Health and Safety Executive

The SACGM Compendium of guidance

Part 2: Risk assessment of genetically modified microorganisms (other than those associated with plants)

HSE Books

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2.1 Risk assessment of genetically modified microorganisms (other than those associated with plants)

Overview

1 Regulation 6 of the Genetically Modified Organisms (Contained Use) Regulations require that *no person shall undertake any activity involving genetic modification of microorganisms unless…a suitable and sufficient assessment of the risks… to human health and the environment has been carried out*. Those wishing to undertake or review risk assessments for the genetic modification of microorganisms are the intended users of this guidance. Users are also directed towards guidance relating to containment and control (see Part 3) and further information regarding the legislation that covers work with genetically modified organisms can be found in Part 1.

Scope

2 This section is intended to give guidance on 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. The guidance covers the modification of both animal and human pathogens but excludes microorganisms associated with plants (such guidance can be found in Part 4).

3 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 GM work involving bacterial or viral systems. This reflects the balance of work that is undertaken in the UK, although the principles of risk assessment set out are valid and applicable to GM activities involving all microorganisms.

4 Specific guidance giving more detailed information regarding aspects of GM work with microorganisms and commonly used systems is also included. The relevant section can be used to supplement the general principles of risk assessment given here. These sections relate to:

- 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.

5 Sample GM risk assessments are also given at the end of this document.

Activities that are likely to raise safety issues

6 There are some types of work where particular caution must be exercised. These are generally cases where the pathogenicity or host-range of a pathogen has been enhanced or altered.

7 GM pathogens that carry genetic inserts that may confer potentially harmful biological activity (eg a known virulence factor, a toxin or a determinant of immune evasion) or have been modified to alter host range (eg viral attachment and entry determinants; bacterial host-range factors), may require a higher containment level compared to the recipient organism or vector construct.

8 Also, careful consideration must be given to procedures that require handling of Genetically Modified Microorganisms (GMMs) in circumstances where standard containment and control practices used in a laboratory may not be possible. Particular attention should be given to the mass production of GMM-based products, especially where this occurs outside of a regular large-scale containment facility (eg manufacture of a GM influenza vaccine in embryonated eggs) and to clinical studies involving the administration of GMMs to humans in a hospital. Further guidance on clinical research studies can be found in Part 6. Guidance on the large-scale manufacture of influenza virus vaccine strains in embryonated eggs can be found in the WHO guidance document 'WHO biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines' (www.who.int/biologicals/publications/ECBS 2005 Annex 5 Influenza.pdf).

Risk assessment and the structure of the guidance

9 Schedule 3 of the Contained Use Regulations describes the risk assessment procedure and sets out the steps that should be included. The following procedure represents a recommended model for GM risk assessments and for the assignment of containment and control measures. The procedure is reflected in the structure of the guidance. This suggested format includes the steps required for risk assessment under the Regulations, although it is not intended to be prescriptive:

- **Overall nature of the intended GMM.** Assessment of the relative risks it may pose to human health and the environment.
- **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 containment level that is sufficient to safeguard human health.
- **Risk assessment for the environment.** The identification of any hazards to the environment, consideration of the potential severity and likelihood of occurrence. Establishment of a containment level that is necessary to protect the environment.
- **Review of procedures and control measures.** Implementation of any additional control measures necessary to safeguard both human health and the environment.
- **Assignment of GM Activity Class 1, 2, 3 or 4.** Containment level to be applied and extra measures or derogations.

10 The majority of GM work involving microorganisms falls into the lowest class of activity and will require minimal assessment. While it is a legislative requirement to assess the risks and employ measures to minimise the chances of exposure, in practice these organisms should be assessed in a way that is commensurate with the actual hazards posed. Therefore, there is a need for an informed and pragmatic approach, rather than an overcomplicated assessment and unwarranted control measures.

11 Much of this guidance has been prepared to aid the risk assessment of activities where uncertainty as to the nature of the intended GMM necessitates more in-depth consideration. 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.

12 Arguments must be clear, but need not be exhaustive. 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 feasible potential hazards should be acknowledged and information should be based upon established scientific knowledge wherever possible. Any uncertainty should be acknowledged and dealt with appropriately; the lack of scientific evidence for a particular hazard being legitimate should not automatically be taken to mean that it does not exist.

13 All GM 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 GM work. All data should be recorded and used to supplement the risk assessment where appropriate. Risk assessments should be kept for 10 years after the work has ceased (storage of materials is also considered to be active work in this case).

14 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.

15 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. The nature of the *intended* GMM will identify whether human health or environmental concerns take priority, as explained below.

Overall nature of the hazards posed by the intended GMM

16 A risk assessment for human health and a risk assessment for environmental protection are required in all cases under the Contained Use Regulations. However, the balance of

the significance given to each section will vary depending on the nature of the organism. For example, GMMs 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 personnel in these cases. Conversely, GMMs based upon animal 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, eg 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.

17 A decision can be made from the outset as to which part of the GM risk assessment is the more applicable and should take precedence.

- For GMMs 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 GMMs 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.

18 This recommended approach to the risk assessment of GMMs is illustrated in Figure 2.1.1.

19 So far, the majority of risk assessments made under the Contained Use Regulations have placed overwhelming significance to the protection of human health, with environmental concerns handled as secondary considerations, irrespective of the nature of the organism or intended GMM. Using the approach suggested here, sufficient emphasis could be placed on the aspects of the risk assessment that will dictate the containment measures needed to handle the organism safely.

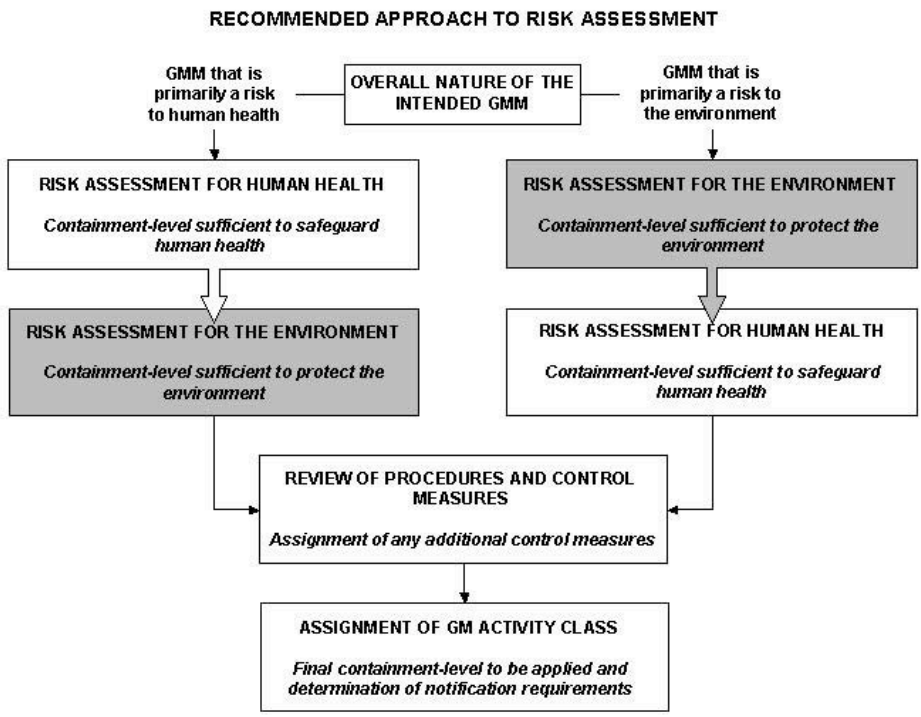

Figure 2.1.1 Illustration of a recommended approach to the risk assessment of GMMs

Risk assessment for human health

20 There is a requirement under the Contained Use Regulations to consider risks to human health posed by the GM activity. The objective is to identify all plausible hazards to human health and then to assess the likelihood and potential severity of the consequences, should the hazards be realised.

Mechanisms by which the GMM might pose a hazard to human health

21 Factors to be considered during the hazard identification process include the pathogenicity of the recipient microorganism, the properties of the sequences to be inserted and the final GMM. The primary consideration will be the pathogenicity of the recipient microorganism. It is accepted that, in most cases, the organism donating genetic material will not actually be handled. However, the potential harmful effects of any inserted genetic material must be assessed and the properties of the sequence in the context of the donor organism may be an important aspect of this. Any biological activity or toxicity of any encoded product should also be considered, as should the nature of any constructs (for example a plasmid or cosmid) used in the modification process.

Hazards associated with the recipient strain

22 Particular care must be given to the assessment of GMMs that have the potential to enter human cells or establish an infection in human hosts. The COSHH Regulations require that all biological agents (any organism that may cause infection, allergy, toxicity or any other hazards to human health) are classified into one of four hazard groups by reference to the ACDP Approved List of Biological Agents (see www.hse.gov.uk/pubns/misc208.pdf) or, if the organism in question does not appear in the Approved List, under classification criteria set out in COSHH. Further guidance can be found in Part 1 of the Compendium and in the latest edition of the ACDP publication 'Categorisation of biological agents according to hazard and categories of containment.' Specific guidance on certain commonly used GM bacterial and viral vector systems is given in Sections 2.3-2.12.

