it should be remembered that adverse effects due to accidental exposure might be similarly delocalised.

v. Pharmacological cytotoxic compounds are often diffusible and can therefore affect cells adjacent to the site of inoculation or to the site of prodrug activation. Protein transduction domains (*e.g.* those derived from HSV tegument protein VP22 and the HIV *TAT* protein) allowing the transport of protein cargo such as prodrug enzymes across cellular plasma membranes have also been used to deliberately increase cytotoxicity beyond the site of the primary effect. Similarly, bacterial toxins in their native form often have membrane transduction properties (*e.g.* Diptheria Toxin) that would allow free toxin to exert its effect on cells other than those in which it was expressed.

vi. Therefore, the potential effects upon non-target tissues and cells should be carefully considered. It is particularly important to assess the hazards that are posed by gene products that have inherent cytotoxicity like bacterial toxins. Such toxins are often highly potent at cell killing and are effective even if poorly expressed (*e.g.* it is estimated that a single molecule of Diphtheria Toxin is sufficient to kill a cell). Biosafety can be improved by using mutated toxins that are still lethal to the affected cell but that are attenuated. Furthermore, many toxins are composed of multiple peptide chains, or subunits. It may be possible to express and utilise only the catalytic subunit of the toxin (*e.g.* Diptheria Toxin alpha chain, Ricin A-moiety and Shiga Toxin A-moiety) and eliminate the plasma-membrane binding and transduction activities (*e.g.* those encoded by the Diptheria Toxin beta chain, Ricin B-moiety and Shiga Toxin B-moiety) restricting the action of the toxin to the cell in which it was expressed.

vii. Consideration should also be given to the potential effects upon

the immune system. Toxins and converting enzymes are often potent antigens and highly immunogenic. The possibility of acute inflammation as a result of inserted gene expression should therefore be considered as a possible pathological side effect of the system.

3.4 CYTOKINES, GROWTH FACTORS AND IMMUNOMODULATORY PROTEINS

i. Growth factors and cytokines that are expressed by GMM have become more commonplace due to the need to boost efficacy and immune responses in GMM-based therapeutics and vaccines. Furthermore, modified mammalian cell lines are used for the purpose of large-scale manufacture of growth factors. As with many other biologically active gene products, the effects of the expressed protein upon human and animal cells as a result of accidental exposure to a GMM expressing such a product should be considered.

ii. By their very nature, exposure to such products may result in false signals leading to inappropriate growth, differentiation or apoptosis of cells. It may be that such an effect is an intentional feature of a therapeutic GMM. For example, many GMM-based vaccines express immunomodulatory growth factors to promote strong and appropriate immune responses to the target antigen (*e.g.* Herpes simplex virus vectors expressing antigens along with granulocyte-macrophage colony stimulating factor). Expression of some growth factors can allow the proliferation of cells that would otherwise be quiescent (*e.g.* Interleukin-2 expression in T-lymphocytes). Affecting the proliferative status and fate of cells in this way is a property of oncogenesis and consideration should be given to the possibility that expression of a growth factor or cytokine might give a growth advantage to a developing tumour. For example fibroblast growth factor 2 is implicated in promoting autonomous proliferation in pancreatic cancers and melanomas.

iii. Growth factors and cytokines may also have teratogenic effects on unborn foetuses. The potential effects upon all cells that may be exposed to the product must therefore be assessed, and not just the effects on the cells that might be normally infected by the GMM. Therefore, the health status of staff will become a higher priority issue when handling GMM expressing such products.

iv. Immunomodulatory growth factors may have the effect of promoting strong and appropriate immune responses in certain systems but careful consideration should be given to the possibility that inappropriate responses to a GMM that is derived from a pathogen may be enhanced. For example, *Mycobacterium bovis*

(BCG) is a vaccine strain that elicits strong immune responses and is normally effectively cleared by the immune system. Expressing some cytokines in this strain might improve the efficacy of the vaccine, but others may result in a suppression of protective immune responses or an enhancement of aberrant responses. The ability of the host immune system to clear the GMM might actually be impaired by the expression of some immune-enhancing cytokines.

3.5 RNA INTERFERENCE

i. RNA interference (RNAi) is an antisense technology that brings about the degradation of double-stranded RNA (dsRNA) molecules and inhibition of translation. Short hairpin RNA (shRNA) or small double-stranded micro RNA (miRNA) molecules give rise to small inhibitory RNA (siRNA) species that are complementary to a targeted cellular mRNA. The targeted mRNA is degraded and the expression of specific mammalian genes or protozoa can therefore be 'knocked-down'. Since the process involves the triggering of a normal biological mechanism for the degradation of dsRNA molecules, siRNA should be considered biologically active.

ii. Careful consideration should be given to a GMM carrying an RNAi cassette that is capable of infecting or invading human, animal or plant cells, particularly vector systems that might result in the permanent modification of the host chromatin (*e.g.* retroviral and lentiviral vectors). The potential outcomes of level of transcription of particular targeted genes should be carefully assessed. For example, knocking down the expression of a tumour suppressor gene in a mammalian cell could contribute to cellular transformation. One of the features of RNAi knockdown system is that the targeted gene is rarely completely silenced. Indeed, it is not unusual to have varying degrees of target gene downregulation within an experimental population and therefore it is important to consider the ramifications of heterogeneous expression of the targeted genes. This is particularly pertinent if the gene encodes a product that is involved in a regulatory network governed by the stoichiometry of its different components.

iii. The effects of the siRNA may have broader effects on the cell than just modulating the expression of a particular gene and its product. There may be areas of sequence homology in other coding sequences within the mammalian genome that are not necessarily linked or closely related to the target. Therefore, it is possible that siRNA targeted to one gene may be able to knock down expression of another gene where there is some sequence homology in the mRNA. It may be helpful as part of the risk assessment procedure to screen human, animal and plant genome databases for sequence

homology. RNAi systems should be designed carefully to minimise the likelihood that there will be unwanted or potentially adverse effects arising from a non-target gene being inadvertently targeted.

iv. RNAi may have deleterious effects upon cellular metabolism due to the triggering of cellular antiviral responses and processes. It has been shown that siRNA molecules (even if less than 30nt in length) can trigger the antiviral response to dsRNA. In addition to the degradation of dsRNA molecules, this results in interferon production that leads to inflammation and the non-specific inhibition of protein synthesis. Accidental exposure to GMM carrying RNAi cassettes might result in inflammation and the ramifications of this to human or animal health should be assessed.

v. The role of various genes in protozoan pathogens is often studied using RNAi techniques and the disruption of gene expression or regulatory pathways may affect virulence. Any potential alteration to the pathogenicity or susceptibility to the host immune system or prophylactic treatment as a result of knocking-down gene expression in these organisms will also require careful assessment.

3.6 TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES (TSEs)

i. TSEs are neurodegenerative disorders of humans (*e.g.* Creutzfeldt-Jakob Disease; Kuru) and animals (Bovine spongiform encephalopathy; Scrapie) and constitute mutant forms of naturally occurring mammalian proteins called Prions. The term prions refers to abnormal pathogenic agents that are transmissible and are able to induce abnormal folding of specific normal cellular proteins called prion proteins that are found abundantly in the brain. These are unconventional agents believed to be 'infectious proteins'. To date, transmission has only been demonstrated via ingestion or percutaneous inoculation of naturally infected material. TSEs agents are classified as RG3 pathogens, GMM expressing TSEs proteins are covered by the *Biosafety Regulations, 2010*. Therefore, genes encoding TSEs agents should be treated in the same way as other genes that encode biologically active proteins, i.e. as potentially hazardous insert sequences. Genes encoding TSEs agents represent unusual inserts as they are classified as pathogens in their own right and the expressed products carry a RG classification. It is therefore possible that a GMM expressing a gene encoding a TSEs agent will have to be handled at BSL3 containment. However, this may not necessarily be representative of the GM activity class and notification requirements.

ii. The "*Biosafety guidelines for Contained use activity of LMO, 2010*" require that a GM activity class be assigned on the basis of the containment and control measures deemed necessary by the GM

risk assessment. The containment of TSEs agents themselves will not necessarily call for the measures required for GM-BSL3. Since these agents are not airborne pathogens, the use of a biological safety cabinet, negative air pressure and HEPA filtration of exhaust air will not be required. Furthermore, as TSEs agents are resistant to fumigation, the laboratory will not require sealability for that purpose. Therefore, TSEs agents themselves only call for the measures required by BSL2 and therefore could conceivably be a class 2 GM activity, although other control measures and more stringent decontamination procedures may be required to ensure safe handling which may not affect classification, but nevertheless must be implemented.

iii. However, an infectious GMM may confer its own properties of transmission upon a TSEs protein that it encodes. Therefore, it follows that the properties of the vector construct and recipient strain will be key considerations for the purposes of risk assessment and the assignment of appropriate control measures. For instance, where a TSEs agent cDNA is inserted into a viral vector the infectious properties of that viral vector will affect the control measures needed. For example, if a TSEs cDNA were cloned into an Adenovirus vector then the possibility of 'aerosol transmission' of the TSEs should be considered. This would require that measures to control the spread biological safety cabinet, HEPA filtration of exhaust air and possibly negative pressure) would be needed. Therefore, the activity would call for the measures required by Containment Level 3 and be GM activity class 3. As TSEs are diseases of neural tissue, whenever viral vectors with neurotropic properties are used (*e.g.* vectors based upon HSV or HIV) a particularly cautious approach should be adopted as transmission directly to neural tissue is much more likely with these vectors.

Note: The use of sharps should be prohibited where such vectors expressing TSEs agents or the TSE agents themselves are present.

iv. For activities in which there is no expression, and where the recipient strain or final GMM is disabled, BSL2 might be sufficient. For example, routine cloning work in *E. coli* K-12 could take place at BSL2 provided no functional TSEs agents can be generated and where non-mobilisible vector constructs and non- colonising bacterial strains are employed.

v. Work involving fragments of TSEs proteins or modified TSEs proteins that are not expected to be pathogenic might also take place at GM-BSL2 depending on the vector used and provided that no harmful biological activity is possible. GMM expressing normal Prion proteins should at a minimum be handled at BSL2 as they

may be pathogenic at high levels and may also become mutated to TSEs forms in GMM vectors, especially in RNA virus vectors as the possibility of mutation is high in these systems.

vi. TSEs agents are extremely durable in the environment and containment measures must also be prescribed to prevent environmental contamination. TSEs agents are also extraordinarily resistant to the decontamination procedures normally used to deactivate GMM and are resistant to fumigation. If TSEs agents are present, it may be necessary to alter the normal procedures and inactivation methods used for GMM to accommodate their unique properties. For example, a higher autoclave heat setting may be required (138°C) and more stringent chemical decontamination (*e.g.* 20,000 ppm Sodium Hypochlorite, or 1M NaOH for 1 hour minimum) should be employed. It is also advised that equipment be dedicated for sole use with materials that might be contaminated with TSEs agents.

3.7 NON-CODING/REGULATORY ELEMENTS

The potential hazards associated directly with a gene and its product, represent the major factors to be considered in the risk assessment. However, it is also important to consider the expression characteristics that this gene may have within the context of the GMM system. Non-coding regions that form part of the expression cassette of which the inserted gene is a component usually confer these characteristics.

These non-coding regions, and other sequence elements that may form part of the system, may affect the potential risks posed by the GMM. The effects of all exogenous non-coding sequences should be carefully assessed.

3.7.1 Promoters / enhancers

i. Expression characteristics, including tissue- or cell-type specificity and the level to which the gene is expressed, will be determined at least in part by the promoter that is used to drive expression. Some commonly used promoter/enhancers, for example the Human Cytomegalovirus (HCMV) Major Immediate-Early enhancer, are already well described in terms of activity in different cell types. The HCMV enhancer directs high-level expression in most cell types although expression level varies between cell and tissue types. Other promoters will drive expression only in certain cell types, for example the prostate specific promoter will only direct expression in prostate cells. Tissue-specific promoters usually exhibit 'basal leakiness' whereby low-level 'break-through' expression occurs, even in non-specific cells. Often, such expression is undetectable, but it can become an issue if the inserted gene product has potent biological activity (*e.g.* a bacterial toxin).

ii. Artificial promoters can be constructed that are tailored to direct expression in cell-types with specific characteristics. For example, if a particular cell type expresses high-levels of a particular transcription factor, then a promoter can be constructed based upon the properties of that transcription factor to exploit the cellular trait. The expression characteristics of novel constructions are likely to be much less well understood and poorly defined in comparison to naturally occurring promoters with documented descriptions of activity. It is advised that expression characteristics of all novel and poorly defined promoters are assessed in cell culture using innocuous reporter genes before a potentially harmful GMM with infectious or invasive properties is generated.

iii. When a regulatory element that is endogenous to the genome of the recipient organism is exploited, it may be reasonable to assume that the characteristics of expression will be comparable to that of the gene that is normally controlled by it. However, different genes may be expressed to different levels, depending on the length and composition of the coding sequences. Furthermore, if endogenous promoters are used but transposed to different areas of the recipient organism's genome, expression characteristics may be affected. For example, genes towards the 3' end of an ssRNA (-) viral genome are expressed at a higher level than those at the 5' end due to the inherent transcriptional mechanisms at play.

iv. It should also be considered that the precise expression characteristics of a particular promoter might differ within the context of the GMM or the experimental system. This could be due to properties inherent to the nucleotide sequence that surrounds the inserted expression cassette. For example, tissue-restricted expression inserted into the E1 region of an adenovirus vector might be overcome by remnants of the viral E1 promoter that necessarily remain in the vector backbone as they are associated with other essential non-coding regions of the virus. Similarly, the expression characteristics of a cassette inserted into the genome of a cell (including prokaryotes, mammalian cells and protozoa) could be altered by sequences flanking the site of insertion. Equally, a strong promoter in an expression cassette in the context of the cellular genome might be able to direct the expression of genes that are in proximity to the site of insertion. These considerations are particularly pertinent to expression cassettes that can be vectored into and inserted into the genomes of mammalian cells by retroviruses and lentiviruses.

3.7.2 Genomic control regions

i. Long-term expression has proven to be problematic in GM mammalian cells as normal cellular mechanisms are prone to silencing expression from the inserted cassette. Genomic Control

Regions could be any non-coding regions (*e.g.* Locus Control Regions, chromatin opening elements and insulator sequences) that can be used to enhance, stabilise or modulate the expression from a promoter. These considerations are relevant to procedures that involve the manipulation of mammalian cell genomes, particularly if an infectious GMM that can modify host-cell chromatin (such as a retroviral or lentiviral vector) will be carrying such an element.

ii. Locus Control Regions and chromatin opening elements have been implicated in the reorganisation of cellular chromatin to permit gene expression. The regions can therefore form a part of an expression cassette in order that the inserted gene might be expressed even if inserted into an area of the host cell chromatin that is transcriptionally silent'. Such elements are often associated with so-called 'housekeeping genes' that are normally transcriptionally active and can have effect, not only on genes that are in close proximity to the element but also to more distal transcriptional units. Therefore, the possible effects of chromatin reorganisation and expression of cellular genes that are normally silent as a result of the integration of such an element into the host cell genome should be considered.

iii. Insulator sequences are used to prevent regions of DNA that flank an integration site from affecting the expression from an inserted cassette. Equally, such regions can be used to prevent sequences present in the cassette from affecting regions of DNA that flank the insert site. Again, these sequences are believed to function by remodeling chromatin and the possibility that such a region might affect the expression of host-cell genes in the area of the integration site should be considered.

3.7.3 Viral post-transcriptional regulatory elements

i. The lentivirus-encoded rev protein interacts with a rev-responsive element (RRE) in the Lentiviral genome, enhancing and stabilising the export of viral mRNAs from the nucleus. In some lentiviral vectors, this has been replaced with heterologous viral sequences with similar function, such as the woodchuck hepatitis B virus (WHV), posttranscriptional regulatory element (WPRE), Human hepatitis B virus regulatory element (HPRE) or the Mason-Pfizer virus constitutive transport element. This negates the need for rev in the lentiviral vector packaging systems, which is intended to improve biosafety by eliminating lentiviral genes from the system. However, it is important that such elements and their associated functions are carefully scrutinised as, in the case of WPRE, there have been unforeseen effects.

ii. In the case of WPRE, some versions of this element are capable of expressing part of the X protein from WHV which may have oncogenic properties, and risk assessments should take into

CHAPTER 4

ROUTINE CLONING AND EXPRESSION WORK USING ATTENUATED *ESCHERICHIA COLI*

4.1 BACKGROUND

The majority of GMM will be generated as a consequence of routine molecular cloning work. This could be defined as the transformation of non-pathogenic recipient microorganisms (usually strains of *E. coli*) with episomal constructs (such as a plasmid) carrying sequences of interest. These GMM are then grown in bulk cultures in order to extract and purify the constructs for use in subsequent procedures.

Risk assessment is a requirement for these activities as it is work involving GMM. The basic principles of hazard identification will be equivalent and measures to minimise the chances of harm occurring to either human health or the environment will be required. However, since routine cloning work usually involves the use of non-pathogenic donor strains of *E. coli*, the majority of these GMM will be low hazard and fall into the lowest class of genetic modification activity. On that basis, there are exemptions of regulatory procedures under the *Biosafety (Approval and Notification) Regulations 2010*. It is likely that these activities will require minimal assessment and these organisms should be assessed in a way that is commensurate with the actual hazards posed. Users should adopt a pragmatic approach and avoid overcomplicated assessments and unwarranted control measures.

4.2 SCOPE

The following is intended as a brief guide to do a risk assessment of low hazard routine cloning work using attenuated *E. coli* strains. The guidance does not make specific reference to low hazard host-vector systems other than attenuated *E. coli* (*e.g. Saccharomyces cerevisiae; Bacillus subtilis*), although the principles will be equivalent. Furthermore, the use of attenuated *E. coli* and other bacterial strains as gene-delivery vectors and vaccines is also not covered here.

4.3 RISK ASSESSMENT FOR HUMAN HEALTH

4.3.1 Hazards associated with the recipient strain

Many derivatives of the *E. coli* K-12 and B strains have been demonstrated to be non pathogenic and have well-understood, stable genetic lesions in the bacterial chromosome. These lesions often render the microorganism auxotrophic and dependent upon nutrients that must be supplied in the culture media. Furthermore, these strains are often rendered incapable of colonising mammalian hosts, either due to introduced biological restrictions or sensitivity to common agents. Many *E. coli* K-12 and B strains have a long history of safe use and most can be handled safely at BSL1 containment. Novel recipient strains should be more carefully assessed and the hazards considered on a case-by-case basis.

4.3.2 Hazards associated with genetic inserts

i. The majority of hazards to human health will arise due to the nature of the inserted genetic material. Therefore, the risk assessment should take into consideration the potential effects of any expressed product. For routine cloning work, most sequences will be carried on episomal constructs, such as plasmids. The mobilisation status of the plasmid backbone should be considered and, as a general rule, non-mobilisable plasmids should be used wherever possible.

ii. Close attention should be paid to inserted genes that encode products with potentially harmful biological activity, for example toxins, cytokines, growth factors, allergens, hormones or oncogenes. In many cases, the product will not be expressed, as there will be no prokaryotic promoter sequences present that could direct transcription. Hence, expression of potentially harmful genes would not be expected in *E. coli* if they were under the control of eukaryotic promoters. Where no expression is anticipated, or where the expressed product is produced in an inactive form, it is unlikely that the gene product will give rise to harm. Eukaryotic gene products are often inactive because prokaryotic host systems lack the required post-translational modification pathways. Furthermore, expressed proteins are often deposited within the cell as insoluble inclusion bodies, or cannot be secreted, and will not pose the same level of risk as they would if expressed in a eukaryotic system.

iii. This is not always the case; for example, many non-glycosylated cytokines are both soluble and biologically active when expressed in *E. coli*. The sequence should be carefully scrutinised to ensure that no cryptic prokaryotic promoters have been generated during the cloning steps or due to sequence optimisation of the control

regions. If expression is possible, or is subsequently observed, then the biological activity and immunogenicity/allergenicity of the products should be considered.

4.3.3 Hazards arising from the alteration of existing traits

A particularly cautious approach is advised when potentially harmful prokaryotic genes are cloned that can be expressed in *E. coli*, especially if it encodes a pathogenicity determinant. For example, a bacterial toxin gene that retains its native regulatory sequences might be expressed, correctly processed and secreted in *E. coli* and this could give rise to a toxigenic derivative that poses a greater risk of harm to human health than the recipient strain. Similarly, expression in *E. coli* of bacterial invasion determinants (*e.g. Yersinia inv* genes) could result in invasive or internalisation qualities and a related increase in pathogenicity compared to the recipient strain. In such cases, additional controls and a higher level of containment might be required.

4.4 RISK ASSESSMENT FOR THE ENVIRONMENT

4.4.1 Survivability and stability

Whether or not a transformed strain of *E. coli* will be able to survive in the environment is a key consideration. Most attenuated strains are auxotrophic for nutrients that will be scarce except in specialised media. These transformants would not be expected to replicate and may not survive in the environment. However, disabled *E. coli* strains have been shown to persist for several days in the environment. The longer the transformant can survive, the greater the likelihood that a genetic transfer event might take place.

4.4.2 Hazards posed by the genetic inserts

i. The frequencies of successful horizontal gene transfer in the environment will be low, especially where non-mobilisable constructs are used. However, genes carried on plasmids require particular consideration, as passive transformation should be considered as a possibility. The finite possibility that any gene may be transferred necessitates the need to focus on the nature of the gene itself. It may be that an 'environmentally harmful' sequence (*e.g.* a drug-resistance marker) may already be present in nature and therefore the impact of transfer will be diminished. However, the consequences of the transfer of inserted genes should be assessed especially if the insert could give an advantage to naturally occurring pathogens or other organisms. In these cases, the focus should be on the possible consequences rather than on the likelihood of transfer.

ii. The biological properties of the expressed gene product, even if they represent a low risk to human health, may be a possible hazard to other species. It is therefore important to consider any potential adverse effects of the encoded products upon non-human species that may come into contact with the GMM.

4.4.3 Hazards arising from the alteration of existing traits

Careful consideration should be given to the cloning of any gene that might enable GM *E. coli* to colonise or adversely affect animal species. A particularly cautious approach should be taken when cloned genes that encode products that might be harmful to animals can be expressed in *E. coli*. In such cases, additional containment measures and a higher level of containment might therefore be required to prevent release of the organism.

4.5 Procedures and control measures

Given that the majority of these GMM will fall into the lowest activity class, BSL1 containment supplemented by the principles of good microbiological practice will be sufficient to protect both human health and the environment. Consideration should be given to the possibility that staff might carry the GMM away from the site of containment. If the GMM poses a risk to other species, or a genetic transfer event with feasible adverse consequences is possible, then measures to minimise dissemination by the staff may be required.

CHAPTER

5

BACTERIAL VACCINES AND GENE DELIVERY SYSTEMS

5.1 BACKGROUND

i. The following guidance is intended for risk assessment associated with the construction and handling of bacterial vaccines and gene delivery systems. It covers the use of attenuated Escherichia coli strains for the purposes of gene delivery and vaccine strain development but does not consider routine cloning work using attenuated *E. coli* strains such as K-12. Users are directed towards more specific guidance for the risk assessment of these activities (see Chapter 1).

ii. Live, attenuated bacteria have been exploited as vaccines for many years. For example, the BCG (Bacille Calmette-Guerin) strain of *Mycobacterium bovis* and Ty21a strain of *Salmonella typhi* are effective vaccines against tuberculosis and typhoid fever respectively. These strains were attenuated using empirical methods resulting in randomly mutated strains that have poorly understood genetic mutations. The advancement of understanding in bacterial biosynthetic pathways and virulence has led to the ability to rationally engineer attenuating mutations into bacteria, to modify such strains to stably express heterologous genes or to deliver plasmid DNA to cells. This has resulted in their development as vaccine and gene therapy vectors.

iii. Much of the developmental work on live vaccines and delivery systems to date has been on rationally attenuated strains of *Salmonella enterica* (serovars *S. typhi* and *S. typhimurium*), *Shigella flexneri* and *Vibrio cholerae* as these bacteria primarily invade gut-associated lymphoid tissue (GALT), giving rise to both mucosal and humoural immunity. The ability of *Salmonella*, *Shigella*, *Listeria*, *Yersinia* and some enteropathogenic strains of *E. coli* to enter mammalian cells and deliver eukaryotic expression cassettes into the cytoplasm has also led to their development as cancer vaccines and gene-therapy vectors. Since these bacterial vector strains are derived from virulent human pathogens, issues are raised regarding biosafety with respect to those who may be exposed and

also to the wider environment. Furthermore, unlike disabled virus systems, these strains have the potential to survive and replicate both independently as well as within the host cell or tissue.

iv. In order to retain the properties of these microorganisms that are desirable for vaccine and gene therapy applications, attenuating mutations that have been engineered into them have largely involved the disruption of determinants directly involved in bacterial virulence, rather than affecting the ability to enter cells, colonise the host or induce immunity. However, since these strains are not obligate parasites like viruses, mechanisms must also be in place to prevent the survival or external spread of the organism and minimise the transfer of any heterologous inserted sequences. Thus strains frequently carry multiple mutations that render them attenuated and auxotrophic, unable to survive for protracted periods outside of the host or specialised environments.

5.2 RISK ASSESSMENT FOR HUMAN HEALTH

i. The rational attenuation and engineering of bacterial strains as potential vaccines or therapeutic vectors can be applied to a number of species. To date, the majority of work in this area has concentrated upon enteric pathogens such as *Salmonella*, *Shigella*, *Vibrio*, *Listeria* and *Yersinia* species. For this reason, this guidance will concentrate mainly on derivatives of these strains, although the aspects covered will be relevant to any similar bacterial system.

ii. Many of the species that will be manipulated in the development of vaccine and vector systems will be human or animal pathogens. Therefore, in order to set an appropriate activity class for the work, it is prudent to begin by considering the hazards posed, the Risk Group and containment level appropriate for the recipient organism.

iii. A list of bacteria that are commonly manipulated as vector strains, as well as some of the hazards posed by the organism can be found in Table 4.

The recipient strain to be manipulated may not have the same characteristics as the wild type pathogen and the associated hazards may differ. For example, attenuated derivatives of *Salmonella typhi* and *Mycobacterium bovis* (strain Ty21a and BCG respectively) are used as vaccines and have a long history of safe use. Therefore, the recipient strain may already be attenuated and may be less hazardous than the pathogen from which it is derived. If it can be demonstrated that the recipient strain is sufficiently attenuated and poses a much lower risk of harm, then the risk assessment could be used to argue the case for lowering the containment level. It is important that the nature of the attenuation

is understood as fully as possible and is supported by relevant scientific data if a downgrading of BSL is sought.

Table 4. Typical symptomatic consequences of infection with wild type bacteria and associated Risk Group (RG)

Recipient	Disease	Intracellular	Risk Group (RG)
Salmonella typhi	Enteric fever (Typhoid fever)	Yes	RG3
Salmonella typhimurium	Enteritis (Salmonellosis)	Yes	RG2
Listeria monocytogenes	Septicaemia; fever; diarrhoea (Listeriosis)	Yes	RG2
Shigella flexneri	Dysentery; fever (Shigellosis)	Yes	RG2
Shigella sonnei	As *S. flexneri*	Yes	RG2
Vibrio cholerae	Gastroenteritis Cholera toxin)	No	RG2
Yersinia enterocolitica	Lymphadenitis, enteritis	Yes	RG2
Yersinia pestis	Plague; enterocolitis	Yes	RG3
Escherichia coli	Gastroenteritis	Yes, some strains	RG2
Mycobacterium bovis	Tuberculosis in cattle	Yes	RG3
M. bovis (BCG)	Tuberculosis vaccine strain	Yes	RG2

5.3 EXAMPLES OF BACTERIAL GENE DELIVERY SYSTEMS

5.3.1 *Salmonella enterica*

i. The natural tropism of *Salmonella enterica* (serovars *S. typhi* and *S. typhimurium*) for the mucous lymphoid tissue of the small intestine has made them interesting vectors for the induction of immunity at these sites. Furthermore, *S. enteric* is able to persist in the phagocytic vacuoles of antigen presenting cells (*e.g.* dendritic cells and macrophages) and deliver plasmid DNA into the cytoplasm by an unknown mechanism. Thus, these bacteria have the ability to induce both humoral and cytotoxic immune responses to

heterologous antigens that are either expressed directly by the bacteria themselves, or by the cell from plasmid-borne eukaryotic expression cassettes.

ii. The ability of *S. enterica* to deliver DNA to the cytoplasm of cells has also made them potentially useable as vectors in gene therapy applications. *S. enterica* can be exploited to deliver therapeutic cargo for the treatment of disease. *Salmonellae* have a natural tropism for solid tumours and could therefore be used to deliver genes with biologically active products to them, either for the purposes of eliciting an immune response (*e.g.* immunomodulatory growth factors and cytokines) or to specifically destroy the cells (*e.g.* toxins or prodrug converting enzymes). Clearly, in addition to any hazards associated with the recipient strain, there may be hazards arising from heterologous genes that are expressed by the vector or delivered to the cytoplasm of infected cells.

iii. The lack of a suitable animal model for S. typhi infection has led to the engineering of attenuated vector strains of *S. typhimurium*, which causes a typhoid-like disease in murine hosts. Attenuating mutations that are characterised in *S. typhimurium* can be extrapolated back to the homologous genes in *S. typhi*. However, caution is advised when using this reasoning, as the only way to confirm attenuation in *S. typhi* is to test the organism in human subjects.

iv. Many of the systems engineered from *S. typhi* have been derived from Ty2, the pathogenic recipient strain from which the Ty21a live typhoid vaccine was generated by chemical attenuation. The rational deletion of genes that are known to be involved in virulence could give rise to attenuated strains and additional mutations in biosynthetic pathways may result in auxotrophs that are unable to survive for prolonged periods outside of the host organism. *S. typhimurium* can also be used as a vector in human systems in its own right as it shares the invasive features of *S. typhi* and has a prolonged intestinal phase, making it of interest in the development of vaccine strains. Examples of genes that can be mutated in *S. typhi* for the purposes of attenuated vector development can be found in Table 5.

5.3.2 *Listeria monocytogenes*

i. *Listeria monocytogenes* has a number of features that has led to its development as a vaccine and gene-delivery system. It is an intracellular pathogen that is internalised by a number of cell types, including splenic macrophages and hepatocytes. Furthermore, *L. monocytogenes* can escape the phagocytic vacuole, replicate in the cytoplasm and spread between cells. It does not generate inflammatory lipopolysaccharide (LPS), although it can cause severe systemic infections in immunocompromised and

pregnant individuals. Therefore, candidate strains engineered for use in humans must be significantly reduced in virulence and carry multiple attenuating mutations. Attenuated phenotypes can be tested in a mouse model but a cautious approach is advised when extrapolating results in animal experiments to potential effects upon humans. Examples of genes that have been mutated in *L. monocytogenes* for the purposes of attenuation can be found in Table 5.

ii. *L. monocytogenes* naturally infects splenic antigen presenting cells and therefore recombinant vaccine strains of *L. monocytogenes* that express and/or secrete heterologous antigens have been shown to be effective at eliciting immune responses. In order for effective delivery of plasmid DNA to the cytoplasm of cells, the bacterial cell wall must be disrupted. This has been achieved by designing self-destructive strains of *L. monocytogenes* by the expression of Listeria-specific cytolysins that result in the preferential lysis of bacteria in infected cells and release of DNA cargo into the cytoplasm.

5.3.3 *Shigella* spp.

i. The enteric pathogens *Shigella flexneri* and *Shigella sonnei* naturally invade the cells of the colonic epithelium and are able to escape the phagocytic vacuole to enter the cytoplasm. From here, the bacteria spread horizontally between cells and elicit immune responses in the GALT. These features make *Shigella* spp. attractive candidates for development as vaccines and for gene-delivery. However, the lack of a reliable animal model and naturally attenuated recipient strains has hampered the development of *Shigella* spp. for these applications and some pathological features of the wild type (for example reactive diarrhoea) are often retained. Rational attenuation using multiple mutations may yield useful strains, although the level of attenuation appears to be proportional to a decrease in effectiveness. Examples of genes that have been mutated in *Shigellae* for the purposes of attenuation can be found in Table 5.

ii. The expression of heterologous antigens by recombinant *Shigella* spp. has demonstrated its effectiveness at eliciting immune responses. Delivery of plasmid DNA to the cytoplasm of cells requires disruption of the bacterial cell wall. Deletion of the *asd* gene, which is required for cell wall biosynthesis, results in strains of *Shigellae* that autolyse in infected cells, releasing the DNA cargo into the cytoplasm.

5.3.4 *Vibrio cholerae*

i. *Vibrio cholerae* is an enteric pathogen that colonises the gastrointestinal mucosa without being internalised and is

highly immunogenic. It is therefore an attractive candidate for development as a vaccine. Furthermore, the virulence of this bacterium appears to be almost entirely related to the expression of Cholera Toxin (CT), a secreted subunit exotoxin encoded by the *ctx* gene on the bacterial chromosome. Cholera toxin activates the adenylate cyclase enzyme in intestinal mucosal cells, leading to increased levels of intracellular cAMP, hypersecretion of ions and water into the lumen of the small intestine resulting in acute diarrhoea.

ii. Mutations and deletions of the *ctx* gene therefore result in attenuated strains of *V. cholerae* that are candidates as a vaccine for cholera itself and can be adapted to express heterologous antigens. Increased secretion of expressed antigens can also be achieved by fusing antigens and epitopes to the B-subunit of CT. The B-subunit of the toxin allows it to bind and transduce cells and therefore it can carry heterologous antigens into cells, resulting in peptide display and an immune response.

5.3.5 *Mycobacterium bovis* (BCG)

i. The *Mycobacterium bovis* (BCG) strain has been attenuated by multiple passage *in vitro* and has been used safely as a tuberculosis vaccine for many years. While the attenuated phenotype and genetic lesions are known, the mechanisms are poorly understood. However, the strain has features that make it a potential GM vaccine.

ii. *M. bovis* (BCG) persists in the phagosome of infected macrophages and elicits a strong cellular immune response. *M. bovis* (BCG) can be modified to secrete or display heterologous antigens and protective immune responses to these have been demonstrated in animal models. Furthermore, the expression of tumour antigens, immunomodulatory cytokines or growth factors could lead to *M. bovis* (BCG) being used in cancer therapy. However, there may be hazards associated with the inserted gene and associated changes to the pathogenicity of the recipient organism.

5.3.6 *Yersinia enterocolitica*

i. There are several known serotypes of *Yersinia enterocolytica* that vary in their natural pathogenesis for humans and animals. However, they are all enteric pathogens that are able to survive and multiply within the GALT. This has led to their development as potential GM vaccines, although wild type strains are able to resist phagocytosis and grow extracellularly. This phenotype is mediated by a secretory system encoded by the virulence plasmid, pYV. 'Curing' strains of this plasmid results in attenuated vector strains that can deliver DNA expression vectors to lymphoid cells and yet still persist for two to three weeks.

ii. There are other virulence-associated genes present of the genomes of *Yersiniae* and these have been exploited previously in the generation of candidate vector strains (see Table 5). The virulence of *Yersiniae* has been extensively studied and is relatively well understood. The scope for the further generation of novel delivery systems is broad and it is important that caution is applied since virulence is complex in this species.

5.3.7 *Escherichia coli*

i. Non-invasive, non-pathogenic *Eschericia coli* strains (*e.g.* DH10B) can be engineered to deliver DNA to the cytoplasm. This offers several advantages as non- pathogenic strains of *E. coli* have been used safely in laboratories for many years; they are efficiently transformed and easy to grow in large amounts. Furthermore, these systems have shown utility for the transduction of cells with both small reporter constructs and large Bacterial Artificial Chromosomes that are over 200 kb in size.

ii. For example, transformation of *E. coli* with an expression plasmid carrying the *inv* gene from *Yersinia pseudotuberculosis* confers the ability to be internalised by cells in culture that express β1 integrins, including primary epithelial and HeLa cells in culture, as well as phagocytic cells in the colonic mucosa. Furthermore, expression of *hlyA* (which encodes Listeriolysin O) from *L. monocytogenes* allows escape from the phagocytic vacuole to enter the cytoplasm.

Table 5. Examples of genes that have been mutated for the purposes of attenuation and development of bacterial vector systems, the function lost and the type of phenotypic effect

Recipient	Mutation	Gene Function Lost	Effect
Salmonella typhi Ty21a	Undefined	Unknown	Attenuation
Salmonella enterica	*phoPQ*	Virulence regulon	Attenuation
(S. typhi; S. typhimurium)	*ssaV* *aroA** *purB*	Virulence-associated Amino-acid biosynthesis Purine biosynthesis	Attenuation Auxotrophy Auxotrophy
Listeria monocytogenes	*dal* *dat* *actA* *plcB*	Alanine racemase (cell wall biosynthesis) D-aminotransferase (cell wall biosynthesis) Actin nucleator (cell-cell spread) Phospholipase B (escape from vacuoles)	Auxotrophy Auxotrophy Attenuation Attenuation

Shigella flexneri	*guaBA*	Guanine monophosphate biosynthesis	Auxotrophy
Shigella sonnei	*sen; set* *icsA* *asd*	Shigella enterotoxins Cell to cell spread Cell wall/diaminopimelic acid biosynthesis	Attenuation Attenuation Auxotrophy
Vibrio cholerae	*ctx*	Cholera Toxin	Attenuation
Mycobacterium bovis (BCG)	Multiple	Various, poorly understood	Attenuated
Escherichia coli	*asd*	Cell wall/diaminopimelic acid biosynthesis	Auxotrophy
Yersinia enterocolitica	*pYV* *yadA-2* *soda* *irp-1*	Resistance to phagocytosis Colonisation; resistance to complement Superoxide dismutase (oxygen radical resistance) Siderophore biosynthesis	Attenuation Attenuation Attenuation Attenuation

* The *aroA* gene mutation has been applied to many vector systems, including vectors derived from *Salmonella* spp., *Shigella* spp. and *Yersinia* spp.

Attenuated strains and gene-delivery systems that can be demonstrated to pose a much-reduced risk of harm compared to the wild type might be handled at a lower containment level. However, hazards arising subsequently due to the insertion of sequences or phenotypic alterations might necessitate additional containment measures.

iii. The recipient strain in these cases will be low hazard and can be handled safely at GM BSL1. However, the insertion of invasion determinants will increase the hazard posed by the GMM and the need for an increase in containment level is likely. Careful assessment of the risks associated with the use of plasmid constructs that carry bacterial invasion determinants such as *Yersinia inv* genes is also required. Horizontal transfer of such constructs to non-invasive, non-pathogenic species such as commensal gut flora could represent a hazard to health. Any selection pressure for the retention or acquisition of this gene can be minimised by using 'balanced lethal' systems.

5.4 HAZARDS ASSOCIATED WITH GENETIC INSERTS

The risk assessment should take into consideration any potential adverse effects of the expressed product or any properties inherent to

the inserted sequence. More detailed guidance on the hazards posed by commonly used genetic inserts can be found in Chapter 3. However, in brief, factors to consider include the following:

5.4.1 Biological properties of the gene product

The expected activities or toxicity of the gene products should be assessed. For example, a bacterial toxin, oncogene or growth factor would represent greater risk of harm than a reporter gene such as Enhanced Green Fluorescent Protein (EGFP) or Luciferase. Furthermore, some proteins may be secreted or displayed on the surface bacterial cell wall whereas others will not. Fusing heterologous sequences to the genes encoding components of bacterial secretory mechanisms (*e.g.* the *sopE* gene of the type III *S. typhimurium* secretory system) may affect the fate of the final product. For example, increased secretion of expressed antigens from *V. cholerae* vectors can also be achieved by fusing antigens and epitopes to the B-subunit of CT. The B-subunit of the toxin allows it to bind and transduce cells and therefore it can carry heterologous antigens into cell, resulting in peptide display and an immune response. The properties of the encoded products or fusions should therefore be considered together with and their potential effects upon individual cell types and tissues that may be affected.

5.4.2 Expression characteristics

i. Heterologous genes in bacterial gene-delivery systems will either be expressed by the bacterium or within the cellular cytoplasm in the context of a eukaryotic expression cassette. The level to which the bacteria will express a heterologous gene will be dependent on the context in which it is present and the regulatory sequences that control it. For example, heterologous genes present on the bacterial chromosome will generally be expressed to a much lower level than those present on plasmids. Furthermore, the expression characteristics of genes inserted into the bacterial chromosome that are under the control of native bacterial regulatory sequences will vary depending on the locus. Assumptions could be made based on known facts regarding the expression of the native gene from that locus. However, it is important that the likely level and kinetics of expression are assessed as fully as possible.

ii. Expression of genes in the context of a eukaryotic expression cassette will also be dependent on the cell type and the regulatory sequences involved. For example, use of the Human Cytomegalovirus Major Immediate-Early enhancer would be expected to direct high-level expression in a broad range of cell types, whereas tissue-specific promoters generally lead to cell-type restricted expression. However, the latter may exhibit 'basal leakiness' whereby low-level expression is observed in non-permissive cells. It is advised that promoter characteristics are

thoroughly assessed where possible using reporter genes in cell culture systems before a GM bacteria is constructed.

5.4.3 Chromosomal insertion

Genes present on plasmids that are delivered to the cytoplasm by bacterial vectors may become inserted into the chromosome of the infected cell. For example, plasmids delivered to the cytoplasm of infected macrophages by *Listeria monocytogenes* vectors become integrated into the host cell chromosome at a frequency of 1 in 10^7. Insertion occurs by a random mechanism and, although insertion is a relatively rare phenomenon, the possible effects should be carefully considered. For example, heterologous promoters or chromatin modulating sequences might affect the natural expression of genes adjacent to the integration site. Furthermore, the infected cell might be permanently modified expressing the heterologous gene and passing the modification to daughter cells.

5.4.4 Alteration of phenotype

It is acknowledged that modifications made in the development of candidate bacterial strains are generally attenuating and therefore the resulting GM organism will pose an equivalent or lower risk of harm than the wild type/recipient. However, there are circumstances where the pathogenicity of the recipient strain could be increased and the potential effects of any modification on the phenotype of the parent organism, whether as a result of chromosomal modification or plasmid transformation, should be scrutinised.

5.4.5 Pathogenicity

i. Bacterial genetics are relatively complex and it is important that enough is known about the modification in order for the risk assessment to accurately claim that it will result in attenuation. Many bacterial genes are co-transcribed in an operon or are a part of a regulatory network and therefore there may be wider implications to a mutation than just the loss of the function of one gene. For example, it may be that deletion of a virulence gene will alter the regulation of other virulence genes that are connected to it genetically or by a regulatory mechanism. While in most cases this will result in attenuation, there is the possibility of inadvertently increasing virulence.

ii. One strategy for generating a bacterial gene-delivery strain might be to confer invasive or internalisation qualities upon a non-pathogenic species. For example, bacterial invasins from an intracellular pathogen such as *Yersinia* could be engineered into non-invasive, attenuated *E. coli* (e.g. K-12). Clearly, this would result in an increase in pathogenicity compared to the recipient strain and, as such, specific containment measures or a higher containment level may need to be implemented.

5.4.6 Genetic stability and sequence mobilisation

i. The genetic stability of modifications made to the chromosome will be much more robust than those present in an episomal form, such as a plasmid or cosmid. Furthermore, the likelihood that any sequences inserted into the bacterial chromosome will be transferred to another organism is also low, although there always remains the finite possibility that the sequence could be transferred. Bacterial genetics are relatively complex and while a mutation may result in disruption of a targeted gene, there may be redundant mechanisms that might compensate for its loss. Moreover, the loss of a gene that impairs an organism's ability to survive will result in a selection pressure to reverse the effects of the lesion. If the modification is a deletion of a gene, then the likelihood of a successful reversion event will be low. Point mutations that disrupt the coding sequences or regulatory regions of a gene will be much less stable and thus the possibility of a reversion will be high.

ii. It is important to consider the potentially harmful consequences of heterologous sequences being transferred to other organisms, or that an attenuated vector may acquire sequences that might increase its pathogenicity. This is particularly pertinent to modified enteric bacterial pathogens carrying heterologous sequences in a mobilisible form (*e.g.* plasmid or cosmid) as sequences could be transferred between the attenuated host strain and the natural gut flora. For example, transfer of constructs carrying determinants of bacterial invasion (*e.g. Yersinia inv* genes) to non-invasive, non-pathogenic commensal bacteria could represent a hazard, both to human health and environmental species. Phage-mediated mobilisation of inserted sequences may be a possibility and should also be considered. The factors that affect the frequency of such events and the likelihood of a harmful consequence may be complex, but these issues must be carefully considered in the risk assessment.

iii. In order for genetic modifications present on a mobilisible construct to be maintained, inherent selection pressure must be present. This can take the form of an antibiotic or drug-resistance marker, or as a gene that complements a stable attenuating mutation inherent to the receiving strain ('balanced lethal' systems). For example, deletions in the *asd* gene in Shigellae and *E. coli* render the organism auxotrophic for diamonopimelic acid and impair bacterial cell wall synthesis. Expression of the *asd* gene on the plasmid transformed into the attenuated strain will complement the mutation and allow the bacteria to grow. Loss of the construct, however, will render the bacterium auxotrophic once more and prevent survival. Modifications using balanced-lethal selection will be more stable, therefore, as loss of the construct will

result in the death of the bacteria. However, constructs carrying antibiotic resistance will be unstable as selection is difficult to maintain *in vivo* and *ex vivo*.

iv. The mobilisation status of a plasmid should be considered. As a general rule, non-mobilisible plasmids should be used wherever possible. It is also important to consider whether there will be any selection pressure *in vivo* that might result in the sequence persisting in commensal bacteria that may acquire it.

5.5 RISK ASSESSMENT FOR THE ENVIRONMENT

5.5.1 Survivability and stability

i. Whether or not an attenuated bacterial strain will be able to survive in the environment in the event of a breach of containment is a key consideration. Most bacterial hosts harbour mutations that render them auxotrophic for nutrients that, while possibly present *in vivo*, will be scarce outside of the host organism except in specialised media. These organisms would not be expected to replicate and may not survive in the environment. However, this may not affect the organism's ability to persist. For example, even disabled *E. coli* can persist for several days in the environment. Furthermore, auxotrophic strains may be able to persist in a vegetative state and begin replicating if acquired by a suitable host. For example, *L. monocytogenes* is persistent in the environment and can be found naturally in soil and water. Similarly, *V. cholerae*, which is primarily transmitted via ingestion of contaminated water, can persist in a vegetative state.

ii. The longer the recombinant strain can survive, the greater the likelihood that a genetic transfer event will be successful in generating a pathogen in the environment. The transfer of genetic information present on the genomes of bacteria is much less likely than if they are present on a mobilisable form (*e.g.* a plasmid or cosmid) and the frequencies of successful horizontal gene transfer in the environment are low. However, the nature of the gene and any associated selection pressures should be considered.

iii. Consideration should also be given to the possibility that humans may carry the bacterium away from the site of containment. Most bacterial systems are based upon human pathogens and therefore staff may harbour attenuated derivatives without overt symptoms. Adherence to the principles of good microbiological practice will be required to minimise the possibility of human exposure and release in this way.

5.5.2 Hazards posed by the genetic insert

i. A potentially 'harmful' sequence could be a heterologous gene insert or a selection marker (*e.g.* antibiotic resistance). If the

gene is already present in nature, the impact of transfer will be diminished. However, any possible adverse effects of sequence transfer should be considered, especially if it could conceivably confer a selective advantage or pathogenic phenotype to naturally occurring bacteria. For example, plasmid constructs carrying bacterial invasion determinants such as *Yersinia inv* genes could be transferred to non-invasive, non-pathogenic species present in nature or commensal species present in the gut of infected humans. This could represent an environmental hazard and a risk to wild and domestic animal species. Selection pressures for the retention or acquisition of this gene might be minimised by using 'balanced lethal' systems rather than antibiotic resistance markers.

ii. The biological properties of the expressed gene product, even if they represent a low risk to human health, may be a possible hazard to other species or ecosystems. It is therefore important to consider any potential adverse effects of the encoded products upon non-human species that may be affected.

5.5.3 Alteration of phenotypic and pathogenic traits

A careful assessment of any modification to an animal pathogen (*e.g. M. bovis*, *Y. pestis*) that might increase its pathogenicity should be made. Furthermore, careful consideration should be given to any modification that might affect the host range of the GMM, giving rise to a novel animal pathogen. Appropriate measures should be in place to prevent environmental release of the GMM, even if minimal containment is required for human health protection.

5.6 PROCEDURES AND CONTROL MEASURES

Staff must be sufficiently protected from the possibility of infection by inoculated experimental animals. Working with animals often involves the use of sharps (*e.g.* hollow needles) and contact with secretions. Furthermore, the possibility that the staff may be bitten or scratched should be considered. Clearly this is important from a human health perspective with regard to working with a human pathogen but there are also environmental considerations. Humans harbouring such an infection could inadvertently release an animal pathogen into the environment. Appropriate control measures and protective equipment should be employed to minimise the possibility that a staff handling an animal could become infected. This might include having standard procedures for the safe use of sharps and the use of animal isolators. When working with larger animals, the use of respiratory protective equipment might be required to protect against infectious aerosols where these cannot be effectively contained by other means.

WORK WITH CELL CULTURES

CHAPTER 6

6.1 BACKGROUND

i. The following guidance covers the risk assessment of GM activities involving the genetic modification of cell cultures. Uncontaminated cell cultures do not appear to present a significant hazard as even direct dermal inoculation may result in only local inflammation. However, the long-term consequences of direct inoculation are uncertain. The main risk presented by cell cultures is as a result of their ability to sustain the survival and/or replication of a number of adventitious agents. The major agents of concern are viruses, but other agents, *e.g.* mycoplasmas such as *Mycoplasma pneumoniae*, should also be considered.

ii. Appropriate baseline containment of different cell types is shown in Table 6. Where a cell line is deliberately infected with a biological agent, or where it is likely that the cell line is contaminated with a particular agent, the BSL used must be appropriate for work with that agent.

iii. The recommendations in Table 6 are based on both the intrinsic properties of the cell culture and the possibility that the culture may be, or inadvertently become, contaminated with pathogens. This is a separate issue from the containment required to protect human health and the environment from the risks associated with a GM cell line, which forms the basis of classification and notification requirements under the Malaysian biosafety regulatory requirements.

iv. Mammalian and insect cells have very stringent requirements for growth and are very susceptible to dehydration and exposure to ultraviolet radiation. Outside of the animals from which they are derived, growth and survival requirements can only be met by using specialised media, the correct temperature range, optimum pH and an adequate oxygen concentration. These constraints mean that cell lines will pose minimal risk to both human health and the environment. In addition, due to immune rejection of non-self tissue, it is highly improbable that accidental exposure would

result in survival and replication in normal healthy individuals (with the possible exception of some tumour cells). Therefore, staff should not conduct genetic modification work with their own cells and use of cells derived from other laboratory staff should be avoided where possible.

v. Unless the modification itself increases the intrinsic risks posed, most modification work with cultured cells can be carried out in a GM-BSL1 laboratory.

6.2 RISK ASSESSMENT

i. The GMM risk assessment should focus on the hazards associated with the cells and their modifications. The genetic modification activity class should reflect this. The GMM risk assessment can also take into account the possibility that adventitious agents might be present and any hazards associated with molecules present in culture media.

Table 6. Recommended baseline containment measures for work with cell cultures

Hazard	Cell type	Baseline containment level
Low	Well characterised or authenticated finite or continuous cell lines of human or primate origin with a low risk of endogenous infection with a biological agent presenting no apparent harm to laboratory workers and which have been tested for the most serious pathogens	**BSL 1**
Medium	Finite or continuous cell lines/strains of human or primate origin not fully characterised or authenticated, except where there is a high risk of endogenous biological agents, *e.g.* blood borne viruses	**BSL 2**
High	Cell lines with endogenous biological agents or cells that have been deliberately infected	Containment level appropriate to the agent. For example, if infected with Hepatitis B virus, BSL3 would be required
	Primary cells from blood or lymphoid cells of human or simian origin	Containment appropriate to the potential risk. A minimum of BSL2 containment is recommended

Note: *Any work that could give rise to infectious aerosols such as with medium or high risk cell lines must be carried out in suitable containment e.g. a biological safety cabinet*

ii. **Adventitious agents**

Primary cell lines, especially those derived from blood or neural tissue, and cell lines that have not been fully authenticated or characterised are most likely to harbour adventitious agents. Where adventitious agents (or gene sequences from them) may be present in the cells, containment measures should be applied which commensurate with the risks. While these risks may be unconnected to the genetic modification, notification of the work will be required in circumstances whereby specific containment measures are required to protect staff from the GMM.

iii. **Human tumour cells**

Many tumour cell cultures will fall into the category of 'well characterised continuous cell lines' and will therefore require minimal containment. As there are few conceivable modifications that could increase the hazards associated with tumour cells, most genetic modification work will be classified as GM BSL1, unless the modification could increase risk (*e.g.* by increasing the rate of tumour growth or metastatic potential). There are concerns regarding primary human tumour cells that have led to recommendations that all work with such cells should be carried out at a minimum of BSL2 containment. In addition to the potential for adventitious agents to be present, these recommendations are also based on the potential for some tumour cells to escape from normal immune surveillance to survive and replicate following accidental inoculation.

iv. **Expression of highly potent secreted proteins**

Where cells are genetically modified to express highly potent biologically active molecules such as cytokines, control measures may be required to minimise the risk of exposure to those molecules. Should the modification lead to the secretion and accumulation of such molecules in the cell medium, then there may be a need to introduce control measures to minimise the risk of exposure to them.

v. **Contamination versus containment**

Many users will automatically use a biological safety cabinet and wear protective gloves to protect the cells from contamination. Similarly, there may be restricted access to culture facilities in order to minimise the possibility of contamination. These measures are specified in the list of controls required for BSL2 but are a separate issue from the containment required to protect human health and the environment from the risks associated with the GMM. The use of such measures for the purposes of protecting the cell culture from contamination should not alter the GM activity class.

Conversely, where restricted access, the wearing of gloves or the use of a biological safety cabinet is required to protect the staff from the modified cell line, the GM activity class should reflect this. It is permissible to use higher containment than indicated by the GM activity class of the GMM, but this will not necessarily mean that a higher classification is required. However, where there is a disparity between the containment level actually being used and the GM activity class identified as being appropriate for the GMM, this should be documented.

ADENO-ASSOCIATED VIRUSES

CHAPTER 7

7.1 BACKGROUND

i. Adeno-associated viruse (AAV) belongs to the family *Parvoviridae* and there is no known link to any human illnesses. AAVs appear to be defective, requiring coinfection with a helper virus (*e.g.* adenovirus or herpes simplex virus) in order to replicate and this has led to their classification as *Dependoviruses*, a discrete genus within this family. Replication can also be induced during cellular stress (*e.g.* in the presence of genotoxic agents or following UV irradiation), suggesting that AAVs are not fully defective but are rather reliant upon certain cellular conditions for replication. Transmission may be via aerosol, the faecal-oral route or direct conjunctival inoculation. In addition to *Avian*, *Bovine*, *Canine*, *Equine* and *Ovine adeno-associated viruses*, there are six known human AAV serotypes that appear to be highly prevalent. For example, over 80% of individuals are seropositive for AAV serotype 2 (AAV-2) and this immunity appears to be long-lasting. The following guidance will focus on the use of human AAVs, however many of the principles will also apply to work involving the animal viruses.

ii. The AAV virion comprises a non-enveloped icosahedral capsid containing a 4.2 kb single stranded DNA genome. The determinants of cell attachment and entry appear to be serotype-specific. For example, cell attachment by AAV-2 is via ubiquitous heparin sulphate proteoglycans and internalisation via endocytosis appears to be mediated by the co-receptors αvβ5 integrins, fibroblast growth factor receptor 1 and the hepatocyte growth factor receptor, c-Met. AAV-5, on the other hand, binds to sialic acid residues and triggers endocytosis via the platelet-derived growth factor receptor. AAV-2, from which most vectors have been derived, is able to transduce both non-dividing and terminally differentiated cells of human, primate, canine, murine and avian origin. The AAV genome contains two gene complexes, *rep* and *cap* that encode nonstructural and structural proteins, respectively, via

mRNA splicing and alternate initiation codon usage. The genome is flanked by two inverted terminal repeats, which contain all the necessary sequences for genome mobilisation and packaging and also serve to prime DNA replication by virtue of its secondary structure (see Figure 3). Following the transduction of cells, AAV can follow one of two distinct pathways (lytic or lysogenic) depending on the presence of a helper virus. Both pathways require the conversion of the single-stranded viral genome into a double-stranded intermediate, which is either mediated by cellular DNA polymerases or occurs as a result of the complementary annealing of positive-sense and negative-sense AAV genomes, both of which are packaged efficiently.

iii. In the absence of a helper virus, AAV enters the lysogenic pathway whereby viral Rep proteins direct the targeted integration of the viral provirus into the host genome at a locus designated AAVS1 on human chromosome 19. The viral replicative gene expression programme is largely suppressed and the virus remains latent with the provirus propagated via host cell division. In the presence of helper functions, however, the lytic pathway is activated and the entire viral replication gene expression programme ensues. This results in replication of viral genomes, the generation of structural proteins from the cap gene complex and the release of infectious virions. Superinfection with an appropriate helper virus results in the excision of proviral AAV genomes and initiation of the lytic cycle in otherwise latently infected cells.

7.2 RISK ASSESSMENT FOR HUMAN HEALTH

7.2.1 Hazards associated with the recipient virus

i. To date, most genetic modification work involving AAVs has involved the development of transduction vectors derived from human AAV-2, although other serotypes are increasingly being used. While it is important to consider the hazards posed by the virus from which these vector systems are derived, since AAVs are defective in nature and not associated with human illnesses, the hazards posed to human health can be expected to be low. The main hazards arising from AAV vectors are likely to arise from the properties of any inserted genetic material.

ii. Wild type AAVs are not categorised by RG Classification of the *Prevention and Control of Infectious Diseases (Importation & Exportation of Human Remains, Human Tissues and Pathogenic Organisms & Substances) Regulations 2006*. However, BSL1 containment will be sufficient and should be adopted as a minimum requirement when handling wild type virus. Most activities with AAVs are low hazard and can take place safely at BSL1. Therefore many GM AAVs will fall into the lowest GM activity class. However,

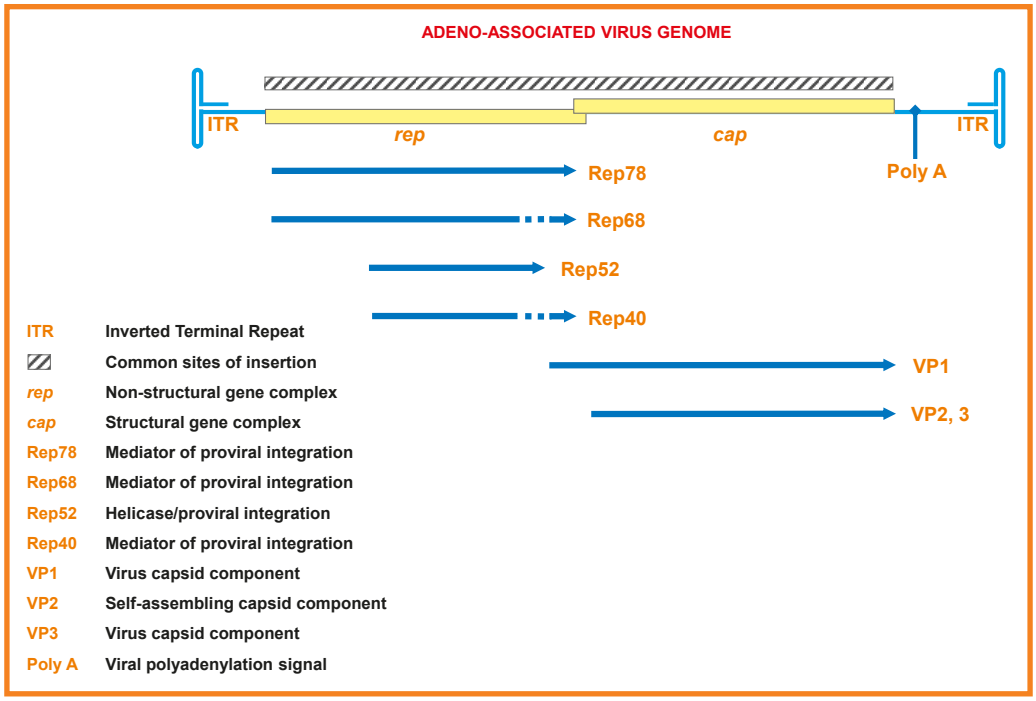


***Figure 3**. Transcription of the adeno-associated virus genome*

hazards arising subsequently due to the insertion of sequences or phenotypic alterations might necessitate additional containment measures. Some activities involving wild type helper viruses (*e.g.* adenoviruses) may need to take place at BSL2. Provided the helper virus is not itself genetically modified, this will not affect the activity classification for the AAV work.

iii. Most AAV-based vector systems to date are typically 'gutless' AAV-2 systems consisting of a plasmid containing the foreign DNA to be transduced into the cell flanked by AAV inverted terminal repeats (ITR) sequences. Cloned *rep* and *cap* genes as well as either wild type adenovirus or expression of Adenoviral genes required for AAV replication (i.e E1, E2A, E4Orf6 and VA RNA) supply helper functions. Clearly, where wild type adenoviruses are used to supply helper functions, the procedures must take place at BSL2 containment since adenoviruses are RG2 pathogens. Other systems have involved using recombinant HSV, Baculoviruses or Adenoviruses to express *rep* and *cap* genes. The hazards associated with such GM virus vectors should be assessed separately from the AAV vector that is the intentional end product. However, any additional hazards posed by the combination of the AAV vector and the helper virus should be considered. Further guidance on the risk assessment of GM Adenoviruses, Baculoviruses and Herpesviruses can be found in Chapter 8, Chapter 9 and Chapter 10 respectively.

iv. A potentially harmful biological agent should be substituted with an agent that is less hazardous or be eliminated entirely, if possible. Therefore, safer helper-virus-free systems should be employed wherever practicable. Alternatively, the hazards associated with different helper viruses should be carefully assessed and the system deemed the safest employed. For example, a baculovirus would arguably pose a lower risk to human health than HSV and, if feasible, should be used in preference.

7.2.2 Hazards associated with genetic inserts

Given the low pathogenicity of the recipient virus, the major hazards that will be posed by recombinant AAV vectors will depend upon the properties of the inserted genetic material and any products that it may encode. The risk assessment should take into consideration any potential adverse effects of the expressed product and the properties associated with non-coding sequences. Guidance on the hazards associated with commonly-used genetic inserts can be found in Chapter 3. In brief, factors to consider include the following:

7.2.2.1 Biological properties of the gene product

The expected activities or toxicity of the products encoded by the gene should be assessed. For example, a bacterial toxin, oncogene or growth factor would represent greater risk than a reporter gene such as EGFP or Luciferase. Properties of the gene products with respect to individual cell types affected should therefore be considered.

7.2.2.2 Expression characteristics

This will be dependent on the cell type and the regulatory sequences used to control expression. For example, use of the Human cytomegalovirus Major Immediate-Early enhancer would be expected to direct high-level expression in a broad range of cell types. Tissue-specific promoters generally lead to cell-type restricted expression. However, they may exhibit 'basal leakiness' whereby low-level expression is observed in non-permissive cells. Promoter characteristics should be thoroughly assessed where possible using harmless reporter genes and low-risk virus-free cell culture systems before a GM AAV vector is constructed.

7.2.2.3 Proviral insertion

i. Wild type AAV-2 integrates into the host cell chromosome at a defined locus. The multiplicity of infection (MOI) affects the rate of integration (5 to 40%) but does not affect the overall rate of infection (which stabilises at 80% above MOI=10). The 'gutless' nature of most AAV vectors means that integration into the AAVS1 locus on human chromosome 19 will not occur, due to the lack of *rep* sequences in the vector backbone needed to target the genome to this locus. Long-term expression of genes transduced

using AAV vectors is seen and this is thought to be mainly due to the maintenance of episomal genomes, which are double-stranded DNA and often concatameric. Non-targeted proviral insertion is seen with AAV vectors. However, unlike the insertion events peculiar to retroviral life cycles, insertion of gutless AAV vector genomes is a passive mechanism that occurs at naturally occurring chromosomal breakpoints.

Approximately 10% of all double stranded genomes are thought to integrate into host chromosomes in this way, and appear to passively target regions of transcriptionally active chromatin.

ii. Insertional mutagenesis has never actually been observed when using an AAV vector system, which includes numerous studies in human clinical trials. However the possibility exists for a mutagenic event and the effects of such integration should be considered. For example, heterologous promoters might activate genes adjacent to the integration site. While no transforming properties have been attributed to AAV vectors, the risk assessment should carefully consider the possibility. This is particularly relevant to 'split gene' approaches with AAV vectors that utilise the natural propensity for AAV genomes to concatamerise, effectively doubling the packaging capacity. In these systems, the expression cassette is split between two recombinant AAV vectors, which concatamerise following transduction to reconstitute the expression cassette. Using this approach, it is likely that one of the AAV vectors will be carrying the promoter and necessary control sequences and it is proviral insertion of this section of the cassette that is more likely to result in insertional activation of cellular genes. Equally, proviral insertion could result in the disruption of a cellular gene.

iii. It is also possible to target a recombinant AAV genome to integrate at a particular site within the host cell genome using homologous recombination. To date, these approaches have proved inefficient. However, where user-targeted integration is sought, the sequence of the AAV genome should be carefully scrutinised and possible effects of the insertion, either at the targeted site or by passive integration, should be evaluated as fully as possible in the assessment.

7.2.3 Alteration of phenotype

i. The non-pathogenic nature of AAVs and the 'gutless' features of their derivative vectors imply that alterations to the pathogenic phenotype of the final vector are unlikely, aside from any hazards associated with the products encoded by the inserted expression cassette. However, it is possible to alter the cell tropism of AAV vectors using a variety of approaches and the effects of such modifications should be carefully considered.

ii. One approach is to pseudotype the vector by substituting the cap genes of the vector strain with those of an AAV serotype with the desired cellular tropism. Another is to modify the cap genes themselves by altering their inherent binding properties or inserting a motif that will interact with a cellular determinant present on the surface of the target cell. Bispecific antibodies or conjugated ligand molecules can also be used to coat the virus, targeting it for endocytic uptake by specific cell types.

iii. Therefore, it is important to consider the susceptibility of various tissues to infection and to evaluate the possible consequences of AAV transduction and expression of the genetic cargo within cell and tissue types that would not normally be infected by the recipient strain.

7.3 RISK ASSESSMENT FOR THE ENVIRONMENT

7.3.1 Survivability and stability

i. AAVs are non-enveloped DNA viruses that are relatively stable and resistant to dehydration. They could potentially survive for protracted periods in the environment. However, they are defective by nature and will be unable to establish an infection in the absence of helper virus or other helper functions. 'Gutless' vectors would also require the provision of *cap* and *rep* genes *in trans* in order to replicate and disseminate.

ii. Most AAV vectors have been derived from human viruses, which are not thought to be able to replicate or cause disease in any animal species. Therefore, it is unlikely that such activities will represent any significant risk to the environment. However, human AAVs are able to enter the cells of many animal species and there may be environmental risk associated with the inserted genetic material that will require assessment. Furthermore, AAVs associated with certain animal types have been identified and work with these viruses may necessitate a more detailed consideration of the potential environmental impact of an accidental release.

7.3.2 Hazards associated with the genetic insert

i. The biological properties of the expressed gene product, even if they represent a low risk to human health, may be a hazard to other species. Furthermore, promoters and control sequences may not show the same expression characteristics or tissue restrictions in other species. It is important to consider any potential adverse effects of the encoded products upon non-human species that may be affected.

7.3.3 Alteration of phenotypic and pathogenic traits

7.3.3.1 Human AAVs can transduce the cells of primate, canine, murine and avian origin, although replication is not possible except in the presence of helper virus or other helper function as supplied by conditions of cellular stress.

7.3.3.2 Modifications that affect the host range of the virus, for example pseudotyping a recombinant AAV with the cap genes of another AAV serotype or modifying the inherent properties of the products encoded by the cap genes themselves, might result in a GM virus capable of transducing the cells of organisms that would not normally be affected. In that event, the expression characteristics and properties of the products encoded by the inserted expression cassette might differ from the effects predicted for human cells, and the possible consequences of such an eventuality should be considered.

7.4 PROCEDURES AND CONTROL MEASURES

i. Most recombinant AAV vectors will be considered low risk genetic modification activity and can be handled in a GM-BSL1 containment laboratory. However, it is important to consider that AAVs are infectious via the airborne route and therefore measures might be required to control aerosol generation and airborne dissemination. Most work with AAVs should be performed within a biological safety cabinet, not only to protect the purity of the culture but also to control aerosol dissemination. Where the risk assessment shows that exposure to airborne GM AAV represents a hazard, these activities should be assigned to GM-BSL2.

ii. The generation of infectious AAV particles may require the use of viable helper viruses. It is likely that these helper viruses will pose a greater risk of harm than the AAV vector that is the focus of the work. It is therefore important to ensure that containment measures appropriate to control the risks posed by the helper viruses are implemented.

iii. Recombinant AAVs are often purified by ultracentrifugation over caesium chloride gradients. Appropriate care should be taken to ensure that centrifugation vessels are properly sealed. High-titre, concentrated virus may require extraction from gradients using hollow needles. Needles should be used with extreme care, only used when absolutely necessary and should never be resheathed, but disposed of directly into a suitable sharps waste container.

CHAPTER

8

ADENOVIRUSES

8.1 BACKGROUND

i. Adenoviruses are ubiquitous pathogens of both mammals and birds. Over 100 serotypes are known, 51 of which infect humans. The following guidance will focus on the use of human adenoviruses. However, many of the principles will also apply to work involving the adenoviruses that infect animals. The severity of these infections varies from acute respiratory disease (ARD) in adults (Ad4; Ad7) to mild respiratory symptoms in children (Ad2; Ad5), gastroenteritis (Ad40; Ad41), conjunctivitis (Ad8; Ad19; Ad37), cystitis or subclinical infection (Ad12). Certain serotypes have also been shown to be tumourigenic in neonatal rats (Ad12; Ad7), although this has never been observed in humans. Primary infection generally occurs in childhood via the airborne or faecal-oral routes and can be persistent with viral shedding continuing for months. Latent infection of lymphoid tissue can also occur and reactivation in the immunocompromised can lead to serious complications. However, the precise mechanism of latency remains unknown. Immunity is thought to be lifelong and over 90% of individuals are seropositive for Ad2 and Ad5.

ii. The adenovirus virion comprises a non-enveloped icosahedral capsid containing a 36 kb double-stranded DNA genome (see Figure 4). Adenoviruses can infect a broad variety of cell types (including non-dividing cells) via interaction between the viral fibre protein and the cellular Coxsackie B Adenovirus Receptor (CAR) - a widely expressed, 46 kDa member of the immunoglobulin superfamily. Following virus adsorption, RGD (Arg-Gly-Asp) motifs on the penton base interact with cell surface αv integrins, stimulating an intracellular signalling cascade and clathrin-mediated endocytosis. Not all serotypes share the same affinity for CAR and some utilise alternate receptors and cell-surface integrins. Adenoviruses replicate in the nucleus.

iii. Viral gene expression is divided into two distinct phases – *Early* and *Late* transcription. Early transcription occurs 6 to 8 hours after

infection, generating early proteins from four major regions, E1, E2, E3 and E4 (see Figure 4). The E1 promoter directs expression of the E1 proteins, E1A and E1B that subvert the cellular environment and control transcription of the other early genes. E1A disrupts cell-cycle regulation by binding to key regulators of transcription and mitosis. This results in the expression of the pro-apoptotic factors, including p53, which is bound and inactivated by an E1B protein. E2 proteins are required for genome replication and packaging. E3 proteins aid the evasion of the immune system by disrupting the processing of class 1 Major Histocompatibility Complexes (MHC) and inhibition of Fas- and Tumour Necrosis factor (TNF) mediated apoptosis. One E3 protein, the so-called Adenovirus Death Protein (ADP), promotes cytolysis and release of progeny virions. E4 proteins further subvert the cellular environment and modulate the activities of E1 proteins. Late transcription, directed by the *Major Late Promoter*, occurs 4 to 6 hours after the onset of early transcription and results in the expression of the structural proteins L1, L2, L3, L4 and L5. The lytic cycle lasts for 24 to 48 hours (depending on subtype and target cell) generating up to $1x10^5$ viral particles per infected cell.

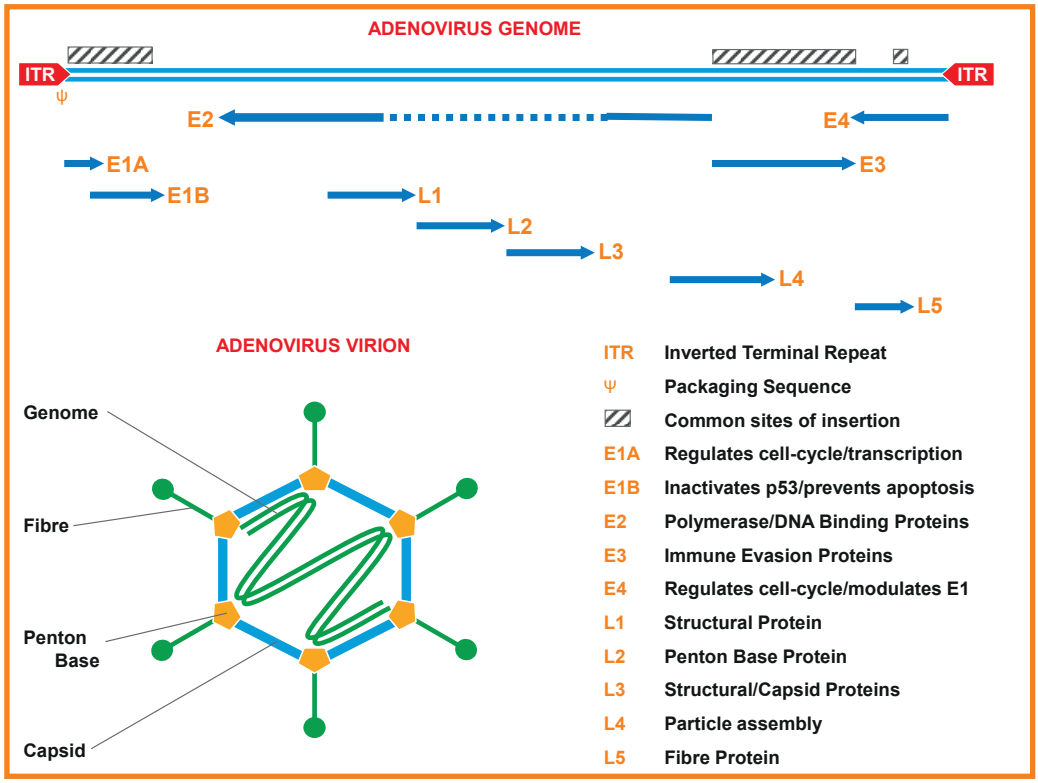


***Figure 4**. Transcription of the adenoviral genome and structure of the adenovirus particle*

8.2 RISK ASSESSMENT FOR HUMAN HEALTH

8.2.1 Hazards associated with the recipient virus

i. Wild type human adenoviruses are RG2 pathogens. Therefore, BSL2 containment should be adopted as a minimum requirement when handling wild type virus. To date, most genetic modification work involving adenoviruses has involved the development of transduction vectors derived from human Ad2, Ad5 and Ad12, although other serotypes are used. While it is important to consider the hazards posed by the virus from which these vector systems are derived, many recipient virus strains will be defective or attenuated and will represent a much reduced risk of harm compared to wild type virus.

8.2.2 Vector systems

8.2.2.1 Disabled vectors

i. 'First Generation' vectors comprise the majority of adenovirus vectors used to date and harbour a genomic deletion that removes the E1 expression cassette.

   E1A and E1B are usually supplied *in trans* using a complementing cell line that contains the E1 expression cassette (such as HEK293 or PerC6). Packaging sequences are retained in order to generate viable progeny. Since adenoviruses have a strict packaging limit (105% of the wild type genome size), the E3 cassette is also commonly deleted since it is dispensable for growth *in vitro*. 'Second Generation' vectors also have much of the E2 cassette deleted, increasing its packaging capacity and further disabling the virus by removing its capability to replicate and process viral DNA. This deletion also virtually eliminates the possibility of a recombination event that might result in Replication Competent Viruses (RCV). 'Third Generation', or 'Gutless' vectors generally retain only packaging sequences and therefore have the largest capacity for inserted genetic material. These vectors require extensive complementation *in trans* from a helper virus and therefore risks associated with the helper must be considered in detail.

8.2.2.2 Replicative vectors

i. Conditionally Replicating Viruses (CRV) are capable of undergoing the full viral lytic cycle, albeit in a restricted fashion. For example, E1B-deleted vectors were proposed only to replicate in cells that do not express p53 or have a disrupted p53 pathway (which encompasses most malignant cell types). Alternatively, the E1A promoter can be replaced by a tissue-specific or inducible promoter, rendering the virus replicative only in a targeted cell type or in response to known stimuli.

ii. The hazards associated with the handling of high titres of replicative virus should be carefully considered. Conditionally Replicating Viruses (CRV) while attenuated, still pose a risk to human health in that lytic infection may occur at unforeseen sites, and recombination resulting in a RCV or wild type virus is a possibility. GM-BSL2 containment level should be adopted as a minimum requirement for these vectors unless the risk assessment or safety data show this to be unwarranted.

8.2.3 Hazards associated with genetic inserts

The risk assessment should take into consideration the potential effects of the expressed product. Guidance on the hazards posed by commonly used genetic inserts can be found in Chapter 3. In brief, factors to consider include the following:

8.2.3.1 Expression characteristics

i. This will be dependent on the cell type and the regulatory sequences used to control expression. For example, use of the Human cytomegalovirus Major Immediate-Early enhancer would be expected to direct high-level expression in a broad range of cell types. Tissue-specific promoters generally lead to cell-type restricted expression, although they often exhibit 'basal leakiness', whereby low-level expression is observed in non-permissive cells. It should also be considered that remnants of the adenovirus E1 promoter (which overlaps with vital viral packaging sequences) might overcome the restriction imposed on genes cloned into the E1 region of the virus. It is advised that promoter characteristics are thoroughly assessed where possible using non-hazardous reporter genes in low-risk virus-free cell culture systems before generating a GM virus.

ii. In most transduced tissues, expression from Ad vectors is transient due to clearance of the virus by the immune system, and lasts only one to two weeks. In some 'immune privileged' tissues, expression may be longer, persisting for a year or more.

8.2.3.2 Integration into host DNA

i. Integration into the host genome represents the only significant mechanism by which long-term expression can be maintained by disabled Ad vectors. This is relatively rare, occurring at a frequency of approximately 1 in 10^5 pfu in human primary cell cultures. The effects of integration in relation to the properties of the insert should be considered.

8.2.4 Biological properties of the gene product

i. The expected activities or toxicity of the gene products should be assessed. For example, a bacterial toxin or growth factor would

represent greater risk of harm than a reporter gene such as EGFP or Luciferase. Properties of the gene products with respect to individual cell types should also be considered.

8.2.5 Alteration of phenotype

i. **Tissue tropism**

Adenoviruses can infect a wide variety of cell types, although individual serotypes have more restricted tropisms. It is often desirable to restrict or retarget a vector and modification or substitution of the viral fibre/penton base genes with those from another serotype can alter tissue tropism. Other methods for retargeting adenoviruses, such as the use of bivalent antibody conjugates, can also retarget the vector. The susceptibility of additional tissues to infection should therefore be considered.

ii. **Immunogenicity and pathogenicity**

Deletions in the viral vector or the genetic insert may alter the immunogenic or pathogenic nature of the virus. For example, proteins derived from the E3 cassette (which is often deleted in adenoviral vectors) are involved in immune evasion strategies *in vivo*. Their deletion, while facilitating the clearance of virus by the host immune system, might result in an increased inflammatory response and increased pathogenicity. Likewise, insertion of immunomodulatory cytokines may have a similar effect.

8.2.6 Recombination

The possibility of recombination that might result in harmful sequences being transferred between related viruses should be considered. This could take place between a vector and a wild type adenovirus or viral sequences present in a cell; for example it has been shown that 20% of normal healthy adults have E1A sequences present in their respiratory epithelium. It is common practice to locate an insert in place of the E1 cassette. Thus, any homologous recombination that restores E1 sequences to the vector will also delete the insert and *vice-versa*. Inserts cloned into other areas of the viral genome could be maintained in the event that E1 sequences are restored, resulting in a GM RCV.

8.2.7 Complementation

The probability of acquisition of sequences from a complementing cell line or helper virus can be minimised if there are no overlapping sequences. For example, HEK293 cells carry 11% of the adenovirus genome containing the E1 cassette; this includes at least 800 bp of sequence present within most E1-deleted adenovirus vectors, providing the potential for recombination that restores the E1 region in the virus. In contrast, PerC6 and similar cell lines have been engineered to express the minimal E1A and E1B genes from

heterologous promoters, and thus have no sequence overlap with most newer E1-deleted vectors, greatly reducing the frequency of generating replication-competent virus.

8.3 RISK ASSESSMENT FOR THE ENVIRONMENT

8.3.1 Survivability and stability

i. Adenoviruses are non-enveloped DNA viruses, relatively stable and resistant to dehydration. Viruses can survive for protracted periods in aerosols and water. Any modifications to the virion that may affect the stability of the virus should be assessed for increased risk to the environment.

ii. Most adenovirus vectors have been derived from human viruses, which are not thought to be able to replicate efficiently in animal cells. Therefore, it is unlikely that activities with these vectors will represent any significant risk to the environment. However, human Ad5 vectors have been shown to enter some animal cells and there may be environmental risk associated with the inserted genetic material that will require assessment. Furthermore, work with animal adenoviruses may necessitate a more detailed consideration of the potential environmental impact of an accidental release.

8.3.2 Hazards posed by the genetic inserts

The biological properties of the expressed gene product, even if they represent a low risk to human health, may be a possible hazard to other species. Furthermore, promoters and control sequences may not show the same expression characteristics or tissue restrictions in other species. It is important to consider any potential adverse effects of the encoded products upon non-human species that may be affected.

8.3.3 Alteration of phenotypic and pathogenic traits

Human Ad5 vectors have been shown to enter (but not replicate efficiently in) cells of mouse, rat and canine origin. This raises the question of whether or not recombination between human and animal adenoviruses might occur, although there is no evidence to suggest that this is possible. Furthermore, modifications that affect the tissue-tropism of the virus or the use of fibre/penton base proteins from other serotypes might result in a GM virus capable of infecting other organisms. In that event, gene products that modulate cell death or the immune system may not function and the pathogenicity of the GM virus in other organisms might, therefore, be greater than in humans.

8.4 PROCEDURES AND CONTROL MEASURES

i. GM adenovirus vectors are generally constructed by molecular

cloning of two overlapping plasmids containing distinct regions of the viral genome. These plasmids are either ligated together prior to transfection or are cotransfected into a complementing cell line whereby viable GM viral genomes are generated by homologous recombination. Other systems require the use of helper viruses. The hazards associated with these should be considered in addition to those associated with the proposed GM virus.

ii. Manipulation of the adenoviral genome is now possible in virus-free systems. Ad genomes have been cloned as Bacterial Artificial Chromosomes and can be manipulated in low-risk bacterial systems prior to the generation of recombinant virus from purified viral DNA. Such systems reduce the risk posed by handling the virus. The use of such systems wherever possible is therefore advised.

iii. Adenoviruses are often purified by ultracentrifugation on caesium chloride gradients. Appropriate care should be taken to ensure that centrifugation vessels are properly sealed. High-titre, concentrated virus is often extracted from gradients using a hollow needle. Needles should be used with extreme care and only used when necessary. Needles should never be resheathed but disposed of directly into a suitable sharps waste container.

iv. A means of monitoring for the presence of RCV in disabled virus stocks should be in place, where appropriate. Permissive, non-complementing cell lines should show signs of productive infection (cytopathic effect, plaque formation) in the presence of RCV and they could be used to test stocks of a disabled GM virus. However, such assays may not be completely reliable as disabled viruses are often cytopathic. The use of molecular detection methods (*e.g.* quantitation of E1 sequences in a purified virus preparation using quantitative PCR) would represent a more reliable method of RCV detection.

v. Adenovirus vector strains that can be shown to pose a much reduced risk of harm compared to the wild type virus might be handled at BSL1 containment. The risk assessment must demonstrate that the recipient is disabled or sufficiently attenuated. Furthermore, the likelihood of a reversion event must be low and the stock should be demonstrably free of any replicative virus. However, hazards arising subsequently due to the insertion of sequences or phenotypic alterations might necessitate additional containment measures.

vi. It is an organisation's responsibility to ensure that a staff health or immune status is sufficient for the activity in question. A system for the monitoring of health and immune status should therefore be implemented where the nature of the work demands it. The health status of staff exposed to the GM viruses should be monitored. For example, those showing signs of a compromised immune system should review their suitability for work.