23 The degree of pathogenicity of the recipient strain and the severity of the consequences of exposure should be estimated. Where the recipient is an known human pathogen, the organism will be assigned to ACDP hazard group 2, 3 or 4. All other microorganisms that are not hazardous to human health can be considered to be hazard group 1 (in practice there is no list of group 1 microorganisms). COSHH requires the prevention of exposure to a biological agent by substituting a biological agent that is less hazardous, where it is reasonably practicable. This can be equated to a statutory requirement to use disabled or attenuated strains with reduced pathogenicity or without a human host range wherever possible.

24 Attenuated derivatives of pathogens may be assigned to a lower hazard group than indicated in the approved list, if it can be demonstrated 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.

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

- Wild type, pathogenic *E. coli* strains are classified as ACDP hazard groups 2 and 3 and as such, should be handled at Containment Level 2 or 3. 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 Containment Level 1.

- Wild-type adenoviruses are ACDP hazard group 2 pathogens and should be handled at Containment Level 2. 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 Containment Level 1.
- Wild-type *Salmonella typhi* is an ACDP hazard group 3 pathogen and should be handled at Containment Level 3. *S. typhi* auxotrophic deletion mutants (for example strain TY21a) have targeted genetic lesions, although the level of attenuation in a human host is difficult to predict. Such strains may be handled at Containment Level 1 if attenuation has been demonstrated in human volunteers. Similarly, some vectors derived from Herpes simplex virus (ACDP hazard group 2) 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 those that have demonstrated a good safety profile (for example DISC; 1716) can be handled at Containment Level 1.
- *Mycobacterium bovis* is an ACDP hazard group 3 pathogen and should be handled at Containment Level 3. The attenuated phenotype of *M. bovis* (BCG) strain is poorly understood but does have a long history of safe use as a vaccine in humans and has been assigned to hazard group 2. Likewise, the MVA strain of Vaccinia virus, while being poorly understood in terms of the nature of attenuating lesions, also has a long history of safe use as a vaccine. This strain can be handled at Containment Level 1 rather than Containment Level 2 as prescribed for wild-type ACDP hazard group 2 isolates of Vaccinia virus.

26 Note that 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, ie deletion mutants are less likely to revert to wild-type than point mutations or conditional-lethal mutants.

27 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.

Hazards associated with genetic inserts

28 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 characteristics of expression anticipated under experimental conditions (ie kinetics and level of expression expected) and the possible consequences of exposure to the GMM carrying the gene.

29 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.

30 Careful consideration should be given to potentially harmful prokaryotic genes expressed in prokaryotic systems (for example 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.

31 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 (eg perhaps if expressed constitutively at high levels). While development of a cancer is acknowledged to be a multistep process requiring a number of genetic lesions to generate a malignant tumourigenic cell, expression of some genes (eg 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.

32 Further specific guidance on the hazards posed by genetic inserts, including oncogenes, can be found in Section 2.2.

Hazards arising from the alteration of existing pathogenic traits

33 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.

34 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:

35 ***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, LPS and capsule that may affect the infectivity and virulence of a bacterial host organism.

36 ***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 signalling pathways may be disrupted, resulting in altered pathogenic traits.

37 ***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 (for example, 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.

38 ***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 (for example Vaccinia virus) have a number of host-range determining genes that bestow the ability to replicate within certain cell types. Modification of viral entry determinants (for example 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.

39 ***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 bestowed upon bacteria during the modification process or the conferring of drug resistance to a virus (for example, 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 workers 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.

Transfer of harmful sequences between organisms

40 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 hazard identification 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.

41 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 cells, and this should not be discounted.

42 ***Sequence mobilisation in bacteria.*** 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.

43 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 (for example, 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 – ie will the sequence give an advantage to naturally occurring pathogens?

44 ***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/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.

45 ***Reassortment between segmented RNA viruses.*** Some RNA viruses have segmented genomes (eg 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 workers that may be harbouring an infection with a wild-type virus.

Likelihood that the GMM will be a risk to human health

46 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.

47 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 (eg the likelihood of gene transfer).

48 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

49 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 acknowledged and a precautionary approach taken.

50 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 organism 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 organism. 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

51 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.

52 In many cases this will not be possible and an approximate, semi-quantitative or descriptive assessment of the frequency, based upon experience with similar GMMs 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, ie the more events that are needed, the less likely it is that reversion will occur.

53 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 proved 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.

54 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 GMMs 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.

Containment level needed to protect human health

55 It is recommended that the minimum containment level (Containment Level 1, 2, 3 or 4) that is necessary to protect human health be set. At this stage, it is only provisional and an estimate of the containment measures that will be required solely for the purpose of protecting those who come into contact with the GMM. This is based upon:

- The ACDP hazard group and/or containment level appropriate to the host organism.
- Any identified hazards arising as a consequence of the genetic modification.
- The severity of any harmful consequences and the likelihood that they might occur.

56 Therefore, a judgement can then be made about whether the GMM will be more hazardous, less hazardous or equivalent to the host 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 (for example 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.

57 Users should judge whether the measures required for the recipient strain in the appropriate table of containment measures in Schedule 8 to the Contained Use Regulations (which are reproduced in the relevant sections of Part 3) are also appropriate for the GMM. If some measures are no longer needed or any extra measures are required then the containment level should be adjusted accordingly to afford sufficient protection for human health.

58 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 Containment Level 1 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 below in the section 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.

Risk assessment for the environment

59 There is a requirement under both the Contained Use Regulations and the Environmental Protection Act to consider risks to the environment. The objective of the risk assessment for environmental protection is to determine the likelihood and the possible consequences of an accidental 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).

60 Clearly, the concern is for GMMs that could feasibly cause harm to the environment. Therefore, GMMs with the potential to infect or colonise animals and plants are of primary concern. Particular attention should be paid to GMMs 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. GMMs 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.

61 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. The Contained Use Regulations require consideration of whether there may be an adverse effect from interactions of the GMM with other organisms at the premises with which it is likely to come into contact. 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.

62 As for the risk assessment for human health, the procedure for environmental risk assessment is to identify hazards to the environment and then to assess the likelihood and potential severity of the consequences, should the hazards be realised. This procedure will be illustrated throughout the following sections using *two* hypothetical model case studies of GMMs that could impact upon the environment in the event of release from containment. These are:

- ***Case Study A.*** Working with a GM bacterium pathogenic for the Grey Seal (*Halichoerus grypus*).
- ***Case Study B.*** Working with a GM Pseudomonad with respect to effects on soil-borne bacterial ecology.

Potential mechanisms by which the GMM might pose a hazard to the environment

63 As for human health risk assessments, the hazard identification process must include considerations of potentially harmful or adverse effects upon the environment that would be mediated by the recipient microorganism, the inserted genetic material and the final GMM.

Hazards associated with the recipient strain

64 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 ACDP hazard group and containment requirements are important preliminary issues for GM work with human pathogens, it is also important to consider the Defra classification of animal pathogens (which are

pathogens of domestic farm animals and poultry) (see: www.defra.gov.uk/animalh/diseases/pathogens/classification.htm). GMMs based upon such pathogens may require Defra licenses in order to handle them (under SAPO) or import them (under IAPO). The containment conditions specified within those licences must be strictly adhered to.

65 For example, *Mycobacterium bovis* and *Mycobacterium tuberculosis* are classified by ACDP as hazard group 3 pathogens, as well as being classified as Defra Group 2 animal pathogens. ACDP Containment Level 3 measures are sufficient to satisfy the Defra containment requirements in this case. Likewise, *Bacillus anthracis* is ACDP hazard group 3 but also a Defra Group 3 specified animal pathogen, hence work with *B. anthracis* requires a SAPO licence and compliance with both ACDP and Defra containment requirements.

66 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 (for example *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 an organism 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.

67 When assessing whether an organism 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, whether or not in a vegetative state, or undergoing active replication.

Hazards associated with genetic inserts

68 GMMs 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) natural flora and fauna (eg expression of a recombinant pesticidal protein in a prokaryotic system).

69 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.

70 Further guidance on the possible hazards associated with inserted genes can be found in Section 2.2.

Hazards arising from the alteration of existing pathogenic traits

71 The recipient strain may not have any inherent properties that pose a hazard to species in the environment or to ecosystems but the genetic modification may bestow 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 and all modifications should be carefully assessed.

72 ***The modification alters stability or survivability.*** As already discussed, the ability of a microorganism to survive in the environment is a key determinant of its potential to cause harm. Therefore, any modification that alters the survivability of the recipient microorganism should be carefully assessed, ie genetic modifications that enhance the ability of a microorganism to resist oxidative stress, 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.

73 ***The modification alters pathogenicity or infectivity.*** 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 organism to host immune systems.

74 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 it is changes in this gene that 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.

75 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.

76 ***The modification alters tissue tropism or host range.*** 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.

77 Altered host range may result from the modification of cellular entry or invasion determinants. Retargeting and/or extending the host range of viral vectors is a common practice and a desirable goal for the development of therapeutic viral GMMs. 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.

78 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. meningitidis* disease at the end of this document).

Transfer of harmful sequences between organisms

79 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.

80 ***Sequence mobilisation in bacteria.*** 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.

81 Once again, the survivability of the organism is a key determinant. It is important to remember than an organism 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.

Phenotypic and genetic stability

82 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 GMMs ability to survive and to spread.

83 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 conditions in which it was intended to exist should be taken into account and consideration given to any detrimental effects this might cause.