BACULOVIRUSES

CHAPTER 9

9.1 BACKGROUND

i. Baculoviruses are a diverse group of insect viruses that have been implicated in causing disease in over 500 different insect species and have been exploited for pest control purposes as well as protein production in insect cells. They are divided into two genera, the nucleopolyhedrosis (NPV) viruses and the granulosis viruses. Individual baculovirus species generally have a very narrow host range, for example *Bombyx mori* nucleopolyhedrosis virus (BmNPV) infects only the mulberry silkworm, the larval form of the moth *Bombyx mori*. Conversely, the *Autographa californica* multi-nucleopolyhedrosis virus (AcMNPV), which has been most extensively studied, can infect the larvae of over 30 different lepitdopteran (butterfly/moth) species, and not just the alfalfa looper, *Autographa californica*, from which it was originally isolated. The virus is lethal to its natural host by literally causing its liquefaction and consequently can be disseminated by aerosol. For the purposes of this Guideline, reference will be made predominantly to AcMNPV, which is the prototypical baculovirus that has been most extensively exploited for biotechnology and research purposes. However, many of the features and aspects covered may also be applicable to other Baculoviruses.

ii. The baculovirus virion consists of a rod-shaped protein capsid, surrounded by a host-cell derived membrane that encases a 134 kb circular double-stranded DNA genome containing over 150 open-reading frames (see Figure 5).

iii. Baculoviruses enter insect cells via receptor-mediated endocytosis, although the cellular factors involved are not known. The viral determinant that mediates cellular attachment and entry is the viral surface glycoprotein gp64. Following entry and uncoating, viral gene expression proceeds in a cascade fashion with early, late and very late kinetics. The majority of transcriptional activity during AcMNPV replication appears to take place from the promoters of the late genes *p10* and *polyhedrin*. This has led to

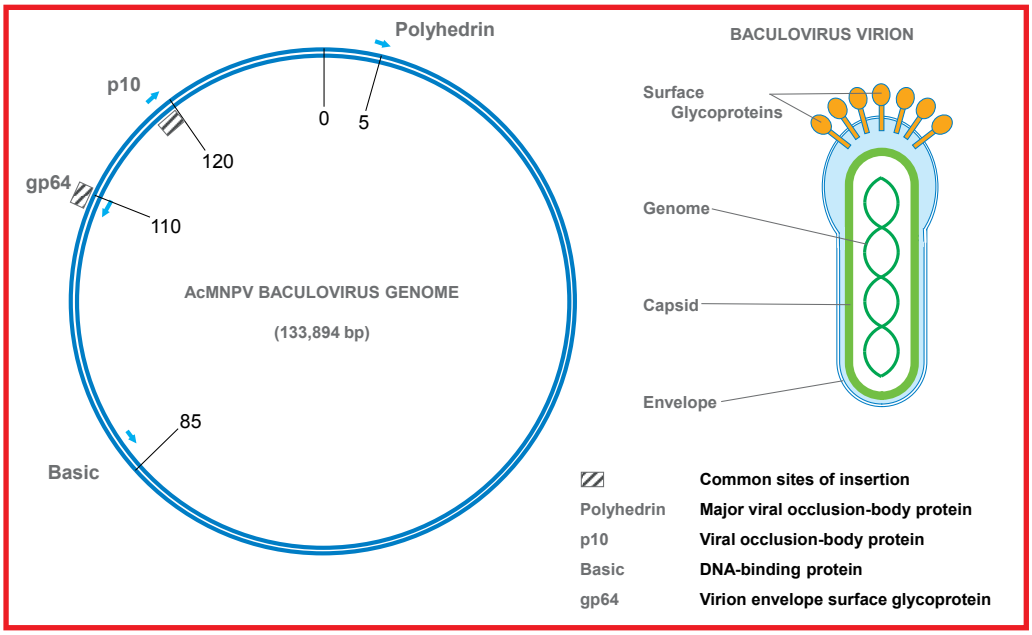


Figure 5. *Representation of baculoviral genome and structure of a baculovirus particle*

these promoters being exploited to direct the expression of foreign genes and recombinant protein production from insect cells.

iv. AcMNPV can infect and replicate effectively in various insect cell lines, notably Sf9 and Sf21 cells that are derived from *Spodoptera fumigans*. Recently, it has also been shown that it can effectively transduce, but not replicate in, a variety of mammalian cells. Transduction of mammalian cells appears to be a general phenomenon, possibly involving common or ubiquitously expressed determinants. While expression of viral genes does not appear to take place, gene expression can be driven by promoter/enhancers that are normally functional in mammalian cells (*e.g.* the Human cytomegalovirus Major Immediate-Early enhancer and the Rous Sarcoma Virus Long Terminal Repeat). Furthermore, AcMNPV appears to be able to transduce both dividing and non-dividing cells and this has resulted in considerable interest in AcMNPVas a potential gene-delivery vector for therapeutic purposes.

9.2 RISK ASSESSMENT FOR HUMAN HEALTH

9.2.1 Hazards associated with the recipient virus

i. To date, most genetic modification work involving baculoviruses has involved the development of gene delivery vectors based upon AcMNPV for the purposes of gene expression from insect cells. Clearly, since baculoviruses are pathogens of insects, the major

hazards posed will be to the natural host in the environment, and measures should be taken to prevent release if susceptible species are present. However, although the original virus was pathogenic for certain lepidoptera, the most commonly used expression systems are based upon strains deleted for the *polyhedron* gene rendering the virus sensitive to insect larval gut conditions and to environmental factors.

ii. The risks to human health posed by baculoviruses are therefore low. However, the ability of AcMNPV to enter mammalian cells and express foreign genes from heterologous promoters means that some risk may arise by virtue of the properties of the genetic insert. Furthermore, although baculoviruses are inactivated rapidly by complement, they have been shown to trigger innate inflammatory responses in mammalian systems. Therefore, inflammation might be a feature of accidental exposure.

iii. Most activities with baculoviruses will be low risk and fall into the lowest class of GM activity. However, hazards arising subsequently due to the insertion of sequences or phenotypic alterations might necessitate additional containment measures.

9.2.2 Hazards associated with genetic inserts

The major hazards that will be posed by baculovirus vectors will arise from the properties of the inserted genetic material and any products that it may encode. AcMNPV can enter a broad range of mammalian cell types and, since they are not inherently cytopathic, the length of time for which they persist and expression of inserted genes may be prolonged. The risk assessment should take into consideration any potential adverse effects of the expressed product or properties inherent to the sequence on human cells, organs or health. Guidance on the hazards posed by commonly used genetic inserts can be found in Chapter 3. In brief, factors to consider include the following:

9.2.2.1 Expression characteristics

i. This will be dependent on the cell type and the regulatory sequences used to control expression. In many cases, the purpose of the GM baculovirus will be for expression of genes in insect cells for protein production purposes. In these cases, insect-cell specific elements, or the baculovirus *p10* or *polyhedrin* promoters, often direct expression. Since these are only functional in insect cells, the nature of the expressed product is not likely to become an issue, despite the ability of baculovirus to transduce a variety of mammalian cells.

ii. The use of heterologous promoters that are functional in mammalian cells may require more careful assessment. For example, use of the Human Cytomegalovirus Major Immediate-

Early enhancer or the Rous Sarcoma Virus LTR would be expected to direct high-level expression in a broad range of mammalian cell types. Tissue-specific promoters generally lead to cell-type restricted expression but they may exhibit 'basal leakiness' whereby low-level expression is observed in non-permissive cells. It is advised that promoter characteristics are thoroughly assessed where possible using harmless reporter genes and low-risk virus-free cell culture systems before a baculoviral transduction vector is constructed. Unless expression in mammalian cells is specifically required, a promoter that is not active in mammalian cells should be used.

9.2.2.2 Biological properties of the gene product

i. The expected activities or toxicity of the gene products should be assessed. For example, a bacterial toxin, oncogene or growth factor would represent greater risk of harm than a reporter gene such as EGFP or Luciferase.

ii. Baculoviruses have been used to transduce heterologous viral genes into cells in order to study their individual functions and also to supply helper functions to other defective vector systems (*e.g.* AAV vectors). The properties of the gene products with respect to individual cell types affected should therefore be considered.

9.2.2.3 Hybrid vector systems

i. Hybrid baculoviruses carrying the genomes of heterologous mammalian viruses have been used to launch productive infections or study viral mechanisms (*e.g.* Hepatitis C Virus and poliovirus). It is important when using such a system that, where viable virus is generated from the inserted genomes, the hazards associated with those viruses are considered in addition to those of the baculovirus vector itself. Containment measures that are appropriate to the virus generated should therefore be selected.

9.2.2.4 Alteration of phenotype

i. The fact that baculoviruses are not human pathogens suggests that alterations to the pathogenic phenotype of the final vector are unlikely, other than any detrimental effects that may arise from the products encoded by the inserted expression cassette. AcMNPV can apparently naturally transduce a broad range of mammalian cell types, including human cells. It is possible, however, to alter the specific tropism of any baculovirus and therefore the effects of such modifications should be carefully considered.

ii. It is possible to pseudotype baculoviral vectors with a heterologous viral surface glycoprotein, for example VSV-G. The full potential of a pseudotyped baculovirus of this kind remains unknown, although it has been shown to increase transduction efficiencies

in certain cell types and also makes the vector more resistant to inactivation by complementation than its non-pseudotyped counterpart. Another approach is to modify the baculovirus gp64 gene by fusing a binding motif that will interact with a cellular determinant present on the surface of a target cell in order to increase transduction efficiencies into that cell type. It is important, therefore, to consider the susceptibility of various tissues to infection and to evaluate the possible consequences of baculovirus transduction and expression of the genetic cargo within cell and tissue types that would normally be infected by the recipient virus.

9.3 RISK ASSESSMENT FOR THE ENVIRONMENT

9.3.1 Survivability and stability

i. Although wild type baculoviruses could infect and pose a potential hazard to lepidopteran species in the environment, most baculoviral vector systems are attenuated by virtue of deletions in the *polyhedrin*, *basic* or *p10* genes. While these deletions permit baculoviruses to replicate efficiently in insect cell culture, it renders them incapable of establishing a productive infection in the host organism. Vector systems such as these are inherently very safe and will require minimal containment. However, work involving wild type, or less attenuated, viruses may require assignment to a higher GM activity class in order to prevent release.

ii. Baculoviruses themselves are quite stable and can survive in the environment for prolonged periods. *Polyhedrin*-negative baculoviruses are more susceptible to desiccation and UV light and have a much-reduced survival time. It is important to assess any modification that might increase the stability of the virus. For example, viruses pseudotyped with the VSV-G glycoprotein may be more stable than those incorporating the native glycoprotein.

9.3.2 Hazards associated with genetic inserts

In many cases, insect-cell specific elements, or the baculovirus *p10* or *polyhedron* promoters, often direct expression. Since these are functional in insect cells, the biological properties of the expressed gene product, even if they represent a low risk to human health, may be a possible hazard to the natural host of the baculovirus if the vector is not suitably attenuated. Where possible, attenuated baculovirus strains should be used. Furthermore, where possible, the insert should be located at the site of an attenuating or disabling mutation so that any reversion event will result in the deletion of the insert.

Heterologous promoters and control sequences may not show the same expression characteristics or tissue restrictions in other species. It is important to consider any potential adverse effects of the encoded products upon any non-human species that may be affected.

9.3.3 Alteration of phenotypic and pathogenic traits

AcMNPV can naturally transduce a wide range of mammalian cells, however replication does not take place. Modifications that affect the host range of any baculovirus (*e.g.* pseudotyping) might result in a GMM capable of transducing the cells of species that would not normally be affected. In that event, the properties of the expression characteristics and properties of the products encoded by the inserted expression cassette might differ from the effects predicted for human cells, and the possible consequences of such an eventuality should be considered.

Consideration should also be given to any work involving the genetic modification of wild type baculoviruses, which may alter the pathogenic or phenotypic traits with respect to the infection of the natural host. The possible consequences upon the natural population of the target organism of an inadvertent release of a virus with altered characteristics should be carefully assessed.

9.4 PROCEDURES AND CONTROL MEASURES

The low-risk nature of most baculoviral systems to both human health and the environment indicates that minimal containment measures will be required. Therefore, most baculovirus work will be classified as RG1 and can be handled at GM-BSL1, and viral preparations could be handled on the open bench. Most work with baculoviruses should take place within a biological safety cabinet. It is acknowledged that this is to protect the purity of the culture and to control aerosol dissemination. The use of a cabinet for these purposes will not necessitate the assignment of the work to GM activity class 2.

However, it is important to consider that baculoviruses may spread via the airborne route and measures might be required to control aerosol dissemination, especially if the virus is not attenuated or it is carrying a potentially harmful insert. If the risk assessment shows that exposure to airborne GM baculovirus represents a hazard, the use of a biological safety cabinet shall be required as a control measure. These activities should be assigned to RG2 and take place at GM-BSL2 containment level.

HERPESVIRUSES

CHAPTER 10

10.1 BACKGROUND

i. Herpesviruses are a diverse family of viruses found in humans and most species of animals. More than 130 species have been identified so far, nine of which are known to infect humans. Although herpesviruses are highly disseminated in nature, individual species appear to be very host-specific. Herpesviruses are classified into three subfamilies, based upon their biological properties and genomic sequence. These are the *Alpha-*, *Beta-* and *Gammaherpesviruses*. Human herpes viruses are also classified as HHV 1 to HHV-8. (see Table 7).

ii. Many features are shared between herpesvirus species; they are morphologically similar, with virions consisting of an icosahedral capsid, which is further surrounded by a proteinaceous *tegument* and bounded by an envelope (the *Alphaherpesvirus*, Herpes simplex is represented in Figure 6). Following entry into the target cell, the linear double-stranded DNA genome circularises and is transported to the nucleus, where replication takes place. Lytic genes are expressed in an ordered cascade, beginning with Immediate-early (IE) followed by Early (E) and Late (L) gene expression. The expression of E genes (which largely encode proteins involved in genome replication, immune evasion and cell process subversion) and L genes (mostly encoding structural components of the virion) are dependent upon the expression of the IE genes. Lytic replication is usually highly cytotoxic and results in the destruction of the target cell. It is this cytotoxicity that is thought to be central to disease causation in most herpesviruses.

iii. All herpesviruses studied so far also have the ability to persist within the host in a latent state. During latency, the majority of viral genes are silenced and small subsets of latent genes are expressed. For example, latency in *Alphaherpesviruses* is ultimately established in the sensory neural ganglia associated with infected peripheral nerves. Latent herpesvirus infection is usually lifelong and incurable and reactivation of the virus is associated with

Table 7. Herpesvirus classification, associated terminology and typical symptoms and diseases associated with herpesvirus infections.

Classification of Herpesviruses	Risk Group (RG)	Disease Association
Alphaherpesviruses		
Herpes simplex virus 1 (HSV-1; HHV-1)	RG2	Oral herpes; genital herpes; encephalitis
Herpes simplex virus 2 (HSV-2; HHV-2)	RG2	Genital herpes; oral herpes; encephalitis
Varicella zoster virus (VZV; HHV-3)	RG2	Chickenpox (Varicella) ; Shingles (Herpes zoster)
Herpesvirus simiae (B virus; CeHV-1)	RG4	Paralysis; death in humans (macaques natural host)
Betaherpesviruses		
Human cytomegalovirus (HCMV; HHV-5)	RG2	Congenital defects; morbidity in immunosupressed
Human herpesvirus 6A (HHV-6A)	RG2	No identified disease association
Human herpesvirus 6B (HHV-6B)	RG2	Exanthum subitum; morbidity in immunosupressed
Human herpesvirus 7 (HHV-7)	RG2	Exanthum subitum; pityriasis rosea
Murine cytomegalovirus (MCMV)	RG1	Mouse model for HCMV infection and disease
Gammaherpesviruses		
Epstein-Barr virus (EBV; HHV-4)	RG2	Proliferative disorders, various malignacies
Kaposi's sarcoma-associated herpesvirus (KSHV; HHV-8)	RG2	Endothelial and B-lymphocytic proliferative disorders
Murine Gammaherpesvirus 68 (MHV68; γMV68)	RG1	Pathogen of wild rodents; model for EBV; HHV8

HHV – human herpesvirus; CeHV – Cercopithecine herpesvirus

subsequent recurrence of symptoms. In the case of the Epstein-Barr virus (EBV), it is the latent immortalisation of infected B-lymphocytes that is the predominant replication cycle and is central to the lympho-proliferative disorders that are associated with infection and reactivation. The individual biological properties and the site of latency vary between species and thus the range of diseases caused by these viruses is therefore broad. Furthermore, although many herpesvirus genes are relatively conserved (either sequentially or functionally), the arrangement of viral genomes also varies along with the precise genetic complement.

iv. The majority of herpesviruses studied to date have been those that infect humans, or animal herpesviruses that share sufficient biological properties with a human equivalent and could constitute a model for human disease. For example, murine *Gammaherpesvirus* 68 and murine Cytomegalovirus are mouse models for Epstein-Barr virus and Human Cytomegalovirus (HCV) infection respectively. Genetic modification work has been carried out on most of these viruses (primarily for the purposes of virological research) and they are all handled in essentially the same way for such work. Herpes simplex virus (HSV) has been more extensively studied and has been developed as a gene-delivery vector system. For this reason, the majority of the following guidance concerns GM work involving HSV and HSV vectorology, although many of the principles outlined for HSV will also be applicable to other herpesviruses.

v. Herpes simplex virus is the prototypical *Alphaherpesvirus* and there are two subtypes; HSV-1 and HSV-2 predominantly cause oral or genital epithelial lesions respectively, although there is causal overlap. In rare cases, the virus enters the central nervous system (CNS) resulting in encephalitis. HSV-1 and HSV-2 are widespread human pathogens that persist latently within sensory ganglia, periodically reactivating as a productive infection with or without symptoms. Primary infection normally occurs in early life via direct contact, the resulting latent infection is life-long and incurable. HSV-1 is more prevalent than HSV-2; it is estimated that around 40% of the population are seropositive for HSV-1 worldwide, although locally it could approach 100%.

vi. HSV gains entry into cells via interaction between viral glycoproteins present in the virion envelope and widely expressed cell surface determinants such as Heparan Sulphate and Nectins. HSV is therefore able to enter a broad range of cell-types, although productive infection is more restricted presumably due to dependence on particular cellular traits. During primary infection this is usually limited to the epithelial cells and the sensory neurons innervating the site. Latency is ultimately established in

the sensory neural ganglia associated with the infected peripheral nerves and following reactivation, virions are transported along the sensory neurons where lytic infection is initiated, often associated with characteristic lesions in the epithelia.

vii. The 150 kb HSV genome encodes approximately 80 proteins, approximately half of which are essential for the lytic cycle. The virion tegument protein VP16, in association with cellular factors, initially activates transcription of five Immediate Early (IE) genes encoding *Infected Cell Polypeptide* ICP4, ICP27, ICP0 (which are indispensable for growth), ICP22 and ICP47. These factors (with the exception of ICP47) direct the expression of the E and L genes. During latency, seemingly all viral gene expression is silenced, with the exception of *Latency Associated Transcripts* (LATs), a family of viral RNAs expressed from *Latency Associated Promoters* LAP1 and LAP2 (see Figure 6).

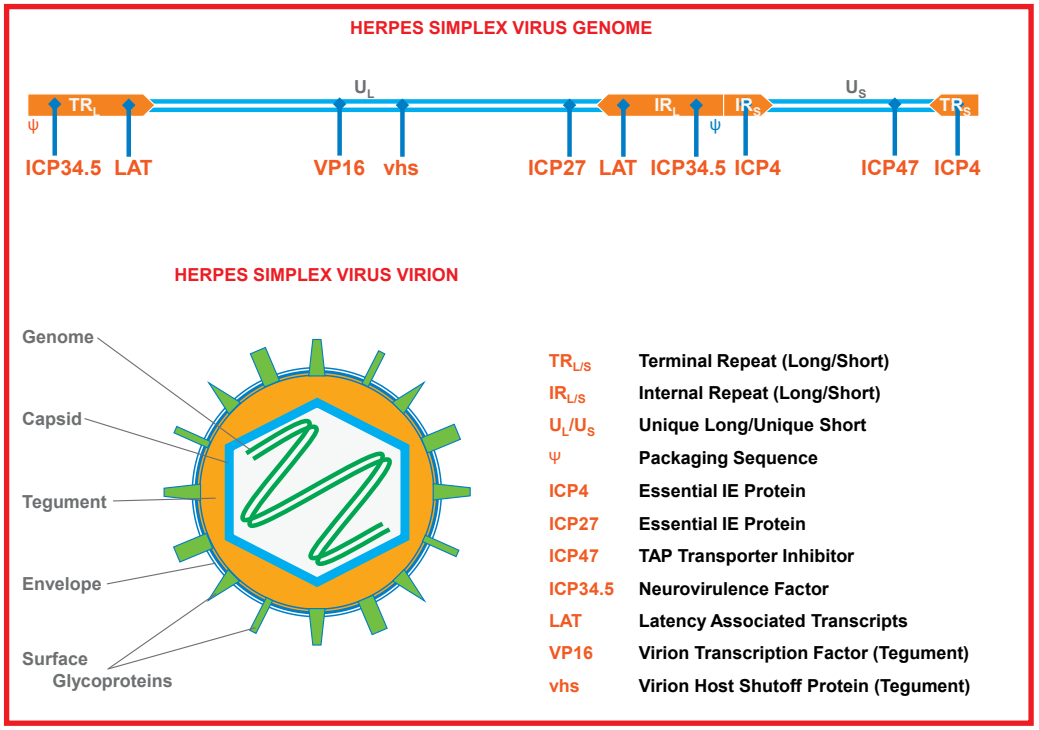


Figure 6. *Representation of the HSV genome and structure of a typical herpesvirus virion*

10.2 RISK ASSESSMENT FOR HUMAN HEALTH

10.2.1 Hazards associated with the recipient virus

Generally, genetic manipulation work on Herpesviruses is undertaken in cell culture by homologous recombination between a wild type virus or a derivative and transfected plasmid DNA, although the use of virus-free systems is becoming commonplace. Most GM HSV vectors have been derived from cell-culture adapted laboratory strains of HSV-1 (e.g. 17+; F). The use of more pathogenic clinical isolates has been documented and the relative hazards of these strains should be carefully weighed. Wild type herpesviruses fall into a range of RG classification (see Table 7). An appropriate BSL should be adopted as a minimum requirement when handling wild type viruses.

10.2.2 Herpes simplex virus vector systems

10.2.2.1 Disabled vectors

i. Deletion of essential IE genes encoding ICP4 and/or ICP27 is sufficient to render HSV replication-defective. The retention of ICP0 and ICP22 sequences, however, maintains the cytotoxic phenotype of the virus. Deletion of ICP0 and ICP22 results in a virus that is defective and non-cytotoxic. The trans-complementation of these viruses in cell culture has proven problematic due to the inherent cytotoxicity of ICP0 and ICP22. Mutation of VP16 in tandem with ICP4/ICP27 deletion results in a defective, non-cytotoxic vector strain that is more easily propagated *in vitro*.

ii. HSV has a large coding capacity and a large number of genes that determine pathogenic traits. With approximately half of the coding capacity of HSV absolutely required for viral growth, there is potential for generating many alternate disabled HSV vector strains. The possible effects of viral gene deletion as well as retention of cytotoxicity/pathogenicity determinants on the resulting GM virus should be carefully considered. Recipient viruses or vector strains that can be shown to pose a much-reduced risk of harm compared to the wild type virus might be handled at a lower containment level. The risk assessment must demonstrate that the recipient is disabled or sufficiently attenuated. Furthermore, the likelihood of a reversion event must be low and the stock should be demonstrably free of wild type virus.

10.2.2.2 Replicative vectors

i. The disruption of many HSV genes will result in a viral strain that is attenuated but remains replication competent. For example, deletion of IE genes encoding ICP0 or ICP22 results in a virus that is defective and able to replicate, albeit with greatly reduced fitness. Removal of other genes has been shown to restrict the

virus biologically, resulting in a CRV. For example, deletion of the gene encoding ribonucleotide reductase, ICP6, or thymidine kinase generates viruses that are unable to replicate efficiently in neuronal cells yet are still highly pathogenic. HSV deleted for the gene encoding ICP34.5, on the other hand, are highly attenuated and appear to replicate specifically in tumour cells (ICP34.5 circumvents the host cell's antiviral block to cellular protein synthesis mediated by interferon; this pathway is commonly disrupted in tumour cells).

ii. The effects of deleting sequences from the viral genome should be considered since regulatory elements adjacent to the deletion site might affect neighbouring viral genes. For example, deletion of the gene encoding ICP47 results in the upregulation of the nearby gene US11. The US11 is one of the Late genes of HSV. The expressed protein US11 is a 21 kDa, highly basic phosphoprotein and is also an RNA-binding protein, post-transcriptional regulator of gene expression. US11 is present in the nucleus, particularly concentrated in the nucleolus, and the cytoplasm and is present in the virion as a component of the tegument. Careful assessment of the nature of an attenuating mutation should be made to determine the degree of biological restriction and the effects on viral systems.

iii. The hazards associated with the handling of high titres of replicative virus should be carefully considered. Conditionally Replicating Viruses (CRV), while attenuated, still pose a risk to human health in that lytic infection may occur at unforeseen sites and that recombination resulting in a RCV or wild type virus is a distinct possibility. However, hazards arising subsequently due to the insertion of sequences or phenotypic alterations might necessitate additional containment measures. GM-BSL2 containment level is likely to be a minimum requirement for these vectors unless the risk assessment can show that this is unwarranted.

10.2.2.3 Amplicons

Amplicons are vectors that retain only HSV packaging sequences and origin of replication and therefore have a large capacity for inserted genetic material. These vectors generally require complementation *in trans* from a helper virus. This is usually an HSV strain or Bacterial Artificial Chromosome (BAC) containing a HSV genome that lacks packaging sequences. Contamination with cytotoxic helper virus has been shown to limit the effectiveness of this approach and the risks associated with the helper virus (which by nature retains most HSV genes) must be carefully considered. A disabled helper virus should be used wherever possible.

10.2.3 Hazards associated with genetic inserts

The risk assessment should take into consideration the potential effects of the expressed product. Guidance on the hazards posed by commonly used genetic inserts can be found in Chapter 3. In brief, factors to consider include the following:

10.2.3.1 Integration into host DNA

Herpesvirus genomes are generally maintained in episomal form and insertion into the host genome is extremely rare. Maintenance of expression long-term using an HSV vector will therefore most likely involve prevention of silencing or use of latency associated promoters.

10.2.3.2 Expression characteristics

i. Viral or cellular regulatory sequences could be employed to control expression in transduced cells. For example, the HCMV Major Immediate-Early enhancer would be expected to direct high-level expression in a broad range of cell types. Tissue-specific promoters generally lead to cell-type restricted expression, although consideration should be given to the possibility that adjacent viral promoters might overcome this restriction. Furthermore, such promoters frequently exhibit 'basal leakiness', whereby low-level expression is observed in non-permissive cells. It is advised that promoter characteristics are thoroughly assessed where possible using low-risk virus-free cell culture systems before generating a GM virus.

ii. The ability of HSV to establish life-long latency in the sensory ganglia indicates that long term expression of a transgene carried by an HSV vector might be possible in neural tissue. The use of LAP1 and LAP2 promoters, or LAP hybrid promoters could be used to drive long-term expression of transgenes, although precise mechanisms of gene expression and silencing in latency remain unknown. However, if long-term expression is sought, this should be a factor in the risk assessment.

10.2.4 Biological properties of the gene product.

i. The expected activities or toxicity of the gene products in any cell type should be assessed. For example, a bacterial toxin, growth factor or cytokine would represent greater risk of harm than a reporter gene such as EGFP or Luciferase.

ii. Since neural tissue is relatively poorly understood and HSV vectors are frequently used to transduce neural tissue, the potential effects of expressed gene products in the CNS or peripheral nervous system should be carefully considered.

10.2.4.1 Alteration of phenotype

a) Tissue tropism

i. Generally speaking, herpesviruses are tissue-specific and can only productively infect cells of certain types and, in many cases, cells at a certain stage of differentiation. The replication characteristics of many herpesviruses appear to be dependent on a particular cellular environment. For example, EBV can productively infect epithelial cells and latently replicate in primary B-lymphocytes. HCMV can productively infect certain fibroblasts and epithelial cells. Macrophages are also permissive for lytic HCMV infection, although their undifferentiated monocyte progenitors are not, but are a site of HCMV latency. Receptor specificity is not the only factor affecting tissue tropism and other cellular and viral mechanisms are involved.

ii. For example, HSV can gain entry to a wide variety of cell types, so the apparent tropism for neural and epithelial tissue is not due to receptor specificity. Generally, mutations in virus surface glycoproteins or other viral determinants (*e.g.* ICP6; ICP34.5) have been shown to narrow the host range rather than extend or alter it.

iii. Any modifications to viral promoters that result in a change of specificity for cellular transcriptional regulators should be assessed with caution. This is especially relevant to IE promoters as the products of this class of genes often direct subsequent expression and such modifications may permit viral gene expression or replication in cells that are normally non-permissive.

b) Immunogenicity and pathogenicity

i. Deletion of viral genes or properties of the genetic insert may alter the immunogenic or pathogenic nature of a virus. Herpesviruses are complex and often have a number of immune-evasion strategies. For example, the HSV IE protein ICP47 is involved in the inhibition of antigen presentation by Class I MHCs. The deletion of this gene or prevention of its expression for example, by VP16 mutation, may result in increased antigen presentation. HSV can enter dendritic cells but prevents their activation via a tegument protein termed the Virion Host Shutoff (vhs) protein. Dendritic cells infected with viruses lacking vhs will therefore present antigen and activate cellular immunity more efficiently.

ii. Increased immune stimulation may be desired for the purpose of generating vaccines and vectors. However, while this might prime the immune system and facilitate the clearance of virus, it could also result in increased inflammation and pathogenicity. Likewise, insertion and expression of immunomodulatory cytokines may have a similar effect. Any potential effects on an immune reaction by a modification should be considered as a possible risk to human health.

c) Recombination

i. Recombination events and spontaneous deletions are a feature of herpesvirus DNA replication and cellular genes have been acquired during the evolution. Homologous DNA recombination has been extensively exploited for the purposes of generating GM herpesviruses. The possibility of a recombination event that might result in harmful sequences being transferred between related viruses should therefore be considered. This could take place between a wild type virus and a GM derivative or between a virus and sequences present in cell culture. A homologous recombination event could result in an RCV expressing a transgene. Furthermore, the possibility of recombination taking place between an Amplicon vector and its helper virus should also be considered.

ii. The likelihood of this occurring can be minimised by ensuring that viral sequences deliberately introduced into cells (*e.g.* for complementation purposes) do not possess any overlapping sequences with the GM virus itself. Furthermore, inserting a transgene at the site of an attenuating mutation would result in the deletion of the inserted sequences in the event of a homologous recombination event restoring competence to the virus.

iii. It should also be considered that many herpesvirus genomes contain repeat regions and therefore contain two copies of some genes. For the generation of a knockout mutant virus, both copies require deletion, but recovery of one copy of the gene may be enough for a reversion event to be successful. For example, some HSV genes commonly deleted for attenuating purposes are present in genome repeat regions (*e.g.* ICP4; ICP34.5). It would therefore be possible for a recombination event to restore one copy of a deleted gene, resulting in a functional heterozygote. In situations where the gene is recovered to one of the repeat regions, the heterozygote formed is usually genetically unstable resulting in either genetic reversion at both sites, or loss of the recovered gene. Any selection pressure that arises as a result of the modification may well determine the outcome. If two copies of one viral gene must be deleted, then a copy of the transgene should be placed at each locus to prevent a revertant RCV being generated that also carries the transgene, unless a virus is attenuated using multiple genetic lesions. Furthermore, in situations where there is a selective advantage in recovering a gene, the possibility that an insertion event will occur at a non-homologous site should also be considered.

d) Complementation

i. The prevalence of HSV and its ability to establish latent infection indicates that accidental infection with a modified HSV vector might pose a special risk. Productive infection with HSV can

occur asymptomatically and might provide helper functions to a defective or attenuated vector. Furthermore, recombination during an *in vivo* co-infection has been demonstrated and could occur in a productively infected individual. The risks associated with such events occurring should be rigorously assessed.

10.3 RISK ASSESSMENT FOR THE ENVIRONMENT

10.3.1 Survivability and stability

Herpesviruses are enveloped and highly susceptible to dehydration, lipid solvents and mild detergents. The viruses are rapidly inactivated outside the host, illustrated by the fact that direct contact is usually required for transmission. Therefore, survivability of herpesviruses is not thought to pose a risk to the environment. However, it is important to assess any modification that might increase the stability of the virus.

10.3.2 Alteration of phenotypic and pathogenic traits

i. Herpesviruses are generally highly species-specific and the factors that affect host range and cellular permissiveness for productive infection are complex. For example, although humans are the only natural host for HSV and it cannot be transmitted between non-human species, other animals can be infected experimentally, notably mice. Other virus species (*e.g.* MCMV and MHV69) have a natural tropism for mice and therefore any effects of accidental exposure of host species to GM derivatives of animal herpesviruses should be considered.

ii. Any modifications that may affect the host range of a virus or allow the transduction of a virus encoded transgenic expression cassette should be carefully considered. For example, modification of the surface glycoproteins may generate a GM virus capable of transducing the cells of organisms that would not normally be affected. In that event, the properties of the expression characteristics and properties of the products encoded by the inserted expression cassette should be considered.

10.3.3 Hazards associated with genetic inserts

The biological properties of the expressed gene product, even if they represent a low risk to human health, may be a possible hazard to other species. Furthermore, promoters and control sequences may not show the same expression characteristics or tissue restrictions in other species.

10.4 PROCEDURES AND CONTROL MEASURES

10.4.1 Operational considerations

i. Genetic manipulation of herpesviruses is often undertaken in

cell culture by homologous recombination between a virus and transfected plasmid DNA containing viral sequences. Contamination with the recipient virus is a feature of this system and therefore repeated purification steps and the serial handling of high-titre stocks is required.

ii. Manipulation of many herpesvirus genomes is now possible in virus-free systems. For example, HCMV and HSV genomes have been cloned as Bacterial Artificial Chromosomes and can be manipulated in low-risk bacterial systems prior to the generation of recombinant virus from purified viral DNA. Such systems reduce the risk posed by handling the virus and *in vitro* recombination events. The use of such systems wherever possible is therefore advised.

iii. HSV 'Amplicon' systems require the use of helper viruses and the hazards associated with these should be considered separately as an individual agent, as well as in conjunction with the proposed vector.

10.4.2 Control measures and monitoring procedures

i. A means for monitoring for the presence of RCV in disabled virus stocks should be in place, where appropriate. Permissive, non-complementing cell lines should show signs of productive infection (cytopathic effect, plaque formation) in the presence of RCV and they could be used to test stocks of a disabled GM virus. However, such assays may not be completely reliable as disabled viruses are often cytopathic. The use of molecular detection methods (*e.g.* the use of PCR to detect the presence of sequences required by the vector for replication) would represent a more reliable method.

ii. There is currently no vaccine against any human herpesvirus infection. Prophylaxis is available in the form of the antiviral drugs Acyclovir, Gancyclovir or Foscarnet. It should be noted that thymidine kinase strains of HSV are resistant to Acyclovir and that natural Gancyclovir resistance in HCMV has been documented. It is an organisation's responsibility to ensure that staff health or immune status is sufficient for the activity in question. A system for the monitoring of health and immune status should therefore be implemented where the nature of the work demands it. The health status of staff exposed to the GM viruses should be monitored. For example, those showing signs of a compromised immune system should be reviewed for their suitability for work. If a staff suspects productive infection with HSV (e.g. has an active orolabial coldsore) then they should consider suspending activities involving a GM HSV vector until the infection is resolved.

CHAPTER 11

POXVIRUSES

11.1 BACKGROUND

i. Poxviruses are complex pathogens that are associated with disease in mammals, birds and arthropods. While some poxviruses have a strict host tropism, many can productively infect other species as intermediate zoonotic hosts. Pustular epidermal lesions typify symptoms, although the severity of the disease is dependent on the host organism and poxvirus species (see Table 8). Infection normally occurs via aerosol or direct contact and results in a vigorous immune response involving innate, humoral and cell mediated mechanisms. Immunity is long lasting and cross-reactive with other poxviruses within the same genus. Since routine vaccination against Variola using vaccinia virus (VV) ceased in the early 1980s, immunity to orthopoxviruses within the population is expected to be sporadic.

ii. Poxviruses consist of a large double stranded DNA genome ranging from 130 to 300 kb in size, enclosed in a complex multi-membraned virion. Cellular entry appears to involve interaction between the virion and ubiquitous cell-surface determinants. Therefore, poxviruses can enter cells promiscuously, irrespective of whether the cell is permissive for replication. Consequently, cellular tropism and the ability to replicate are determined by the expression of viral host range genes in concert with host-cell characteristics.

iii. Replication of DNA viruses takes place in the cytoplasm of permissive cells and all enzymes required to initiate viral gene transcription are packaged within the virion. Expression occurs in three waves beginning with the early genes (largely encoding proteins involved in genome replication), followed by the Intermediate genes and then the Late genes (predominantly encoding virus structural proteins). Virions are assembled in a complex morphogenic pathway into various intracellular and extracellular forms, which are all infectious, yet have discrete structural differences.

11.2 RISK ASSESSMENT FOR HUMAN HEALTH

11.2.1 Hazards associated with the recipient virus

i. Generally, genetic manipulation work on poxviruses is undertaken in cell culture by homologous recombination between recipient virus and transfected plasmid DNA. To date, this has largely involved strains that have been extensively attenuated by passage in cell culture. However, the use of more virulent viruses might be more desirable for certain applications. Deliberate inoculation with attenuated VV strains during the Smallpox vaccination campaign showed that adverse reactions occur at a relatively high rate of 1:1000, with severe complications at a rate of 1:50,000. However, more recent data obtained following the inoculation of military personnel has suggested that, while adverse reactions are common, they occur below these historical rates. There is variability in the relative virulence of different strains (*e.g.* Western Reserve strain of VV is more virulent than Copenhagen strain) and the individual hazards associated with these strains should be carefully weighed.

ii. Wild type poxviruses fall into a range of RG. An appropriate BSL should be adopted as a minimum requirement when handling wild type viruses causing human diseases or that are classified as animal pathogens in the *Animal Act 1953 (Revision of Laws (Rectification of Animals Act 1953) Order 2006.*

11.2.2 Disabled and attenuated vectors

i. Poxviruses have a large number of genes, many of which are dispensable for growth *in vitro* and cause attenuation when disrupted. Defective strains of vaccinia have been used extensively in humans during vaccination campaigns and often form the basis for GM vector derivatives. Modified Vaccinia Ankara (MVA) has been attenuated by serial passage in chicken embryo fibroblasts; approximately 31 kb of the genome has been lost resulting in a viral strain that can no longer replicate in mammalian cells. Similarly New York Vaccinia virus (NYVAC), which is derived from the Copenhagen strain, contains multiple deletions that render it severely impaired for replication in human cells. Avipoxviruses are inherently replication defective in mammalian cells and can therefore be considered as attenuated in mammals. These have been have been used for the expression of heterologous genes in human cells. Fowlpox virus (TROVAC, FP9) and Canarypox virus (ALVAC) have proved avirulent in human clinical trials and in other mammalian pre-clinical and veterinary trials. It is important that the nature of the attenuation is understood as fully as possible, particularly if a downgrading of containment level is sought.

ii. It is always advisable that a potentially harmful biological agent be substituted with an agent that is less hazardous or be eliminated

Table 8. Host range of poxviruses and the typical symptomatic consequences of infection.

Poxvirus	Risk Group (RG)	Risk Group (RG)	Risk Group (RG)	Primary Host
Orthopoxviruses				
Variola virus	RG4	Humans	None	Smallpox
Vaccinia virus (wild type)	RG2	Unknown	Humans, cows, rabbits	Localised epidermal lesions, eczema*, encephalitis*, vaccinia necrosum*
Cowpox virus	RG2	Rodents	Humans, cows, cats and foxes	Localised epidermal lesions
Monkeypox virus	RG3	Squirrels	Humans, monkeys	Smallpox-like
Camelpox virus	RG2	Camels	None	Smallpox-like (in camels)
Mousepox virus	RG2	Rodents	Laboratory mice	Infectious ectromelia in lab mice
Molluscipox viruses				
Molluscum contagiosum virus	RG2	Humans	None	Localised epidermal lesions
Parapoxviruses				
Orf virus	RG2	Ungulates	Humans, cats	Localised epidermal lesions
Yatapox viruses				
Yaba monkey tumour virus / Tanapoxvirus	RG2	Unknown	Humans, monkeys	Localised epidermal lesions
Avipoxviruses				
Fowlpox / Canarypox	RG2	Birds	Humans as vaccine vector	Localised epidermal lesions in birds
Leporipoxviruses				
Myxoma virus	RG2	Rabbits	None	Myxomatosis
Suipoxviruses				
Swinepox virus	RG2	Pigs	None	Epidermal lesions, acute but mild

Capripoxviruses				
Lumpy Skin Disease virus	RG2	Cattle	None	Epidermal lesions. Occasionally fatal
Sheeppox / Goatpox virus	RG2	Sheep and goats		Lesions on mucous membranes and exposed skin, fever, paralysis

**Less common adverse reactions to vaccinia virus inoculation in humans*

entirely, if possible. Therefore, safer virus systems or less virulent strains should be employed wherever practicable. For instance, the use of attenuated vaccinia strains (*e.g.* MVA; NYVAC; Lister; WYETH; Copenhagen) should be used in preference to more virulent strains (such as Western Reserve) wherever possible. Some attenuated poxvirus strains that can be shown to pose a much-reduced risk of harm compared to the wild type virus might be handled at BSL1. However, the risk assessment must demonstrate that the recipient is sufficiently attenuated. Furthermore, the likelihood of a reversion event must be low and the stock should be demonstrably free of wild type virus. It is advisable to conduct the activity in BSL2.

iii. However, hazards arising subsequently due to the insertion of sequences or phenotypic alterations might necessitate additional containment measures.