Case studies – overview

Case Study A. Consider the accidental release of a GM bacterial pathogen of the Grey Seal. If that GMM were unable to survive even for a short time in the environment, then the only likely environmental hazard would be transfer of the genes encoding pathogenic traits to other, indigenous bacteria. However, should the GMM be able to survive in the environment, then the inherent pathogenic traits of the organism would pose an additional hazard.

Case Study B. Consider a pseudomonad isolated from soil and subsequently transformed with a promiscuous conjugative plasmid incorporating a gene encoding a bacteriocin that is toxic to a wide range of soil-borne bacteria. Accidental release of this GMM would constitute a hazard due to potential for gene transfer to soil-borne bacteria and because the expression of the toxin could have adverse effects on the bacterial ecology of the soil.

Likelihood that the GMM will be a risk to the environment

84 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.

85 It is therefore important to considering 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.

86 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.

87 A determination of the risk of harm posed by a GMM can be estimated using a risk determination matrix (see Table 2.1.1). Risk can be expressed as ‘high’, ‘medium’, ‘low’ or ‘effectively zero’ 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.

Assessment of likelihood

88 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, for example, climatic, geographical or soil conditions and the types of potential host species present. For the purposes of using the risk determination matrix, likelihood can be expressed as 'high', 'medium', 'low' or 'negligible'.

89 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, GMMs derived from animal pathogens of non-UK hosts would have limited capacity to become disseminated within the UK even if it could survive for extended periods. Similarly, the transmission of some pathogens requires an intermediate vector that might not be present in the UK. For example, species of *Leishmania* parasites require phlebotomid sandflies for transmission. Although *Leishmania* are pathogens of humans and animals, sandflies are not present in the UK and therefore the organism could not be transmitted and become disseminated (see the example risk assessment on the analysis of helminth immune evasion genes by expression in *Leishmania* at the end of this document). 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 the UK, for example, as a result of climate change. However, in general, the risk that such GMMs could be a hazard to the environment will be negligible.

90 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 hazard will be realised will often depend on the number of GMMs that are being handled and, consequently, the number that could escape.

Case studies – assessment of likelihood

Case Study A. In the case of the Grey Seal pathogen, the assessment of likelihood would depend on where the work was carried out. For example, if the facility was located in an area far removed from marine environments and there was no access to watercourses, the likelihood that Grey Seals would be affected would be 'negligible', even if the GMM escaped in low numbers. However, if the same work were carried out at a coastal laboratory on the

North Sea, the likelihood that Grey Seals would be affected might be 'high'. Other factors may also affect this determination, for example season, rate of dilution and local seal population sensitivity.

Case Study B. In the case of the GM pseudomonad, exposure to the soil in the vicinity of the laboratory is a possibility. The likelihood of gene transfer and of expression of the bacteriocin toxin would need to be assessed and might be deemed 'high'. The likelihood might be reduced, however, if a minimum of Containment Level 2 had already been set for human health protection.

Assessment of the possible consequences

91 After the likelihood of all the hazards has been assessed, the consequences of each hazard being realised should be estimated. 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 determination matrix, consequences could be described as being 'severe', 'modest', 'minor', or 'negligible'.

92 It should be borne in mind that even if the consequences of a hazard being realised are deemed 'severe', if the probability of the hazard being manifested at all was 'negligible' then there is 'effectively zero' risk of harm. Likewise if the consequence of a hazard were 'negligible' or 'minor', then even if the probability of its manifestation were 'high', the risk of harm would still be 'low' (See Table 2.1.1).

93 However, a precautionary approach to risk determination is advised. In situations where the probability of the hazard being manifested was 'negligible', should there be a 'severe' consequence to the identified hazard then it is unlikely that Containment Level 1 would be appropriate, even though there is an 'effectively zero' risk of harm. A balanced view of the risks is therefore required.

Case studies – assessment of the possible consequences

Case Study A. For the Grey Seal pathogen, the consequences of contact with the host species would probably be 'severe'. Even small fluctuations in the seal populations due to infection with the pathogen should be deemed as a 'severe' consequence. Given that the likelihood of the hazard being realised is 'high' at a coastal facility but 'negligible' at an inland one, the resultant risk of harm would be either 'high' or 'effectively zero' respectively.
Case Study B. For the GM pseudomonad, if the bacteriocin gene were expressed, the effects of toxin production on other soil-borne micro-organisms might lead to 'severe', 'modest', 'minor' or 'negligible' consequences, depending on the magnitude of the effects on the soil

ecology. While some fluctuation in the normal microbial population of the soil would be acceptable, major changes in the numbers of microbes would be deleterious to the ecosystem and most likely be irreversible. Given the level of uncertainty, the worst should be assumed and the consequences of a release deemed to be 'severe'. Assuming that containment for human health purposes is insufficient to prevent release into the environment, the resultant risk of harm would be 'high'.

94 The risk determination matrix is a tool and should not be seen as definitive. It is important that uncertainty is acknowledged 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 severe. For example, in relation to Case Study A: The Grey Seal Pathogen, it is assumed that the pathogen cannot be carried by other species such as dogs or even humans. This would mean that the pathogen could not be carried from an inland facility to coastal regions. If it could be carried by another species, this may have a bearing on the level of risk at an inland facility. The basis of any assumption should be explained and the robustness of the argument supporting it should be acknowledged.

95 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 'effectively zero'. Further guidance on containment measures to protect both the environment and human health can be found below.

		LIKELIHOOD OF HAZARD			
		High	**Medium**	**Low**	**Negligible**
CONSEQUENCE OF HAZARD	**Severe**	High	High	Medium	Effectively Zero
	Modest	High	Medium	Medium/Low	Effectively Zero
	Minor	Medium/Low	Low	Low	Effectively Zero
	Negligible	Effectively Zero	Effectively Zero	Effectively Zero	Effectively Zero

Table 2.1.1 Risk determination matrix

Containment level needed to sufficiently protect against harm to the environment

96 It is recommended that the minimum containment level (Containment Level 1, 2, 3 or 4) 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:

- The Defra group or containment measures required by any SAPO license needed for work on the recipient organism.
- The ACDP hazard group for the recipient organism if it is pathogenic for humans.
- Any identified hazards arising as a consequence of the genetic modification, the severity of any harmful consequences and the likelihood that they might occur (determination of the risk of harm, see above).

97 If there are no prescribed containment measures for the recipient organism (ie not on the ACDP Approved List or covered by SAPO), 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 'effectively zero' 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 'effectively zero'.

98 Users should judge whether the measures required for the recipient strain in the appropriate table of containment measures in Schedule 8 to the Contained Use Regulations (which are reproduced in the relevant section of Part 3) are also appropriate for the GMM. If some measures are no longer needed or any extra measures are required then the containment level should be adjusted accordingly to safeguard the environment. In cases where the GMM is derived from a human pathogen, a minimum containment level necessary to protect human health should have been assigned and it should therefore be considered whether these measures are also sufficient to protect the environment.

Review of procedures and control measures

99 The requirements of the final containment level 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 containment levels 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.

100 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 activity class and containment level.

Consideration of the nature of work and procedures to be used

101 The nature of the activity will affect the level of risk. In particular, any non-standard operations that are not accounted for in the general requirements for a given containment level should be considered as increased risks might arise from certain procedures. For example:

- Inoculating animals with the GMM and the use of sharps for administration or post-mortem analysis. The use of sharps increases the likelihood of an exposure that might lead to infection. Furthermore, the chances of recombination or reversion may be enhanced when work *in vivo* is undertaken, as compared to work *in vitro*
- Procedures that will generate aerosols.
- Large-scale manufacture of a GMM or GMM-derived product and/or exposure to large amounts or high titres of the GMM.

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

Assignment of additional measures to minimise risks

103 The Contained Use Regulations set out the underlying principles of containment and control measures for all GMMs. These include the principles of Good Microbiological Practice and Good Occupational Safety and Hygiene (similar measures are also required for work with biological agents under COSHH). Furthermore, the detailed containment requirements are described in the relevant section of Part 3. Additional measures may be needed to ensure safety, especially where the organism is pathogenic for humans or able to infect human cells. For example, the use of sharps should be minimised when working with retroviruses and oncogenic material.

104 ***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.

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

106 ***Monitoring.*** 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) can be used to detect the presence of sequences deleted from the GMM.

107 In certain circumstances, it may be possible to monitor for the presence of a GMM outside of primary containment (for example 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 GMMs from the containment facility.

108 ***Management issues.*** The person responsible for the work 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 a BSO or other competent person to ensure that the local rules are satisfactorily implemented.

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

110 ***Preventing release into the environment.*** 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 GMMs that pose an environmental hazard are adequately inactivated and appropriately disposed of. Further guidance on waste inactivation and disposal can be found in Part 3, Section 3.5.

111 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 workers may inadvertently carry the GMM out of containment on contaminated equipment or clothing.

GM activity classification (Class 1, 2, 3 or 4)

112 A GM activity class must be assigned in relation to the control measures needed to protect both human health and the environment. The measures that are indicated as necessary by the risk assessment must be applied.

113 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, Containment Level 1 measures must be applied as a minimum. For class 2 activities, Containment Level 2 and so on. The only exception to this is when the user has the agreement of the Competent Authority to not apply the full corresponding containment level.
- It determines the notification requirements for the activity (see Part 1 for further guidance as well as the Guide to the Regulations cited above).