11.2.3 Conditionally replicative vectors

i. Targeted deletion of viral genes can alter the growth requirements of poxviruses and restrict replication to certain cell types. For example, the VV Viral Growth Factor (*vgf*) gene is required for the stimulation of mitosis in cells surrounding the site of infection. Deletion of this gene restricts viral replication to cells that are actively dividing. Also, the VV thymidine kinase gene is required for nucleotide biosynthesis. Deletion of the thymidine kinase gene results in a strain that is attenuated and requires cells with naturally high levels of free nucleotides for efficient replication. VV that are deleted for both *vgf* and thymidine kinase appear to replicate specifically in tumour cells. Poxviruses also carry a number of so-called host range genes, deletion of which will generally attenuate the recipient strain and limit tissue tropism. It should be noted, however, that instances of accidental inoculation of laboratory staff have demonstrated that thymidine kinase deleted strains of VV retain the ability to establish an infection and cause lesions in humans. Virulence mechanisms, and hence, the attenuation of poxviruses is complex and a cautious approach is advised when handling VV and other poxviruses with the ability to infect humans.

ii. The risks associated with the handling of high titres of replicative virus should be carefully considered. Conditionally Replicating Viruses (CRV) while attenuated, still pose a risk to human health in that lytic infection may occur at unforeseen sites. An appropriate BSL should be adopted for these vectors.

11.2.4 Hazards associated with genetic inserts

The risk assessment should take into consideration the potential effects of the expressed product. The factors to consider include the following:

11.2.4.1 Expression characteristics

Using poxvirus-derived Early, Intermediate or Late promoters can broadly control the timing of expression in poxvirus systems. The use of heterologous promoters is largely ineffective due to the fact that poxvirus replication is restricted to the cytoplasm. However, since poxviruses can enter virtually any cell, damage to 'untargeted' tissue due to transgene expression is a possibility that should be considered.

11.2.4.2 Proviral insertion

Poxvirus replication occurs in the cytoplasm of infected cells using virion-associated and virus-encoded machinery. Insertion of viral DNA into the host genome would therefore be exceptionally rare. Poxviruses have been used to vector recombinant retrovirus genomes, which will insert into the host genome. The effects of integration in such chimaeric systems should be considered (guidance on the use of retroviruses can be found in Chapter 12).

11.2.4.3 Biological properties of the gene product

The expected activities or toxicity of the gene products should be assessed. For example, a bacterial toxin or growth factor would represent greater risk of harm than a reporter gene such as EGFP or Luciferase. Properties of the gene products with respect to individual cell types should also be considered.

11.2.4.4 Alteration of phenotype

i. Tissue tropism

As previously discussed, poxviruses can enter virtually any cell and may cause damage to non-permissive tissues. Replication, however, is far more cell-type specific and individual poxviruses have their own array of host range genes that influence the ability to replicate in certain cell types (see Table 9). These genes might alter tissue tropism when deleted or heterologously inserted into a poxvirus genome and the susceptibility of additional tissues to productive infection should therefore be considered.

Table 9. Poxvirus host range genes

Poxvirus gene	Host range
Vaccinia E3L	HeLa Cells; Chicken Embryo Fibroblasts
Vaccinia K3L	Baby Hamster Kidney Cells
Vaccinia C7L	Hamster Dede Cells
Vaccinia K1L	Rabbit Kidney Cells
Vaccinia SPI-1/B22R	Human Keratinocytes; Human Epithelial Lung Cells
Mousepox p28	Mouse Macrophages
Cowpox C9L/CP77	Chinese Hamster Ovary Cells

ii. Immunogenicity and pathogenicity

a. Poxviruses have multiple strategies for evading the host immune response and the genes encoding the proteins that mediate these properties are often dispensable for growth *in vitro* (see Table 10). Since a vigorous immune response is characteristic of many poxvirus infections and important for the eventual clearing of virus, deletion or insertion of such genes might alter the immunopathological nature of the virus. The consequences of such a modification should be considered in the context of a possible risk to human health. Similarly, the insertion and expression of genes encoding immunomodulatory products might affect pathogenesis.

b. For example, the cellular immune response to an infection is often characterised by a polarisation of the CD4 helper T-lymphocyte population so that Th1 or Th2 subsets of these cells predominates. Th1 cells are primarily involved in the generation of CD8+ cytotoxic T-cell responses to bacterial and viral infections, whereas Th2 cells are involved in priming B-lymphocytes and the generation of antibody responses to parasitic infections. The polarised population arises due to a reciprocal negative regulation of these subsets, mediated by the cytokines generated by each.

c. Interleukin-4 (IL-4) is an immunomodulatory cytokine generated by Th2 cells. As a consequence, poxviruses that are modified to express IL-4 are less efficiently cleared by the host immune system as Th1-induced cytotoxic T-lymphocyte response is inhibited. Therefore, these poxviruses have increased pathogenicity.

Table 10. Examples of poxvirus immune-evasion genes and their function

Poxvirus gene	Viral Protein	Viral Protein
Vaccinia virus C3L	Complement binding protein	Binds C3b/C4b, inhibits complement activation
Vaccinia virus B8R	Soluble IFN-γ receptor	Binds and antagonizes IFN-γ
Vaccinia virus B19R	Soluble IFN-α/β receptor	Binds and antagonizes IFN-α/ IFN-β
Vaccinia virus B15R	Soluble IL-1β receptor	Binds and antagonizes IL-1β
Vaccinia virus C12L; Mousepox virus p13/16; MCV MC54L	Secreted IL-18 binding protein	Binds and antagonizes IL-18
Cowpox virus crmA-E	Soluble TNF receptor	Binds and antagonizes TNF-α
Vaccinia virus 35 kd	Secreted chemokine binding protein	Binds and antagonizes CC chemokines
Vaccinia virus E3L	dsRNA binding protein	Prevents PKR activation
Vaccinia virus K3L	EIF-2α homologue	Inhibits PKR function
MCV MC148	Secreted chemokine homologue	Binds and antagonizes CC chemokine receptor 8
MCV MC80R	MHC Class I homologue	Binds β2-Microglobulin
MCV MC159	FLIP (FLICE-like inhibitory protein)	Prevents Fas and TNF mediated apoptosis

iii. Recombination

a. Homologous recombination has been extensively exploited for the purposes of generating GM poxviruses, therefore the possibility of recombination that might result in harmful sequences being transferred between related viruses should be considered. Homologous recombination in poxviruses is dependent upon viral DNA replication and therefore coinfection or DNA transfection of productively infected cells would be required. Naked Poxvirus DNA is not infectious, and since poxvirus infections are non-persistent and the only naturally occurring orthopoxvirus infections of humans are Cowpox (a rare occurrence, most likely transmitted from rodents via cats) or Monkeypox (which is geographically restricted to Central Africa), the probability of recombination occuring *in vivo* is expected to be low.

b. The likelihood of undesirable recombination *in vitro* could be minimised by placing the insert at the site of an attenuating mutation. This would result in the deletion of the inserted sequences in the event of a recombination event restoring competence or virulence to the virus. Insertions are routinely made at the thymidine kinase locus, and meet this criterion. However, the need to express multiple antigens means that recombinants carrying insertions, frequently at non-attenuating loci, are becoming more common. Under these circumstances, it would be important to conduct the risk assessment assuming that transfer of the inserted gene to a wild type virus were possible, if very unlikely.

11.3 RISK ASSESSMENT FOR THE ENVIRONMENT

11.3.1 Survivability and stability

i. Poxviruses are highly stable and resistant to dehydration; infectious virus can be stored in dried powder form. Transmission is usually via aerosol or direct contact and infectious virus can survive for protracted periods in dried scab material shed from epidermal lesions. Risk assessments should therefore consider that, in the event of any release into the environment, GM poxviruses might persist and could be transmitted to other humans or animal species.

ii. Some poxviruses (Cowpox virus and avipoxviruses, but not VV) are capable of forming A-type inclusion bodies, which are believed to be mechanisms for enhanced survival of viruses shed into the environment in desquamated epithelium. Disruption of the genes (equivalent to Cowpox virus 158 & 159; Fowlpox virus 190 & 191) responsible for formation of the inclusion may reduce environmental persistence of the intracellular form of the recombinant (shed as dust from skin lesions and conceivably in blood leucocytes) but would not affect stability of the extracellular virion released into culture media.

11.3.2 Hazards associated with genetic inserts

Even if the biological properties of the expressed gene product, represent a low risk to human health, it may be a possible hazard to other species. These considerations are particularly applicable to poxviruses as they are able to gain entry to most cells, irrespective of host range or tissue tropism. Expression from the viral genome is therefore possible in cells that would not normally express the particular products (although the outcome of poxvirus infection is normally cytotoxic, irrespective of heterologous gene expression).

11.3.3 Alteration of phenotypic and pathogenic traits

While some poxviruses have a narrow host range and tissue tropism, others can productively infect other organisms (see Table 9). This is pertinent when evaluating GM Vaccinia or Cowpox viruses as they can establish productive infection in a variety of animals. Furthermore, poxviruses have host range determining genes that could confer the ability to infect an otherwise refractory host organism, as well many genes governing virulence and pathogenic determinants. A careful assessment of any modification in the context of altered pathogenicity or host range must be made and the risks posed to the wider environment evaluated. For example, poxviruses (*e.g.* Mousepox virus, Myxoma virus) that are modified to express IL-4 have increased pathogenicity as they inhibit the appropriate anti-viral immune response. These viruses are less easily cleared by the host immune system. Furthermore, they cause disease in normally resistant hosts and previously immune animals. In the case of the Mousepox virus, this is particularly pertinent as normally only laboratory animals are susceptible, whereas all mice are potentially susceptible to an IL-4 expressing derivative.

11.4 PROCEDURES AND CONTROL MEASURES

11.4.1 Operational considerations

i. Genetic manipulation work on poxviruses is undertaken in cell culture by homologous recombination between recipient virus and transfected plasmid DNA. Contamination with parental virus can occur with this procedure and therefore repeated purification steps are required. Vaccinia genomes have been cloned as Bacterial Artificial Chromosomes and can be manipulated in low-risk bacterial systems prior to the generation of recombinant virus from purified viral DNA (in the presence of a poxvirus helper). Such systems reduce the risk posed by handling the virus and *in vitro* recombination events. The use of such systems is advised wherever possible. However, since poxvirus DNA is not infectious, a helper virus is still required in order to recover the recombinant. The hazards associated with the helper virus should be considered in addition to the intended recombinant and appropriate containment and control measures implemented.

ii. Poxviruses are generally highly cell associated and the preparation of high-titre viral stocks often involves repeated freeze-thaw and sonication to release virions. It is important that the vessel used for freeze-thaw is sufficiently robust so that no breakage occurs due to extreme temperature variation (*e.g.* polypropylene rather than polystyrene). Sonication generates aerosols that can disseminate infectious virus and should be performed only if necessary in sealed vessels using a waterbath or cuphorn sonicator. Probe

sonicators should not be used unless otherwise contained, for example in a sealed cabinet.

iii. Another possible route of accidental infection with a poxvirus will be via inadvertent percutaneous inoculation. Several cases of laboratory-acquired infections with VV have occurred due to needlestick injury during animal handling procedures. Therefore, hollow needles should be used with extreme care, and only when necessary. Needles should never be resheathed, but disposed of directly into a suitable sharps waste container.

11.4.2 Control measures and monitoring procedures

i. Poxviruses are robust and transmitted effectively via aerosols, droplets and direct contact, even if disabled or attenuated. A rigorous approach to risk assessment must be adopted and appropriate control measures implemented. Procedures that minimise aerosol formation should be employed. Some attenuated poxviruses could, in principle if not in practice, be handled on the open bench. It is good laboratory practice, however to handle these viruses under appropriate conditions and, if necessary within a biological safety cabinet to safeguard human health and to prevent environmental release.

ii. Where the risk assessment shows that exposure to airborne GM poxvirus represents a hazard, the use of a biological safety cabinet is required as a control measure. These activities should be assigned to GM-BSL2 or higher and take place at an appropriate BSL.

iii. The Smallpox vaccine should not be administered unless: (i) a staff requests for it; (ii) if work involves Monkeypox virus; or (iii) if the risk assessment says it is required due to the GM virus representing a specific hazard.

11.4.3 Health surveillance and staff training

i. It is an organisation's responsibility to ensure that a staff health or immune status is sufficient for the proposed activity. A system for the monitoring of health and immune status should therefore be implemented where the nature of the work demands it. The health status of staff exposed to GM poxviruses should be monitored. For example, those showing signs of a compromised immune system or with a special medical status should review their suitability for work.

ii. There have been several documented laboratory-acquired VV infections, many of which have occurred due to needlestick injury or as a result of inadequate protective measures. Therefore, there is a need for instruction and training of staff in the correct operating procedures for handling virus, especially for animal handling work.

Furthermore, staff should be trained to recognise poxvirus lesions, so that any infection can be detected early and the appropriate remedial action taken. Prophylaxis for VV infection is available in the form of anti-vaccinia virus immunoglobulin. The antiviral drug Cidofovir has been shown to be effective against poxvirus infection, although it is currently unlicensed. All laboratory-acquired infections should be reported to the IBC and the Occupational Health and Safety Committee.

iii. It is well known that vaccinia and other poxviruses have the capacity to survive for considerable periods in dried material such as detached vaccination scabs, but it is less well appreciated that survival in aqueous solutions can be for several weeks. Live virus can also be isolated from solid surfaces and fabric for as long as two weeks after contamination. For laboratory staff, ingestion, inoculation via needles or sharps, and droplet or aerosol exposure of mucous membranes or broken skin are possible routes of infection. Laboratories working with VV and other poxviruses should have suitable local rules to control these potential sources of infection, including suitable procedures for decontamination of equipment and surfaces.

iv. As work with RG2 organisms such as VV requires restricted access, ideally only those who work with the virus should have access to the areas where the virus is used. Where VV are used in multi-user facilities, all users must be familiar with the risks associated with VV and be trained to recognise the signs of vaccinia virus infection.

11.4.4 Risk awareness

i. Vaccinia virus is categorised by Ministry of Health, Malaysia as a RG2 biological agent. It is recognised that VV may cause particularly severe disease during pregnancy, in people with active skin disorders such as eczema or psoriasis, or in immuno-compromised individuals such as those infected with HIV. Indeed a number of vaccinia virus vaccine associated deaths of HIV positive individuals have been reported. It is well documented that VV can be passed to close contacts of vaccine recipients generally with little adverse consequence. Therefore, although an individual with a laboratory-acquired infection is unlikely to receive the virus dose given for vaccination purposes, close contacts, particularly those with contraindications for vaccination, may also be at risk. All staff who work with VV should be:

- trained to recognise VV infection;
- made aware of the possibility of human-to-human transmission; and
- be aware of the increased risk to those with eczema, and those who are immuno-compromised, or those who are pregnant.

RETROVIRUSES

CHAPTER 12

12.1 BACKGROUND

i. Retroviruses form a diverse and extensive family affecting both human and animal species. Many retroviral infections are subclinical or benign, although some cause significant disease, the majority of which are haematopoietic disorders. However, the range of manifested symptoms is broad (see Table 11). Retroviruses are characterised by a unique replication mechanism involving reverse transcription of the viral RNA genome, giving rise to a DNA provirus. This inserts into host chromosomal DNA and acts as a template for viral mRNA and genome copies. Individual viral genomes are bounded by Long Terminal Repeats (LTRs) containing viral transcriptional promoter and enhancer regions (the U3 region) that control viral gene expression. All retroviruses contain the same three gene clusters: *gag* (encoding structural proteins), *pol* (encoding reverse transcriptase and integrase) and *env* (encoding the envelope glycoproteins). More complex retroviruses, such as deltaretroviruses, spumaviruses and lentiviruses, contain additional sequences encoding accessory proteins that enhance or modulate replication that might be involved in viral pathogenesis (see Figure 7).

ii. Retrovirus virions contain two positive sense copies of the RNA genome, encased within a capsid that is surrounded by a host-cell derived envelope. Cellular entry involves interaction between the Surface (SU) subunit of the virion envelope glycoprotein and cell-surface determinants. These interactions are generally specific and are believed to be the principal factor affecting cell- type and species specificity. Receptor binding triggers membrane fusion mediated by the Transmembrane (TM) subunit of the virion envelope glycoprotein, resulting in delivery of the virus capsid to the target cell.

iii. Several retroviruses are oncogenic and can cause malignant disease, either by insertional mutagenesis into the host genome or as a consequence of having acquired host genomic DNA, the

sequence of which has been incorporated into the RNA genome of the virus. Oncogene acquisition is generally at the expense of viral sequences and results in defective, but acutely transforming strains dependent on a helper virus for replication. Retroviruses are generally transmitted via exposure to contaminated body fluids or percutaneous inoculation resulting in persistent infections. Most of these viruses are strongly immunogenic, but host immunity usually suppresses replication rather than clearing the infection altogether.

12.2 RISK ASSESSMENT FOR HUMAN HEALTH

12.2.1 Hazards associated with the recipient virus

i. To date most genetic modification work involving retroviruses has involved the development of transduction vectors derived from competent oncogenic retroviruses and lentiviruses. Many such retroviral transduction systems are manipulated in cDNA form and give rise to defective vectors. It is important to consider the hazards posed by the virus from which these vector systems are derived in order to make an accurate assessment of the risks posed to human health.

ii. Wild type retroviruses fall into a range of RG (see Table 12). The appropriate BSL should be adopted as a minimum requirement when handling wild type viruses causing human diseases or that are classified as animal pathogens in the *Animal Act 1953 (Revision of Laws (Rectification of Animals Act 1953) Order 2006*.

Table 11. Typical diseases associated with commonly studied retroviruses

Retroviruses	Disease
Alpharetroviruses	
Avian leukosis virus	Lymphoid leukaemia and wasting syndromes in chickens
Rous sarcoma virus	Sarcoma in chickens (encodes *v-src* oncogene)
Avian myeloblastosis virus	Myeloid leukaemia in chickens (defective - encodes *v-myb* oncogene)
Betaretroviruses	
Mouse mammary tumour virus	Epithelial mammary tumours in mice

Gammaretroviruses	
Feline leukaemia virus	Feline immunodeficiency / Lymphoid Leukaemia in cats
Moloney murine leukaemia virus	T-cell leukaemia in mice/rats
Moloney murine sarcoma virus	Sarcoma in mice/rats (defective – encodes *v-mos* oncogene)
Deltaretroviruses	
Human T-cell lymphotrophic virus -1	Adult T-cell Leukaemia in humans (Long latency period)
Human T-cell lymphotrophic virus - 2	Hairy-cell leukaemia in humans; CNS disease
Bovine leukaemia virus	T-cell leukaemia in cattle
Epsilonretroviruses	
Walleye dermal sarcoma virus	Fish retrovirus
Lentiviruses	
Human immunodeficiency virus 1 and 2	Acquired Immune Deficiency Syndrome; CNS disease
Simian immunodeficiency virus	Non pathogenic in monkeys, immunodeficiency in old-world primates
Feline immunodeficiency virus	Immunodeficiency in cats
Equine infectious anaemia virus	Chronic haemolytic anaemia in horses
Caprine arthritis-encephalitis virus	Arthritis, pneumonia and wasting in goats
Visna/maedi virus	Pneumonia, wasting and paralysis in sheep
Spumaviruses	
Chimpanzee foamy virus	Non-pathogenic

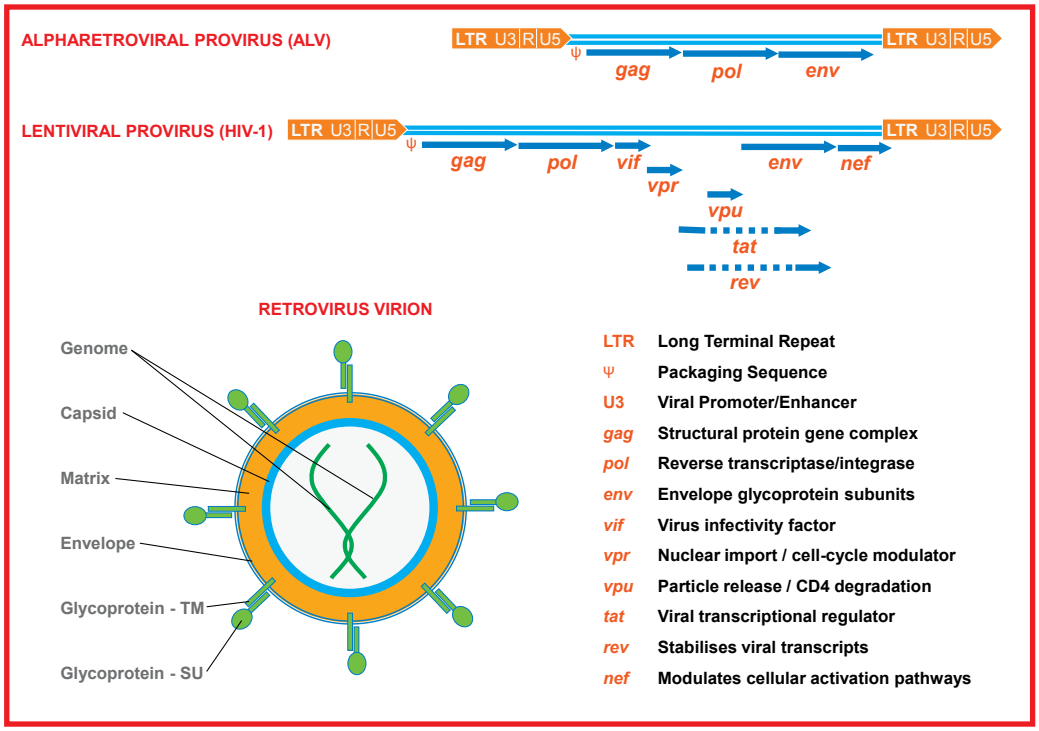


Figure 7. *Diagram of retroviral genomes and structure of a retrovirus particle*

Table 12. Risk Group (RG) classification of commonly studied retroviruses

Retroviruses	Risk Group (RG)
Avian leukosis virus (ALV)	RG1
Moloney murine leukaemia virus (MoMLV)	RG1
Mouse mammary tumour virus (MMTV)	RG1
Feline leukaemia virus (FeLV)	RG1
Bovine leukaemia virus (BLV)	RG1 (Animal pathogen GA-BSL2)
Human T-cell lymphotrophic virus 1 and 2 (HTLV-1/-2)	RG3
Human immunodeficiency virus 1 and 2 (HIV-1/-2)	RG3
Simian immunodeficiency virus (SIV)	RG3
Feline immunodeficiency virus	RG1
Equine infectious anaemia virus (EIAV)	RG1 (Animal pathogen GA-BSL2)
Visna/maedi virus (VISNA)	RG1
Chimpanzee foamy virus (CFV)	RG1

12.2.1.1 Vector Systems and their design

i. Oncogenic retroviral and lentiviral vector systems generally consist of two main components: a transfer vector and a packaging system. The transfer vector is usually a proviral cDNA in which viral coding sequences have been deleted and foreign DNA inserted. The packaging system commonly consists of one or more helper constructs that express viral genes needed to generate infectious viral particles. The generation of RCV and insertional mutagenesis as a result of proviral integration poses major safety issues when handling retroviral vectors.

ii. RCV can be generated by recombination events between the vector and the components of the packaging system (including both the packaging constructs themselves and endogenous proviruses present in the cell line used). Proviral integration can result in the activation of cellular genes adjacent to the integration site or insertional disruption of tumour-suppressor functions (features central to oncogenesis by retroviruses not carrying a cellular oncogene). Retroviral vector systems have therefore been developed and refined in order to reduce the likelihood of RCV generation and proviral transactivation. Consequently, there is a range of systems that vary in their safety profile.

A. Oncogenic Retroviral Vectors

i. The majority of these vectors have been derived from competent oncogenic retroviruses, such as ALV, MoMLV and FeLV that efficiently infect actively dividing cells. "First Generation" retroviral vectors contain a packaging system that is essentially a retroviral cDNA itself, encoding viral *gag*, *pol* and *env* genes but with its packaging sequence deleted. This construct is either co-transfected with the transfer vector, or is stably incorporated into the host-cell chromosomes generating a helper cell line. Such systems are inherently the most hazardous since a single recombination event would be sufficient to generate RCV.

ii. The 3' LTR is deleted in "Second Generation" packaging systems, improving biosafety by reducing the possibility that the packaging construct will be mobilised as well as reducing the likelihood of RCV generation, as two recombination events are required.

iii. With "Third Generation" systems, the 5' LTR is also deleted and the packaging sequences are divided between two constructs, with *gag/pol* encoded by one construct and *env* by the second. This significantly reduces the likelihood of RCV generation, by increasing the number of recombination events that are required to reconstitute a competent viral genome. Two-component packaging systems of this type should be used wherever possible. Additional biosafety can also be achieved by using "*self-inactivating (SIN) transfer vectors*".

B. Lentiviral Vectors

i. Lentiviral vectors have become widely used due to their ability to infect non-dividing cells, which gives them an advantage over oncogenic retroviral vectors for certain applications. Furthermore, unlike oncogenic retrovirus vectors, transformation has not been seen when using lentivirus systems in a broad range of *in vitro* studies and animal studies using both *in vivo* and *ex vivo* protocols. However, in common with AAV and MLV vectors, liver tumours have been observed following administration of lentiviral vectors to foetal or neo-natal animals. This is based on limited data and the mechanism by which these tumours arise has not been elucidated. For example, it is not clear whether or not this is due to vector activity.

ii. "First Generation" lentiviral vectors resemble third generation oncogenic retroviral systems, and are composed of a transfer vector containing all viral components except *gag*, *pol* and *env* which are provided *in trans* by two helper constructs. Several of the lentiviral accessory genes are deleted in "Second Generation" transfer vectors (*vif, vpr, vpu* and *nef*) since they are not required for *in vitro* replication and the products they encode have cytotoxic activities. In "Third Generation" vectors, the *tat* gene is also deleted and the Tat-responsive promoter present in the 5' LTR is replaced with heterologous promoters, for example with the Rous sarcoma virus U3 region. Additional biosafety is achieved by deletion of the *rev* gene from the transfer vector and expressing this from a third packaging construct as well as employing the *SIN* principle.

C. Hybrid vectors (Viral Shuttle Vectors)

i. Another strategy employed is to use other viral vector systems to deliver retroviral vector or packaging constructs to cells (*e.g.* VV or HSV). These approaches are designed to improve the efficacy and scale-up potential of retroviral vector production over transfection methodologies or the use of stable packaging cell lines. When assessing the hazards associated with such chimaeric viruses or shuttle vectors, they should be considered as a separate, distinct GMM from the intended retroviral vector. However, it should also be assessed as an integral part of the retroviral system.

12.2.1.2 Vector Choice

Clearly, there is a variety of vector systems and a spectrum of safety profiles. It is therefore important to choose a system that both fulfils the requirement of the task it is to perform as well as offering a high degree of safety for the user. For example, third-generation lentiviral systems have a much-improved biosafety profile when compared to First or Second generation oncogenic retroviral systems. Safety versus functionality considerations should therefore be carefully weighed and the safest system possible should ultimately be employed.

12.2.1.3 Proviral insertion

i. Integration of viral cDNA into the host cell genome is essential for retroviral replication and it is this feature that makes them attractive for stable cell transduction and gene therapy applications. The effects of integration upon the infected cell should be considered. For instance, promoter/enhancer sequences present in the provirus can activate genes adjacent to the integration site or, alternatively, insertion may disrupt genes and prevent their expression. Deletion of the retroviral enhancer in SIN systems reduces the risk of activation but not of disruption.

ii. The potential effects of other exogenous non-coding sequences within the vector should be carefully assessed. Retroviral infection might have permanent effects upon a cell and this can include tumourigenesis (*e.g.* in mouse models and in children receiving retrovirally transduced bone marrow cells). However, the risk of transduction leading to tumourigenesis or other untoward harm following exposure is related in part to the titre of the viral vector; and exposure of the staff to quantities of virus high enough to cause such effects would be unlikely during standard laboratory-based manipulations of retroviral vectors.

12.2.1.4 The woodchuck hepatitis B virus (WHV) post-transcriptional regulatory element (WPRE) encoding X protein

Some vectors contain sequences from WHV to increase retroviral vector titre and gene expression. Some versions of this WPRE element are capable of expressing part of the X protein from WHV, which may have oncogenic properties. This should be taken into account in the overall risk assessment. For example, vectors containing X protein expressing forms of WPRE may therefore need to be assigned to BSL2.

12.2.1.5 Sequence Manipulation

i. Careful manipulation of the sequence of both the vector and packaging constructs can reduce the probability of recombination and insertional mutagenesis events. Splitting the packaging sequences between as many constructs as possible and careful sequence manipulation to reduce homology between those constructs will significantly reduce the likelihood of recombination events giving rise to RCV. For example, the packaging sequence and 5' region of the *gag* gene is usually the only remaining region of homology in many of the systems in current use. Using vectors with altered codon usage in this region effectively eliminates the likelihood of RCV generation.

ii. Further refinements of retroviral vectors have involved the generation of *SIN* systems. This takes advantage of a feature of

retroviral replication whereby the U3 region of the 3' LTR (which contains the major viral promoters and enhancers) is copied to the 5' end of the provirus during reverse transcription. Thus, deletion of enhancer and promoter elements from the 3' U3 region in the vector construct will result in a provirus that is entirely devoid of U3 enhancer sequences, therefore reducing the potential for *trans*-activation of cellular genes as a result of insertion. Furthermore, such vectors are not easily mobilisable as a result of a superinfection with wild type virus. Whilst the effects of the viral LTRs are negated in these vectors, *trans*-activation by heterologous transcription-regulatory sequences (enhancers, promoters etc.) used to drive expression of inserted genes remains a possibility and the risks should be carefully considered.

iii. Acquisition of oncogenes by retroviruses (oncogene capture) is a natural phenomenon that is characteristic of retrovirus evolution, albeit a rare occurrence. The resulting recombinant viruses are usually replication defective (but not always *e.g.* Rous sarcoma virus) but acutely transforming in cells of specific hosts due to expression of the transduced oncogene. The mechanism for oncogene capture is thought to be transcriptional read-through from the provirus into flanking cellular genes generating a chimeric RNA transcript that is subsequently packaged into a virion. For replication defective viruses (particularly *SIN* vectors), oncogene capture is minimised. However, where replication competent viruses are used, such as in experimental tumour therapies, the potential for this rare event should be considered.

12.2.1.6 Packaging Cells

The use of cell lines stably expressing the packaging sequences will also reduce the likelihood of recombination resulting in RCV generation. Cotransfection methodologies bring high-levels of plasmid DNA together within cells and therefore increase the probability of DNA homologous recombination giving rise to a competent viral genome. Stable packaging cell lines should therefore be employed wherever possible. Furthermore, using cell lines that have been screened for endogenous proviruses will reduce the likelihood of recombination events and mobilisation of endogenous proviruses by superinfection with the vector.

12.2.2 Hazards associated with genetic inserts

The risk assessment should take into consideration the potential effects of the expressed product. Guidance on the hazards posed by commonly used genetic inserts can be found in Chapter 3 of this Guideline. In brief, factors to consider include:

12.2.2.1 Biological properties of the gene product

The expected biological activities or toxicity of the gene products should be assessed. For example, an oncogene or cytokine/growth factor would represent greater risk of harm than a reporter gene such as EGFP or Luciferase; with a spectrum of inserts of varying biological activity between these extremes (*e.g.* siRNA, signalling molecules). Properties of the gene products with respect to individual cell types should also be considered and whether effects are localised or systemic.

12.2.2.2 Expression characteristics

This will be dependent on the cell type and the regulatory sequences used to control expression. For example, use of the human cytomegalovirus Major Immediate-Early enhancer would be expected to direct high-level expression in a broad range of cell types. Tissue-specific promoters generally lead to cell-type restricted expression. However, tissue specific promoters may exhibit 'basal leakiness', whereby low-level expression is observed in non-permissive cells. A further level of control can be achieved using inducible promoter systems (*e.g.* tetracycline responsive promoters) whereby transgene expression is controlled by the presence of the inducer. Where the promoter is ill-defined, it is advised that promoter characteristics are thoroughly assessed where possible using low-risk virus-free cell culture systems before a vector is generated. Properties of the gene products with respect to cell types and tissues that could be affected should therefore be considered.

12.2.3 Alteration of phenotype

i. Retroviruses are able to replicate in a wide variety of cell types. However, tissue tropism and host range is restricted by the specificity of the surface glycoprotein molecules encoded by the *env* gene. For this reason, it is often desirable to alter or extend the specificity of virus vectors. This commonly involves the substitution of the *env* gene with the glycoprotein gene from another virus ("pseudotyping") or modification of the native env gene such that specificity is altered. The vectors are often classified as ecotropic (infectious for the cells of the host species), xenotropic (infectious for the cells of another species, but not the host cell species) or amphotropic (able to infect the cells of the host and other species).

ii. Vectors can be pseudotyped with the glycoprotein of another virus that possesses the desired specificity. It is also possible to produce vector particles with a broad, amphotropic nature by using the vesicular stomatitis virus (VSV) G protein, for example. The susceptibility of additional tissues and organisms to infection should therefore be considered and it is an important factor in

determining the BSL and control measures appropriate to the intended virus vector.

iii. Furthermore, it has been shown that pseudotyping viruses can alter the stability and potentially alter the transmission properties compared to the wild type virus from which it is derived. For instance, some pseudotyped retroviral and lentiviral vectors could possibly be transmissible via aerosols as well as the recognised routes. Equally, changes to the envelope protein may also result in changes in the virus susceptibility to host complement. All potential changes to the properties of the vector as a result of such modifications should be considered and specific containment measures may need to be implemented to account for any increased risk. A precautionary approach should be adopted when using pseudotyped viruses where there are no clear data regarding their properties of transmission and appropriate control measures (*e.g.* restricted access, the use of a safety cabinet) employed.

12.2.4 Immunogenicity and Pathogenicity

i. Deletion of elements from the viral vector backbone (or expression of heterologous elements in the genetic insert) may alter the immunogenic or pathogenic nature of the virus. This is particularly relevant to lentiviral vectors, where accessory genes have normally been deleted in order to improve vector capacity and biosafety.

ii. Retroviruses incorporate host cell-derived proteins into virions during packaging and these will be delivered to the target cells. These proteins may be cellular proteins or viral proteins expressed by the packaging system. For example, lentiviral proteins Nef, Vpr and Vif, as well as a number of cellular proteins, are incorporated into viral particles and may enhance the immunogenic nature of the vector. The potential harmful effects on host immune response from incorporating proteins such as these should also be considered.

12.3 RISK ASSESSMENT FOR THE ENVIRONMENT

12.3.1 Survivability and stability

Retroviruses are enveloped viruses that are highly susceptible to dehydration. However, they can survive for long periods in high protein media. Retroviruses are rapidly inactivated outside the host, as illustrated by the fact that close contact is required for transmission. Furthermore, many oncogenic retroviruses require high titre inoculations to establish an infection. Therefore, the survivability of retroviruses is not thought to pose a risk to the environment, but it is important to assess any modification that might increase the environmental or clinical stability of the virus.

12.3.2 Hazards associated with genetic inserts

The biological properties of the expressed gene product, even if they represent a low risk to human health, may be a possible hazard to other species. Furthermore, heterologous control sequences may not show the same expression characteristics or tissue restrictions in other species. These considerations are particularly applicable to amphotropic vectors as they are able to gain entry to many cell types across many species.

12.3.3 Phenotypic and pathogenic traits

i. Whilst retroviruses generally have a narrow host range and tissue tropism, amphotropic and xenotropic vectors will be able to infect cells of other species. Careful assessment of any modification in the context of altered pathogenicity or host range must be made and the risks posed to the wider environment evaluated.

ii. It is also important to pay particular attention to the potential environmental hazards when handling vectors derived from different species. For example, whilst a defective oncogenic retroviral vector based on Avian leukosis virus (ALV) or a lentiviral vector based upon Feline immunodeficiency virus FIV might be generated for use in human cells, it is possible that it could be mobilised by naturally occurring retroviruses present in its natural (or a related) host.

12.4 HAZARD ASSESSMENT SUMMARY

Hazards associated with these vectors are summarised as: stable expression of transgenes, insertional mutagenesis and potential for generation of replication competent virus. Replication defective vectors that cannot infect human cells may generally be considered GM-BSL1 from the risk assessment. Factors supporting this classification will include: low risk of generation of RCV (*e.g.* a third generation packaging system), self inactivating (*SIN*) LTR and non-harmful insert. For replication defective retroviruses and lentiviruses capable of infecting human cells, even if the risk assessment demonstrates they are adequately attenuated, it is advisable to designate the activity as BSL2. Contaminated sharps represent a significant hazard and their use should be excluded for vectors that can infect human cells.

12.5 PROCEDURES AND CONTROL MEASURES

12.5.1 Control measures and monitoring procedures

i. The most likely route of accidental infection with a retrovirus will be via inadvertent percutaneous inoculation. Consequently, the direct use of sharps (*e.g.* needles, blades, glass Pasteur pipettes) for virus manipulation would be incompatible with BSL1 designation

even if risk assessment justifies for a lower risk containment activity for retroviruses and lentiviruses that are replication defective but capable of infecting human cells.

ii. Percutaneous injury is the most commonly reported route of worker exposure. Therefore the use of sharps for manipulation of retroviruses and lentiviruses increases the possibility of staff exposure to the extent that any inherent hazards associated with the vector may be realised. Where the use of sharps for work with these viruses cannot be avoided, it is incumbent upon the user to formulate a policy for the control of sharps to minimise inadvertent exposure. This should include documented training of staff for safe use and disposal and the work classified as BSL2. Hollow needles and other sharps should be used with extreme care and only when necessary. Needles should never be re-sheathed, but rather disposed of directly into an appropriate sharps waste container for autoclaving or incineration.

iii. Staff exposure can occur via other routes, which become significant where the laboratory work involves large volumes/titres of viral vectors and/or the use of aerosol generating procedures; in which case exposure via a mucosal or inhalation route needs to be carefully assessed. Where exposure via this route is deemed significant, use of a biological safety cabinet is appropriate and the work should be classified as BSL2. Where product sterility is the sole purpose for necessitating the use of a biological safety cabinet (and indeed gloves), then this should not be used as the basis for classification.

iv. Some retroviral vector systems may use helper viruses, although this approach is becoming less widely exploited in favour of virus-free helper systems. The hazards associated with any helper viruses should be considered in addition to those relating to the proposed GM virus.

v. Where possible, systems using multiple plasmids with minimum sequence homology should be used to generate vectors, thereby minimising the risk of RCV generation. Where this is not the case, it may be important to ensure that RCV do not occur in the production procedure. Direct plating of vector stock onto permissive cell lines and observing for indications of viral replication (*e.g.* syncytia formation or other cytopathic effects) could be used to detect RCV. However, these approaches do not always give a clear result and specific molecular detection methods could be employed to supplement these tests or as an alternative. For example viral protein expression could be detected by immunostaining, or *gag*, *pol* or *env* DNA proviral sequences could be detected by PCR.

vi. The risk of exposure to lentiviral/retroviral vectors in transduced cells subsequently used for other activities (*e.g.* non-fixed flow

cytometry; inoculation of animals; *in vitro* propagation; clinical gene therapy applications) is negligible where the cells do not incorporate any helper function and where residual virus titres have been reduced by replacing the potentially infectious cell supernatant medium. In those circumstances, no additional containment measures beyond those needed for the non-transduced cells are required. For experimental animals that have been inoculated with non-replicating virus, the risk of staff exposure from the infected animals is minimal as is the potential for virus shedding. It is therefore appropriate to house these animals in a GA-BSL1 facility but adhering to BSL2 safety practices. Where the animals are used for post mortem procedures, classification should be assessed on a case-by-case basis.

12.5.2 Activity Based Classification Summary

The degree of control needed, and therefore classification, should be determined by the risk assessment on a case-by-case basis. The risk of insertional mutagenesis, an inherent hazard associated with lentiviral/retroviral vectors, is difficult to quantify given the current available data. However, the potential likelihood of this hazard, along with others conferred by the transgene, being realised is increased where work involves the use of sharps to deliver the viral vector. Other specified control measures such as restricted access to authorised and trained staff, written training records and the use of appropriate PPE are deemed necessary to facilitate their control necessitating a minimum classification of GM-BSL2. A biological safety cabinet is necessary to control exposure of the staff via mucosal or inhalation routes.

CHAPTER

13

RNA VIRUSES AND REVERSE GENETICS

13.1 BACKGROUND

i. For the purposes of this guidance, the term reverse genetics is used to describe approaches whereby a cloned copy of a viral genome is manipulated and used to generate new viruses. This terminology is commonly associated with the manipulation of viruses with single-stranded RNA (ssRNA) genomes, which do not naturally have a DNA step during virus replication. The ability to manipulate double-stranded RNA viruses (*e.g. reoviruses* and *orbiviruses*) in such a way has so far proved elusive and for these reasons, the following guidance will concentrate upon ssRNA viruses. Issues relating to reverse genetics approaches are also appropriate to other virus types (*e.g. Adenoviruses* and *Retroviruses*) and aspects covered will be applicable to the manipulation of any viral system in this way., Specific guidance relating to the risk assessment of other virus systems (*e.g. adenovirus, poxvirus* and *retrovirus*) can be found in the respective sections in this Guideline.

ii. Reverse genetics approaches allow the generation of GM viruses that are precisely engineered, which has expedited the advancement of virological knowledge, vaccine design and the development of new virus-based technologies. For example, viral genes can be removed, modified or substituted in order that gene function may be elucidated and studied. Known determinants of pathogenicity can be modified or removed in order that the virus might be rationally attenuated for the purposes of vaccine development. Furthermore, viruses can be engineered to express heterologous genes (*e.g. cytokines, immunomodulators* or *antigens*) for the purposes of improved vaccine performance and GM-virus-based therapy development.