114 To decide on the final classification, users should compare the measures warranted by the risk assessment with the appropriate table of containment measures in Schedule 8 to the Contained Use Regulations (these tables are also reproduced in the relevant section of Part 3. Where the required containment measures correspond to those from a single level of containment this process will be simple: a GM activity requiring Containment Level 2 will be class 2.

115 There will be cases where the required containment measures are a mixture from two levels. For instance, Containment Level 2 with the use of negative air pressure may be required but with the addition of HEPA filtration of the extracted air. Therefore, the measures selected in this case are a mixture of those from Containment Levels 2 and 3. Where there is such a mixture of containment measures the GM activity class will correspond to the higher level of containment indicated (which in this case is class 3) and must be notified accordingly. Even if only a single measure corresponds to a higher containment level, the class must reflect this. However, derogation may be sought from HSE at notification to exclude those measures required for the higher containment level that are shown to be superfluous by the risk assessment. Further explanation of the classification system can be found in the Guide to the Genetically Modified Organisms (Contained Use) Regulations 2000.

116 It is possible that some control measures deemed necessary by the risk assessment are not actually in the Schedule 8 containment level tables. The class is determined only by those items listed in the tables and indicates the minimum containment level that must be applied (unless derogation has been agreed by HSE). 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 GMMs 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.

117 Class 1 activities are described in the Contained Use Regulations as being of ‘no or negligible risk’. It is unlikely that any non-disabled human or animal pathogen could be deemed to be of ‘no or negligible risk’ (except where the host species is absent from the receiving environment) and such work will always be class 2 or higher. Since work with pathogens will almost invariably require at least some of the measures required at Containment Level 2 (eg an autoclave in the building; restriction of access) it would not normally be possible to assign the activity to class 1. *Even if no Containment Level 2 measures were justified by the risk assessment, assignment of pathogen work to class 1 would be inappropriate and the activity must be notified to HSE as class 2.* If a GMM that

is, or could be, pathogenic to humans or animals is assigned as a class 1 activity, then it is probable that the risk assessment is inadequate.

118 Remember that classification into a GM activity class does not necessarily mean that you will always have to apply all the measures from the associated containment level. If it is adequately justified by the risk assessment derogation may be sought from HSE to exclude unwarranted measures.

119 It should be reiterated that other legislation might come to bear on the measures needed for environmental protection. For example, pathogens covered by SAPO require a license from Defra and the containment measures prescribed within that license must be implemented. In situations where the containment measures required for protection of human health and the measures needed to protect the environment differ, the more stringent of those measures should be applied.

2.2 Guidance on hazards posed by inserted sequences

Overview

1 The Genetically Modified Organisms (Contained Use) Regulations require that, for the purposes of risk assessment, any potentially harmful effects associated with any exogenous genetic material inserted into a microorganism or GM vector system should be taken into account. The following section concerns exogenous inserted sequences that may have harmful biological activity (for example toxins, cytokines and growth factors), 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). Sequences which alter tissue tropism, host range or virulence are not covered in this section but further details can be found in Sections 2.1, and in specific guidance Sections 2.3-2.12.

2 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.

3 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 cell 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.

4 The elements that control gene expression in GMMs 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.

5 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.

Oncogenes

6 Oncogenesis is the process leading up to a cell losing the ability to effectively regulate its own growth or survival and becoming tumourigenic or *transformed*. It is a multi-step process requiring mutations in more than one gene; 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 (ie 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.

7 It is recognised that there is no precise definition of an oncogene. There are some genes that encode mitogenic factors with demonstrable oncogenic properties (for example *c-myc*), which, when expressed, result in deregulated growth. A transformed phenotype usually requires expression of an oncogene in conjunction with another gene (*c-myc* and *c-ras* co-expression can result in stable transformation of cells, for example) or in cells with impaired tumour-suppressor function or apoptotic pathways (eg 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 signalling or extra-cellular interactions could be potentially oncogenic. In addition, any gene that confers a phenotype upon the cell that is associated with transformation (for example deregulated growth, loss of contact inhibition, density- or anchorage-dependent growth) could have oncogenic properties.

8 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.

9 It is also prudent to avoid the use of recipient strains that are able to infect or colonise human hosts. For example, workers 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 (eg retroviruses and lentiviruses) represent an immediate hazard to the operator and appropriate controls will be required.

Toxins and cytotoxic genes

10 The development of GMMs 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 (for example 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.

11 There are different approaches to generating cytotoxic GMMs. One is the use of an inserted gene with a product that is inherently toxic, for example a plant toxin (eg Ricin) or a bacterial toxin (eg 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.

12 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.

13 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.

14 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 ie a *bystander effect*. In the past, the development of therapeutic GMMs 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.

15 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 (for example 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 (eg Diptheria Toxin) that would allow free toxin to exert its effect on cells other than those in which it was expressed.

16 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 (for example, 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 (eg Diptheria Toxin alpha chain, Ricin A-moiety and Shiga Toxin A-moiety) and eliminate the plasma-membrane binding and transduction activities (eg 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.

17 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.

Cytokines, growth factors and immunomodulatory proteins

18 Growth factors and cytokines that are expressed by GMMs 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.

19 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 (for example, 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 (for example, 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.

20 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 workers will become a higher priority issue when handling GMMs expressing such products.

21 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.

RNA interference

22 RNA interference (RNAi) is an antisense technology that exploits a normal cellular antiviral response to bring about the degradation of double-stranded RNA (dsRNA) molecules and inhibition of viral protein synthesis. 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.

23 Not all RNAi work will be covered by the Contained Use Regulations, for example, if the molecules are delivered directly into an organism, or where they are delivered by inert carriers (eg liposomes). RNAi cassettes that are delivered by an organism (for example a bacterial or viral vector), are covered by the Contained Use Regulations.

24 Careful consideration should be given to a GMM carrying an RNAi cassette that is capable of infecting or invading human or animal cells, particularly vector systems that might result in the permanent modification of the host chromatin (for example retroviral and lentiviral vectors). The potential outcomes of downregulating 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 systems is that the targeted gene is rarely completely silenced. Indeed, it is not unusual for there to be 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.

25 The effects of the siRNA species that are generated in these systems 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 and animal 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.

26 RNAi experiments 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 GMMs carrying RNAi cassettes might result in inflammation and the ramifications of this to human or animal health should be assessed.

27 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.

Transmissible Spongiform Encephalopathies (TSEs)

28 TSEs are neurodegenerative disorders of humans (eg Creutzfeldt-Jakob Disease; Kuru) and animals (Bovine spongiform encephalopathy; Scrapie) and constitute mutant forms of naturally occurring mammalian proteins called *Prions*. 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. However, TSE agents are classified as ACDP hazard group 3 pathogens and are therefore subject to the provisions of COSHH as biological agents. The following guidance is not intended to give advice on the handling of TSE agents themselves even if they are purified as a result of GM activities as this falls outside the scope of the Contained Use Regulations. Users are referred to the ACDP and the Spongiform Encephalopathy Advisory Committee (SEAC) document *Transmissible spongiform encephalopathy agents: Safe working and the prevention of infection*, published 15 December 2003, for guidance on the handling of TSE agents.

29 Microorganisms that have been genetically modified to express TSE proteins are covered by the Contained Use Regulations. Therefore, genes encoding TSE agents should be treated in the same way as other genes that encode biologically active proteins, ie as potentially hazardous insert sequences. Genes encoding TSE agents represent unusual inserts as they are classified as pathogens in their own right and the expressed products carry an ACDP hazard group classification. It is therefore possible that a GMM expressing a gene encoding a TSE agent will have to be handled at Containment Level 3. However, this may not necessarily be representative of the GM activity class and notification requirements.

30 The Contained Use Regulations 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 TSE agents themselves will not necessarily call for the measures required for containment–level 3. Since these agents are not airborne pathogens, the use of a safety cabinet, negative air pressure and HEPA filtration of exhaust air will not be required. Furthermore, as TSE agents are resistant to fumigation, the laboratory will not require sealability for that purpose. Therefore, TSE agents themselves only call for the measures required by Containment Level 2 and therefore could conceivably be a class 2 GM activity, although other control measures may be required to ensure safe handling which may not affect classification, but nevertheless must be implemented.

31 However, a GMM with infectious characteristics may confer its own properties of transmission upon a TSE 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 TSE 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 TSE cDNA were cloned into an adenovirus vector then the possibility of ‘aerosol transmission’ of the TSE should be considered. This would require that measures to control the spread (microbiological safety cabinet, HEPA filtration of exhaust air and possibly negative pressure) would be needed in addition. 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 (for example 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.

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

32 Recent evidence suggests that TSE agents generated by recombinant systems are non-functional and disease transmission to laboratory animals using recombinant TSE agents has not been demonstrated. This would indicate that a lower containment level would be sufficient for such work provided that there is no expectation that pathogenic TSE agents will be generated. A precautionary approach to the handling of GMMs carrying these sequences is advised and any derogation from the measures required for the handling of TSE agents themselves should be fully justified by the risk assessment.

33 For activities in which there is no expression, and where the recipient strain or final GMM is disabled, Containment Level 1 or 2 might be sufficient. For example, routine cloning work in *E. coli* K-12 could take place at Containment Level 1 provided no functional TSE agents can be generated and where non-mobilisible vector constructs and non-colonising bacterial strains are employed.

34 Work involving fragments of TSE proteins or modified TSE proteins that are not expected to be pathogenic might also take place at Containment Level 2 or Containment Level 1, depending on the vector used and provided that no harmful biological activity is possible. GMMs expressing normal Prion proteins should be handled at Containment Level 2 as they may be pathogenic at high levels and may also become mutated to TSE forms in GMM vectors, especially in RNA virus vectors as the possibility of mutation is high in these systems.

35 TSE agents are extremely durable in the environment and containment measures must also be prescribed to prevent environmental contamination. TSE agents are also extraordinarily resistant to the decontamination procedures normally used to deactivate GMMs and are resistant to fumigation. If TSE agents are present, it may be necessary to alter the normal procedures and inactivation methods used for GMMs to accommodate their unique properties. For example, a higher autoclave heat setting may be required (138 °C) and more stringent chemical decontamination (for example 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 TSE agents. For further information on the handling and inactivation material contaminated with TSE agents, users are referred to ACDP/SEAC guidance at www.dh.gov.uk.

Non-coding/regulatory elements

36 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.

37 ***Promoters/enhancers.*** 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 (eg a bacterial toxin).

38 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.

39 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.

40 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.

41 ***Genomic control regions.*** Long-term expression has proved 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 (for example, 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.

42 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.

43 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 remodelling 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.

44 ***Viral post-transcriptional regulatory elements.*** 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) 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.

45 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 account the possible harmful effects of this sequence. Vectors containing these forms of WPRE should be assigned to class 2 or higher. This highlights the need for rigorous scrutiny of the possible effects of regulatory sequences present in vectors.

2.3 Routine cloning and expression work using attenuated *Escherichia coli*

Overview

1 The majority of GMMs 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 GMMs are then grown in bulk cultures in order to extract and purify the constructs for use in subsequent procedures.

2 The requirements for risk assessment under the Contained Use Regulations are the same for these activities as they are for GM work with any microorganism. 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 GMMs will be low hazard and fall into the lowest class of GM activity. Therefore, it is likely that they 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.

Scope

3 The following is intended as a brief guide for those wishing to undertake risk assessment of low hazard routine cloning work using attenuated *E. coli* strains. This guidance is intended to supplement more general guidance that can be found in Section 2.1. The guidance does not make specific reference to low hazard host-vector systems other than attenuated *E. coli* (eg *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. Specific guidance for these applications can be found elsewhere (see Section 2.4).

Risk assessment for human health

Hazards associated with the recipient strain

4 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.

Wild type, pathogenic *E. coli* strains are classified as ACDP hazard groups 2 and 3 and as such, should be handled at Containment Level 2 or 3.

Many *E. coli* K-12 and B strains have a long history of safe use and most can be handled safely at Containment Level 1.

Novel recipient strains should be more carefully assessed and the hazards considered on a case-by case basis.

Hazards associated with genetic inserts

5 Given that the recipient strain is likely to be attenuated and non-pathogenic for humans, 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. Guidance on the hazards posed by commonly used genetic inserts can be found in Section 2.2.

6 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.

7 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.

8 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.

Hazards arising from the alteration of existing traits

9 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 (eg *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.

Risk assessment for the environment

Survivability and stability

10 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.

Hazards posed by the genetic insert

11 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 (for example, 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.

12 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.

Hazards arising from the alteration of existing traits

13 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.

Procedures and control measures

14 Given that the majority of these GMMs will fall into the lowest activity class, Containment Level 1, 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 workers 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 workers may be required.

2.4 Bacterial vaccines and gene delivery systems

Overview

1 The following guidance is intended for use by those wishing to assess the risks 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 Section 2.3).

2 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.

3 Much of the developmental work on live vaccines and delivery systems to date has been on rationally attenuated strains of *Salmonella enterica* (serovars *typhi* and *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 *Escherichia 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 (see Table 2.4.1). 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.

4 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.

Risk assessment for human health

Hazards associated with the recipient strain

5 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.

6 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 and the ACDP or Defra hazard group and containment level appropriate for the recipient organism. 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 2.4.1.

RECIPIENT	DISEASE	FACULTATIVE INTRACELLULAR PATHOGEN	HAZARD GROUP
Salmonella typhi	Enteric fever (Typhoid fever)	Yes	ACDP 3
Salmonella typhimurium	Enteritis (Salmonellosis)	Yes	ACDP 2
Listeria monocytogenes	Septicaemia; fever; diarrhoea (Listeriosis)	Yes	ACDP 2
Shigella flexneri	Dysentery; fever (Shigellosis)	Yes	ACDP 2
Shigella sonnei	As *S. flexneri*	Yes	ACDP 2
Vibrio cholerae	Gastroenteritis (Cholera - Cholera toxin)	No	ACDP 2
Yersinia enterocolitica	Lymphadenitis, enteritis	Yes	ACDP 2
Yersinia pestis	Plague; enterocolitis	Yes	ACDP 3; Defra 3
Escherichia coli	Gastroenteritis	Yes, some strains	ACDP 2 or 3
Mycobacterium bovis	Tuberculosis in cattle	Yes	ACDP 3; Defra 2
M. bovis (BCG)	Tuberculosis vaccine strain	Yes	ACDP 2

Table 2.4.1 Typical symptomatic consequences of infection with wild-type bacteria and associated ACDP and Defra hazard groups

Bacteria that are engineered to become vaccine vectors or gene-delivery systems fall into a range of ACDP and/or Defra hazard groups. An appropriate containment level should be adopted as a minimum requirement when wild-type microorganisms will be handled.

7 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 containment level is sought.

Examples of bacterial gene delivery systems

8 ***Salmonella enterica.*** The natural tropism of *Salmonella enterica* (serovars *typhi* and *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. enterica* are able to persist in the phagocytic vacuoles of antigen presenting cells (for example dendritic cells and macrophages) and deliver plasmid DNA into the cytoplasm by a hitherto unknown mechanism. Thus, these bacteria have the ability to induce both humoural 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.

9 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 (for example immunomodulatory growth factors and cytokines) or to specifically destroy the cells (for example 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 (see below).

10 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.

11 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 2.4.2.

12 ***Listeria monocytogenes.*** *L. monocytogenes* has a number of features that have led to its development as vaccine and gene-delivery systems. 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 2.4.2.

13 *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.

14 ***Shigella* spp.** 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 have 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 2.4.2.

15 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.

16 ***Vibrio cholerae.*** *V. 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. 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.

17 ***Mycobacterium bovis (BCG).*** The *M. 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.

18 *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 (see below).

19 ***Yersinia enterocolitica.*** 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.

20 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 2.4.2). 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.

21 ***Escherichia coli.*** Non-invasive, non-pathogenic *E.coli* strains (eg 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.

22 For example, transformation of *E.coli* with an expression plasmid carrying the inv gene from *Yersinia pseudotuberculosis* bestows the ability to be internalised by cells in culture that express β_1 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.

23 The recipient strain in these cases will be low hazard and can be handled safely at Containment Level 1. However, the insertion of invasion determinants will increase the hazard posed by the organism 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 (see below).

RECIPIENT	MUTATION	GENE FUNCTION LOST	EFFECT
Salmonella typhi Ty21a	Undefined	Unknown	Attenuation
Salmonella enterica	*phoPQ*	Virulence regulon	Attenuation
(*S. typhi; S. typhimurium*)	*ssaV*	Virulence-associated	Attenuation
	*aroA**	Amino-acid biosynthesis	Auxotrophy
	purB	Purine biosynthesis	Auxotrophy
Listeria monocytogenes	*dal*	Alanine racemase (cell wall biosynthesis)	Auxotrophy
	dat	D-aminotransferase (cell wall biosynthesis)	Auxotrophy
	actA	Actin nucleator (cell-cell spread)	Attenuation
	plcB	Phospholipase B (escape from vacuoles)	Attenuation
Shigella flexneri	*guaBA*	Guanine monophosphate biosynthesis	Auxotrophy
Shigella sonnei	*sen; set*	Shigella enterotoxins	Attenuation
	icsA	Cell to cell spread	Attenuation
	asd	Cell wall/diaminopimelic acid biosynthesis	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*	Resistance to phagocytosis	Attenuation
	yadA-2	Colonisation; resistance to complement	Attenuation
	sodA	Superoxide dismutase (oxygen radical resistance)	Attenuation
	irp-1	Siderophore biosynthesis	Attenuation

Table 2.4.2 Examples of genes that have been mutated in for the purposes of attenuation and development of bacterial vector systems, the function lost and the type of phenotypic effect
*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.

Hazards associated with genetic inserts

24 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 Section 2.2. However, in brief, factors to consider include the following.

25 ***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 (eg 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.

26 ***Expression characteristics.*** 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.

27 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.

28 ***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.

Alteration of phenotype

29 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.

30 ***Pathogenicity.*** 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 (a regulon) 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.

31 One strategy for generating a bacterial gene-delivery strain might be to bestow 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* (eg 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.

32 ***Immunogenicity.*** Expression of cytokines or immunomodulatory growth factors may alter the immune response to infection, and could exacerbate an inappropriate response or result in the failure to elicit a protective immune reaction to the parent strain, making the recombinant strain more pathogenic. Also, in the interests of attenuation and immunogenicity, it might be desirable to disrupt genes that may be involved in evasion of the immune system. While this might result in an increased immune response and effective clearing of the organism from the infected host (resulting in attenuation), there may be changes in pathology. For example, acute inflammation or abscess formation that would not normally be a consequence of exposure to the recipient may be a feature of contact with the GM organism. Such potential phenotypes should be carefully assessed.