13.2 PRINCIPLES OF REVERSE GENETICS OF ssRNA VIRUSES

The ssRNA viruses can be subdivided into two main groups on the basis that their genomes are either positive-strand (i.e. the genome in its

native form is translatable) or negative-strand (i.e. viral proteins must first replicate the genome to generate a positive strand intermediate to allow gene expression). For reverse genetics approaches, this has certain ramifications. Positive-strand RNA, or ssRNA(+), viral genomes are infectious in their native state, meaning that *in vitro* transcribed viral RNA (or a cDNA copy of a viral genome under the control of a suitable promoter) can be transfected into cells to recover viable virus. Negative-strand, or ssRNA(-), viral genomes are only infectious as *ribonucleoprotein* (RNP) and must be complexed with virally-encoded nucleoprotein and polymerase molecules, either *in vitro* or within the cell, for virus to be recovered. Furthermore, ssRNA(-) viral genomes can be either segmented (*e.g.* Influenza, which has 8 viral genome segments) or non-segmented (*e.g.* Measles, which has all its genes present on one RNA molecule). The genomes of Influenza and Measles viruses are shown diagrammatically in Figure 8 and a summary of different reverse-genetics approaches can be found in Figure 9.

Some important pathogens that are significant causes of illness and mortality in humans and animals can be manipulated using reverse genetics. Circulating immunity for some of these viruses will be widespread, either as a result of natural exposure to virus or as a result of vaccination (effective vaccines are available for several viruses, e.g. Measles, Influenza and Rabies). As RNA viruses, however, their replication is dependent upon RNA-dependent RNA polymerases, which are error-prone. This can lead to antigenic drift resulting in novel quasi-species of virus that may not be susceptible to vaccine-induced immunity.

13.3 REPLICONS

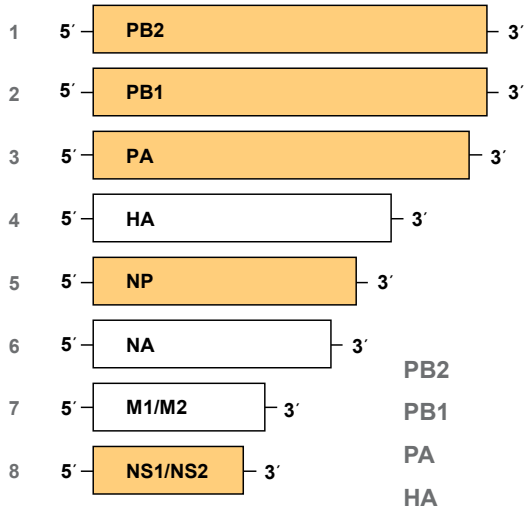
i. Some ssRNA(+) viruses may be engineered and handled as Replicons – self-replicating RNA molecules derived from viral genomes that do not give rise to viable virus. Replicons based upon human or animal viruses have been derived from the *Togaviridae (Alphaviruses)*, *Coronaviridae*, *Flaviviridae*, and *Picornaviridae* families, many of which are responsible for human and animal diseases that pose significant risks to both health and the economy (*e.g.* Dengue, SARS, FMDV). Consequently, they have been the focus of considerable study and Replicon technology has permitted virological research without the need to handle infectious material.

ii. The genomic organisation of these viruses is such that genes involved in viral RNA replication and genes encoding virion structural proteins lie in distinct regions. All Replicons to date are based on the same fundamental principle – the deletion of viral genes encoding structural proteins from the genome, resulting in an RNA molecule that is capable of replicating, but lacking the ability to package itself into a virion. Replicons are typically

NEGATIVE-STRAND RNA VIRUSES NON-SEGMENTED GENOME (MEASLES VIRUS)

N	Nucleocapsid (RNP component)
P	Phosphoprotein (RNP component)
C	Accessory protein
V	Accessory protein
M	Matrix protein (viral structural protein)
F	Fusion protein (viral entry determinant)
H	Haemagglutinin (cell binding and entry)
L	Large polymerase (RNP component)

NEGATIVE-STRAND RNA VIRUSES SEGMENTED GENOME (INFLUENZA VIRUS)



PB2	RNP component
PB1	Endonuclease/RNP component
PA	RNP component
HA	Haemagglutinin
NP	Nucleocapsid
NA	Neuraminidase
M1/M2	Matrix proteins
NS1/NS2	Anti-inflammatory proteins

Figure 8. *Diagram illustrating the representative segmented and non-segmented ssRNA(-) genomes (Measles and Influenza viruses)*

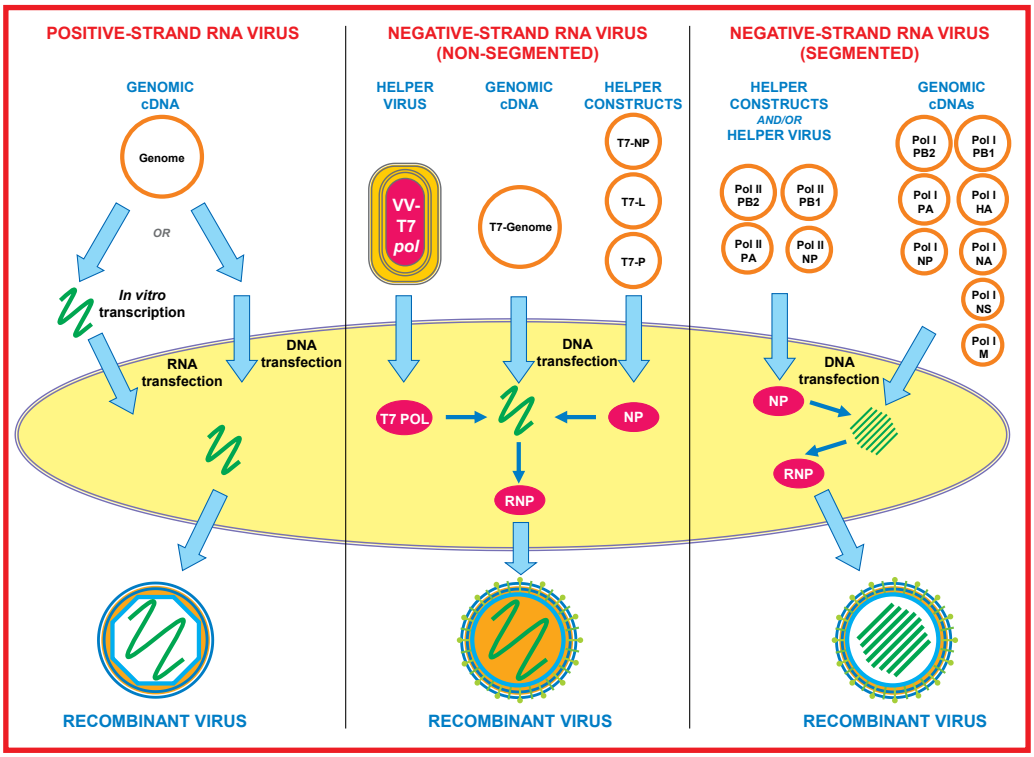


Figure 9. *Examples of methods used to recover virus using reverse genetics. The recovery of segmented negative strand RNA virus from cloned DNA is exemplified using Influenza virus as a model.*

VV – Vaccinia virus; T7pol - bacteriophage T7 polymerase gene; T7 POL – T7 polymerase; NP- nucleoprotein; RNP – ribonucleoprotein; T7 – T7 polymerase-specific promoter; Pol I – RNA polymerase I promoter; Pol II – RNA polymerase II promoter

engineered as a cDNA, transcribed in vitro and RNA transfected into cells where the RNA is translated generating viral proteins that mediate RNA replication.

iii. Replicons are, therefore, powerful research tools since the 'replication apparatus' of a virus can be studied without the need to handle infectious material, reducing the inherent hazards compared to the actual pathogen. Furthermore, Replicons can be used to assay candidate antiviral therapies and study viruses that have been difficult to culture so far (*e.g.* Hepatitis C virus).

iv. The deletion of the structural genes also affords the ability to incorporate heterologous gene expression cassettes into a Replicon. The self-amplifying nature of the Replicon makes them attractive mediators of heterologous protein production and can be used to generate stable cell lines, provided they are carrying a resistance marker. Furthermore, if the structural genes from the viral genome are provided *in trans*, Replicons can be packaged into

virions generating viable but defective GM virus vectors that can be used to deliver and express a therapeutic gene to a target cell (see Figure 10).

13.4 RISK ASSESSMENT FOR HUMAN HEALTH

13.4.1 Hazards associated with the recipient virus

i. Genetic modification using reverse genetics approaches can be applied to a wide range of virus species that are pathogens of humans and in order to set an appropriate activity class for the work, it is important to consider the hazards posed by the virus that is to be manipulated. It is therefore prudent to begin by considering the RG classification and GM_BSL containment appropriate to the wild type virus. A list of viruses that are commonly manipulated using reverse genetics methodology can be found in Table 13 and Table 14.

ii. Single-stranded RNA viruses fall into a range of RG. An appropriate BSL should be adopted as a minimum requirement when wild type viruses will be handled. Where wild type viruses cause human diseases or are classified as animal pathogens in the *Animal Act 1953 (Revision of Laws (Rectification of Animals Act 1953) Order 2006*, they should be handled using the appropriate prescribed containment measures. Organisms subject to licensing under MAQIS Act 2011 or Department of Agriculture must be handled in accordance with the licence conditions.

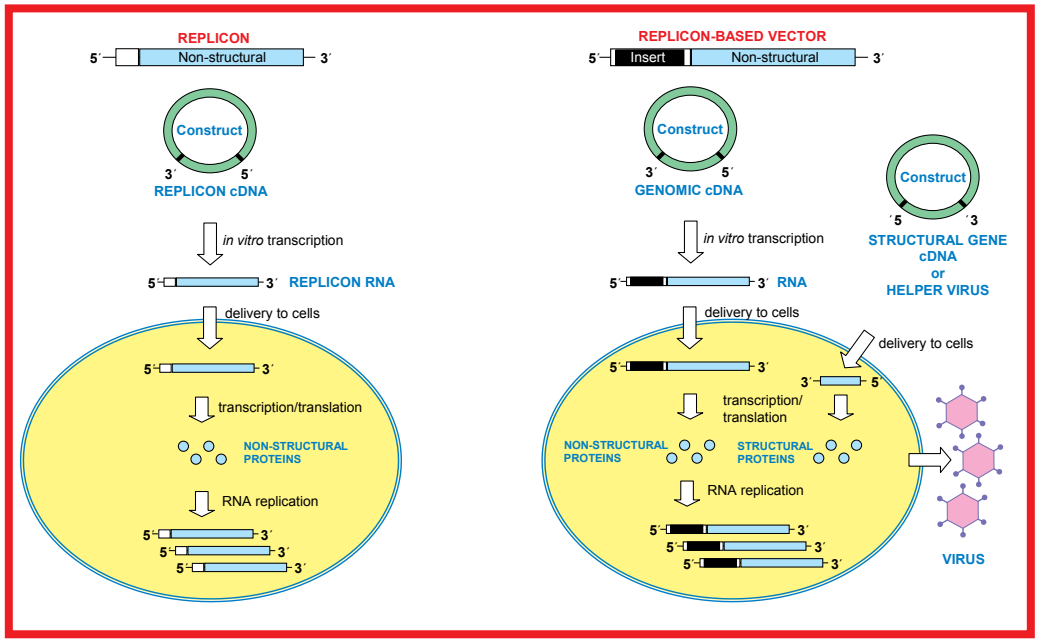


***Figure 10**. Schematic representation of Replicon and Replicon-based vector systems*

Table 13. Containment requirements and vaccine availability for Positive sranded RNA viruses commonly manipulated using reverse genetics.

Family/Genus	Species	Risk Group (RG)
POSITIVE-STRAND RNA VIRUSES		
Paramyxoviridae		
Alphaviruses	Chikungunya virus Sindbhis virus Semliki Forest virus	RG2 RG2 RG2
Flaviviridae *Flaviviruses*	Yellow fever virus Dengue 1, 2, 3, 4	RG3 RG2
Pestiviruses	Classical Swine Fever virus	RG3
Hepaciviruses	Hepatitis C virus	RG3
Picornaviridae		
Enteroviruses *Rhinoviruses*	Poliovirus Human Rhinovirus	RG2 RG2
Coronaviridae		
Coronaviruses	Human Coronavirus SARS-Coronavirus	RG2 RG3

Table 14. Containment requirements for negative strand viruses commonly manipulated using reverse genetics.

Family/Genus	Species	Risk Group (RG)
NEGATIVE-STRAND RNA VIRUSES NON-SEGMENTED GENOMES *(MONONEGAVIRALES)*		
Paramyxoviridae		
Morbilliviruses	Measles virus Nipah virus Hendra virus Canine distemper virus Rinderpest virus Peste de petits ruminants virus	RG2 RG3 RG4 RG1 RG4 RG4

Rubulaviruses	Mumps virus Newcastle Disease virus	RG2 RG2
Respiroviruses	Sendai virus	RG1
Pneumoviruses	Human Respiratory Syncytial virus Bovine Respiratory Syncytial virus	RG2 RG1
Paramyxoviruses	Human parainfluenza virus 1, 2, 3, 4	RG2
Rhabdoviridae		
Lyssavirus *Vesiculoviruses*	Rabies virus Vesicular stomatitis virus	RG3 RG2
Bornavirida		
Bornaviruses	Borna Disease virus	RG3
Filoviridae		
Filoviruses	Ebola virus Marburg virus	RG4 RG4
NEGATIVE-STRAND RNA VIRUSES SEGMENTED GENOMES		
Orthomyxoviridae		
Orthomyxoviruses	Influenza Type A, B, C Highly Pathogenic Avian Influenza V.	RG2 RG4
Bunyaviridae		
Bunyaviruses	Bunyamwera	RG2
Arenaviridae		
Arenaviruses	Lassa fever Lymphocytic choriomeningitis virus	RG4 RG3

iii. The recipient strain may not have the same characteristics as the wild type pathogen and the associated hazards may differ. For example, attenuated derivatives of human pathogens may be used as disabled vectors (*e.g.* alphavirus replicon-based vectors) or vaccine strains (*e.g.* Influenza A/PR/8, Measles virus Edmonston strain and genomically re-ordered Vesicular Stomatitis Viruses). Furthermore, reverse genetics methodology allows for the rational attenuation of a particular virus, for example, some mutations in the L gene or ablation of C and V genes in Human Parainfluenza virus 3 (hPIV-3) results in attenuation. Generally speaking, the containment measures prescribed for the wild type virus will still be applicable. However if the recipient strain is demonstrably attenuated, then the risk assessment could be used to justify a decision to lower the containment level.

iv. Many reverse genetics methods for recovering virus from cloned DNA rely solely on the transfection of cells with the appropriate nucleic acids. Some systems require the provision of viral proteins in trans and this has led to methods that have involved the use of helper viruses (*e.g.* VV that expresses the bacteriophage T7 polymerase). The hazards associated with the use of such helper viruses should be assessed separately and control measures appropriate for the handling of the helper virus should be in place, irrespective of those required by the recipient strain or the intended final GM virus.

13.4.2 Vector systems

13.4.2.1 Replicons

In situations where Replicons are being used to study viral replication without the generation of viable virus, they can be considered a much safer alternative to handling the pathogen from which they are derived and their use in this capacity should be encouraged. A researcher could, therefore, study the mechanisms of a hazardous virus at a level of containment that is lower than that which is applicable to the wild type pathogen. However, there are mechanisms by which infectious virus could be inadvertently generated. Should this occur while handling Replicons derived from dangerous or economically harmful pathogens that would ordinarily demand a high level of containment, the potential ramifications could be severe. Examples include the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and the Foot and Mouth Disease Virus (FMDV). For this reason, there is a need for thorough assessment of the risks and the implementation of control measures designed to minimise the likelihood of inadvertently releasing hazardous virus.

13.4.2.2 Replicon-based vectors

i. Viruses with ssRNA(+) genomes have restricted capacity for genetic inserts, therefore Replicons that are derived from these viruses lack structural genes and have increased coding capacity. Post-translation processing signals are required in order to generate a functional product (for example an internal ribosome entry site and/or FMDV 2A protease cleavage site; and structural genes must be provided in trans in order to generate a viable defective vector. The general principles of Replicons and Replicon-based vectors is summarised in Figure 10.

ii. These vector systems retain the general safety features of Replicons, since the virus particles generated are defective. However, hazards may arise from the properties of the inserted gene and there are mechanisms by which infectious virus could be inadvertently generated. A thorough assessment of the risks and the implementation of control measures are required to minimise the likelihood of inadvertently releasing hazardous virus.

13.4.2.3 Negative-strand RNA virus vectors

i. Viruses with ssRNA(-)also have restricted capacity as heterologous inserts need to be linked to vital viral genes and also need post-translational processing signals in order that a functional product can be generated. Non-segmented ssRNA(-) viruses, on the other hand, can tolerate the introduction of large genetic inserts, provided that the heterologous gene is flanked by the appropriate viral sequences.

   Clearly, if wild type viruses are to be modified to carry a heterologous gene, then the full containment level appropriate to wild type virus should be employed. One non-segmented ssRNA(-) virus that has been exploited as a potential vector is Sendai virus. Sendai is a murine parainfluenza virus that is apparently non-pathogenic to humans, yet can transduce human and animal cells. Clearly, such a virus poses a minimal risk to human health. However, hazards may arise due to the properties of the genetic insert and since it is an animal pathogen there may be environmental risk issues.

ii. Attenuating mutations have been engineered into Sendai virus that, while not affecting its replicative ability, serve to reduce its ability to spread within the host organism. For example, removal of the F gene, which encodes the virion surface fusion protein, results in a 'transmission' incompetent vector. The F protein must be supplied *in trans* during vector production but cannot transmit to any cells other than those initially transduced following administration to non-complementing cells or the host. Equally, deletion of the M gene that encodes the viral matrix protein results in a virus that

is defective for budding from the cells in which it is replicating, resulting in a virus that can only spread by cell-cell contact.

iii. Recipient viruses or vector strains that can be shown to pose a much-reduced risk of harm compared to the wild type might be handled at a lower containment level, where the risk assessment shows that this is justified. However hazards arising subsequently due to the insertion of sequences or phenotypic alterations might necessitate additional containment measures.

13.4.3 Hazards associated with genetic inserts

The risk assessment should take into consideration any potential adverse effects of the expressed product or any properties inherent to the inserted sequence. More detailed guidance on the hazards posed by commonly used genetic inserts can be found in Chapter 3. However, in brief, factors to consider include:

13.4.3.1 Expression characteristics

Most ssRNA viruses replicate in the cytoplasm of an infected cell (Orthomyxoviruses are a notable exception to this as they replicate in the nucleus) and gene expression involves viral mechanisms that are intrinsic to a particular virus species. The expression characteristics of a heterologous gene will usually be determined by these mechanisms and subsequently vary depending on the specific virus carrying it. Furthermore, the level to which viral genes are expressed in non-segmented ssRNA(-) viruses is influenced by their position within the genome - genes towards the 3' end of the genome are expressed at a higher level than those at the 5' end. The level to which a heterologous gene is expressed will therefore also depend upon the site of insertion within the viral genome.

13.4.3.2 Biological properties of the gene product

The expected activities or toxicity of the gene products should be assessed. For example, a bacterial toxin, oncogene or growth factor would represent greater risk of harm than a reporter gene such as EGFP or Luciferase. Properties of the gene products and the potential effects upon individual cell types that may be transduced or otherwise affected by the vector should therefore be considered.

13.4.3.3 Proviral insertion

Since most ssRNA viruses replicate in the cytoplasm using viral factors and (with the notable exception of the retroviruses) there is no genomic DNA intermediate generated, proviral insertion is not a feature of the biology of these viruses and can be expected to be extremely rare.

13.4.4 Alteration of phenotype

13.4.4.1 Tissue tropism

i. It is often desirable to alter the surface properties of a virus, either for the intentional targeting of a vector to a particular cell or tissue type, or to develop vaccine strains by displaying antigens for a pathogenic virus on the surface of another virus that is less harmful or attenuated.

ii. The structural genes of ssRNA(+) viruses can often be interchanged with those of a related virus. For example, putative vaccine strains for the flaviviruses Dengue, West Nile virus and Japanese encephalitis virus can be constructed by substituting the structural genes of the live, attenuated vaccine strain Yellow fever virus (YFV 17D) with those of the target virus. The resulting strains have the attenuated phenotype of YFV 17D but are antigenically similar to the donor viruses. Similarly, the structural genes of SARS-CoV could be used to pseudotype Human coronavirus strain 229E. Furthermore, Replicons can be encapsidated *in trans* by supplying the structural genes of a related virus that has distinct properties to those from which the Replicon itself is derived.

iii. Since the structural genes are involved in cell adhesion and virus entry, chimaeric viruses of this sort will most likely have the cell tropism and infectious characteristics of the donor virus. The effects of accidental exposure to an encapsidated Replicon is expected to be localised since it would be defective. However, if the chimaeric strain is competent and able to establish an infection, the pathology will be undefined due to the combination of factors from two distinct, albeit related viruses.

iv. Viruses with ssRNA(-) genomes have a versatile envelope structure that permits the substitution or inclusion of heterologous surface glycoproteins. For example, VSV can be modified to express the env genes of HIV, which are incorporated into the viral envelope for the purposes of eliciting protective immune responses against HIV. VSV has also been modified to incorporate CD4 and CDXR4 (the determinants of HIV entry into CD4 T cells) in order to retarget VSV to destroy HIV infected cells. Similarly, Measles virus Haemagglutinin (H) surface glycoprotein (one of the determinants of Measles virus cell entry) can be modified to incorporate peptide domains that will allow entry into otherwise refractory cells (*e.g.* Measles pseudotyped with an anti-CD38 antibody fusion fused to H could be used to target CD38-positive myeloma cells). The manipulation or exchange of other viral genes might be involved in the ability to replicate efficiently within certain cell types (*e.g.* Morbillivirus P genes) and should also be carefully assessed.

v. Altering the structural properties or genetic complement of a virus may have a bearing upon the cells and tissue types that will become susceptible to the modified virus. It is important, therefore, to consider the susceptibility of various tissues to infection and to evaluate the possible consequences of transduction and expression of the genetic cargo within cell and tissue types that would not normally be infected by the wild type virus.

13.4.4.2 Pathogenicity

i. Reverse genetics methodology is a powerful tool for the study of viral pathogenesis since genes that may have a role in virulence can be knocked-out or substituted with similar genes from a related virus with relative simplicity. Furthermore, the regulatory mechanisms controlling expression can be manipulated, for example, reordering the genes of ssRNA(-) viruses within the genome will affect the level to which a given gene is expressed. Particular attention should be paid to any modification that may increase the virulence or pathogenic phenotype of the modified virus and appropriate measures taken to ensure worker safety.

ii. It is acknowledged that, generally speaking, manipulations of this sort will attenuate rather than exacerbate the virulence of a virus. However, the possibility that the process may generate high-virulence derivatives of the virus or novel pathogens of humans (or animals) should be carefully considered. Furthermore, modifications that result in attenuation in culture may not reflect pathogenicity *in vivo* (*e.g.* Measles C and V proteins are dispensable in culture but are pathogenicity determinants *in vivo*). It should not be assumed that virulence will be, at worst, comparable to the wild type virus or a donor virus and suitable measures to protect the health of workers should be employed.

iii. For example, different morbilliviruses affect a range of different species. While Measles virus is pathogenic to humans, Canine distemper virus is not, although it is capable of infecting humans subclinically. The importation of equivalent sequences that may be involved in host range or virulence from one virus species to another could give rise to a novel human or animal pathogen and, consequently, additional containment measures may be required.

13.4.4.3 Immunogenicity

i. The immunogenic nature of the virus may be altered by the deletion of viral genes or insertion of genes encoding products with immunomodulatory activity. The ability to elicit or evade an immune response can be a key determinant in the pathogenicity of a virus and any modification that will alter these properties should be assessed with care.

ii. For example, the ability of influenza virus to spread and cause disease is, in part, due to the host immune system being naive to the virus surface glycoproteins, Haemagglutunin (HA) and Neuraminidase (N). Manipulation of the sequence of HA or N or exchanging the HA or N genes could therefore generate a novel pathogen.

iii. Some viral genes may also be involved in evading the host immune system. For example, Influenza NS1, paramyxovirus C and V and Respiratory Syncytial virus NS genes all encode proteins involved in evading the host innate inflammatory response to viral infection. Such genes are often targeted for deletion as they are dispensable for growth *in vitro* and are attenuated *in vivo* as the viruses are cleared more effectively by the host immune response. Increased immune stimulation may be desired for the purposes of generating vaccine strains and it should be considered that acute inflammation could be a feature of accidental exposure to such a virus. Likewise, insertion and expression of immunomodulatory cytokines may have similar effects. Any potential effects on the immune reaction by a modification should therefore be considered as a possible risk to human health.

13.4.4.4 Genetic stability

The replication of ssRNA virus genomes is mediated by RNA-dependent RNA polymerases, which lack proofreading functions. Replication is therefore error-prone and gives rise to quasi-species that will be distinct in sequence from the virus that was originally engineered. The ramifications of this are that attenuating mutations may be rapidly lost if they are deleterious to the virus or if reversion would give a selective advantage. Furthermore, natural changes to the sequence of influenza virus HA and N genes may generate antigenically novel viruses (antigenic drift) that may be able to evade the immune response and be pathogenic. It is important to assess whether or not a strain will remain disabled and the possibility of reversion or antigenic drift should be considered. The likelihood of reversion will depend on the mechanism of attenuation; deletion mutants are less likely to revert than point mutations or conditional lethal mutants. However, where there is a likelihood that the modified virus will revert to a pathogenic state, containment and control measures appropriate to the reverted virus should be employed.

13.4.4.5 Recombination

i. Recombination does occur between related ssRNA(+) viruses in nature and is an important mechanism for promoting genetic variation. However, this is most usually restricted to related virus groups, virus species or even serotypes. For example, poliovirus recombination with the closely related enterovirus C is

commonly observed, but not with other enterovirus groups. Nor is it seen between enterovirus groups. Recombination between Coronaviruses in nature has also been observed, occurring at 'hotspots' - areas of the viral genome that appear to be prone to recombination events.

ii. Recombination *in vitro* is a possibility if the sequence similarity is sufficient. Copy-choice 'strand-switching' during RNA replication is thought to be the major means by which recombination takes place in these viruses; the viral RNA polymerase detaches from the template during synthesis and re-associates with another RNA strand with a similar sequence before completing the transcript. A recombination event that would restore competency to a disabled vector, reverse an attenuating deletion or restore the coding capacity for capsid genes to a Replicon system pose the primary risks.

iii. Reconstitution of a viable virus from a Replicon would require the infection of Replicon - carrying cells with the wild type or a related virus. Heterologous genes would probably be lost from a Replicon-based vector where the structural gene cassette is the site of insertion. However, the possibility of recombination with transcripts derived from structural gene sequences provided *in trans* is a possibility. The resulting virus would probably have the cell tropism characteristics of the 'donor' virus and could be able to establish an infection with an undefined pathology.

iv. Recombination events do not appear to be a feature of ssRNA(-) virus biology. However homologous recombination could conceivably occur between cDNA genomic and RNP protein-expression constructs that could cause the reversion of an attenuating mutation. It is possible for the virions of non-segmented ssRNA(-) viruses to contain more than one copy of a genome without loss of infectivity. This could give rise to functional heterozygotes in cells that have been co-infected with distinct, but related viruses and the dominant pathogenic phenotype may not be attributable to the intended recombinant virus.

13.4.4.6 Reassortment

i. Reassortment of genomic segments can take place in cells coinfected with different strains of ssRNA(-) viruses with segmented genomes (*e.g.* a cell coinfected with two distinct A-type influenza viruses). This could generate a virus that is either novel antigenically or have novel pathogenic characteristics. Particular care should be taken when using helper viruses to supply viral functions during reverse genetics procedures that require them.

ii. Given the possible ramifications of a highly pathogenic virus being generated by a recombination or reassortment event, it is

important to minimise any risks by implementing suitable control measures to prevent cross-contamination.

13.5 RISK ASSESSMENT FOR THE ENVIRONMENT

13.5.1 Survivability and stability

i. The survivability of ssRNA viruses will vary depending on the species. Some viruses in this group are known to survive for some time in the environment, for example influenza viruses can persist for several hours on surfaces and can be transmitted by manual inoculation of mucosae. It is important, therefore, to consider the ability of the recombinant virus to persist and be transmitted and this will probably be comparable to the properties of the wild type or recipient strain.

ii. Consideration should also be given to the ability of the virus to be vectored away from the site of containment by humans. Some animal viruses may be able to persist within human hosts (*e.g.* the morbillivirus Canine distemper virus may subclinically infect humans) and, therefore, humans harbouring a subclinical infection could inadvertently release an animal pathogen into the environment. Appropriate control measures should be adopted to minimise the possibility of human exposure and release of the virus in this way.

iii. Viruses with ssRNA genomes are also genetically unstable and mutant quasi-species arise naturally both during *in vitro* and *in vivo* infections. Attenuating mutations may be lost and revertant viruses may become dominant, particularly if there is selective pressure. For example, a virus attenuated by serial passage in monkey kidney cells or an attenuated virus that is maintained in mammalian cells could adapt to growth in avian species if they are grown in avian cells. The possibility of such adaptation occurring should be considered and, where necessary, additional control measures be taken to prevent the exposure of susceptible species.

13.5.2 Hazards associated with genetic inserts

i. The biological properties of the expressed gene product, even if they represent a low risk to human health, may be a possible hazard to other species. It is therefore important to consider any potential adverse effects of the encoded products upon non-human species that may be affected.

ii. The possibility that the process of generating novel virus strains by reverse genetics could give rise to novel animal pathogens should be carefully assessed. For example, importing known virulence determinants from one species or strain of an animal morbillivirus to another could result in a novel chimaeric animal pathogen

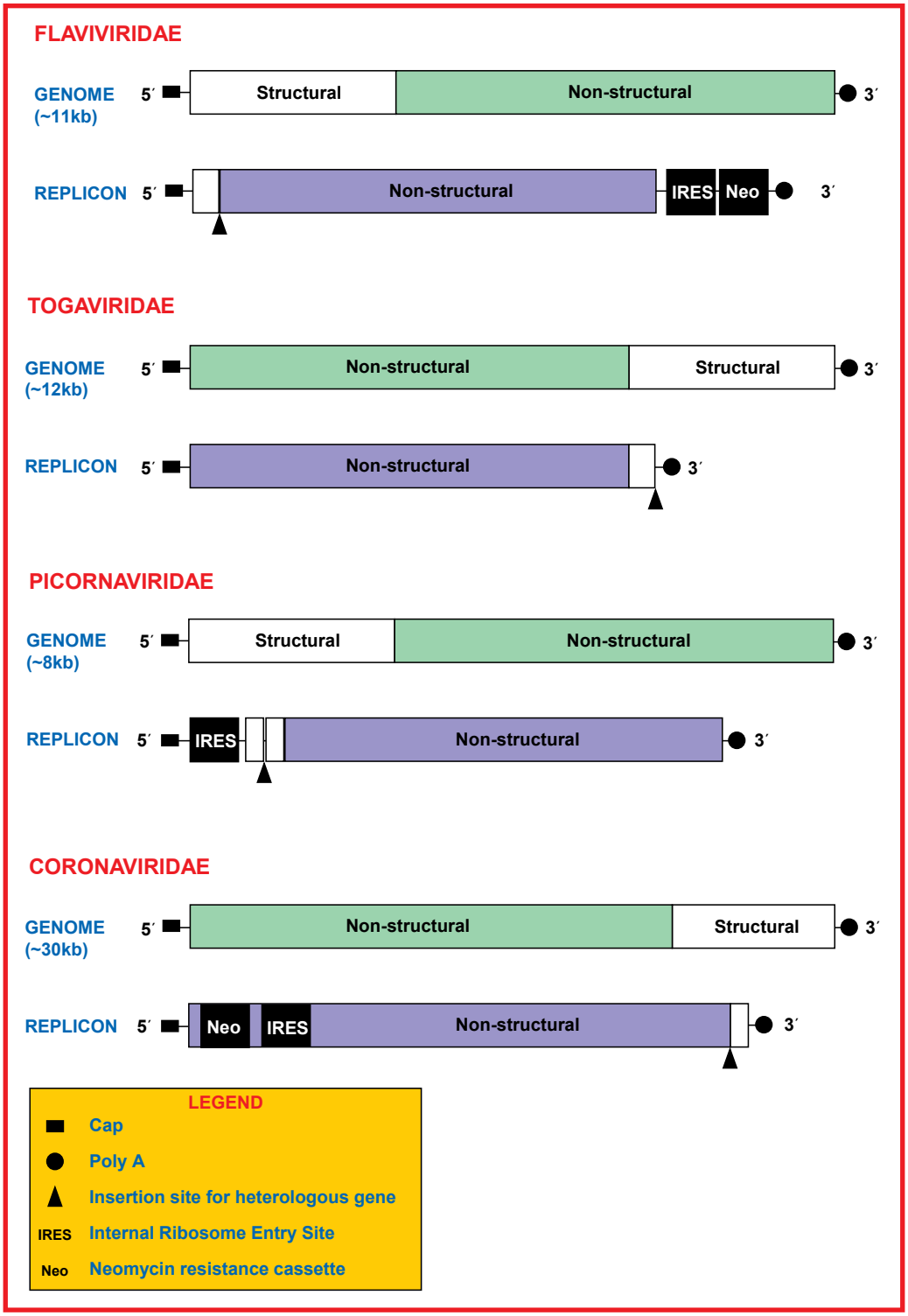


Figure 11. *Diagram illustrating the construction of 'typical' Alphavirus, Coronavirus, Flavivirus, and Picornavirus Replicons. It should be noted that not all flavivirus genomes have endogenous poly A sequences*

or a strain of the virus with enhanced pathogenicity. Similarly, pseudotyping SARS-CoV with the structural genes from a feline coronavirus could give rise to a novel pathogen of cats.

iii. Another example is that of the Influenza virus HA glycoprotein. The sequence of the HA gene in high pathogenicity and low pathogenicity influenza viruses differs. High pathogenicity viruses have a motif known as the 'polybasic' region - a series of basic aminoacids that is absent from low pathogenicity strains. Manipulations of the HA polybasic cleavage motif could, therefore, increase or decrease virulence. Furthermore, certain mutations in the influenza PB2 and NS1 genes are known to affect the efficiency of replication in certain host species. A modification that might increase the virulence of a virus should be carefully assessed and may require additional containment measures or an increase in containment level.

13.6 PROCEDURES AND CONTROL MEASURES

13.6.1 Operational considerations

i. Work with wild type human or animal pathogens must always take place in accordance with the prescribed containment measures. When working with attenuated derivatives of high-risk pathogens of humans and animals, the risk assessment can be used to justify the use of a BSL below that of the wild type pathogen. However, control measures might be required to prevent cross-contamination that could result in recombination or reassortment events that could generate wild type or novel pathogenic viruses. In essence, this means that activities involving such attenuated derivatives might require separation using temporal, physical and/or chemical means.

ii. For example, work involving GM viruses derived from high-risk pathogens (*e.g.* SARS or FMDV) in the same facility as materials that could enable the generation of a pathogenic derivative (*e.g.* plasmids containing virus sequences) must be appropriately contained or separated. If potential cross-contamination with compatible viruses cannot be reasonably prevented, the BSL appropriate to the most pathogenic donor/recipient or possible recombinant must be applied.

iii. Where the use of a separate laboratory or facility is not feasible, then separate equipment that is dedicated to specific viruses could be used. For example, separate incubators or safety cabinets could be dedicated to tasks with certain viruses or materials.

iv. Replicon cDNA and capsid-gene constructs should be stored separately. Materials containing Replicons or associated constructs should be clearly labelled to prevent accidental misuse or

contamination. In cases where the Replicons are derived from pathogens that represent a significant health or environmental risk, then dedicated freezers or storage boxes should be used and access restricted to the materials, either by the use of locks or by situating the storage facility in an area where admittance is controlled. Replicons and related viruses should not be stored together in liquid nitrogen to prevent the possibility of cross-contamination. It is advised that an up-to-date inventory is kept regarding the location and nature of the materials to prevent accidental cross-contamination and to facilitate appropriate disposal when the materials are no longer required. Waste should be segregated to prevent possible cross-contamination of Replicons and related viruses.

v. Many ssRNA viruses can be spread by aerosol, for example: Influenza virus; Measles virus, Human rhinovirus; Coronaviruses. Therefore, work involving these viruses (or viruses derived from them) may require containment within a biological safety cabinet or equivalent isolation equipment. Some viruses may be arthropod-borne (*e.g.* Dengue; West Nile virus), and while the intermediate vector may not be able to transmit the virus, transmission could occur as a result of percutaneous inoculation. The use of sharps should therefore be avoided or prohibited, commensurate with the conclusions of the risk assessment.

13.6.2 System design

13.6.2.1 Sequence manipulation

i. When working with high-risk pathogens, it is important to scrutinise the sequence and, where possible, engineer the virus so that it poses the lowest possible hazard. For example, a larger Replicon is more likely to contain more recombination 'hotspots', therefore the smallest possible viral subfragment should be used. However, the nature of the sequences it contains should be considered since the viral genes retained in the Replicon might encode certain pathogenic or virulence determinants. The known biological properties encoded by the genes that are present should be considered and, where possible, known pathogenicity or virulence genes should be deleted.

ii. It may also be possible to alter non-coding sequences to increase the biosafety of an engineered virus. For example, it might be possible to delete sequences involved in RNA packaging so that a Replicon cannot be encapsidated. Alternatively, it might be feasible to relocate cis-acting sequences required for packaging or replication purposes to an area of the genome previously occupied by structural genes. Thus, in the event that recombination takes place restoring structural gene coding capacity, the capabilities for

replication and/or packaging will be lost or impaired.

iii. The sequences of engineered viral genomes and constructs should be tailored as far as possible to minimise the likelihood of hazards being realised or to maximise attenuation. Careful manipulation of the sequence to reduce homology between those constructs can reduce the probability of recombination events and splitting helper sequences between as many constructs as possible will significantly reduce the likelihood of recombination events giving rise to an RCV or unintended virus.

13.6.2.2 Use of helper viruses.

Some reverse genetics systems have involved the use of helper viruses (e.g.VV) to supply helper functions. Where possible or practicable, the use of a biological agent should be eliminated or substituted with one that is less hazardous. Therefore, helper viruses should not be used if another system can be usefully and effectively employed. For example, some reverse genetics approaches to generating engineered influenza viruses have involved the use of a helper influenza strain. Virus-free reverse genetics systems for the production of engineered influenza exist, and a system that requires no helper functions at all *in trans* has been developed (Pol I/Pol II eight-plasmid system). It is a general requirement that the safest practicable system be employed.

13.6.3 Control measures and monitoring procedures

13.6.3.1 Vaccination

i. If the risk assessment shows that there is a risk of exposure to biological agents for which effective vaccines exist, then these should be offered if the staff is not already immune. Effective vaccines are available for several viruses that can be manipulated using reverse genetics approaches, for example Measles, Influenza and Rabies. The pros and cons of immunisation/non-immunisation should be explained when making the offer to the staff.

ii. Therefore, vaccination could be offered to protect staff handling such a virus. Vaccination should not be viewed as a primary control measure but rather as a supplementary precaution. Sufficient control measures and procedures should be implemented to minimise accidental exposure to a virus. Furthermore, a vaccine should only be used as a control measure if its ability to protect the staff has been established. For example, there is some evidence to suggest that exposure to a morbillivirus elicits cross-protective immunity within the genus. However, it should not be automatically assumed that Measles vaccine would offer protection to all morbilliviruses. If the risk assessment relies to any extent upon the immune status of a staff as a control measure, it is important that

it is stated and that the immune status is checked and verified in practice and an appropriate vaccine offered if necessary.

13.6.3.2 Health surveillance

It is an organisation's responsibility to ensure that staff health or immune status is sufficient for the proposed activity. A system for the monitoring of health and immune status should therefore be implemented where the nature of the work demands it. Periodic monitoring of immune status may be required and it may be necessary for staff to monitor their own health when working with certain virus systems. For example, co-infection of a human with different strains of influenza virus can result in reassortment generating viruses with a novel genetic (and therefore antigenic) complement. Therefore, those working with influenza reverse genetics systems that suspect they may be harbouring an influenza infection should review their suitability for work.

13.6.3.3 Animal experimentation

Staff must be sufficiently protected from the possibility of infection by inoculated experimental animals. Clearly this is important from a human health perspective with regard to working with a human pathogen, but there are also environmental considerations. Some animal viruses may be carried by humans or infect them subclinically. Therefore, staff could inadvertently release such an animal pathogen into the environment. Appropriate control measures and protective equipment should be employed to minimise the possibility that a staff handling an animal could become infected. A 'cool-off' period should be implemented, whereby staff that could be conceivably harbouring a subclinical infection with, or carrying, a potential animal pathogen should not interact with susceptible animals outside of containment for a period of time (determined based on scientific knowledge regarding the virus in question). For example, workers with FMDV are required to avoid contact with susceptible animals for three days.