Genetic stability and sequence mobilisation

33 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.

34 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 (eg 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 (eg *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.

35 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*.

36 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.

Risk assessment for the environment

Survivability and stability

37 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.

38 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 (eg 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.

39 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 workers 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.

Hazards posed by the genetic insert

40 A potentially 'harmful' sequence could be a heterologous gene insert or a selection marker (for example 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 bestow 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.

41 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.

Alteration of phenotypic and pathogenic traits

42 A careful assessment of any modification to an animal pathogen (for example *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 organism, giving rise to a novel animal pathogen. Appropriate measures should be in place to prevent environmental release of such an organism, even if minimal containment is required for human health protection.

Procedures and control measures

Operational considerations

43 ***Animal experimentation.*** Workers must be sufficiently protected from the possibility of infection by inoculated experimental animals. Working with animals often involves the use of sharps (for example hollow needles) and contact with secretions. Furthermore, the possibility that the worker 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 worker 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.

2.5 Work with cell cultures

Overview

1 The following guidance covers the risk assessment of GM activities involving the genetic modification of cell cultures. Genetically modified cell cultures fall within the scope of the Contained Use Regulations and this guidance is intended to aid users in preparing GM risk assessments and assigning an appropriate activity class.

2 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, eg mycoplasmas such as *Mycoplasma pneumoniae*, should also be considered.

3 Cell cultures themselves could be considered to be GMMs, but unlike true microorganisms, they are not classified in the ACDP Approved List of Biological Agents. COSHH requires that the risks associated with adventitious agents be considered and Table 2.5.1 is adapted from guidance given by ACDP on appropriate containment of different cell types. 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 containment level used must be appropriate for work with that agent.

4 The recommendations in Table 2.5.1 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 Contained Use Regulations.

5 Mammalian and insect cells have very stringent requirements for growth and are very susceptible to dehydration and to 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, workers should not conduct genetic modification work with their own cells and use of cells derived from other laboratory workers should be avoided where possible.

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	Containment Level 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, eg blood borne viruses	Containment Level 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, Containment Level 3 would be required
	Primary cells from blood or lymphoid cells of human or simian origin	Containment appropriate to the potential risk. A minimum of Containment Level 2 is recommended
NB: 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, eg a microbiological safety cabinet		

Table 2.5.1 Recommended baseline containment measures for work with cell cultures

6 Unless the modification itself increases the intrinsic risks posed, most modification work with cultured cells will be GM activity class 1 and will not require notification.

Risk assessment

7 The GM risk assessment should focus on the hazards associated with the cells and their modifications. The GM activity class should reflect this and, in turn, determine the notification requirements. The GM risk assessment can also be used to satisfy the COSHH requirement to take into account of the possibility that adventitious agents might be present and any hazards associated with molecules present in culture media.

8 ***Adventitious agents.*** Primary cell lines, especially those derived from blood or neural tissue, and cell lines that have not been 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 are 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 workers from the GMM (see below).

9 ***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 GM work will be classified as GM activity class 1 and will not require notification unless the modification could increase risk (for example, 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 Containment Level 2. 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.

10 ***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.

11 ***Contamination versus containment.*** Many users will automatically use a microbiological 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 Containment Level 2 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 and the associated notification requirements.

12 Conversely, where restricted access, the wearing of gloves or the use of a microbiological safety cabinet is required to protect the worker from the modified cell line, the GM activity class should reflect this and notification may be required.

13 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 or notification 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.

2.6 Adeno-associated viruses

Overview

1 Adeno-associated viruses (AAV) belong 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 (for example 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 (for example 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.

2 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 heparan sulphate proteoglycans and internalisation via endocytosis appears to be mediated by the co-receptors $\alpha_v\beta_5$ 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.

3 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 2.6.1). 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.

4 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.

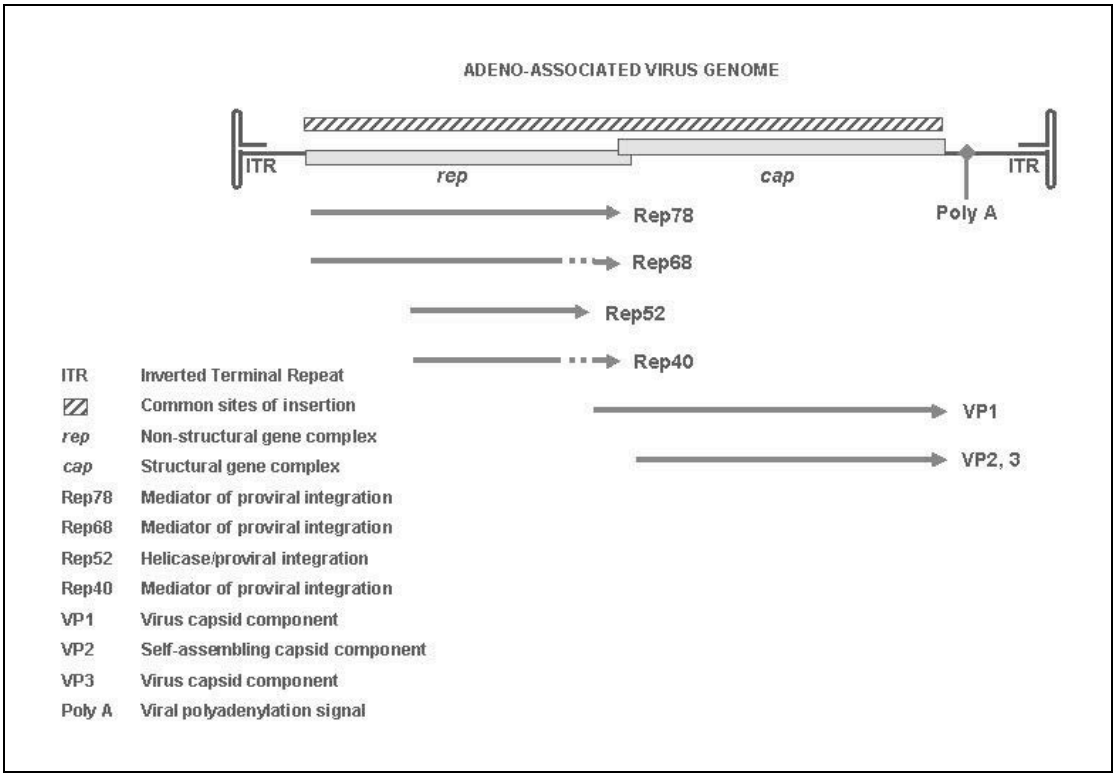

Figure 2.6.1 Transcription of the adeno-associated virus genome

Risk assessment for human health

Hazards associated with the recipient virus

5 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.

6 Wild-type AAVs are not categorised by ACDP and therefore, Containment Level 1 will be sufficient and should be adopted as a minimum requirement when handling wild-type virus.

7 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 ITR sequences. Cloned *rep* and *cap* genes as well as either wild type Adenovirus or expression of Adenoviral genes required for AAV replication (ie 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 Containment Level 2 since adenoviruses are ACDP Hazard Group 2 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 Section 2.7, Section 2.8 and Section 2.9 respectively.

8 It is a requirement of the COSHH Regulations that a potentially harmful biological agent 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 Herpes simplex virus and, if feasible, should be used in preference.

Most activities with AAVs are low hazard and can take place safely at Containment Level 1. Therefore many GM AAVs will fall into the lowest GM activity class.

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

Some activities involving wild-type helper viruses (for example, Adenoviruses) may need to take place at Containment Level 2. Provided the helper virus is not itself genetically modified, this will not affect the GM activity classification for the AAV work.

Hazards associated with genetic inserts

9 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 Section 2.2. In brief, factors to consider include the following.

10 ***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 Enhanced Green Fluorescent Protein (EGFP) or Luciferase. Properties of the gene products with respect to individual cell types affected should therefore be considered.

11 ***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.

12 ***Proviral insertion.*** 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.

13 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.

14 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.

Alteration of phenotype

15 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.

16 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.

17 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.

Risk assessment for the environment

Survivability and stability

18 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.

19 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.

Hazards associated with the genetic insert

20 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.

Alteration of phenotypic and pathogenic traits

21 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. 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.

Procedures and control measures

Operational considerations

22 Most recombinant AAV vectors will be considered low risk GM activity class 1 and can be handled at Containment Level 1. While this means that viral preparations could be handled on the open bench, 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 will take place within a microbiological safety cabinet. It is acknowledged that this is to protect the purity of the culture and not 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, where the risk assessment shows that exposure to airborne GM AAV represents a hazard, the use of a cabinet might be required as a control measure. These activities should be assigned to GM activity class 2 and take place at Containment Level 2, unless derogations are obtained from the competent authority.

23 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.

24 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 waste container.

Class 1 activities are described in the Contained Use Regulations as being of 'no or negligible risk'. It is unlikely that any non-disabled human or animal pathogen could be deemed to be of 'no or negligible risk' (except where the host species is absent from the receiving environment) and such work will always be class 2 or higher. Since work with pathogens will almost invariably require at least some of the measures required at Containment Level 2 (eg an autoclave in the building; restriction of access) it would not normally be possible to assign the activity to class 1. *Even if no Containment Level 2 measures were justified by the risk assessment, assignment of pathogen work to class 1 would be inappropriate and the activity must be notified to HSE as class 2.* If a GMM that is, or could be, pathogenic to humans or animals is assigned as a class 1 activity, then it is probable that the risk assessment is inadequate.

Remember that classification into a GM activity class does not necessarily mean that you will always have to apply all the measures from the associated containment level. If it is adequately justified by the risk assessment, derogation may be sought from HSE to exclude unwarranted measures.

2.7 Adenoviruses

Overview

1 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.

2 The adenovirus virion comprises a non-enveloped icosahedral capsid containing a 36 kb double-stranded DNA genome (see Figure 2.7.1). 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 motifs on the penton base interact with cell surface α_v 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.

3 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 2.7.1). 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 and inhibition of Fas- and 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 - 48 hours (depending on subtype and target cell) generating up to $1x10^5$ viral particles per infected cell.

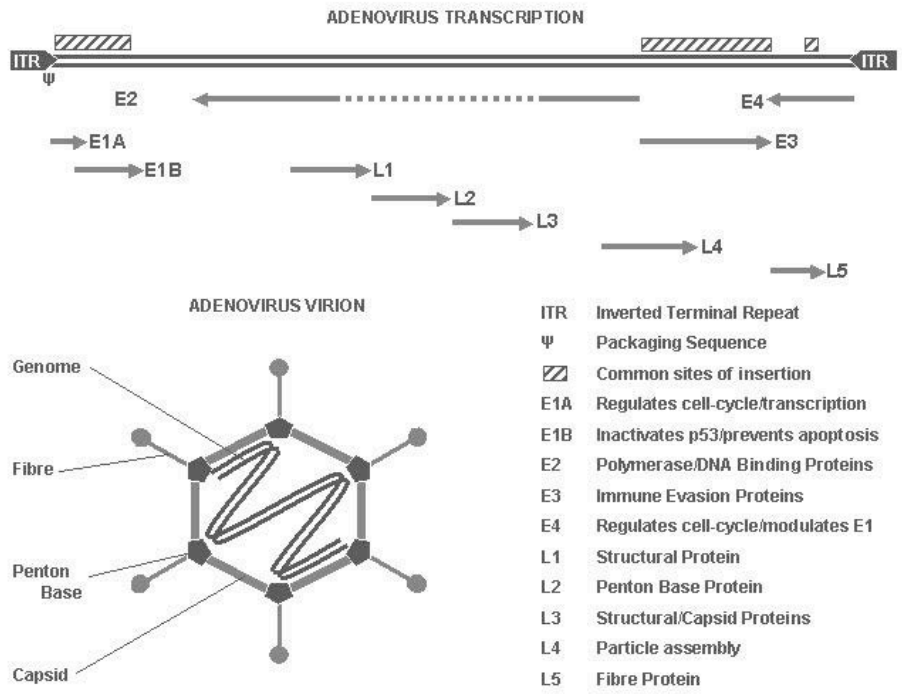

Figure 2.7.1 Transcription of the adenoviral genome and structure of the adenovirus particle

Risk assessment for human health

Hazards associated with the recipient virus

4 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.

Wild-type Human Adenoviruses are ACDP Hazard Group 2. Therefore, Containment Level 2 should be adopted as a minimum requirement when handling wild-type virus.

Vector systems

5 ***Disabled vectors.*** '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.

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 Containment Level 1. 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.

6 ***Replicative vectors.*** 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.

The hazards associated with the handling of high titres of replicative virus should be carefully considered. Conditionally Replicating Viruses (CRVs) 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. Containment Level 2 should be adopted as a minimum requirement for these vectors unless the risk assessment or safety data show this to be unwarranted.

Hazards associated with genetic inserts

7 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 Section 2.2. In brief, factors to consider include the following.

8 ***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, 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.

9 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.

10 ***Integration into host DNA.*** 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.

11 ***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 Enhanced Green Fluorescent Protein (EGFP) or Luciferase. Properties of the gene products with respect to individual cell types should also be considered.

Alteration of phenotype

12 ***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.

13 ***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.

Recombination

14 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.

Complementation

15 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.

Risk assessment for the environment

Survivability and stability

16 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.

17 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.

Hazards associated with genetic inserts

18 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.

Alteration of phenotypic and pathogenic traits

19 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.

Procedures and control measures

Operational considerations

20 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.

21 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 all but eliminate contamination with the recipient virus and therefore reduce the risks posed by handling virus and *in vitro* recombination events. The use of such systems wherever possible is therefore advised.

22 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 waste container.

Control measures and monitoring procedures

23 Many adenovirus vectors will be considered low risk GM activity class 1 and can be handled at Containment Level 1. This means that virus preparations could be handled on the open bench. However, adenoviruses are robust and transmitted effectively in aerosols and droplets, even if disabled or attenuated. Therefore, measures might be required to control aerosol generation and airborne dissemination.

Most work with adenoviruses will take place within a microbiological safety cabinet. It is acknowledged that this is to protect the purity of the culture and not to control aerosol dissemination. The use of a cabinet for these purposes *will not in itself* necessitate the assignment of the work to GM activity class 2 or higher.

However, where the risk assessment shows that exposure to airborne GM adenovirus represents a hazard, the use of a cabinet might be required as a control measure.

These activities should be assigned to GM activity class 2 or higher and take place at an appropriate containment level, unless derogations are obtained from the competent authority.

24 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 (for example quantitation of E1 sequences in a purified virus preparation using quantitative PCR) would represent a more reliable method of RCV detection.

25 It is an employer's responsibility to ensure that a worker's 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 workers exposed to the GM viruses should be monitored. For example, those showing signs of a compromised immune system should review their suitability for work.

Class 1 activities are described in the Contained Use Regulations as being of 'no or negligible risk'. It is unlikely that any non-disabled human or animal pathogen could be deemed to be of 'no or negligible risk' (except where the host species is absent from the receiving environment) and such work will always be class 2 or higher. Since work with pathogens will almost invariably require at least some of the measures required at Containment Level 2 (eg an autoclave in the building; restriction of access) it would not normally be possible to assign the activity to class 1. *Even if no Containment Level 2 measures were justified by the risk assessment, assignment of pathogen work to class 1 would be inappropriate and the activity must be notified to HSE as class 2.* If a GMM that is, or could be, pathogenic to humans or animals is assigned as a class 1 activity, then it is probable that the risk assessment is inadequate.

Remember that classification into a GM activity class does not necessarily mean that you will always have to apply all the measures from the associated containment level. If it is adequately justified by the risk assessment derogation may be sought from HSE to exclude unwarranted measures.

2.8 Baculoviruses

Overview

1 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 guidance, 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.

2 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 2.8.1). 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 these promoters being exploited to direct the expression of foreign genes and recombinant protein production from insect cells.

3 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 (for example, 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 AcMNPV as a potential gene-delivery vector for therapeutic purposes.

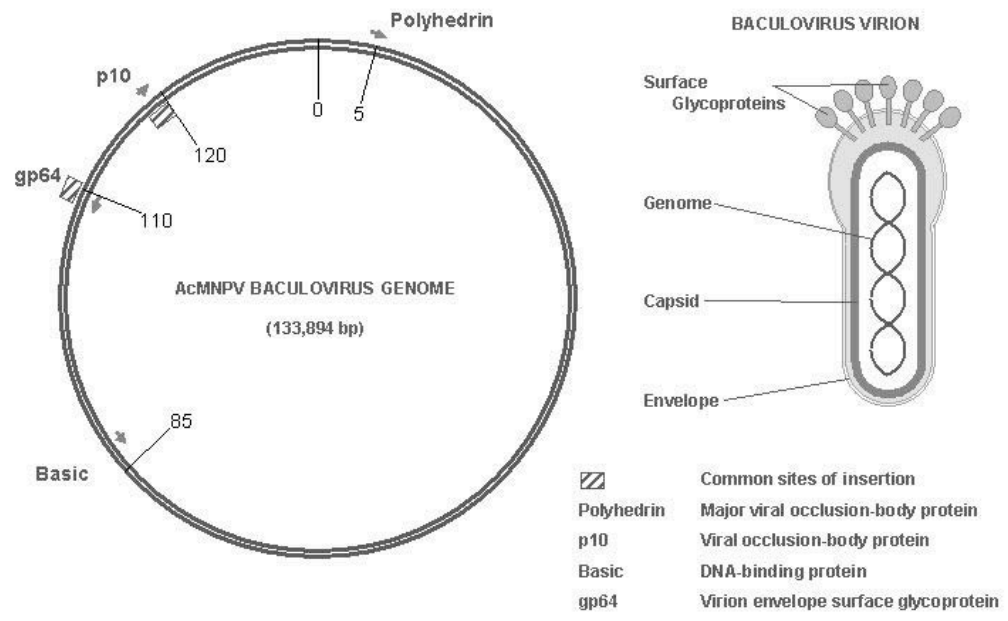

Figure 2.8.1 Representation of baculoviral genome and structure of a baculovirus particle

Risk assessment for human health

Hazards associated with the recipient virus

4 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 (see below). However, although the original virus was pathogenic for certain lepidoptera, the most commonly used expression systems are based upon strains deleted for the *polyhedrin* gene rendering the virus sensitive to insect larval gut conditions and to environmental factors.

5 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.

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.

Hazards associated with genetic inserts

6 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 Section 2.2. In brief, factors to consider include the following.

7 ***Expression characteristics.*** 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. However, 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.

8 ***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. 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 (for example AAV vectors). The properties of the gene products with respect to individual cell types affected should therefore be considered.