SECTION

B

RISK ASSESSMENT OF GENETICALLY MODIFIED MICROORGANISMS ASSOCIATED WITH PLANTS

INTRODUCTION

CHAPTER 14

14.1 SCOPE

i. The following guidance concerns the risk assessment of activities involving GMM that are associated with plants. Plant associated microorganisms include viroids, virusoids, viruses, bacteria, fungi, protozoa and algae that have a benign, beneficial or symbiotic association with plants such as certain Rhizobium species and microorganisms known to be pathogens. Furthermore, microorganisms that have been engineered for use as biological control agents are also covered by this guidance.

ii. The use of genetic modification has permitted the study of interactions between microorganisms and their host plants. This includes research into the mechanisms of pathogenesis, symbiosis and mutualism and the elucidation of plant gene functions. GM plant viruses in particular, have been exploited for both research and biotechnology applications. This is largely because transformation is only possible in a restricted number of plant species. However, plant viruses can be used to inoculate a wide range of plant species and host range can be altered. Furthermore, the use of plant virus vectors in this way overcomes the problem of position effect variegation, which occurs in GM plants that are modified by transformation.

iii. For example, GM plant viruses can be used in the study of plant functional genetics by exploiting post-transcriptional gene silencing. Inoculation of a virus vector carrying a copy of the gene to be silenced triggers plant RNA-mediated defence mechanisms that counter viral threats resulting in the silencing of both the vectored gene and the cellular equivalent. This system has been dubbed virus-induced gene silencing (VIGS). GM plant viruses have also been heavily exploited for biotechnological purposes. GM plant viruses have been used to transform plants for the purposes of metabolic engineering and the expression of foreign genes, such as antigens for vaccine production and novel therapeutic products.

iv. For most activities involving the genetic modification of plant associated GMM, the primary considerations of the risk assessment will be given to the effects the GMM may have on plant species in the environment should there be any unintended release. This is likely to be the case for genetic modification activities relating to the study of microbe–host interactions and plant functional genetics, as the potential ramifications for human health will be negligible. However, human health implications will require greater emphasis where activities involve genes that encode biologically active products, or products that may be toxic or allergenic. Therefore, the potential environmental impact of any GMM that can infect or interact with a plant or otherwise impact upon any environmental ecosystem (including microbial populations) will require careful assessment and control. However, it is important not to overlook the possible effects on staff or other humans who may be exposed.

v. Each part of the risk assessment will involve the following steps:
   - hazard identification;
   - assessment of likelihood of hazards being realised, including an assessment of the relative fitness of the GMM;
   - assessment of the consequences of hazards being realised;
   - determination of risk that hazards will be realised; and
   - assignment of BSL.

vi. The risk assessment process should also include a consideration of the nature of the work and a review of the procedures, with additional control measures implemented where necessary. From this, the minimum containment requirements will be evident and a GM-BSL activity class must be set. This will determine the notification requirements for the work.

vii. The scientific knowledge and experiences gained from monitoring will complement the risk assessment process. Thus, the results of monitoring provide opportunities to update the risk assessment continually in the light of any new knowledge.

14.2 RISK ASSESSMENT FOR THE ENVIRONMENT

The objective of the risk assessment is to determine the likelihood and the possible consequences of an unintentional release of a GMM from containment into the environment. In a properly maintained and managed facility with the correct containment measures in place, the likelihood of such a release will be low. However, it is important to identify all possible hazards and consider any routes by which the GMM could be released (including waste disposal, equipment failure and spread by humans).

The risk assessment should consider both the environment surrounding the containment facility as well as the wider environment, especially if

there is a possibility that the GMM could survive and disseminate.

Mechanisms by which the GMM might pose a hazard to the environment.

During the risk assessment process, the factors to consider will include:

- risks associated with the recipient microorganism. This will be particularly relevant where the organism being modified is a plant pathogen or is not indigenous to Malaysia and could disrupt microbial ecological balance.
- risks associated with the inserted gene or element. This will be particularly relevant if the insert encodes a toxic product and could have adverse effects on animals, plant and soil ecology.
- risks arising from the alteration of existing traits. This concerns the effects of the modification and will centre upon changes to the survivability and interactions with the host plant or other environmental organisms.

14.2.1 Risks associated with the recipient organism

i. The characteristics of the recipient strain that will be of relevance to the final GMM include pathogenicity, virulence, infectivity, toxicity, symbiosis, ability to colonise and ability to compete with indigenous microbes. If the recipient organism is pathogenic or mutualistic, then the GMM may also exhibit the same features, albeit potentially altered by the modification.

ii. Particular care must be given to the assessment of work with pathogens that infect plants that are indigenous to Malaysia. Clearly there may be major economic risks to consider if work is undertaken on pathogens of plants that are grown commercially. Similarly, work on pathogens that infect indigenous plants or those grown ornamentally may also pose significant hazards to the environment.

iii. In the event of a release, there is potentially a fine balance between the reduced pathogenicity of an attenuated pathogen and the ability to contain an outbreak of a virulent one. Clearly, if the host organism is present in the receiving environment, then an attenuated strain should be used if possible or otherwise practicable, as this will reduce the impact of pathological effects in the event of a release. Should a virulent microorganism be used, then careful consideration should be given to the possibility that the pathogen may persist in the environment.

    A pathogen with increased virulence that causes severe disease might fail to persist, as the disease will be 'self-limiting' due to local 'fade-out' of the host plant population. Conversely, a less virulent strain might be more able to persist and therefore spread further.

If a virulent pathogen is to be constructed or used, then this should be fully justified by the risk assessment and suitable controls implemented. These activities carry with them the risk of serious environmental impact and effects upon population structure and density of the host organism, as well as impact upon the wider ecology. Such considerations need to be carefully weighed and all hazards, including the possibility of severe disease and persistence, should be fully accounted for in the risk assessment.

iv. Where appropriate, a number of modification strategies that can be employed to disable a plant pathogen or study mechanisms of host interactions more safely. These approaches include:

- deletion or mutation of genes that are essential for growth or replication;
- deletion or mutation of genes involved in pathogenesis;
- eliminate intermediate vector transmission by using non-transmissible isolates altering/removing sequences required; and
- study molecular mechanisms without using whole pathogen. For instance study self-propagating viral RNAs (*replicons*).

v. The origin and mechanism of such attenuation should be well understood and will form an important part of the risk assessment. In assessing whether a GM plant pathogen is adequately disabled, the possibility of reversion or complementation should be considered. Furthermore, it should be confirmed that the GMM is disabled, or remains so, after modification.

vi. The stability of the genetic modification should also be considered, particularly where there is the possibility that an attenuated or disabled GMM might revert to a wild type or pathogenic phenotype and become an environmental hazard. The likelihood of reversion will be dependent upon the mechanism of attenuation; deletion mutants are less likely to revert than point mutations or conditional lethal mutants. Therefore, the genetic stability of the modification is linked to phenotypic stability, especially where the modification restricts the GMM's ability to survive and to spread.

vii. An organism with a restricted capacity to survive will be under stress in the environment and there will be a strong selection pressure for the reversion of attenuating and disabling genetic lesions. The possibility that a GMM will be genetically unstable outside of the controlled conditions in which it was intended to exist should be taken into account and consideration given to any detrimental effects this might cause. In particular, careful consideration should be given to the use of disabled GM plant viruses in conjunction with transgenic plants engineered to complement the genes which are deleted from the viral genome (thus effectively using a helper plant). Such an approach could be

used to generate disabled virus vectors, providing an enhanced measure of biological containment. This approach may, however, lead to a selective pressure for recombinant viruses to reacquire the essential genes from the transgenic plant.

viii. Survivability of the organism will be a key attribute. If an organism is not capable of surviving for significant periods in the environment, as may be the case for many of the disabled organisms used in containment, then none of the other hazard areas are likely to come into play. In many cases, a disabled GMM can probably be considered safe from an environmental standpoint as they are biologically, if not physically, contained. Conversely, if an organism can survive and perhaps disseminate in the environment, then other possible hazards should be considered. This means that alterations in pathogenicity, possible adverse effects of any inserted gene products will also need to be considered.

ix. When assessing whether organism GMM might survive in the environment, it should be remembered that this includes all types of association with living organisms, as well as the possibility of persisting in soil, water or other sites.

14.2.2 Risks associated with inserted the genes

i. GMM might be a hazard to the environment by virtue of the properties inherent to the genetic insert, even if the recipient microorganism poses no specific risk. For instance, the products of the inserted sequences may have the desired effect in the intended experimental system but nevertheless kill or be detrimental for environmental plant, animal or microbial species. This is particularly relevant for GMM that could infect plants and express the inserted gene within plant tissues.

ii. Careful assessment will also be required for recipient microorganisms that can remain viable outside of a plant host and secrete potentially hazardous products into soil or water. It is important to consider any potentially harmful (or beneficial) effects that a GMM could have on microorganisms in the soil environment. For instance, a soil-borne bacterium expressing and secreting anti-fungal compounds could kill mycorrhizal fungi if it escaped and became established. Similarly a plant infected with a GMM encoding a product that could disrupt mechanisms of mutualism could harm the ecology.

iii. It is also important to assess the potential for an encoded product to cause adverse effects in animal populations. These considerations primarily apply to those genes encoding products with biological activities, particularly if they are novel and not normally found in plants. Examples of such genes would include those encoding industrial, pharmaceutical, immunogenic, toxic or allergenic products, such as antigens from human or animal pathogens

expressed for vaccine development. Such products could have adverse effects on humans and animals in the environment. In particular, if an infectious GMM could lead to expression of a gene encoding a toxic product in a plant eaten for food by animals, then populations might be reduced.

iv. It is important to consider the properties inherent to the products of a heterologous gene insert in conjunction with the expected characteristics of expression. For instance, the gene product might be allergenic or toxic to animals. If the gene is expressed in the leaves or edible parts of an infected plant, then an adverse effect due to contact with or ingestion by animals or humans might be possible. Should the expression of that product be restricted to root tissue, then the potential risks posed to grazing animals might be reduced. However, toxic products secreted by root systems or micorrhizae might have adverse effects on soil microbial populations, symbiotic organisms and plant health. The non-coding regulatory regions and signal sequences present in the insert will affect the characteristics of expression. It is important that the effects of these are considered in addition to the biological activity of the expressed product.

v. Inserted genes might encode products with no specific activity, but nevertheless have a potentially harmful action within the GMM or due to interactions with the host. For instance, an inserted gene could encode a pathogenicity or virulence determinant. This could exacerbate a potentially harmful phenotype of a plant pest or confer pathogenicity on an organism that is otherwise harmless (see Part 2.3: *Risks arising from the alteration of existing traits* below). Furthermore, the insertion of an essential gene from the host plant into a GM virus vector can cause the modified virus to have harmful effects due to post-transcriptional gene silencing. If the virus is carrying an essential gene, this could have adverse effects on the growth of infected plants, overcome inherent resistance mechanisms or alter environmental tolerances.

14.2.3 Risks arising from the alteration of existing traits

i. The modification may lead to adverse effects arising as the result of alteration of existing traits. This could represent an exacerbation of a pathogenic phenotype or disruption of a mechanism that is beneficial to plant, animal or microbial populations. This may arise as the result of the product of inserted gene acting alone (see *Part 2.2: Risks associated with genetic inserts* above) or in combination with other microbial determinants. Alternatively it is possible that modification of normal microbial genes may also alter pathogenicity. In identifying any hazards associated with the modification to a microorganism, the following points should be considered (the list is not exhaustive):

a) The modification alters survivability or stability

A key question will be whether the modification could alter the GMM's ability to survive in the environment and this will affect whether or not other potential risk factors will come into play. Organisms will have varying degrees of survivability. However, modifications may impact upon tolerances to UV, temperature fluctuations and dehydration.

b) The modification alters infectivity or pathogenicity

Consideration should be given to modifications that might affect the pathogenic mechanisms of a GMM. For instance, the insertion of a known pathogenicity or virulence determinant into a microorganism might increase the potential for that organism to cause harm in the event of environmental exposure. Special consideration should be given to the insertion of genes encoding products involved in pathogenesis into microorganism that are not normally harmful.

There are many possible mechanisms by which the inherent pathogenicity of the host organism can be affected and these may not be directly related to the harmful properties of the encoded products. Unforeseen effects may also be observed while making seemingly innocuous alterations to the genes of the organism. This is particularly relevant to complex systems such as bacteria where genes are often part of a cluster or encode a component of a regulatory network. Fungal gene regulation systems are also complex, but are poorly understood compared to bacteria. The modification or deletion of one gene may have ramifications beyond the loss or alteration of the known functions of the encoded products. The expression of other genes may be affected and biosynthetic or signalling pathways may be disrupted, resulting in altered traits.

c) The modification affects host plant defence mechanisms

The modification of genes that are involved in subverting host defence mechanisms might affect the susceptibility of plants to infection, constituting an alteration in pathogenesis. For instance, products that are secreted by bacteria can be important determinants of pathogenesis in bacteria and may suppress plant defence mechanisms.

d) The modification alters tissue tropism or host range

Modifications that could alter the types of plant tissue affected, or alter host range will require careful consideration. There are many factors that might change the natural tropism or host range of a microorganism. Pathogenic bacteria may also have determinants that affect host range or the ability

to colonise certain sites. During the risk assessment, careful consideration should be given to the possible effects on tissues or host plants not normally affected or colonised by the recipient organism and whether the normal route of transmission of the organism has been altered. It is recognised that the consequences of changes in tropism or host range are difficult to predict. In assessing the risk of manipulations designed to modify tropism, particularly in the case of replication competent viruses, it should be assumed that they would require higher level of containment as compared to the recipient strain until the properties of the GMM are better understood.

e) The modification alters transmissibility

A clear distinction should be drawn between the movement of a microorganism within a plant, and transmission between plants. Both may present a hazard, although the risk assessment of the two scenarios may be very different.

14.2.4 Transfer of harmful sequences between organisms

i. In general, the insertion of gene sequences that are known to facilitate the migration of plant-associated microorganism within a host will potentially create a GMM that is more harmful. Careful consideration should also be give to modifying sequences that will affect the transmission between plants, for example, the DAG motif (aspartic acid-alanine-glycine) in potyvirus capsid proteins. Generally speaking, modifications that are expected to confer additional transmissibility functions should be assumed to result in a GMM that is more hazardous.

ii. There are many mechanisms by which sequences may be transferred between organisms and the factors that affect the frequency of such events and the likelihood of a harmful consequence are complex. Such issues must be carefully considered in the risk assessment. It is important to consider the potentially harmful consequences of sequences inserted into a GMM being transferred to other organisms, or that the GMM itself may acquire sequences that might result in adverse effects in the environment.

iii. With the notable exception of viruses, the transfer of genetic information present on the genomes of microorganisms is much less likely than if they are present on an episomal form, such as a plasmid or cosmid. The frequencies of successful horizontal gene transfer in the environment are low, even for genes located on plasmids. However, there is a finite possibility that any gene may be transferred, even if the mechanism is just a passive one involving release of DNA from senescing cells. Therefore, the primary

consideration needs to concentrate on the possible consequences, rather than on the likelihood of transfer.

iv. The survival of a GMM in the environment, either independently or in association with a plant host, may affect the likelihood of nucleic acid sequence transfer to another organism. Consideration should be given to the possibility that there could be selective pressure in the environment that might contribute to the persistence of a sequence or gene and its acquisition by an organism. There are a number of mechanisms whereby sequences could be transferred or acquired. The possibility that one or more of the following mechanisms might contribute to a potentially harmful sequence being acquired by another organism should be considered:

14.2.4.1 Sequence mobilisation in bacteria

This is particularly pertinent to sequences that are present in a mobilisible or episomal form, such as a bacterial plasmid. Sequences present on bacterial chromosomes are less likely to be transferred.

14.2.4.2 Introduction of sequences into plant cells

Transformation of plants with *Agrobacterium* results in stable integration of genetic material into plant chromosomes. The genomes of some DNA plant viruses can also become inserted into plant genomic DNA.

14.2.4.3 Recombination between related viruses

While the phenotype of the GM virus that is under construction is the primary consideration, some thought should also be given to the possibility that harmful sequences may be transferred as the result of a recombination event. Recombination between plant viruses is common and could lead to persistence of an inserted sequence in a replication competent virus. For example, recombination is observed in geminiviruses and has been correlated with enhanced pathogenicity. Interspecies hybrids will often result in a less virulent virus but some may be more virulent than their progenitors. If a recombination event could give rise to a harmful derivative of a GM plant virus by restoring previously deleted or mutated genes, then great care should be taken to prevent cross-contamination in the laboratory or plant growth areas.

14.2.4.4 Reassortment between segmented plant viruses

Some viruses have segmented genomes and can achieve genetic variability in nature by 'swapping segments' with related viruses. It is important to consider that cross-contamination in the laboratory or co-infection of the GMM with a wild type virus in the environment could result in the generation of novel strains that could be regarded as harmful.

14.2.5 Phenotypic and genetic stability

The stability of the genetic modification should also be considered, particularly where there is the possibility that a GMM attenuated or disabled for growth might revert to a wild type or pathogenic phenotype and become an environmental hazard. Therefore, the genetic stability of the modification may be linked to phenotypic stability, especially where the modification restricts the GMM's ability to survive and to spread.

The loss of an inserted gene from a GMM is unlikely to constitute a hazard. However, inherent genetic instability leading to incorporation of genes elsewhere in the genome of the same GMM could be hazardous. An organism with a restricted capacity to survive will be under stress in the environment and there will be a strong selection pressure for the reversion of attenuating and disabling genetic lesions. The possibility that a GMM will be genetically unstable outside of the controlled conditions in which it was intended to exist should be taken into account and consideration given to any detrimental effects this might cause.

14.3 LIKELIHOOD THAT THE GMM WILL BE A RISK TO THE ENVIRONMENT

i. The initial stages in the risk assessment process thus far involve identifying those features of the GMM that have the potential to cause harm and the mechanisms by which these hazards could be realised. While it may be possible to draw up theoretical scenarios whereby the GMM may be hazardous to the environment, the chances of them being realised should be evaluated and understood.

ii. It is therefore important to consider the likelihood that the identified hazards will be manifested. Factors that come into play are: (i) judgement of the overall fitness of the GMM; (ii) the probability that rare events may occur (*e.g.* the likelihood of gene transfer); and (iii) the severity of the possible consequences.

iii. Estimating the likelihood of a harmful consequence being realised will be difficult where there is no firm data on which to base a judgement. In general, the weight given to information used in these considerations should reflect the quality of the supporting data. Where the likelihood of harm is poorly understood, a cautious approach is recommended until evidence to the contrary has been obtained.

14.3.1 Assessment of likelihood

i. A key factor in whether or not the hazard will be realised is the environment into which the GMM would be released. It is therefore important to consider the nature of the GMM in relation

to the receiving environment. There may be characteristics of the receiving environment that will contribute to the likelihood of the hazard being manifested, for example the presence of a suitable host species or soil conditions. For the purposes of using the risk estimation matrix, likelihood can be expressed as 'highly likely', 'likely', 'unlikely' or 'highly unlikely'.

ii. Even if the GMM could conceivably survive, become established and disseminate in the environment, it may be that the environment itself would not be able to support it. For example, GMM derived from pathogens of plants that are not present in Malaysia would have limited capacity to become disseminated, even if it could survive for extended periods. Similarly, the transmission of some pathogens may require an intermediate vector that might not be present in Malaysia. Where possible, unknown hosts or intermediate vectors should be accounted for, as should the longer-term possibility that such hosts and vectors will become native to Malaysia, for example, as a result of climate change. However, in general, the risk that such GMM could be a hazard to the environment will be 'low' or 'negligible'.

14.3.2 Consideration of the ability of the GMM to become established

i. An assessment should be made as to the ability of the GMM to become established, how efficient it will be and its ability to spread within a host, population or ecosystem. This represents an evaluation of the 'fitness' of a GMM and should be based upon available scientific knowledge. Any uncertainty should be taken into consideration in the risk assessment and the precautionary principle followed.

ii. The concept of fitness is difficult to define but will clearly be important in assessing the potential for a GMM to cause harm if there were to be a breach of containment. For instance, over-expression of a toxin in a bacteria or fungus may make the GMM more hazardous than the recipient strain, but the over-expression of that toxin might be deleterious to the metabolism of the organism.

iii. An example relating to fitness has been demonstrated with a number of GMM systems, as there is a tendency for inserted sequences to be deleted. The loss of a gene that confers environmental tolerances would therefore reduce the potential for spread and render the virus less fit. However, extra gene carriage should not automatically be presumed to reduce GMM fitness.

14.3.3 Consideration of the probability that rare events will occur

i. It is often possible to assign a frequency to a given event, for example, mutation, recombination or sequence mobilisation rates. Often, this can take the form of a precise numerical frequency obtained in-house or through published data. In many cases, precise evaluation will not be possible or properly supported. An approximate, semi-quantitative or descriptive assessment of the frequency, based upon experience with similar GMM or techniques, could be used in these cases. For example, the likelihood of an attenuated or disabled GMM reverting to wild type status can be assessed on the basis of the number of discrete events that would need to take place, i.e. the more events needed, the less likely it is that reversion will occur.

ii. However, it should not be assumed that failure to observe an event is evidence that it does not occur. As part of such considerations, it should be recognised that microorganisms often have extremely short generation times and adapt to specific environments and selective pressures rapidly.

iii. Mutant genomes are continually being generated and the effects of selection pressures should be assessed. For example, although variants will be often be maintained at low frequencies by negative selection, in a situation where a microorganism can replicate in an environment that differs from that in which it is normally found, the probability of one of the genetic variants becoming dominant will be increased. When undertaking risk assessments of GMM, it is important to have some awareness of this genetic variability. Even if the GMM that is initially constructed is not well adapted to growth in a particular environment or host, there is a possibility that it will adapt as new variants arise. Therefore, it is necessary to proceed with caution and use defective recipient strains wherever possible. This will virtually eliminate problems arising from genetic variability.

iv. When estimating the probability and frequency of events, consideration should also be given to the number of organisms that might be involved in the incident. This will depend on the nature of the experiment. However the probability that a hazard will be realised will often depend on the number of GMM that are being handled and, consequently, the number that could escape.

14.3.4 Assessment of the possible consequences

i. After the likelihood of all hazards is assessed, the consequence of each hazard should be estimated. Again, the consequence will depend to a very large extent on the potential receiving environment. In particular, the presence of compatible host plants

or species with which the GMM may be able to compete will be an important consideration.

ii. Evaluation of the magnitude of potential consequence is difficult since there is inevitably a degree of judgement involved, although a qualitative appraisal of the impact on other species or ecosystems should be possible. For the purposes of using the risk estimation matrix in Table 15, consequences could be described as being 'major', 'intermediate', 'minor', or 'marginal'. The following descriptions may help:

Major consequence: a major change in the numbers of one or more species leading to negative effects on the functioning of the ecosystem and/or other connected ecosystems (*e.g.* significantly altering the turnover of biomass or supply of nutrients to crops). It is unlikely that the changes would be easily reversible.

Marginal consequence: minimal or no measurable change in any population e.g. plant, animal or microbial, in the environment or in any ecosystem function. (This does not preclude some fluctuation in indigenous populations as long as this is within the range of that which could be expected naturally).

iii. It should be borne in mind that even if the consequences of a hazard being realised are deemed 'major', if the probability of the hazard being manifested at all was 'highly unlikely' then there is 'moderate' risk of harm. Likewise if the consequence of a hazard were 'marginal' or 'minor', then even if the probability of its manifestation were 'highly likely' the risk of harm would still be 'negligible' (see Table 15).

iv. However, a cautious approach to risk estimation is advised. In situations where the probability of the hazard being manifested was 'highly unlikely', should there be a 'major' consequence to the identified hazard, then more stringent containment than would otherwise be appropriate for a 'negligible' risk of harm might be prudent. A balanced view of the risks is therefore required.

Table 15: Risk estimation matrix

		RISK ESTIMATE			
Likelihood of Hazard	4 Highly likely	Low	Moderate	High	High
	3 Likely	Low	Low	Moderate	High
	2 Unlikely	Negligible	Low	Moderate	Moderate
	1 Highly unlikely	Negligible	Negligible	Low	Moderate
		Marginal	Minor	Intermediate	Major
		Consequence of Hazard			

14.4 ESTIMATION OF RISK

The risk estimation matrix in Table 15 can be used to estimate the level of risk. This matrix is provided as a tool and is not intended to be a definitive measure of risk.

14.5 CONTAINMENT LEVEL NEEDED TO PROTECT AGAINST HARM TO THE ENVIRONMENT

i. It may be necessary to evaluate whether any specific control measures are required to adequately protect the environment. Containment measures should be applied until the risk of harm is 'negligible'.

ii. The main points of concern that should be reviewed are the following:

- Cross-contamination between compartments or growth chambers. i.e. movement of GM material from one compartment or growth chamber to another compartment or growth chamber
- Unintended release of GM material to the environment
- Cross contamination of plant pathogens (pests and disease) between compartments or growth chambers
- Unintended release of plant pathogens (arthropod vectors, microorganisms) to the environment (endemic organisms or quarantine organisms)

To evaluate these concerns, an analysis of the microorganisms (GMM or wild type) intended to be used is carried out as well as features of the facility, waste management and staff training that could mitigate or aggravate potential risks.

iii. It is recommended that the minimum GM-BSL that is necessary to protect the environment be set. At this stage, it is only an estimate of the containment measures that will be required solely for the purpose of preventing release of the GMM or to minimise the likelihood that it will become a threat to the environment. Factors that may be relevant to this include:

- containment measures required by any other licenses (such as by the Department of Agriculture) needed for work on the recipient microorganism where it is an unmodified plant pathogen.
- any identified hazards arising as a consequence of the genetic modification, the severity of any harmful consequences and the likelihood that they might occur (estimation of the risk of harm, see Part 4).

iv. If there are no prescribed containment measures for the recipient organism, then a judgement should be made about whether the GMM will be a risk to the environment. If all risks are deemed to be 'low' or 'negligible' then no specific measures will be required. However, if any risk exceeds this level then control measures should be implemented such that the risk of harm to the environment is reduced to 'low' or 'negligible'.

v. Users should judge which measures listed in the appropriate containment measures in the *Biosafety guidelines for Contained use of LMO, 2010*, are appropriate for containment of the GMM. The BSL can be set accordingly to safeguard the environment. It is recognised that there is a degree of judgement required in setting 'risk values' and containment measures.

14.6 RISK ASSESSMENT FOR HUMAN HEALTH

It is recognised that for many activities with GMM associated with plants, the risk to humans will automatically be 'low' or 'negligible'. The objective is to identify any potential hazards to human health and then to assess the likelihood and potential severity of the consequences, should the hazards be realised. Where a hazard is identified, this will most likely be associated with modifications that result in production of a toxin or allergen.

14.6.1 Mechanisms by which the LMO could be a risk to human health

i. Similar to environmental risk assessment, the risk assessment process must include considerations of potentially harmful or adverse effects upon human health that would be mediated by the recipient organism, the products of any inserted genes or the predicted properties of the final GMM. However, assessments should concentrate on hazards arising from modification, rather than those associated with the recipient organism.

ii. The majority of human health hazards will most likely arise where toxic products are secreted by a GMM. Alternatively, hazards may arise as a result of modifications that alter properties of an infected plant. Using a GMM as a vector in plants that express biologically active compounds might make them more toxic or allergenic.

iii. Where a potential for harm to humans is identified, consideration should be given to whether direct contact with GMM-contaminated material, or with transduced plant materials (*e.g*. leaves, sap or pollen) might represent a hazard. Consideration may also be needed to be given to the potential for the products to be expressed in different plant tissues, the consequent routes of exposure and the possibility that these may be altered.

iv. Consideration should also be given to the possibility that microbial or plant post-translational processing may differ from mammalian cells. Therefore, potentially toxic or allergenic human or animal products expressed in microbial or plant systems might be processed differently and there may be unexpected effects due to presentation of novel confirmations.

14.6.2 Likelihood that the GMM will be a risk to human health

i. For each identified hazard, an estimation of the likelihood of it being manifested and the seriousness of the consequence should be made in a similar way to the assessment of environmental risks outlined above. The GMM may have characteristics that might lead to a potential health hazard, but the chances of them being realised should be evaluated and understood. The risk estimation matrix can be used as a tool to evaluate the magnitude of the hazards. This will require an estimation of both the likelihood and consequence of exposure. This matrix is not intended to be a definitive measure of risk and the specifics of each case should be carefully considered.

ii. Once again, estimating the likelihood of a harmful consequence being realised will be difficult where there is no firm data on which to base a judgement and the weight given to information should reflect the quality of the supporting data. Where the likelihood of harm is poorly understood, a precautionary approach is recommended until evidence to the contrary has been obtained. For the purposes of using the risk estimation matrix, likelihood can be expressed as 'highly likely', 'likely', 'unlikely' or 'highly unlikely'.

iii. Similarly, evaluation of the magnitude of potential consequence may be difficult as it is inevitable that this will involve a degree of judgement. However, a qualitative appraisal of the impact on humans should be possible. For the purposes of using the risk estimation matrix, consequences could be described as being 'major', 'intermediate', 'minor', or 'marginal'.

14.7 BIOSAFETY LEVEL NEEDED TO SUFFICIENTLY PROTECT HUMAN HEALTH

i. It is recommended that the minimum GM-BSL containment level that is necessary to protect human health be set. At this stage, it is only an estimate of the containment measures that will be required solely for the purpose of safeguarding the well-being of those who may come into contact with the GMM.

ii. The measures implemented for environmental protection may be adequate to protect human health. In many cases, the principles of good occupational safety and hygiene and good microbiological practice will also be sufficient for this purpose. However, it may

be necessary to evaluate whether any specific control measures are required to protect human health. If necessary, containment measures should be applied until the risk of harm is 'negligible'

iii. Users should apply the practices listed in the "*Biosafety Guidelines for Contained Use Activity of LMO, 2010*" required to minimise harm to staff exposed to the GMM. The BSL can be set accordingly.

14.8 REVIEW OF PROCEDURES AND CONTROL MEASURES

i. The requirements of the final BSL must be sufficient to control all the potential harmful properties of the GMM and offer sufficient protection for both the environment and human health. All risks must be reduced to 'low' or 'negligible'. The containment and control measures identified so far for environmental and human health protection only broadly define those needed as a function of the properties of the GMM itself.

ii. The nature of the activity will also affect the level of risk. Therefore, it is important to take into account the nature of the work or any non-standard operations that might increase the likelihood of release or risk of exposure. For example, large-scale growth or harvest of a GMM will often mean that large amounts of the GMM will be handled, which may result in increased likelihood of release and/or exposure.

iii. If any such operations or activities are likely to generate risks that are not accounted for in the minimum containment measures (already applied in reaction to the risk assessments for the environment and human health), then additional control measures should be applied. Equally, it may be that as a result of the nature of the activity, the nature of a risk that is inherent to the GMM itself is diminished. For example, if GMM are cultured in a sealed system, then exposure to staff might be much less likely. In these cases, certain control measures might not be required.

iv. The principal investigator or laboratory supervisor responsible for the work should be satisfied that the local rules covering the use of laboratories or plant growth facilities are in line with regulatory requirements under the Biosafety Act and are adequate to minimise or prevent viable GMM being released from the containment facility. Moreover there should be a programme of internal inspections by IBC and/or active monitoring to ensure that the local rules are satisfactorily implemented.

14.9 ASSIGNMENT OF BSL FOR GMM CONTAINMENT

i. An Activity Class must be assigned in relation to the control measures needed to protect both the environment and human

health (GMBSL 1, 2, 3 or 4) for work with GMM. The measures that are indicated as necessary by the risk assessment must be applied.

ii. The importance of the final activity classification is twofold: It determines the minimum containment and control measures that must be applied. For Class 1 activities, GM-BSL1 measures must be applied as a minimum. For Class 2 activities, GM-BSL2 and so on.

iii. The risk assessment must be used to determine the appropriate control measures that are needed to afford maximum protection to both human health and the environment.

iv. For activities with plants that involve handling GMM, in addition to the containment measures set out for GM-BSLs, i.e. activities involving genetic modification of microorganisms in laboratories, the relevant containment measures for the category of activities involving genetic modification of microorganisms in plant growth facilities, GP-BSLs must also be applied. Therefore, users may wish to read the "*Biosafety Guidelines for Contained Use Activities of LMOs, 2010*" in conjunction with this Guideline. However, the table represented in this Guideline has been integrated such that all relevant measures for activities with GMM associated with plants are shown.

v. To decide on the final classification, users should therefore compare the measures warranted by the risk assessment with the integrated table of containment measures (Table 16 and Table 17). Where the required containment measures correspond to those from a single level of containment this process will be simple: a GM activity requiring BSL2 will be GP-BSL2. There will be cases, however, where the required containment measures are a mixture from two levels, for instance, BSL2 with the addition of one or two measures from BSL3. In these cases, the higher level of containment will determine the GM activity class and must be applied. A request can be made to the NBB, at the time of notification for permission to use the mixture of two levels identified, but unless and until the written agreement is obtained the lower containment level than that corresponding to the GM Activity Class should not be used.

vi. The risk assessment must always take precedence and all measures identified as necessary must be applied (there is a general requirement for the exposure of humans and the environment to GMM to be as low as reasonably practicable and the principles of good microbiological practice and of good occupational safety and hygiene must also be applied).

CONTAINMENT AND CONTROL OF ACTIVITIES WITH GMM IN A PLANT FACILITY

CHAPTER 15

15.1 CONTAINMENT LEVELS FOR GMM IN A PLANT FACILITY

The following guidelines outline physical containment and work practices suitable to conduct experiments with plants associated with GMM. The main objective of plant containment is to avoid the unintentional release of rDNA derived microorganisms associated with plants from the facility. The "*Biosafety Guidelines for Contained Use Activity of LMO, 2010*" describe four BSL of containment for GMM, GM-BSL1, GM-BSL2, GM-BSL3 and GM-BSL4; Three levels of plant containment are described which falls under GP-BSL1, GP-BSL2 and GP-BSL3. The BSL4 is not represented for plants in this Guideline. No such facility currently exists in Malaysia and it is not envisaged that any work involving GMM in association with plants will warrant the use of GP-BSL4. If such work is proposed, or the construction of such a facility is planned, then the person initiating it is strongly advised to discuss the details of containment requirements, management control and design of the facility etc. in advance with the Department of Biosafety, National Biosafety Board at the Ministry of Natural Resourses and Environment, Malaysia.

15.2 BIOSAFETY LEVEL 1 CONTAINMENT

GM-BSL1 must be applied for Class 1 activities involving GMM that fall under RG1. All the required measures, in addition to the principles of good microbiological practice and good occupational safety must be applied.

15.2.1 Plant Facility (GP-BSL 1)

i. GP-BSL1 facilities provide the basic containment and include structures comprising greenhouses, screen houses and flexible film plastic structures. Where the facility is a glasshouse, it shall have a continuous waterproof covering.

ii. The facility floor may be composed of gravel or other porous

material. At a minimum, impervious (*e.g.* concrete) walkways are recommended that are easy to clean in order to maintain good hygiene levels. Plastic sheet, or other flooring material should be used to reduce the spread of GM material within the facility.

iii. Permanent structures must have self-closing, lockable outer doors and be located on a site designed to prevent the entry of surface run-off water. It is recognised that most facilities of this type have doors that are not self-closing.

iv. There is no regulatory requirement for a GP-BSL1 facility to be physically separated from other areas of the building. There should be adequate space provided and the working area should be a safe, comfortable environment that takes full account of work practices and equipment present.

v. A GP-BSL1 facility does not need to be sealable for the purposes of fumigation. However, there may be a requirement for specific disinfection procedures to be in place.

15.2.2 Equipment

i. Bench surfaces should be easily cleaned, impervious to water and resistant to acids, alkalis, solvents, disinfectants and other decontamination agents that may be in use. It is recognised that the benching commonly used within plant growth facilities may not be impervious to water. Such benching is frequently made of mesh in order to permit the free drainage of water. Where such benching is used, run-off water should be controlled by alternative means, example using saucers and trays.

ii. There is no regulatory requirement for the use of a biological safety cabinet or other similar equipment and all work can take place on the open bench. It is acknowledged that containment equipment such as a biological safety cabinet is recommended to prevent contamination of the genetic modification work or products being handled.

iii. An autoclave is required to be available on the site, but not necessarily in the same building. However, it is recognised that, in some cases, autoclaving will not be appropriate and alternative waste inactivation procedures should be used. In these cases, derogation must be requested from the NBB detailing the alternative waste management procedures in place.

iv. Where the risk assessment identifies that a GMM could be disseminated via the drainage system, control measures must be used to control run-off water. No plants should be planted directly into the ground. All higher plants should be grown in pots, trays or similar containers. All lower plants should be grown in physical containers such as flasks, tanks or fermenters. This could be

supplemented with appropriate filters/mesh covers to limit the amount of soil, plant material and water entering the drains. The facility is not required to have a dedicated drainage system and therefore soakaways may be sufficient.

15.2.3 Work practices

i. There is no requirement for access to GP-BSL1 containment laboratories to be restricted. However, those permitted to work in the laboratory should be competent, trained and properly informed.

ii. Procedures should be carried out in such a way as to keep aerosol production to a minimum but there is no requirement for specific measures to control aerosol dissemination. Care should be taken to ensure that contact of the GMM with people and the environment is minimised.

iii. There is no requirement for a showering facility to be present and staff are not required to shower when entering or leaving the facility. However, good hygiene should be maintained, and a hands-free sink should be provided in the facility. Hands should be washed immediately if contamination with a GMM is suspected, after handling viable GMM or before leaving the laboratory.

iv. There is a regulatory requirement for suitable protective clothing, such as laboratory coats or overalls, to be worn for all activities involving GMM. Where practicable, these should be left within the facility on exit, particularly where staff are required to go outdoors.

v. Suitable gloves should be used to prevent contamination of the genetic modification work or to protect staff against other chemical or biological contaminants.

vi. A pest control programme should be implemented to control undesired species that could disseminate the GMM (*e.g.* weed, birds, rodent or arthropod pests) by methods appropriate to the organism. For example, a polytunnel is unlikely to offer appropriate protection against invertebrate or fungal vectors. Where the risk assessment shows that these should be controlled, a permanent structure, such as a glasshouse, is more appropriate.

vii. Where the risk assessment shows that it is required, the dissemination of GMM in plant pollen, seeds and other plant material must be effectively controlled. The use of a certain degree of biological containment is inherent to all facilities. GMM within the facility will be unable to infect plants in the receiving environment, either because there are no suitable host species or because the environmental conditions are unfavourable. The facility should be dedicated to experimental plants only and the growing of ornamental plants for decorative purposes is not allowed.

viii. When transferring GM material between different facilities on site, there is a regulatory requirement that the dissemination of GMM be minimised. Secondary containment, for example the use of double bagging or a box, should provide a suitable means of containment.

ix. It is a regulatory requirement for specified disinfection procedures to be in place where the risk assessment shows that they are required. Effective disinfectants should always be available for immediate use in the event that GMM-contaminated material is spilled.

15.2.4 Waste disposal

i. All GMM contaminated materials and waste must be inactivated by a validated means prior to disposal. In plant growth facilities, this may include growing media, pots and tools, as well as plant material and other incidentally contaminated items. Autoclaving will generally provide the best assurance of inactivation, but it may not be appropriate for all contaminated materials. When autoclaving, the equipment should be operated so as to comply with the manufacturers' instructions. For example, small amounts of plant material may be inactivated using 121ºC for 15 minutes but appropriate times and temperatures may vary.

ii. Larger volumes of waste may necessitate a longer holding time or higher temperature. The key requirement is that the system is validated to ensure sufficient steam penetration to the centre of the load for the required time period is achieved. Incineration is an appropriate alternative, although the risk assessment should detail the risk management procedures in operation. Where the incinerator is located off site, there is a regulatory requirement that the incinerator premises be registered as a facility that is handling GMM waste. Waste material should be double bagged and placed in a suitable container for transfer to waste management facilities.

15.2.5 Other safety measures

i. There is no requirement to have equipment solely dedicated for use in GP-BSL1 facilities. However, equipment may need to be decontaminated before removal, repair or servicing.

ii. A window or alternative method of observing the laboratory occupants might be required where the risk assessment indicates that it is necessary. It is unlikely that such a system will be required for safety reasons in GP-BSL1 facilities, although it may offer additional protection or reassurance for staff working alone.

iii. If the risk assessment indicates that it is needed, GMM cultures should be stored in appropriate refrigerators or freezers, be clearly labelled and be stored within the facility or nearby (so far as is reasonably practical).

iv. Relevant staff should receive information, instruction and training in the procedures conducted in the laboratory. All written records, for example, staff training, accidents and incidents should be recorded.

15.3 BIOSAFETY LEVEL 2

Biosafety Level 2 (GM-BSL2) must be applied for Class 2 activities involving RG 2 associated GMM, if deemed sufficient through a risk assessment. All the required measures, in addition to the principles of good microbiological practice and good occupational safety practices must be applied.

15.3.1 GP-BSL2 Plant facility

i. The plant growth facility should be a permanent fixed structure with walls, a roof and a floor. Where the facility is a glasshouse, it shall have a continuous waterproof covering. The facility must have self-closing, lockable outer doors and be located on a site designed to prevent the entry of surface run off water. It is recognized that most facilities of this type have doors that are not self-closing. Users may consider this requirement to be met if doors are not left open when the facility is not in use. It is likely that the majority of such facilities will be a standard research glasshouse, although recent technological advances in alternatives to glass may mean other structures are suitable. Facilities should be designed to withstand the local weather conditions and the potential for breakage through other activities, e.g vandalism.

ii. There is no requirement for a BSL2 facility to be physically separated from other areas of the building. There should be adequate space provided and the working area should be a safe, comfortable environment that takes full account of work practices and equipment present.

iii. A BSL2 facility does not need to be sealable for the purposes of fumigation. However, there is a requirement for specific disinfection procedures to be in place.

15.3.2 Equipment

i. Bench surfaces should be easily cleaned, impervious to water and resistant to acids, alkalis, solvents, disinfectants and other decontamination agents that may be in use. It is recognised that the benching commonly used within plant growth facilities may not be impervious to water. Such benching is frequently made of mesh in order to permit the free drainage of water. Where such benching is used, run-off water should be controlled by alternative means, e.g using saucers and trays. Although not required to have a permanent floor, the facility should be easy to clean in order to

maintain good hygiene levels. Where appropriate, measures to reduce the spread of mechanically transmitted GMM should be implemented. Plastic sheeting or other flooring material should be used in conjunction with clearly defined walkways to reduce the dissemination of GMM material within the facility.

ii. Good hygiene should be maintained and a hands-free sink should be provided for hand washing to control against the dissemination of GMM from the hands of staff. These should be located near the exit door.

iii. Where the risk assessment shows that entry to the facility should be via a separated room with two interlocking doors, this can be achieved by either having a dedicated entrance lobby/vestibule (*e.g.* to a stand-alone glasshouse facility) or by using a shared header house area within a larger facility. Where access is via a lobby/vestibule, at its simplest, containment can be achieved by staff being trained not to have the two doors open at the same time. Permanent structures must have lockable outer doors and be located on a site designed to prevent the entry of surface run off water. It is good practice to lock the facility when unattended to prevent unauthorised access.

iv. The risk assessment may show that it is important to maintain the facility at a negative pressure with respect to the surrounding areas if the GMM can be passively transmitted via the air; but is unlikely to be required for mechanically transmitted or vectored organisms. Where negative pressure is employed, a system to monitor the status of the pressure differential (*e.g.* Magnehelic gauges) should be installed so that any associated failure in containment can be detected.

v. When the risk assessment shows that it is required, a biological safety cabinet (or similar containment equipment) may be used. For example, if the GMM can be disseminated in the air, then procedures that might generate aerosols (*e.g.* vigorous shaking or sonication) should take place within a biological safety cabinet or similar containment equipment. Where such equipment is used, and where there is a risk of harm from not doing so, exhaust or recirculated air should be HEPA filtered. Procedures should be in place to limit the production and dissemination of aerosols and such equipment can be employed for this purpose.

vi. An autoclave is required to be available and located within the same building as the plant growth facility. Where the containment facility is accessed via a header house area, it is expected that the autoclave will be positioned here. Where the autoclave is not in the same building as the plant growth facility, or alternative waste inactivation procedures are used, derogation will need to be applied to the NBB detailing the alternative arrangements in place.

vii. Control measures must be taken to minimise the dissemination of GMM material via run off water (i.e. the drainage route). It is recognised that the benching used may not be impervious to water and saucers or trays should be used, supplemented with appropriate hygiene measures to limit the amount of soil, plant material and water entering the drains. For example, hand watering systems are likely to be in place as opposed to automatic systems and appropriate filters/mesh covers could be fitted to the floor drains.

15.3.3 Work practices

i. There is a requirement that access to the facility is restricted to authorised staff. This is most easily achieved via outer doors that are locked at all times or by using digital keypad or electronic swipe-card entry systems. Those permitted to work in the laboratory should be competent, trained and properly informed. Entry into the contained area for maintenance purposes can be minimised by locating control panels and engineering access points outside the restricted parts of the facility.

ii. When handling GMM, there is a regulatory requirement to control aerosols such that airborne dissemination is minimised. It is recognised that activities resulting in aerosol generation are likely to be limited within a plant growth facility, but nevertheless the risk assessment should determine what measures are appropriate. For example, the use of containment equipment, such as a biological safety cabinet or, for centrifugation, sealed rotors or buckets.

iii. There is no requirement for a showering facility to be present and staff are not required to shower when entering or leaving the facility. However, good hygiene should be maintained and hands should be washed immediately if contamination with a GMM is suspected, after handling viable GMM or before leaving the laboratory.

iv. There is a requirement that suitable personal protective equipment (PPE) such as laboratory coats, be worn for all activities involving GMM. This is particularly important for mechanically transmitted GM plant pathogens in order to prevent human-mediated release from (or dissemination of the organism within) the facility. Where practicable, PPE should be removed upon exiting the facility and prior to washing hands and left within the facility, particularly where staff are required to go outdoors.

v. Gloves are required to be worn where indicated by the risk assessment. This will be particularly important for mechanically transmitted GM plant pathogens in order to prevent human-mediated release from (or dissemination of the organism within) the facility.

vi. A pest control programme should be implemented to control potential disease vectors (including invertebrates) that could disseminate the GM plant pathogens from the facility. Vents should have a mesh screen appropriate to the invertebrate species to be excluded. Caulking materials should be used to seal any gaps, such as those between glass panes and service pipes, and brushes or pneumatic strips should be fitted around the edges of doors. In addition, an efficient control regime should be used involving monitoring traps (such as sticky traps) and where necessary, appropriate chemical control. Where biological control agents are to be introduced into the facility, the risk assessment should consider the possibility of these agents themselves disseminating the GMM. If the risk assessment has identified soil-borne organisms (such as nematodes and fungi) as vectors for the GMM, the control of these should be achieved using similar measures to those described for run off water and soil.

vii. The dissemination of GMM in plant pollen, seeds and other plant material must be minimised. In addition, the dissemination of GMM in other plant material (including plant sap) should be minimised and suitable measures employed to prevent the spread of mechanically transmitted GM pathogens. Gloves should be worn at all times when handling the GMM and potentially infected plants, and should be removed before leaving the laboratory. Appropriate PPE should be worn which is removed on exiting the main facility. Care should be taken when watering plants with lances or cans. Since Class 2 GMM have been identified as being able to infect species in the environment, the growth of plants in the immediate vicinity of the facility should be restricted in order to control against potential GMM hosts and compatible relatives of the GM plants. This can be reasonably achieved by employing a paving or gravel barrier around the facility, in conjunction with herbicide treatment regimes. Where necessary, there should be different compartments within the facility for genetic modifiction and non-genetic modification work. Where the sharing of compartments between different activities is unavoidable, the risk assessment should clearly outline the likelihood of contamination, taking into account susceptibility of plants to infection with the GMM and sexual compatibility.

viii. When transferring GM material between different facilities on site, there is a regulatory requirement that the dissemination of GMM be minimised. Secondary containment (*e.g.* a bag or box) should be used in conjunction with a transfer container, such as a wheelie bin. Where the risk assessment has identified that there is a risk of the dissemination of the GMM during transfer, a more robust secondary container that should contain the GMM in the event of an accident should be used.

ix. There is a regulatory requirement that specific disinfection procedures are in place within the facility for use against GMM. Effective disinfectants should be available for routine disinfection and for immediate use in the event of a spillage. The disinfectants selected should be validated and local rules should be in place governing their use.

15.3.4 Waste disposal

i. There is a regulatory requirement that all GMM contaminated materials and waste must be inactivated by a validated means prior to disposal. In plant growth facilities, this may include growing media, pots and tools, as well as plant material and other incidentally contaminated items. Autoclaving will generally provide the best assurance of inactivation, but it may not be appropriate for all contaminated materials. When autoclaving, the equipment should be operated so as to comply with the manufacturer's instructions. For example, small amounts of plant material may be inactivated using 121°C for 15 minutes but appropriate times and temperatures may vary.

ii. Larger volumes of waste may necessitate a longer holding time or higher temperature. The key requirement is that the system is validated to ensure sufficient steam penetration to the centre of the load for the required time period is achieved. Incineration is an appropriate alternative, although derogation and details of the risk management procedures will be required. Where the incinerator is located off site, the incinerator premises have to be registered as a facility handling GMM, since RG2 GMM have been identified as being able to infect plants in the environment.The transportation of the material to the site should follow the "*Biosafety Guidelines for Contained Use Activity of LMO, 2010*".

iii. The containers used for transporting to the incinerator should be sufficiently robust. Where small amounts are involved, validated containment vessels (*e.g.* incinerator bins) may be sufficient. One-way burn bins may also be appropriate, but for larger volumes burn bags contained within wheelie bins are acceptable. Local rules should be used to clearly outline the expected fate of all material within the facility and GM and non-GM material may have to be subject to the same waste inactivation measures unless fully justified in the risk assessment.

15.3.5 Other safety measures

i. There is no requirement to have equipment solely dedicated for use in BSL2 facilities. However, equipment may need to be thoroughly decontaminated before removal, repair or servicing.

ii. A window or alternative method of observing the laboratory occupants might be required where the risk assessment indicates

that it is necessary. It is unlikely that such a system will be required for safety reasons in GP-BSL 2 plant growth facilities, although it may offer additional protection for staff working alone.

iii. There is a regulatory requirement for safe storage of GMM, which may include plant material that is either infected or contaminated. Appropriate vessels should be used which are labelled and stored in an appropriate facility, such as a locked freezer. Where numerous different GMM are constructed, consideration should be given to a management system of recording all the lines stored and cross-referencing them to the relevant risk assessment.

iv. Formal written records of staff training are required if the risk assessment indicates that it is necessary. Laboratory staff should receive information, instruction and training in handling of GMM. All accidents and incidents should be recorded and reported internally. If human health or the environment could have been harmed then this must be reported to the IBC and Occupational Safety and Health Committee.

15.4 BIOSAFETY LEVEL 3

Biosafety Level 3 (GM-BSL3) must be applied for Class 3 activities involving GMM, if deemed sufficient through a risk assessment. All the required measures, in addition to the principles of good microbiological practice must be applied.

15.4.1 GP-BSL 3 Plant facility

i. The facility should be a permanent fixed structure with walls, a roof and a floor. It is likely that a GP-BSL3 containment plant growth facility will comprise either a highly engineered glasshouse or, more likely, growth rooms or cabinets within a controlled environment suite. Where a glasshouse or similar structure is used, an increased level of containment is expected when compared to an equivalent GP-BSL2 facility. For example, all joints, overlapping panels etc. should be effectively caulked and, at the highest level, break-resistant glazing/polycarbonate sheeting should be used. The facility must have self-closing, lockable outer doors and be located on a site designed to prevent the entry of surface run off water.

ii. The facility should be isolated. Where the plant growth facility is a controlled environment suite within a secondary building, restricting access and ensuring that communal corridors etc. do not compromise separation can achieve this. Similarly, in a larger glasshouse facility, a small section or wing may be dedicated to BSL3 and as such, access can be restricted in order to maintain isolation. There should be adequate space provided and the

working area should be a safe, comfortable environment that takes full account of work practices and equipment present.

iii. The facility should be sealable for fumigation. This is so that they can be appropriately decontaminated in the event of a significant unintentional release or where the local rules require fumigation to be undertaken. Sealability also protects humans outside the facility from the potentially toxic effects of the fumigant. It is recognised, however, that fumigation against plant pathogens is not routine within plant growth facilities and may not even be possible in a glasshouse. Where the facility is not sealable and fumigation is not to be used, derogation will be required along with detailing alternative means of decontamination. For example, washing the facility down with a validated chemical disinfectant may be appropriate. Specific disinfection procedures are required to be in place.

15.4.2 Equipment

i. Bench and floor surfaces should be easily cleaned, impervious to water and resistant to acids, alkalis, solvents, disinfectants and other decontamination agents that may be in use. It is recognised that the benching commonly used within plant growth facilities may not be impervious to water. Such benching is frequently made of mesh in order to permit the free drainage of water. Where such benching is used, run off water should be controlled by alternative means, *e.g.* using saucers and trays. Where appropriate, measures to reduce the spread of mechanically transmitted GMM should be implemented. Plastic sheeting, or other flooring material should be used in conjunction with clearly defined walkways to reduce the dissemination of GM material within the facility. Good hygiene should be maintained and hand-washing facilities should be provided to control against the dissemination of GMM on the hands of staff. These should be located near the exit door and it should be a hands-free sink.

ii. Entry to the facility should be via a separate room with two interlocking doors, where the risk assessment shows that this is necessary. It is expected that a GP-BSL3 plant growth facility handling moderately hazardous GMM in association with plants will be entered via a lobby/vestibule with self-closing doors. Entry via an airlock with a separate chamber with showering and changing facilities is not required. Consideration should be given to a system (*e.g.* audio/visual alarm or electronic interlock) that ensures that the two doors are not open at the same time. Within the lobby area, there should be space to store laboratory coats dedicated to the facility and hand-washing facilities should be provided with a hands-free sink.

iii. GP-BSL3 c facilities should be maintained at a negative pressure relative to the immediate surroundings. This is to control the movement of airborne GMM, particularly those disseminated as the result of a spillage or an aerosol release. This should be achieved by appropriate air handling systems in conjunction with appropriate seals. A system to monitor the status of the pressure differential (*e.g*. Magnehelic gauges) should be installed so that any associated failure in containment can be detected. While negative pressure is a requirement, it is recognised that this may not always be possible or appropriate within a plant growth facility. For example, positive pressure may be required to prevent ingress of an intermediate vector species. Where this is the case, or where a negative pressure gradient is not appropriate, derogation can be requested from the NBB. Alternative measures in place to afford an appropriate level of containment should be fully detailed in the risk assessment.

iv. Exhaust air extracted from a GP-BSL3 facility should be HEPA filtered. This is to prevent release of an airborne GMM, particularly that are disseminated as a result of any spillage or an aerosol release. It is recognised that HEPA filters may not be appropriate for use in glasshouse facilities where large volumes of air need to be exchanged. Where this is the case, and where HEPA filters are not used, this should be fully detailed in the risk assessment and a derogation should be requested from the NBB outlining the alternative measures in place to afford an appropriate level of containment. For example, alternative air filters can be used to prevent a GMM being disseminated via pollen, a filter of the G4 standard is likely to be appropriate. This is because these filters are rated as being over 90% efficient at arresting larger airborne particles, including the majority of plant pollens. In addition, they tend to comprise pleated panels that are more likely to prevent the movement of insects. Where the risk assessment identifies that HEPA filters offer the most effective measures for containing the GMM material, for example airborne spores derived from GM fungi, in the majority of cases it is likely that such work will be restricted to a controlled environment facility.

v. A biological safety cabinet should be present in a GP-BSL3 containment facility and all procedures involving infective GMM should be undertaken within it. While a biological safety cabinet should be used for handling stock cultures of the GMM and for the infection of small volumes of plant material (such as detached leaf material in petri dishes), it is recognised that they may be inappropriate for other activities within a plant growth facility. Where a biological safety cabinet is not in place or used, derogation must be requested from the NBB detailing the alternative measures in place to afford an appropriate level of containment. Procedures

should be in place to prevent the production and dissemination of aerosols and such equipment can be employed for this purpose.

vi. An autoclave is required to be available and located within the GP-BSL3 containment facility. Where the autoclave is outside the connected suite, or alternative waste inactivation procedures are used, derogation should be requested from the NBB outlining the alternative transfer and risk management procedures in place. Validated procedures for the safe transfer and inactivation of material will be required that provide an equivalent level of protection to having an autoclave positioned within the GP-BSL3 containment facility.

vii. It is a requirement to control run off water within a GP-BSL3 facility so as to prevent the dissemination of GMM material. In addition to placing all pots on impervious trays or lining all benches with impervious plastic sheeting, the floor of the facility should be impervious to water. Where practicable, the facility should have no drainage or the drains should be blocked throughout the course of the activity with an appropriate system in place to collect and treat any large volumes of water. Where the facility remains connected to the drains throughout the course of the activity, it should be connected to an appropriate 'kill tank' for the validated inactivation of any potential GMM material that may enter the system.

15.4.3 Work practices

i. Access to the facility should be restricted to authorised staff only. This is most easily achieved via outer doors that are locked at all times or by using digital keypad or electronic swipe-card entry systems. In addition, access should also be restricted to individual compartments, cabinets etc. within the facility. Those permitted to work in the laboratory should be competent, trained and properly informed. Entry into the contained area for maintenance purposes can be minimised by locating control panels and engineering access points outside the restricted parts of the facility.

ii. Specific measures should be adopted to prevent aerosol dissemination of GMM within the containment facility. It is recognised that activities resulting in aerosol generation are likely to be limited within a plant growth facility, but nevertheless the risk assessment should determine what measures are appropriate. It is expected that the measures in place will be of a higher standard than at BSL2 and may involve some of the measures previously outlined, such as the use of a biological safety cabinet or, for centrifugation, sealed rotors or buckets.

iii. Where the risk assessment indicates that it is required, staff must shower before leaving the facility. Good hygiene should be maintained at all times and hands should be washed immediately

if contamination with a GMM is suspected, after handling viable GMM or upon exit.

iv. Suitable PPE and clothing, such as laboratory coats, should be worn for all activities involving GMM. This is particularly important for mechanically transmitted GM plant pathogens in order to prevent human-mediated release from (or dissemination of the organism within) the facility. Protective clothing may be of the disposable type which are discarded for autoclaving and disposal or alternatively may be reused. If reused, PPE should be dedicated to the BSL3 facility, be removed prior to washing hands and left in the lobby area on exit. The use of differently coloured lab coats may help in the management of this. Protective clothing should be decontaminated prior to laundering, usually via autoclaving.

v. Gloves are required to be worn at all times when handling GMM materials. This is particularly important for mechanically transmitted GM plant pathogens in order to prevent human-mediated release from (or dissemination of the organism within) the facility. They are particularly important for controlling the dissemination of mechanically transmitted GM plant pathogens.

vi. A pest control programme should be implemented to control potential disease vectors (including invertebrates) that could disseminate the GM plant pathogens from the facility. Caulking materials should be used to seal any gaps, such as those between glass panes and service pipes, and brushes or pneumatic strips should be fitted around the edges of doors. In addition an efficient control regime should be used involving monitoring traps (such as sticky traps) and where necessary, appropriate chemical control. It is expected that a GP-BSL3 glasshouse would not have open vents. Instead the temperature regimes will be maintained by air conditioning or air-handling arrangements. Soil-borne vectors should be controlled using the arrangements described above to prevent dissemination by run off water. It is likely that GP-BSL3 containment facilities will be the only ones in which the deliberate, experimental transmission of GMM material using invertebrate vectors are permitted. However, the use of a specialist insectary facility containing growth cabinets in which plants can be grown is encouraged. Within such a facility, temperature and light gradients can be used to provide additional barriers to control the movement of invertebrates. The experiments should involve the minimum number of plants, should be short term and ideally should be undertaken when the environmental conditions outside of the facility are less likely to permit the survival of the vector.

vii. Where the risk assessment shows that it is required, the dissemination of GMM in plant pollen and seeds must be prevented. In addition, it is also a requirement to prevent the dissemination

of GMM in other plant material (including plant sap). This should be minimised and suitable measures employed to prevent spread of mechanically transmitted GM pathogens. The most appropriate measures for controlling such dissemination are based on ensuring good levels of hygiene. Gloves must be worn at all times when handling infectious GMM or infected plants. Contamination of work surfaces and door handles etc. should be controlled with chemical disinfection regimes. Appropriate protective clothing must be worn and dedicated protective footwear, sticky floor mats or a footbath containing an appropriate validated chemical disinfectant could be used to control the dissemination of the GMM on the feet of staff.

viii. Where GMM have been identified as being able to infect species in the environment, the growth of plants in the immediate vicinity of the facility should be restricted in order to control against potential GMM hosts and compatible relatives of the GM plants. This can be reasonably achieved by employing a paving or gravel barrier around the facility, in conjunction with herbicide treatment regimes. There should be different compartments within the facility for genetic modifiction and non-genetic modification work. Where the sharing of compartments between different activities is unavoidable, the risk assessment should clearly outline the likelihood of contamination, taking into account susceptibility of plants to infection with the GMM and sexual compatibility.

ix. When transferring GM material between different facilities on site, steps should be taken to prevent the dissemination of the GMM. Secondary containment (*e.g.* a bag or box) should be used in conjunction with a robust, leak-proof secondary container that should contain the GMM in the event of an accident.

x. Specific disinfection procedures should be in place within the facility for use against GMM. Effective disinfectants should be available for routine disinfection and for immediate use in the event of a spillage. Whatever disinfectant is selected, it should be validated and local rules should be in place governing their use.

15.4.4 Waste disposal

i. Inactivation of GMM in effluents from washbasins and showers might be required in the GP-BSL3 containment facility, where the risk assessment shows that this is necessary. Where this is required, effluents should be collected in a sump and inactivated, or should pass through a 'kill tank'.

ii. All GMM-contaminated materials and waste should be inactivated by a validated means prior to disposal. In plant growth facilities, this may include growing media, pots and tools, as well as plant material and other incidentally contaminated items. Autoclaving

will generally provide the best assurance of inactivation, but it may not be appropriate for all contaminated materials. When autoclaving, the equipment should be operated so as to comply with the manufacturer's instructions. For example, small amounts of plant material may be inactivated using 121°C for 15 minutes but appropriate times and temperatures may vary. Larger volumes of waste may necessitate a longer holding time or higher temperature. The key requirement is that the system is validated to ensure sufficient steam penetration to the centre of the load for the required time period is achieved. Incineration might be an appropriate alternative, although derogation and details of the risk management procedures will be required. Where the incinerator is located off site, there is a regulatory requirement that the incinerator premises be registered as a facility handling GMM and the transportation of the material to the site should follow the "*Biosafety Guidelines for Contained Use Activity of LMO, 2010*". Given the hazardous nature of the material, the containers used for transporting to the incinerator should be sufficiently robust. One-way burn bins should be sufficient. Local rules should be used to clearly outline the expected fate of all material within the facility and GM and non-GM material may have to be subject to the same waste inactivation measures unless fully justified in the risk assessment.

15.4.5 Other safety measures

i. GP-BSL3 facilities should contain all its own equipment so far as is reasonably practicable. This is to reduce the movement of experimental materials between different facilities and thereby reduce the likelihood of GMM dissemination. Equipment should be thoroughly decontaminated before removal, repair or servicing.

ii. A window or alternative method of observing the laboratory occupants is required. This is in order to be able to view any operatives working in the facility as a safety measure.

iii. Cultures should be safely stored in appropriate vessels, be clearly labeled and be, so far as is reasonably practical, stored within the laboratory or laboratory suite. Ideally, viable materials requiring BSL3 containment should only be stored and handled within the GP-BSL3 laboratory itself. Refrigerators and freezers used for storage outside of the laboratory should be kept locked.

iv. Formal written records of staff training are required. Laboratory staff should receive information, instruction and training in handling of GMM. Other staff who may need to access the contained areas should also receive an appropriate degree of training, particularly if they need to enter the facility while work is in progress. All accidents and incidents should be recorded

and immediately reported internally. If human health or the environment could have been harmed, then this must be reported to the IBC and Occupational Safety and Health Committee.

Table 16. Plant Containment measures showing Facility design and work practices

Containment measures		Containment Levels		
		GP-BSL1	GP-BSL2	GP-BSL3
Plant Facility				
1	Permanent structure	Required to extent RA shows it is required	Required	Required
2	Laboratory suite: isolation	Not required	Not required	Required
3	Laboratory: sealable for fumigation	Not required	Not required	Required
Work practices				
4	Access restricted to authorised staff only	Not required	Required	Required
5	Specific measures to control aerosol dissemination	Not required	Required to minimise	Required to prevent
6	Shower	Not required	Not required	Required to extent the RA shows it is required
7	Protective clothing	Suitable protective clothing required	Suitable protective clothing required	Suitable protective clothing required; footwear required where the RA shows it is required
8	Gloves	Required	Required	Required
9	Effective control of disease vectors such as insects, rodents, arthropods which could disseminate the GMM	Required	Required	Required

10	Effective control of pollen, seeds and other plant material which could disseminate the GMM	Required to extent the RA shows it is required	Required to minimise dissemination	Required to prevent dissemination
11	Procedures for transfer of living material between the plant growth facilities, protective structure and laboratory to control dissemination of GMM	Required to minimise dissemination	Required to minimise dissemination	Required to prevent dissemination
12	Specified disinfection procedures in place	Required to extent the RA shows it is required	Required	Required
13	Written records of staff training	Not required	Required to extent the RA shows it is required	Required

RA= Risk assessment

Table 17. Containment measures showing equipment, waste disposal and other safety measures

Containment measures		Containment Levels		
		GP-BSL1	GP-BSL2	GP-BSL3
Equipment				
1	Surfaces impervious to water and resistant to acids, alkalis, solvents, disinfectants, decontamination agents and easy to clean	Required for bench	Required for bench	Required for bench and floor
2	Entry via an airlock or a separate room with two interlocking doors	Not required	Required to extent the RA shows it is required	Required where and to extent the RA shows it is required
3	Negative pressure relative to the pressure of the immediate surroundings	Not required	Required to extent the RA shows it is required	Required
4	Extract and input air from the laboratory should be HEPA filtered	Not required	Not required	HEPA filters required for extract air
5	Biological safety cabinet/enclosure	Not required	Required to extent the RA shows it is required	Required and all procedures with infective materials required to be contained within a cabinet/enclosure
6	Autoclave	Required on site	Required in the building	Required in the laboratory suite
7	Control of contaminated run off water	Required where and to extent the RA shows it is required	Required so as to minimise run off water	Required so as to prevent run-off water

Waste disposal				
8	Inactivation of GMM in effluent from handwashing sinks and showers and similar effluents	Not required	Not required	Required to extent the RA shows it is required
9	Inactivation of GMM in contaminated material and waste	Required by validated means	Required by validated means	Required by validated means with waste inactivated in the laboratory suite
Other Safety measures				
10	Laboratory to contain its own equipment	Not required	Not required	Required, so far as is reasonably practicable
11	An observation window or alternative is to be present so that occupants can be seen	Required to extent the RA shows it is required	Required to extent the RA shows it is required	Required
12	Safe storage of GMM	Required to extent the RA shows it is required	Required	Required

RA= Risk assessment

APPENDIX 1

NATIONAL LEGISLATIONS AND RELEVANT DOCUMENTS

Relevant documents, including those mentioned in this Guideline, are listed below.

1. Relevant Legislation

1.1 Biosafety Act 2007 and Biosafety (Approval & Notification) Regulations 2010
1.2 Environmental Quality Act 1974
1.3 Environmental Quality (Scheduled Wastes) Regulations 1989
1.4 Plant Quarantine Act 1976
1.5 Malaysian Quarantine and Inspection Services (MAQIS) Act, 2011
1.6 National Forestry Act 1984
1.7 Occupational Safety & Health Act 1994
1.8 Occupational Safety & Health (Notification of Accidents Dangerous Occurrences, Occupational Poisoning & Occupational Diseases) Regulations 2004
1.9 Prevention & Control of Infectious Diseases Act 1988
1.10 Prevention & Control of Infectious Diseases (Importation & Exportation of Human Remains, Human Tissues and Pathogenic Organisms & Substances) Regulations 2006
1.11 Pathology Laboratory Act 2007
1.12 The Animal Ordinance 1953
1.13 Animal Rules 1962
1.14 Animal Importation Order 1962
1.15 Animal Act 1953 (Revision of Laws (Rectification of Animals Act 1953) Order 2006

2. Relevant Local Guidelines

2.1 Guidelines for Institutional Biosafety Committees: Use of Living Modified Organisms and Related Materials. (Published by the Ministry of Natural Resources and Environment, Malaysia)
2.2 Biosafety Guidelines for Contained Use Activity of Living Modified Organism, 2010 (Published by the Ministry of Natural Resources and Environment, Malaysia)
2.3 Principles and Guide to Ethical Use of Laboratory Animals, 2000 (Published by the Ministry of Health, Malaysia)

3. Relevant Biosafety Guidelines and References

3.1 The SACGM Compedium of Guidance Part 2: Risk assessment of genetically modified microorganisms (other than those associated with plants). Published by Health and Safety Executive (HSE), United Kingdom: www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp/part2.pdf
3.2 The SACGM Compedium of guidance Part 4: Genetic modification work that involves plants (including plant-associated genetically modified microorganisms) Published

by Health and Safety Executive (HSE), United Kingdom: www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp/index.htm

3.3 Singapore Biosafety Guidelines for Research on Genetically Modified Organisms (GMOs). Published by Genetic Modification Advisory Committee Singapore www.gmac.gov.sg/pdf/Biosafety%20Guidelines%20for%20GMO%20Research-Final-2008Nov.pdf

3.4 Guidance notes for risk assessment outlined in Annex 3 of council directive 90/219/EEC on the contained use of genetically modified micro-organisms; Official Journal of the European Communities - 12.10.2000 - No L 258 P. 0043 – 0048. COMMISSION DECISION 2000/608/EC www.hse.gov.uk/biosafety/gmo/acgm/ecrisk.htm

3.5 The International Air Transportation Association (IATA), *Dangerous Goods Regulations*; (IATA online store –www.iataonline.com/Store/default.htm).

3.6 Laboratory Biosafety Manual, 3rd Edition, World Health Organization, 2004, WHO/CDS/CSR/LYO/2004.11

3.7 Biorisk Management: Laboratory Biosecurity Guidance, World Health Organization, 2004, World Health Organization. WHO/CDS/EPR/2006.6

APPENDIX 2

CLASSIFICATION OF MICROORGANISMS INTO RISK GROUPS

Appendix 2 describes the list of microorganisms according to RG. It is based on existing international norms for the grouping of pathogenic organisms.

Safety considerations in the application of biotechnology are imperative since possible risks in research and development involving different microorganisms have been recognised. According to their possible risks to health and environment, the microorganisms have been classified into different RG (Reference: *Prevention & Control of Infectious Diseases Act 1988, Prevention & Control of Infectious Diseases (Importation & Exportation of Human Remains, Human Tissues and Pathogenic Organisms & Substances) Regulations, 2006*). RG1 contains organisms that cause no risk to health and environment (as per definition). However, Good Laboratory Practices have to be followed. The list should ease the grouping and identification of specific strains. It does not compensate the responsibility of the scientists.

Note: ***This is not a complete list.*** For the purpose of the the Prevention and Control of Infectious Diseases Act and Regulations 2007, any organism not listed in RG 2, 3 or 4, should not be classified in RG1, until its characteristics and pathogenicity are verified in consultation with the Expert Committee on Prevention and Control of Infectious Diseases, Ministry of Health, Malaysia.

BACTERIA: RG1	
• *Acetobacter spp.* • *Actinoplanes spp.* • *Agrobacterium spp.* • *Alcaligenes aquamarinus / A.eutrophus / A.latus* • *Aquaspirillum spp.* • *Arthrobacter spp.* • *Azotobacter spp.* • *Bacillus spp., except B.cereus and B.anthracis* • *Bifidobacterium.spp., except B.dentium* • *Brdyrhizobium spp.* • *Brevibacterium spp.* • *Caryphanon spp.* • *Clavibacter spp. Except C. michiganensis and C.sepedonicus* • *Clostridium aceticum / C.acetobutylicum/ C.acidiurici/ C.cellobiparum / C.kluyveri / C.thermoaceticum / C.thermocellum / C.thermosulfurogenes* • *Corynebacterium glutomicum/lilium* • *Enterococcus facium ATCC 4043* • *Escherichia coli ATCC 9637, CCM28,* NCIB 8743, B, K12 and derivatives • *Erwinia spp. Except E.chrysanthemi, E.amylovora and E.herbicola* • *Gluconobacter* • *Klebsiella planticola*	• *Lactobacillus acidophilus / L.bauaricus / L.breuis / L.bucneri / L.casei / L.cellobiosis / L.fermentum / L.ermentum / L.helveticum / L.sake* • *Lactococcus lactis* • *Leuconostoc spp.* • *Lysobacter spp* • *Methanobacter spp.* • *Methylomonas spp.* • *Micrococcus spp.* • *Pediococcus spp.* • *Pseudomonas gladioli / P.fluorescens / P.syringae, except P. pathotype persicae* • *Ralstonia spp.* • *Rhizobium spp.* • *Rhodobacter spp.* • *Rhodopseudomonas spp.* • *Staphylococcus carnosus* • *Rickettsiella spp.* • *Streptococcus salivarius-thermophilus* • *Streptomyces spp., except S.somaliensis* • *Thermobacteroides spp.* • *Thermus spp.* • *Thiobacillus spp.* • *Vibrio diazotrophicus / V.fischeri*
BACTERIA, CHLAMYDIA AND MYCOPLASMA: RG2	
• *Acinetobacter baumannii (Acinetobacter calcoaceticus)* • *Acinetobacter lwoffi* • *Actinobacillus actinomycetemcomitans* • *Actinomadura madurae* • *Actinomadura pelletieri* • *Actinomyces spp.* including: – *Actinomyces gerencseriae* – *Actinomyces israelii*	• *Actinomyces pyogenes (Corynebacterium pyogenes)* • *Aeromonas hydrophila* • *Afipia spp – Agrobacterium radiobacter* • *Alcaligenes spp.* • *Amycolata autotrophica* • *Archanobacterium haemolyticum (Corynebacterium haemolyticum)* • *Arizona spp* - all serotypes

• *Bacillus cereus* • *Bacteroides spp.* Including – *Bacteroides fragilis* • *Bartonella bacilliformis (Rochalimaea bacilliformis)* • *Bartonella quintana (Rochalimaea quintana)* • *Bartonella henselae (Rochalimaea henselae)* • *Bartonella vinsonii (Rochalimaea vinsonii)* • *Bordetella bronchiseptica – Bordetella parapertussis* • *Bordetella pertussis* • *Borrelia spp.* including: – *B. burgdorferi / B. Duttonii / B. recurrentis* • *Brucella ovis* • *Burkholderia spp.* including: – *Burholderia cepacia / B.mallei (Pseudomonas mallei) B.pseudomallei (Pseudomonas pseudomallei)* – *Campylobacter spp. including:* – *Campylobacter coli / C. Fetus / C. jejuni*	• *Capnocytophaga spp.* • *Cardiobacterium hominis* • *Chlamydia pneumoniae* • *Chlamydia psittaci (non avian strains)* • *Chlamydia trachomatis* • *Citrobacter spp.* • *Clostridium spp.* including: – *Clostridium botulinum / C. chauvoei / C. haemolyticum / C.histolyticum / C. novyi / C.perfringens / C.septicum / C.tetani* • *Corynebacterium spp.* including: – *Corynebacterium diphtheriae / C. minutissimum / C. pseudotuberculosis / C. Renale* • *Dermatophilus congolensis* • *Edwardsiella tarda* • *Enterobacter spp.* including: – *Enterobacter aerogenes / E.cloacae* • *Enterococcus spp.*
BACTERIA, CHLAMYDIA AND RICKETTSIA: RG3	
• *Bacillus anthracis* • *Brucella spp. (except B. ovis, listed in Risk Grp 2):* – *Brucella abortus* – *Brucella canis* – *Brucella melitensis* – *Brucella suis* • *Burkholderia (Pseudomonas) mallei* • *Burkholderia (Pseudomonas) pseudomallei* • *Chlamydia psittaci* (avian strains) • *Coxiella burnetii* • *Ehrlichia spp.* including: – *Ehrlichia sennetsu (Rickettsia sennetsu)* • *Eikenella corrodens*	• *Francisella tularensis* (Type A) • *Mycobacterium bovis (except BCG strain, refer to Risk Group 2)* • *Mycobacterium tuberculosis* (multi-drug resistant strains) • *Pasteurella multocida type B - "buffalo" and other virulent strains* • *Rickettsia spp.* including: – *Rickettsia akari / R.australis / R.canada / R. conorii / R. prowazekii / R.rickettsii / R.sennetsu* (refer to *Ehrlichia sennetsu) / R. siberica / R. Tsutsugamushi / R. typhi (Rickettsia mooseri*) • *Yersinia pestis*
BACTERIA, CHLAMYDIA, MYCOPLASMA AND RICKETTSIA: RG4	
NONE	

VIRUSES: RG1

- attenuated viral strains which are accepted vaccines. Only a limited number of passages in defined cell-culture or host-systems are allowed
- apathogenic viral strains
- viral strains from fungal or bacterial systems, provided they do not contain virulence-factors and are described as apathogenic for higher animals and human beings
- Baculoviruses of insects

VIRUSES: RG2

- Adenoviridae
  – Adenoviruses, all serotypes
- Arenaviridae
  – Lymphocytic choriomeningitis virus complex (LCM); non-neurotropic strains; Ippy, Mobala
  – Tacaribe virus complex: Ampari, Latino, Parana, Pichinde, Tacaribe, Tamiami
- Hepatitis delta virus
- Astroviridae
  – Human astrovirus
- Bunyaviridae
  – Genus: Bunyavirus
    Bunyamwera virus, California encephalitis group, including LaCrosse virus
- Genus: Phlebovirus
  – all species, except Rift Valley fever virus (refer to RG3), includes: Rift Valley fever virus vaccine strain MP-12, Sandfly fever virus, Toscana, Uukuvirus
- Genus: Nairovirus
  – Hazara virus, Dugbe virus
- Caliciviridae
  – all isolates including Norwalk virus, Sapovirus and Hepatitis E virus
- Coronaviridae
  – Human coronaviruses (serotypes, 229E and OC43) *except SARS coronavirus (refer to RG 3)*
- Flaviviridae
  – Genus: Flavivirus (Group B Arbovirus): Dengue virus serotypes 1, 2, 3, 4
  Yellow fever virus vaccine strain 17D
  Japanese encephalitis virus
  – Genus: Hepacivirus
  Hepatitis C virus
- Orthomyxoviridae
  – Influenza viruses types A, B, and C, *except Avian Influenza A, H5N1, (Refer to RG3*)
  – Other tick-borne orthomyxoviruses such as Dhori and Thogoto
- Papillomaviridae
  – Genus: Papillomavirus all human papilloma viruses
- Paramyxoviridae
  – Genus: Paramyxovirus
  all isolates including Human parainfluenza viruses types 1, 2, 3 and 4, and Newcastle disease virus
  – Genus: Pneumovirus
  all isolates including Respiratory syncytial virus
  – Genus: Morbillivirus
  all isolates including measles virus
  – Genus: Rubulavirus
  Mumps virus
  – Genus: Metapneumovirus
  Human metapneumovirus
- Parvoviridae
  – Genus: Parvovirus
  all isolates including human parvovirus (B19)
- Picornaviridae
  – Genus: Aphthovirus
  – Genus: Cardiovirus
  – Genus: Enterovirus
  Coxsackie viruses types A and B
  – Echoviruses
  – Polioviruses
  – Enterovirus serotypes 68 – 71
  – Genus: Rhinoviruses
  – Genus: Hepatovirus
  Hepatitis A

• Hepadnaviridae Hepatitis B virus • Herpesviridae all Herpesviruses, *except Herpesvirus simiae (refer to RG 4)*: – Cytomegalovirus – Epstein Barr virus – Herpes simplex types 1 and 2 – Herpes varicella-zoster – Human herpesvirus type 6 (HHV 6) – Human herpesvirus type 7 (HHV 7) – Human herpesvirus type 8 (HHV 8) • Rhabdoviridae – Genus: Lyssavirus Rabies virus (fixed virus / vaccine strain) • Genus: Vesiculovirus – Vesicular stomatitis virus - laboratory adapted strains including VSV-Indiana, San Juan and Glasgow, Piry, Chandipura	• Polyomaviridae all isolates including BK and JC viruses, Simian virus 40 (SV 40) • Poxviridae all types, *except Monkeypox virus and restricted poxviruses such as Alastrim, Smallpox, and Whitepox (refer to RG 3 and 4)* includes viruses: – Buffalopox, Cowpox, Milker' s nodule, Molluscum contagiosum, Orf, Vaccinia, Yabapox and Tanapox • Reoviridae – Genus: Coltivirus all types including Colorado tick fever virus – Genus: Rotavirus all human Rotaviruses – Genus: all isolates of Orthoreovirus and Orbivirus • Togaviridae – Genus: Alphaviruses - Group A
	• Arboviruses – Bebaru, Barmah forest virus, Chikungunya, O'nyong-nyong, Ross river virus, Semliki forest virus, Sindbis, Venezuelan equine encephalomyelitis vaccine strain TC-83 only – Genus: Rubivirus Rubella virus

VIRUSES AND PRIONS: RG3	
• Arenaviridae – Flexal, Mopeia – Lymphocytic choriomeningitis virus (LCM) (neurotropic strains) • Bunyaviridae – Genus: Hantaviruses – Hantaan virus (Korean haemorrhagic fever), Seoul, Sin Nombre virus, Belgrade, Puumala and unclassified Bunyaviruses – Genus: Nairovirus – Bhanja – Genus: Phlebovirus – Rift Valley fever virus • Coronaviridae SARS Coronavirus • Flaviviridae - Group B Arboviruses – Genus: Flavivirus – Yellow fever virus (wild type), West Nile fever virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, Ntaya virus group: Israel turkey meningitis virus Modoc virus group: Sal Vieja virus, San Perlita virus Tentative species: Rocio, Spondweni, Wesselsbron Tick-borne encephalitis virus group: Hanzalova, Absettarov, Hypr, Kumlinge, Louping Ill, Negishi, Powassan • Orthomyxoviridae – Avian Influenza virus A, H5N1	• Paramyxoviridae – Genus: Henipah – Hendra (Equine morbillivirus), Nipah virus • Poxviridae • Monkeypox virus • Togaviridae - Group A Arboviruses – Genus: Alphavirus – Semliki Forest virus, Getah, Mayaro, Middleburg, Ndumu – Eastern equine encephalomyelitis, Western equine encephalomyelitis, Venezuelan equine encephalomyelitis virus (except the vaccine strain TC-83), Sagiyama, Tonate, Mucambo • Retroviridae – Human immunodeficiency virus (HIV) types 1 and 2 – Human T cell lymphotropic virus (HTLV 1 and 2) – Simian immunodeficiency virus (SIV) • Rhabdoviridae – Rabies virus (Street virus) • Unclassified Viruses –Chronic infectious neuropathic agents (CHINAs). • Prions –Transmissible spongioform encephalopathies (TME) agents: Bovine spongiform encephalopathy (BSE), Creutzfeldt-Jacob disease (CJD), Variant Creutzfeldt-Jacob disease, Fatal familial insomnia, Gerstmann-Straussler-Scheinker syndrome and Kuru

VIRUSES: RG4	
• Arenaviridae – Genus: Arenaviruses – Lassa, Guanarito, Junin, Machupo, and Sabia • Bunyaviruidae – Genus: Nairovirus – Crimean-Congo hemorrhagic fever virus • Filoviridae – all Ebola viruses and Marburg virus • Flaviridae (Togaviruses) - Group B Arboviruses – Tick-borne encephalitis virus complex including Central European encephalitis, Kyasanur Forest disease, Omsk hemorrhagic fever, and Russian spring-summer encephalitis viruses	• Herpesviruses (alpha) – Herpesvirus simiae (Herpes B or Monkey B virus) • Poxviridae – Variola major, variola minor, whitepox, alastrim *(Importation of organisms, including alastrim, smallpox (variola) and whitepox is strictly prohibited. All activities, including storage of variola and whitepox, are restricted to a single facility (World Health Organization Collaborating Center for Smallpox Research, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America)*. • Hemorrhagic fever agents and viruses as yet undefined
PARASITES: RG2	
• *Acanthamoeba spp* • *Ancylostoma* human hookworms including: – *Ancylostoma duodenale, Ancylostoma. ceylanicum* • *Angiostrongylus spp.* • *Anisakis simplex* • *Ascaris including: – Ascaris lumbricoides, Ascaris suum* • *Babesia including: – Babesia divergens, Babesia microti* • *Balantidium coli* • *Blastocystis hominis* • *Brugia filaria* worms including: – *Brugia malayi, Brugia timori* • *Capillaria spp.* • *Coccidia* • *Contracaecum osculatum* • *Cryptosporidium spp.* including: – *Cryptosporidium parvum* • *Cyclospora spp* incl. – Cyclospora cayetanensis • *Cysticercus cellulosae* (hydatid cyst, larva of *Taenia solium*) • Dicrocoelium dendriticum • Dientamoeba fragilis • Dracunculus medinensis	• Fasciola gigantica / *Fasciola hepatica*, • *Fasciolopsis buski* • *Giardia spp.* Including – *Giardia lamblia (Giardia intestinalis)* • *Heterophyes spp*. • *Hymenolepis diminuta / Hymenolepis nana* • *Isospora belli* • *Leishmania* spp. (mammalian) *except Leishmania braziliensis and Leishmania donovani (Refer to Risk Group 3)* includes: – *Leishmania ethiopia, Leishmania major, Leishmania mexicana, Leishmania peruvania, Leishmania tropica* • *Loa loa* filaria worms *Mansonella spp*. such as: – *Mansonella ozzardi, Mansonella perstans, Mansonella streptocerca* • *Metagonimus spp.* • *Microsporidium spp.* • *Naegleria spp.* • *Necator human hookworms* including: – *Necator. americanus* • *Onchocerca* filaria worms including, *Onchocerca volvulus*

• Entamoeba histolytica • Enterobius vermicularis • Enterocytozoon bieneusi	• *Opisthorchis felineus* • *Opisthorchis sinensis (Clonorchis sinensis)* • *Opisthorchis viverrini (Clonorchis viverrini)* • *Paragonimus spp* including: *P. westermani*
PARASITES: RG2	
• *Plasmodium spp*. (human and simian) including: – *Plasmodium cynomologi, Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax* • *Sarcocystis suihominis* • *Schistosoma spp*. Incl.: – *Schistosoma haematobium, Schistosoma intercalatum, Schistosoma japonicum, Schistosoma mansoni, Schistosoma mekongi* • *Strongyloides spp.* Incl. – *Strongyloides stercoralis* • *Taenia saginata / Taenia solium* • *Toxocara spp*. Incl. – *Toxocara canis* • *Toxoplasma spp.* Incl. : *Toxoplasma gondii*	• *Trichinella nativa / Trichinella nelsoni* • *Trichinella pseudospiralis / Trichinella spiralis* • *Trichomonas vaginalis* • *Trichostrongylus spp*. including *Trichostrongylus orientalis* • *Trichuris trichiura* • *Trypanosoma brucei sub-spp. except Trypanosoma brucei rhodesiense and Trypanosoma cruzi (Refer to Risk Group 3)* includes *Trypanosoma brucei gambiense* • *Wuchereria bancrofti* filaria worms
PARASITES: RG3	
• *Echinococcus spp. such as:* *– Echinococcus granulosis, Echinococcus multilocularis, Echinococcus vogeli*	• *Leishmania braziliensis,* • *Leishmania donovani* • *Trypanosoma brucei rhodesiense* • *Trypanosoma cruzi*
PARASITES: RG4	
NONE	
FUNGI: RG1	
Agaricus bisporus *Acremonium chrysogenum / strictum / elegans* *Actinomucor elegans* *Ashyba gossypii* *Aspergillus oryzae* *Aureobasidum pullulans* *Blakeslea trispora* *Brettanomyces bruxellensis* *Candida boindinii / shehateae/utilis* *Chaetonium globosum*	*Mortierella vinacea* *Mucor circnelloides / mucedo / plumbeus / rouxii* *Myrothecium verrucaria* *Neurospora crassa / sitophilla* *Nigrospora sphaerica* *Oxyporus populinus* *Pachysolen tannophilus* *Paecilomyces varioti/lilacinus* *Penicillium camemberti / chrysogenum / funiculosum*

Cladosporium cladosporioides *Claviceps paspali / purpurea* *Coprinus cinereus* *Cunninghamella blakesleana / elegans* *Curvularia lunate* *Cyathus stercoreus* *Debaryomyces hansenii* *Dacrymyces deliquescens* *Engyodontium album* *Geotrichum candidum* *Hansenula anomala / polymorpha* *Hypholama fasciculare / roseonigra* *Engyodontium album* *Geotrichum candidum* *Hansenula anomala / polymorpha* *Hypholama fasciculare / roseonigra* *Kloecdera corticis* *Lentinus edodes* *Lipomyces lipofer / sarkeyi* *Metarhizium anisopliae* *Monascus pupureus / ruber* *Moniliella suaveolena*	*Phycomyces blakesleanus* *Pichia farinosa / guilliermondii / membranae faciens/stipitis* *Pleurotus ostreatus* *rhizoctonia solani* *Rhizopus oryzae / stolonifer* *Rhodosporidum toruloides* *Rhodotorual glutinis* *Saccharomyces cerevisiae* *Schizoasccharomyces pombe* *Schwanniomyces occidentalis* *Sordaria macrpsopra* *Thanatephorus cucumeris* *Trametes vesicolor* *Trichoderma harzianum / longibrachiatum / viridae* *Trigonopsis variabilis* *Verticillium lecanii* *Volvariella volvacea* *Wallernia sebi* *Xeromyces bisporus* *Zygorhynus moelleri* *Zygosaccharomyces bailii / rouxii*
FUNGI: RG2	
• *Asperigillus fumigatus* • *Asperigillus flavus* • *Candida albicans* • *Candida tropicalis* • *Cryptococcus neoformans var neoformans (Filobasidiella neoformans var neoformans)* • *Cryptococcus neoformans var gattii (Filobasidiella bacillispora)* • *Dactylaria galopava (Ochroconis gallopavum)* • *Emmonsia parva var parva* • *Emmonsia parva var crescens* • *Epidermophyton spp.* including: – *Epidermophyton floccosum*	• *Exophiala (Wangiella) dermatitidis* • *Fonsecaea compacta / Fonsecaea pedrosoi* • *Madurella grisea* • *Madurella mycetomatis* • *Microsporum spp* • *Neotestudina rosatii* • *Penicillium marneffei* • *Scedosporium apiospermum (Pseudallescheria boydii)* • *Scedosporium proliferans (inflatum)* • *Sporothrix schenckii* • *Trichophyton spp.* including: – *Trichophyton rubrum*

FUNGI: RG3	
• *Blastomyces dermatitidis (Ajellomyces dermatitidis)* • *Cladophialophora bantiana (Cladosporium bantianum, Xylohypha bantiana)* • *Cladosporium trichoides* • *Coccidioides immitis*	• *Histoplasma capsulatum spp*. including: – *Histoplasma capsulatum var capsulatum* – *Histoplasma capsulatum var farcinimosum* – *Histoplasma. capsulatum var. duboisii* • *Paracoccidioides braziliensis*
FUNGI: RG4	
NONE	

APPENDIX 3

HOST / VECTOR SYSTEMS PROVIDING BIOLOGICAL CONTAINMENT

The objective of biological containment is to minimise both the survival of the host and vector outside the laboratory, and the transmission of the vector from the propagation host to a non-laboratory host. This Appendix lists the host/vector systems which are currently accepted as providing a level of biological containment *(Reference: Singapore Biosafety Guidelines for Research on GMOs 2006).*

	Host	Vector
Bacteria	*Escherichia coli* K12 or *E. coli* B derivatives which do not contain conjugative or generalized transducing phages *Bacillus subtilis* or *B. licheniformis*, Asporogenic strains with a reversion frequency of less than 10 -7 *Pseudomonas putida* Strain KT 2440 *Streptomyces* specified species, *S. coelicolor, S. lividans, S. parvulus, S. griseus*	1. Non-conjugative plasmids 2. Bacteriophage – lambda – lambdoid – Fd or F1 (e.g. M13) Indigenous *Bacillus* plasmids and phages whose host range does not include *B. cereus* or *B. anthracis* 1. Certified plasmids: – pKT 262, pKT 263, pKT 264 2. Certified plasmids: SCP2, SLP1, SLP2, PIJ101 and derivatives 3. Actinophage phi C31 and Derivatives
Fungi	*Neurospora crassa*, laboratory strains *Saccharomyces cerevisiae, Pichia pastoris* *Schizosaccharomyces pombe*	No restriction No restriction No restriction No restriction
Slime Moulds	*Dictyostelium* species	*Dictyostelium* shuttle vectors, including those based on the endogenous plasmids Ddp1 and Ddp2

Tissue Culture	Mammalian (including human) cells Avian cells Plant cell cultures Insect cell cultures such as *Spodoptera frugiperda*	Non-viral vectors or defective viral vectors (including retrovirus or retroviral-helper combinations) that cannot infect human cells. Avipoxvirus vectors Non-tumorigenic disarmed Ti plasmid vectors in *Agrobacterium tumefaciens* and non-pathogenic viral vectors* * Baculovirus (*Autographa californica* nuclear polyhedrosis virus)

Notes: The above approved hosts may also be used in experiments where DNA is inserted into the host cell without the use of a biological vector (e.g. by mechanical, electrical or other means), provided that the DNA:

- is not derived from microorganisms able to cause disease in humans, animals or plants, unless the DNA to be introduced is fully characterized and will not increase the virulence of the host or vector.
- does not code for a toxin for vertebrates with an LD50 of less than 100 ˜g/kg, and is not an oncogene.
- does not comprise or represent more than two-thirds of the genome of a virus and is not being used in an experiment in which the genetic material missing from the viral genome and essential for producing infection is available in the cell into which the incomplete genome is introduced, or is made available by subsequent breeding processes.
- Any commercially available Host-Vector Systems. Such a system with an approved host and the DNA meeting these conditions would constitute an approved host/vector system for the purposes of this Guideline.
- Any other plants expression vectors available either derived from *Agrobacterium* species or other commercially available vector systems.

APPENDIX 4

EXAMPLES OF GMM RISK ASSESSMENTS

The following risk assessments give an example format and are for illustrative purposes only. They are not intended to prescribe how GM risk assessments are to be carried out.

Furthermore, they are not exhaustive and under each section, advice is given on the type of information that should be included to provide a comprehensive document that should enable a reviewer (PI, IBC or external) to determine whether the risk assessment is suitable and sufficient.

Example 1: Construction of an Adenoviral vector with a modified tissue tropism

1. Overview

The aim of this project is to develop a replication competent adenoviral vector with a modified fibre gene that targets the virus to leukaemic cells. The long-term aim is to use the virus in the treatment of leukaemia.

An amount of background information regarding the purpose of the work should be included. For example, the long-term aim is to use the virus in the treatment of leukaemia. This will ultimately be evaluated in animal and human studies.

2. Nature of the risks

The work involves the genetic modification of a human pathogen. Therefore, risk assessment for human health will take precedence.

3. Risk assessment for human health

3.1 Mechanisms by which the GMM might pose a hazard to health

What are the hazards associated with the recipient virus?

The vector under development will be based on adenovirus serotype 5 (Ad5), which is a RG2 pathogen. It will be replication competent. Specific details of the nature of adenovirus pathogenesis should be incorporated. For example, it is known to cause mild respiratory symptoms in children and is transmitted via aerosol and the faecal-oral route. Over 90% of individuals are seropositive for Ad5 and immunity is thought to be life-long.

What hazards are associated with the inserted genetic material?

The insert will be the modified segment of the adenovirus fibre gene. This protein will not in itself be inherently harmful. Relevant facts regarding the insert and expression characteristics

should be included here. For example, the modification involves the adenovirus L5 gene and it is expected that expression will be equivalent to wild type L5.

Have the pathogenic traits of the recipient virus been altered?

The project will involve the replacement of the L5 fibre gene with a version in which the sequence of the knob region has been modified. The modification will involve the insertion of a binding site for a protein present at high levels on the surface of leukaemic cells. This will mean that the virus should have an altered tissue tropism *in vivo*. It is predicted that GMM will specifically target, replicate within and destroy leukaemic cells. This surface protein may also be present at lower levels on normal lymphocytes and therefore the GMM may infect normal lymphocytes at a higher efficiency than wild type adenovirus, which does not normally infect blood cells *in vivo*.

This section would benefit from the addition of extra background information. For example, the role of the fibre protein in normal adenovirus infection and the principles behind retargeting the virus to infect lymphocytes should be expanded upon. This section could be reinforced with experimental data and/or references.

Could the GMM or other organisms acquire harmful sequences?

The DNA sequence corresponding to the modified fibre gene could represent a hazard if it were to recombine into a wild type adenovirus as the recombinant would be able to infect lymphocytes.

Inadvertent recombination would generate a virus that would represent a similar hazard to the intended GMM. Therefore it is unlikely that specific containment measures will be required to prevent cross-contamination.

3.2 Likelihood that the GMM will be a risk to human health and safety

Could the GMM establish an infection in vivo and how efficient would it be?

The GMM may be able to infect normal lymphocytes and replicate within them. Given that the virus cannot infect cells to which it is naturally targeted, this infection is likely to be less efficient.

Uncertainty must be taken into consideration in the risk assessment. There is no experimental evidence presented to support the supposition that the GMM will be less fit than wild type Ad5, nor is there any to demonstrate that only lymphocytes are susceptible. Moreover, it is unlikely there will be selective pressure for deletion of the modified sequences since this would leave the virus without a receptor-binding site.

How severe might the consequences be?

There is a likelihood of harm arising in the event of an individual becoming exposed in that

the virus could productively infect lymphocytes and destroy them. The ramifications of this consequence are potentially severe and should be expanded upon. Infection of lymphocytes with a replication-competent adenovirus could result in immunosuppression. Furthermore, this could impair the ability of the individual's immune system to clear the virus.

3.3 Containment level needed to sufficiently protect human health

The parental virus is listed as a RG2 pathogen. An important additional hazard that will arise as a result of the genetic modification is the possible alteration of tissue tropism. Therefore GM-BSL3 may needed to sufficiently safeguard human health.

This step will often involve considering the BSL necessary to control the risk of the recipient virus and making a judgement about whether the modification will result in a GMM that is more hazardous, less hazardous or approximately equivalent. Sometimes it may help to compare the GMM with the relative hazard presented by other organisms.

4. Risk assessment for the environment

4.1 Mechanisms by which the GMM might pose a hazard to the environment

Has the stability or survivability of the recipient virus been altered?

Adenoviruses are non-enveloped DNA viruses, relatively stable and resistant to dehydration. The modifications proposed are not expected to affect stability compared to the wild type virus. Human adenoviruses have been shown to enter some animal cells although they are not thought to replicate efficiently. Therefore, it is unlikely that the GMM will be able to survive or disseminate in the environment.

Has the infectivity, pathogenicity or host range of the recipient been altered?

None of the modifications proposed are expected to alter the host range or infectivity of the vector.

Does the inserted gene pose a risk to other organisms?

The inserted sequences are not expected to represent a hazard to other organisms.

Could the GMM or other organisms in the environment acquire harmful sequences?

Exchange of genetic material is unlikely, as human adenoviruses do not normally productively infect other species. It is assumed that the modified tropism of the GMM is specific for human lymphocytes. No data is presented to demonstrate this. There is a finite possibility that an animal adenovirus could recombine with the GMM within animal cells and acquire an altered tropism. Measures may be required to minimise the possibility of release, although in this case the GMM already commands a high level of containment for human health purposes.

4.2 Likelihood that the GMM will be a risk to the environment

What is the likelihood that the hazards will be manifested?

The likelihood of the GMM constituting a hazard to the environment is UNLIKELY.

What will be the consequences if the hazard is realised?

The effects of accidental exposure could be transfer of the inserted genetic material to an animal virus, although it is anticipated that this recombinant would be non-infectious. The consequences of exposure are therefore MINOR.

4.3 Containment level needed to protect the environment

GM-BSL2 is sufficient to prevent release and protect the environment. A higher level of containment has been assigned to protect human health, the environmental risk is essentially NEGLIGIBLE.

5. Review procedures and control measures

Implement measures to safeguard human health and the environment

What operations and procedures might increase risk of exposure?

The GMM will be transmissible by an airborne route and cell culture procedures may generate aerosols and therefore pose a specific risk of exposure.

The GMM will be concentrated over caesium chloride gradients and purified using dialysis cassettes. These operations require the use of hollow needles and this increases the risk of stick injury and inadvertent inoculation.

What control measures and monitoring procedures are to be used?

The virus will be replication competent and it is appropriate for all manipulations involving this virus to be undertaken within a biological safety cabinet to contain aerosols. High-speed centrifugation will take place in sealed vessels within a removable rotor.

An inward airflow will be required and the laboratory will be sealable for fumigation. Access to the laboratory will be restricted to authorised staff only.

Any standard procedures or guidelines should be outlined here. Full details of standard procedures can be appended in full to the risk assessment. For example, new staff will be specifically trained in the safety aspects of this work with written training records being kept.

Are the potential routes of environmental release known and managed?

The most likely routes for the release of the virus into the environment are via aerosol dissemination and contaminated waste. These routes are known and managed.

Details of the waste disposal procedures and the effectiveness of inactivation methods would enhance this section. For example, all waste materials will be autoclaved using equipment situated within the laboratory suite. Liquid waste will be inactivated using a hypochlorite disinfectant according to the manufacturer's instructions.

6. GM BSL Containment

Assign final GM activity class - 1, 2, 3 or 4

The measures required to prevent exposure or release indicate that GM-BSL3 is required. There is no case for seeking derogation on any of the measures that are specified.

The activity is therefore assigned to GM-BSL3.

Any additional measures or derogations from the standard GM-BSL3 conditions should be outlined here.

Example 2: Laboratory production of recombinant adenovirus vector

1. Overview

The aim of this project is to produce recombinant adenovirus viral vector. The background information on the use of the Ad vector should be included. For example the phase 2 of the project will be application of the vector as a vaccine delivery tool which will be evaluated in animal studies by induction of systemic and mucosal immune responses in rats immunized with the Ad vector expressing different antigens.

2. Nature of risks

The work involves the genetic modification and the propagation of a human pathogen. The risk assessment for human health will take precedence.

3. Risk assessment for human health

3.1 Mechanisms by which the GMM might pose a hazard to health

What are the hazards associated with the recipient virus?

The recipient organism is human Adenovirus type 5 (Ad5). It is a human pathogen, a RG 2 virus. The Ad genome is divided into Early (E) and Late (L) genes, expressed respectively

before and after replication of the viral chromosome. E1 gene products are involved in the control of viral gene transcription, shut-off of cellular proteins and cellular transformation. E3 gene codes for proteins that interfere with the host immune responses against virus infection. The complete E1 region and the majority of the E3 region of the genome have been removed. The adenovirus vector is replication defective by virtue of deletion of the E1 and E3 regions, rendering the viral vector replication deficient.

What are the hazards associated with the construction vector?

Construction of recombinant adenovirus is a two step process in which the desired expression cassette is first assembled into a pUC vector and subsequently transferred into the adenoviral genome by homologous recombination. pUC vectors have a history of safe use.

What are the hazards associated with the inserted genetic material?

The relevant facts regarding the insert xyz and expression characteristics of the protein should be included here. For example the expressed protein will not be inherently harmful.

3.2 Likelihood that the GMM will be a risk to human health and safety

Could the GMM establish an infection in vivo and how efficient would it be?

The recombinant adenovirus is replication deficient and therefore can only replicate in cells which carry complementing regions of the E1 genes. It will not replicate in other *in vivo* or *in vitro* cells. The modified virus is is less pathogenic than the wild type since it is replication deficient and there is minimal capacity for colonisation. If it is exposed to the environment via aerosols, it is unlikely to survive for extended periods.

A replication competent Adenovirus has the potential to be produced. The host cell used in the project is PER.C6 cell line, derived from primary human embryonic retinoblasts, transformed with the E1 region of Ad5. Since the Adenoviral vector is replication deficient, recombinant adenovirus can only grow in complementing cells such as PER.C6 which contain the appropriate E1 sequences. PER.C6 cells have limited homology with Ad5 viral sequences and reduces or eliminates the emergence of recombinant competent adenovirus. Therefore in order to generate a replication competent adenovirus, two non homologous recombination events would have to occur. A revertant regaining the E1 gene would still be devoid of the E3 gene since PER.C6 does not contain the E3 gene. The absence of the E3 gene would reduce the fitness of the virus as an infective agent.

How severe might the consequences be?

Wild type ad5 is ubiquitous, causes a mild upper respiratory tract infections in humans that is self limiting and does not require any specific treatment. Similarly there is no association with allergic or toxic effects. As explained above, the modified virus is is less pathogenic than the wild type since it is replication deficient.

3.3 Containment level needed to sufficiently protect human health

The parental virus is listed as a RG2 pathogen. The adenovirus viral vectors are replication defective owing to the removal of the E1 and the E3 regions. Even if replication competent adenoviruses were generated, the risk associated is low since human adenoviral infection is very common and the majority of adults may have already been infected. However, GM adenovirus may mimic some of the characteristics of the wild type or may pose a risk to immuno compromised individuals. Since the project involves production of large quantities of virus GM-BSL2 may still be needed to sufficiently safeguard human health.

4. Risk assessment for the environment

4.1 Mechanisms by which the GMM might pose a hazard to the environment

Has the stability or survivability of the recipient virus been altered?

Adenoviruses are non-enveloped DNA viruses, relatively stable and resistant to dehydration. The modifications proposed are not expected to affect stability compared to the wild type virus

Has the infectivity, pathogenicity or host range of the recipient been altered?

None of the modifications proposed are expected to alter the host range or infectivity of the vector.

Does the inserted gene pose a risk to other organisms?

The inserted sequences are not expected to represent a hazard to other organisms.

4.2 Likelihood that the GMM will be a risk to the environment

What is the likelihood that the hazards will be manifested?

The likelihood of the GMM constituting a hazard to the environment is UNLIKELY. The host cells, PER.C6 die rapidly outside the artificial environment created within the laboratory. There is little likelihood of the recombinant PER.C6 cells proliferating or surviving in the environment and therefore poses little risk to animal/plant health or the environment

4.3 Containment level needed to protect the environment

GM-BSL2 is sufficient to prevent release and protect the environment.

5. Review procedures and control measures

5.1 Implement measures to safeguard human health and the environment

What operations and procedures might increase risk of exposure?

The GMM will be transmissible by an airborne route. Working with large scale volumes, manipulations with viral cultures and cell culture procedures, for example, repeated freeze-thawing of cell cultures to release viruses, may generate aerosols and therefore pose a specific risk of exposure.

What control measures and monitoring procedures are to be used?

Work practices as in *Biosafety guidelines for Contained use activity of LMO, 2010* should be outlined here. Full details of standard operating procedures can be appended in full to the risk assessment. For example, staff training and competency in the safety aspects of this work with written training records being kept.

All manipulations involving this virus will be undertaken within a Class II biological safety cabinet to contain aerosols. High-speed centrifugation will take place in sealed vessels within a removable rotor.

Access to the laboratory will be restricted to authorised staff only.

Are the potential routes of environmental release known and managed?

The most likely routes for the release of the virus into the environment are via aerosol dissemination and contaminated waste. These routes are known and managed.

Details of the waste disposal procedures and the effectiveness of inactivation methods would enhance this section. For example, all waste materials will be autoclaved using equipment situated within the laboratory suite. Liquid waste will be inactivated using a hypochlorite disinfectant according to the manufacturer's instructions.

6. GM BSL Containment

Assign final GM activity class - 1, 2, 3 or 4

The measures required to prevent exposure or release indicate that GM-BSL2 is required. The activity is therefore assigned to GM-BSL2.

Example 3: Development of an animal model for *Neisseria meningitidis* disease

1. Overview

The aim of the project is to develop a new animal model for the study of Neisseria meningitidis disease processes by replacing the genes encoding transferring binding proteins (TbpA and TbpB) with those encoded by the pig pathogen *Actinobacillus pleuropneumoniae*.

An amount of background information regarding the nature of the proposed GMM and the purpose of the work should be included. For example, the roles of the Tbp proteins in the pathogenesis of *N. meningitidis* should be outlined. It should also be stated that there is

currently no animal model for *N. meningitidis* disease processes and that the work will involve both laboratory manipulations of the organism and large animal experimentation.

2. Nature of the risks

The activity involves the genetic modification and handling of a human pathogen. Therefore risk assessment for human health will take precedence.

In this case, the resulting GMM also poses a significant risk to the environment and ultimately, it is the environmental concerns that set the activity class. Risk assessment for the environment could legitimately take precedence here.

3. Risk assessment for human health

Mechanisms by which the GMM might pose a hazard to health

What are the hazards associated with the recipient strain?

N. meningitidis is classified as a RG2 bacteria and is a specific human pathogen.

Specific details of the nature of *N. meningitidis* pathogenesis should be incorporated. For example, it is a commensal organism that is transmitted by aerosol and direct contact. It is normally carried asymptomatically but is the cause of meningococcal septicaemia and meningitis in children.

What hazards are associated with the inserted genetic material?

The transferrin binding proteins encoded by the genetic inserts are not believed to be inherently toxic to humans as they specifically bind porcine transferrin. Relevant facts known about the functions of the encoded products and their expression characteristics should be included here. For example, the *A. pleuropneumoniae* genes will be expressed in the GMM from the endogenous *fur* promoter that regulates native *N. meningitidis tbp* genes. The promoter is not considered to be strong.

Have the pathogenic traits of the recipient strain been altered?

Pathogenicity for human hosts will be reduced since the GMM will be rendered unable to sequester iron from human transferrin.

Since prophylaxis for *N. meningitidis* infection is antibiotic treatment, any antibiotic resistance that is conferred during the construction of the GMM should be stated and assessed here.

Could the GMM or other organisms acquire harmful sequences?

Exchange of genetic material is possible between the GMM and commensal *Neisseria*.

The risk assessment would benefit from extended considerations as to the mechanism and likelihood of genetic transfer to commensal strains. For example, it is known that *Neisseriacae* are naturally competent and thus genetic exchange is likely in the event that the GMM and commensal organisms interact. The use of any techniques that would prevent exchange should be stated and assessed.

Likelihood that the GMM will be a risk to human health and safety

Could the GMM establish an infection in vivo and how efficient would it be?

The ability of the GMM to infect and colonise human hosts is expected to be unchanged. However, pathogenicity will be diminished reducing the fitness of the organism due to the inability to scavenge iron from human transferrin.

How severe might the consequences be?

The consequences would not be expected to be more severe than infection with wild type *N. meningitidis* and most likely, less so.

Containment level needed to sufficiently protect human health

No new hazards are apparent therefore GM-BSL2 is sufficient.

4. Risk assessment for the environment

Mechanisms by which the GMM might pose a hazard to the environment

Has the stability or survivability of the recipient strain been altered?

The stability of the organism will be unchanged as *N.meningitidis* is an obligate pathogen and cannot survive outside the host organism.

Inclusion of scientific knowledge or data relating to the longevity of *N. meningitidis* survival outside the host could enhance this assessment. The genetic stability of the modification itself should also be considered and that the GMM could survive within human or porcine carriers as a commensal organism and possibly be disseminated.

Has the infectivity, pathogenicity or host range of the recipient been altered?

The modification is expected to enable *N. meningitidis* to infect and cause disease in pigs. This statement should ideally be qualified. For example: The replacement of the *tbp* genes of *N. meningitidis* with those of *A. pleuropneumoniae* will allow the GMM to scavenge iron from porcine transferrin and therefore may become pathogenic for pigs.

Does the inserted gene pose a risk to other organisms?

The products of the inserted gene are not considered to be inherently toxic. However, the expression by the GMM may result in disease in porcine hosts.

Could the GMM or other organisms in the environment acquire harmful sequences?

Exchange of genetic material is possible between the GMM and strains in the environment. The risk assessment would benefit from extended considerations as to the mechanism and likelihood of genetic transfer to other strains, taking into account the known natural competency of *Neisseriae*.

Likelihood that the GMM will be a risk to the environment

What is the likelihood that the hazards will be manifested?

The likelihood that the GMM will be released into the environment under the requirements of the containment level to protect human health is HIGHLY UNLIKELY.

There are two aspects to the work outlined in this risk assessment. The first involves laboratory manipulations of *N. meningitidis* to generate GMM and these operations are sufficiently contained at BSL2. The second aspect is the use of the GMM in large animal studies and with procedures such as these, it is more difficult to maintain containment. Furthermore, there may be a regional context if the animal work is to take place in a locality where there is domestic pig farming or wild pig colonies. In light of this, it may be more accurate to state that the likelihood is LIKELY to UNLIKELY.

What will be the consequences if the hazard is realised?

The consequences of GMM dissemination in the environment could be INTERMEDIATE to MAJOR.

The consequences could be qualified. The GMM could represent a novel pig pathogen that could disseminate in both domestic and wild pigs. This could have significant environmental and economic impact that should be both assessed and addressed.

Containment level needed to protect the environment

GM-BSL2 is sufficient to prevent release into the environment.

Again, this may be true for the laboratory stages of the work but less applicable or sustainable with large animal studies. If this is the case, this should be qualified and addressed in procedures and control measures.

Review procedures and control measures

Implement measures to safeguard human health and the environment

What operations and procedures might increase risk of exposure?

Some laboratory procedures may result in the aerosolisation and the use of hollow needles for experimental inoculation of animals increases the likelihood percutaneous inoculation.

What control measures and monitoring procedures are to be used?

The standard operating procedures used for handling *N. meningitidis* will also be appropriate for handling the GMM. Specific animal handling procedures will minimise the risk to staff.

Any standard operating procedures or guidelines should be outlined here. For example, Class II biological safety cabinets will be used to control aerosols in the laboratory and all animal work will take place on a downdraft autopsy table. If appropriate, full details can be appended to the risk assessment.

Are the potential routes of environmental release known and managed?

Colonisation of human hosts with the GMM and waste disposal are the major routes by which the GMM could be released. These routes are known and managed and the risk of harm to the environment is NEGLIGIBLE.

Details of the waste disposal procedures and the effectiveness of inactivation methods would enhance this section.

GM-BSL Containment

Assign final GM activity class - 1, 2, 3 or 4

The proposed genetic modifications will generate *N. meningitidis* that is attenuated for humans but with the potential to infect pigs. The work is therefore assigned to be handled at GM-BSL3. Any additional measures or derogations from the standard GM-BSL3 conditions should be outlined here.

Example 4: Analysis of helminth immune evasion genes by expression in Leishmania

1. Overview

The aim of this project is to express immune modulating genes from the Helminth parasite *Brugia malayi* in the protozoan *Leishmania major*. This will be used to characterise the modulation of immune responses to GM *Leishmania*.

Relevant information pertinent to the nature of the proposed GMM and the purpose of the work should be included, for example, information regarding the pathogenesis of the donor organism *B. malayi* and how the functions of the immune modulating genes to be inserted may be involved. The rationale for the experiment (i.e. *L. major* is a more tractable system for

modification and immune studies than helminths) and the nature of the work (i.e. laboratory manipulations and small animal experimentation) should also be outlined.

2. Nature of the risks

The activity involves the genetic modification and handling of a human pathogen. Therefore risk assessment for human health will take precedence.

3. Risk assessment for human health

Mechanisms by which the GMM might pose a hazard to health

What are the hazards associated with the recipient strain?

L. major is an RG 2 agent and is a pathogen of both humans and animals.

Specific details of the nature of *L. major* pathogenesis should be incorporated. For example, it causes cutaneous lesions (cutaneous Leishmaniasis) that are normally self-healing and is naturally transmitted only by an intermediate vector (i.e. phlebotomid sandflies).

What hazards are associated with the inserted genetic material?

The *B. malayi* genes to be inserted are modulators of the immune system. They are Macrophage Inhibitory Factor (MIF) 1 and 2 and Cystein Protease Inhibitor (CPI) 1 and 2

Significant facts about the functions of the encoded products and the likely expression characteristics in the GMM should be included here. For example, the biological activities of the *B. malayi* genes are known, even if the precise role in pathogenesis is unclear. Furthermore, the genes will be inserted into a conserved ribosomal RNA gene locus and expression driven by the innate promoters present.

Have the pathogenic traits of the recipient strain been altered?

The GMM may acquire a more pathogenic phenotype, as it may be able to modulate the host's immune system. However the inserts do not encode known virulence determinants so it is unlikely that there will be a significant shift in pathogenicity.

There is uncertainty as to the pathogenic phenotype of the GMM and this should be taken into consideration in the risk assessment. The possible nature of any increase in pathogenicity should be postulated based upon known scientific facts, for example lesions may heal more slowly or parasite numbers may reach a higher peak.

Could the GMM or other organisms acquire harmful sequences?

There is no possibility of the sequences being transferred to other organisms as linearised plasmid DNA is integrated into the host genome. The risk assessment would benefit from a reasoned argument as to why sequence transfer is impossible. For example, the organisms

are transfected with linearised plasmid DNA, precluding episomal maintenance. The plasmid is non mobilisible and there is no means of excision or independent replication following integration into the *L. major* genome.

Likelihood that the GMM will be a risk to human health and safety

Could the GMM establish an infection in vivo and how efficient would it be?

The GMM could establish an infection in a human host and will likely be at least as efficient as wild type *L. major*.

How severe might the consequences be?

It is possible that the genetic modification could suppress the normal immune response to *L. major* and therefore alter the outcome of the infection.

The possible alteration in outcome could be usefully elaborated upon. For example, the infection may not resolve at all or the organism may gain the ability to visceralise.

What is the probability that rare events will occur?

No probabilistic considerations are given in the risk assessment, possibly due to the lack of precise information with which to calculate them. A qualitative assessment could be made regarding the likelihood of inadvertent infection and that the impact of the outcome would be diminished due to the availability of antimonial drugs.

Containment level needed to sufficiently protect human health

No new hazards are apparent therefore GM-BSL2 is sufficient.

4. Risk assessment for the environment

Mechanisms by which the GMM might pose a hazard to the environment

Has the stability or survivability of the recipient strain been altered?

The survivability and stability of the GMM can be assumed to be comparable to wild type *L. major.*

Inclusion of known scientific knowledge or data relating to the longevity of *L. major* outside a host could enhance this assessment and it could be stated that transmission is not possible from contact with environmental matrices.

Has the infectivity, pathogenicity or host range of the recipient been altered?

The infectivity and host range of the GMM can be assumed to be comparable to wild type L. major.

The risk assessment would benefit from more information regarding susceptible organisms. Humans are at risk from infection, as are wild and domestic animals that may serve as a natural reservoir. However, since the intermediate host/vector (phlebotomid sandflies) is not present in Malaysia, infection would not spread beyond the primary host.

Does the inserted gene pose a risk to other organisms?

Humans as well as wild and domestic animals are at risk from infection and therefore may be affected by the immune modulating proteins encoded by the insert.

Likelihood that the GMM will be a risk to the environment

What is the probability that the hazards will be manifested?

Phlebotomid sandflies, the intermediate vector of L. major, is not present in Malaysia and therefore the likelihood of the hazards being manifested is HIGHLY UNLIKELY.

What will be the consequences if the hazard is realised?

The consequences of environmental release is MARGINAL.

Containment level needed to protect the environment

The risk to the environment is NEGLIGIBLE. GM-BSL1 is sufficient.

Review procedures and control measures

Implement measures to safeguard human health and the environment

What operations and procedures might increase risk of exposure?

Inoculating animals with the GMM using hypodermic needles increases the risk of infection via accidental percutaneous infection.

What control measures and monitoring procedures are to be used?

Specific standard operating procedures for the safe handling of and the injection of mice with *L. major* are in place and will be followed. All waste is autoclaved before disposal using a validated cycle.

Any standard operating procedures or guidelines should be outlined here. For example, the use of sharps is minimised for all operations and specific standard operating procedures for the injection of mice with *L. major* are in place and will be followed. All staff are trained in the standard operating procedures for animal work with *L. major*. Records of training are kept. Standard operating procedures stipulate the wearing of double-gloves, laboratory coats and eye protection.. Contaminated syringes are disposed of in a sharps waste container with the needle unsheathed.

Are the potential routes of environmental release known and managed?

Since the intermediate vector is not present in Malaysia, no transmission can occur. Thus, with the containment and waste disposal measures provisionally in place, the risk to the environment is NEGLIGIBLE.

GM BSL Containment

Assign final GM activity class - 1, 2, 3 or 4

No additional containment measures are required to control the risks to human health and the environment. The activity is therefore assigned to be handled at GM-BSL2 for both laboratory work and animal work. Any additional measures or derogations from the standard BSL2 conditions should be outlined here.

Example 5: Expression of peptides in plants using a plant virus

1. Overview

The aim of the project is to express the human endostatin peptide in the plant species *Nicotiana benthamia* using Potato virus X (PVX).

An amount of background information regarding the purpose of the work should be included. For example, the longer-term aim for the work might be to develop a system for production and manufacture of a therapeutic product, which would be handled in large numbers and possibly marketed. It would be helpful to state this here in the risk assessment.

Nature of the risks

The work involves the genetic modification of a plant pathogen and therefore risk assessment for environment will take precedence.

2. Risk assessment for the environment

Mechanisms by which the GMM might pose a hazard to the environment

Can the GMM survive, establish and disseminate?

PVX occurs naturally in many parts of the world including Malaysia, causing disease in potatoes. The recipient strains are naturally occurring Malaysian field isolates. The burden of the inserts is likely to reduce the fitness of the GMM in the wider environment, and it is anticipated that the inserts will be rapidly lost. Therefore, it will be assumed that the GMM constructed will retain the ability to establish infections in the Malaysian plant hosts.

Further information about the nature of the recipient strain should be included, for example its host range, properties of transmission and mechanisms of spread. Statements regarding fitness and the potential loss of inserts must be qualified, perhaps by using references

to scientific data and the literature. Where there is uncertainty, a precautionary approach should always be taken. For work like this that involves novel methods for the production of pharmaceutically active products, the regulatory authorities will require greater detailed evidence regarding the safety of the GMM.

What hazards does the inserted material pose?

The insert will encode human endostatin peptide, which is normally produced in humans and animals during wound healing. The gene will be expressed at high levels in plants via a duplicated subgenomic promoter for the PVX coat protein. The plant material will not be consumed by humans or animals in the laboratory and, as such, is not anticipated to pose a hazard.

The expressed product would not normally be present in the receiving environment in the context of the GMM or infected plant. Once again, a precautionary approach must be taken, as there is unlikely to be substantial evidence of how this product will affect the environment. Any assertions as to the safety of the product in the environment will need to be justified and are likely to be closely scrutinised.

Have the pathogenic traits of the recipient strain been altered?

The expression of the peptide is not anticipated to alter the pathogenicity of the virus, or its routes of transmission. If the virus was to escape and infect plants, endostatin could be expressed in the field. It is not expected that this in itself will be harmful.

These statements would need to be fully justified, using a reasoned argument. The regulators would not accept simple statements such as ‘it is not anticipated’ or ‘is not expected to be harmful’ without proper justification and supporting evidence.

Could the GMM or other organisms acquire harmful sequences?

No.

Further details and justification for this answer should be included here. For example, information about the potential for PVX to recombine with viruses in the field or stable transfer of the inserted sequence to the plant genome would be expected.

Is the GMM phenotypically/genetically stable?

Yes.

Statements like this must be justified. In this case, it is hard to justify the statement as arguments have already been presented that the insert will be rapidly lost from the virus. Therefore, the GMM is not genetically stable, even if the consequences of such an event are considered to be negligible.

Likelihood that GMM will be a risk to the environment

What is the likelihood that the hazards will be manifested?

There are no intermediate vectors (*e.g.* arthropods) known for PVX and the main route of environmental exposure is likely to be mechanical transmission via infected plant material. Given that host plants are not grown in the vicinity of the facility, the likelihood of the GMM escaping and infecting potato plants is unlikely.

Clearly, the likelihood on environmental release and dissemination of the GMM will be much higher if host plants are grown commercially or privately in the immediate environs of the facility. This is unlikely if the facility is in an urban area, but the likelihood of escape and dissemination will be higher in a rural setting.

What will be the consequences if the hazard is realised?

Should the GMM escape and find a suitable host, it is assumed that it will be able to initiate an infection and express endostatins in plants. While it is not expected that the disease symptoms elicited by the GMM will be any different from those associated with the wild type organism, a novel protein will be expressed. Therefore, the consequences can be considered to be INTERMEDIATE.

Given that the expressed product will be novel in the context of the GMM or host plant, there is a high degree of uncertainty and a precautionary approach should always be taken.

Determine risk level to the environment

Using the risk estimation matrix, the risk to environment is MODERATE or LOW.

Containment level needed to protect the environment

All laboratory work will be undertaken at GM-BSL2. This will reduce the risks to the environment to NEGLIGIBLE.

A brief explanation as to why GM-BSL2 is appropriate for this work and what specific measures are to be used should be included.

3. Risk assessment for human health

Mechanisms by which the GMM might pose a hazard to health

Are there any health hazards associated with the GMM?

Endostatin is naturally occurring in the human body. It will be expressed to high levels in experimental plants, but these will not be consumed. It is not anticipated that exposure to the peptides in the sap of infected plants will increase the allergenic or toxic hazards associated with the plants.

These statements would need to be fully justified, using a reasoned argument. The regulators would not accept simple statements such as 'it is not anticipated' without proper justification and supporting evidence. As endostatins are in therapeutic use, information on the toxicology of the product should be readily available.

Likelihood that GMM will be a risk to human health

What is the likelihood that the hazards will be manifested?

Likelihood that humans will be exposed to hazards associated with the GMM is UNLIKELY.

Justification for this assertion is required and will depend upon the likely route of exposure. For example, if the product is a potential allergen and humans may be exposed through handling the plants, then specific control measures (*e.g.* gloves, coveralls) may need to be assigned as control measures.

What will be the consequences if the hazard is realised?

Even if humans were to be exposed, no harmful effects are anticipated. Therefore, the consequences of exposure are considered to be MARGINAL.

A reasoned argument as to why there are no anticipated harmful effects is required here and will depend upon the toxicology of the product.

Estimation of risk to human health

Using the risk estimation matrix, the risks to human health are NEGLIGIBLE.

Containment level needed to protect human health

As no harmful effects are anticipated in the event of exposure, GM-BSL1 would be sufficient.

The use of protective clothing or gloves may be indicated as part of the environmental risk assessment to prevent release of the GMM into the environment. Such measures may be sufficient to protect against staff exposure. However, if RA indicates the need, a biological safety cabinet, may be used for protection of human health.

Review procedures and control measures

Implement measures to safeguard human health and the environment

What operations and procedures might increase risk of exposure?

None.

What control measures and monitoring procedures are to be used?

Measures are implemented for environmental protection. Standard good practice in a GP-BSL2 containment, glasshouse facility will be sufficient, including measures to control mechanical transmission of the GMM.

Details of the control measures used should be included. For example, the plants will be grown in pots and stored on trays within a locked room in a glasshouse for three weeks before being harvested. During this time only trained, authorised staff will enter the facility. All watering will be via a watering can, proper care should be taken so that the virus is not spread between plants. All infected waste (including plants, pots and soil) will be autoclaved and strict hygiene measures will be observed in the growth room, including the wearing of gloves, which will be disposed of through autoclaving and removal.

Are the potential routes of environmental release known and managed?

The most likely routes for release of the virus into the environment are via contaminated waste plant material and human mechanical transmission. These routes are known and managed.

GM BSL Containment

Assign final GM activity class – 1, 2, 3 or 4

The GP-BSL2 measures described above for environmental protection are considered appropriate for ensuring that all risks are NEGLIGIBLE.

The activity is therefore assigned to GP-BSL2.