9 ***Hybrid vector systems.*** Hybrid baculoviruses carrying the genomes of heterologous mammalian viruses have been used to launch productive infections or study viral mechanisms (for example 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.

Alteration of phenotype

10 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.

11 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 complement 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.

12 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.

Risk assessment for the environment

Survivability and stability

13 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.

14 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.

Hazards associated with genetic inserts

15 In many cases, insect-cell specific elements, or the baculovirus *p10* or *polyhedrin* 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.

16 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.

Alteration of phenotypic and pathogenic traits

17 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 (for example pseudotyping) might result in an organism 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.

18 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.

Procedures and control measures

Operational considerations

19 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 GM activity class 1 and can be handled at Containment Level 1, and viral preparations could be handled on the open bench. It is important to consider that baculoviruses may be 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.

Most work with baculoviruses will take place within a microbiological safety cabinet. It is acknowledged that this is to protect the purity of the culture and not 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, where the risk assessment shows that exposure to airborne GM baculovirus represents a hazard, the use of a cabinet might be required as a control measure. These activities should be assigned to GM activity class 2 and take place at Containment Level 2, unless derogations are obtained from the competent authority.

Class 1 activities are described in the Contained Use Regulations as being of 'no or negligible risk'. It is unlikely that any non-disabled human or animal pathogen could be deemed to be of 'no or negligible risk' (except where the host species is absent from the receiving environment) and such work will always be class 2 or higher. Since work with pathogens will almost invariably require at least some of the measures required at Containment Level 2 (eg an autoclave in the building; restriction of access) it would not normally be possible to assign the activity to class 1. *Even if no Containment Level 2 measures were justified by the risk assessment, assignment of pathogen work to class 1 would be inappropriate and the activity must be notified to HSE as class 2.* If a GMM that is, or could be, pathogenic to humans or animals is assigned as a class 1 activity, then it is probable that the risk assessment is inadequate.

Remember that classification into a GM activity class does not necessarily mean that you will always have to apply all the measures from the associated containment level. If it is adequately justified by the risk assessment derogation may be sought from HSE to exclude unwarranted measures.

2.9 Herpesviruses

Overview

1 Herpesviruses are a diverse family found in humans and most species of animals. More than 130 species have so far been identified, 9 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* (see Table 2.9.1).

2 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 2.9.1). 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.

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

Table 2.9.1 Herpesvirus classification, associated terminology and typical symptoms and diseases associated with herpesvirus infections. HHV – human herpesvirus; CeHV – Cercopithecine herpesvirus

3 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 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.

4 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.

5 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 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.

Herpes simplex virus

6 HSV 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 CNS resulting in encephalitis. HSV-1 and -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%.

7 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.

8 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 IE genes encoding 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 2.9.1).

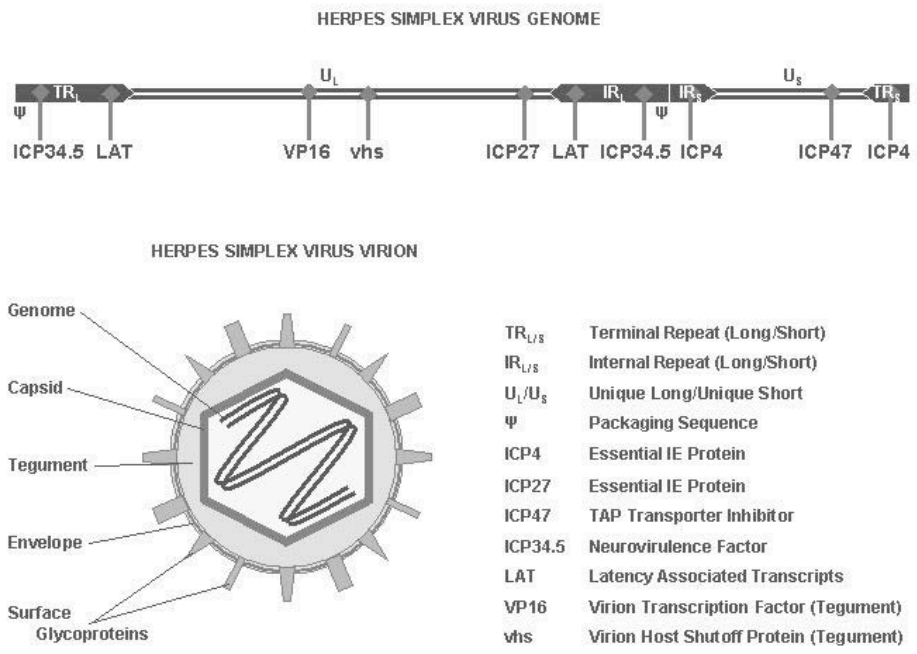

Figure 2.9.1 Representation of the HSV genome and structure of a typical herpesvirus virion

Risk assessment for human health

Hazards associated with the recipient virus

9 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 (see below). Most GM HSV vectors have been derived from cell-culture adapted laboratory strains of HSV-1 (eg 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 ACDP hazard groups (see Table 2.9.2). An appropriate containment level should be adopted as a minimum requirement when handling wild-type viruses.

HERPESVIRUS	HAZARD GROUP
Alphaherpesviruses	
Herpes simplex virus 1 (HHV-1)	ACDP Hazard Group 2
Herpes simplex virus 2 (HHV-2)	ACDP Hazard Group 2
Varicella zoster virus (HHV-3)	ACDP Hazard Group 2
Herpesvirus simiae (B-virus)	ACDP Hazard Group 4
Betaherpesviruses	
Human cytomegalovirus (HHV-5)	ACDP Hazard Group 2
Human herpesvirus 6A (HHV-6A)	ACDP Hazard Group 2
Human herpesvirus 6B (HHV-6B)	ACDP Hazard Group 2
Human herpesvirus 7 (HHV-7)	ACDP Hazard Group 2
Murine cytomegalovirus (MCMV)	ACDP Hazard Group 1
Gammaherpesviruses	
Epstein-Barr virus (HHV-4)	ACDP Hazard Group 2
Kaposi's sarcoma-associated herpesvirus (HHV-8)	ACDP Hazard Group 2
Murine *Gammaherpesvirus* 68 (MHV68; γMV68)	ACDP Hazard Group 1

Table 2.9.2 Hazard Group Classification of herpesviruses

HSV vector systems

10 ***Disabled vectors.*** 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*.

11 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.

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

12 ***Replicative vectors.*** 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 conditionally replicative virus (CRV). For example, deletion of the gene encoding ICP6 (Ribonucleotide Reductase) or Thymidine Kinase (TK) 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). 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. 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.

13 The hazards associated with the handling of high titres of replicative virus should be carefully considered. Conditionally Replicating Viruses (CRVs), 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 (see below).

Containment Level 2 is likely to be a minimum requirement for these vectors unless the risk assessment can show that this is unwarranted.

14 ***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 an 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.

Hazards associated with genetic inserts

15 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 Section 2.2. In brief, factors to consider include the following.

16 ***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 (see below).

17 ***Expression characteristics.*** Viral or cellular regulatory sequences could be employed to control expression in transduced cells. For example, 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 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.

18 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.

19 ***Biological properties of the gene product.*** 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 Enhanced Green Fluorescent Protein (EGFP) or Luciferase.

20 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 PNS should be carefully considered.

Alteration of phenotype

21 ***Tissue tropism.*** 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.

22 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 (eg ICP6; ICP34.5) have been shown to narrow the host range rather than extend or alter it.

23 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.

24 ***Immunogenicity and pathogenicity.*** 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 (by VP16 mutation, for example) may result in increased antigen presentation. HSV can enter Dendritic Cells (DCs) but prevents their activation via a tegument protein termed the Virion Host Shutoff (vhs)

protein. DCs infected with viruses lacking vhs will therefore present antigen and activate cellular immunity more efficiently.

25 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.

Recombination

26 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.

27 The likelihood of this occurring can be minimised by ensuring that viral sequences deliberately introduced into cells (eg 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.

28 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 (eg 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.

Complementation

29 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 wt 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.

Risk assessment for the environment

Survivability and stability

30 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.

Alteration of phenotypic and pathogenic traits

31 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 (for example, 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.

32 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.

Hazards associated with genetic inserts

33 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.

Procedures and control measures

Operational considerations

34 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.

35 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 all but eliminate contamination with the recipient virus and therefore reduce the risks posed by handling virus and in vitro recombination events. The use of such systems wherever possible is therefore advised.

36 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.

Control measures and monitoring procedures

37 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 (for example, the use of PCR to detect the presence of sequences required by the vector for replication) would represent a more reliable method.

38 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 TK^- strains of HSV are resistant to Acyclovir and that natural Gancyclovir-resistance in HCMV has been documented.

39 It is an employer's responsibility to ensure that a worker's 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 workers 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 worker suspects productive infection with HSV (eg has an active orolabial coldsore) then they should consider suspending activities involving a GM HSV vector until the infection is resolved.

Class 1 activities are described in the Contained Use Regulations as being of 'no or negligible risk'. It is unlikely that any non-disabled human or animal pathogen could be deemed to be of 'no or negligible risk' (except where the host species is absent from the receiving environment) and such work will always be class 2 or higher. Since work with pathogens will almost invariably require at least some of the measures required at Containment Level 2 (eg an autoclave in the building; restriction of access) it would not normally be possible to assign the activity to class 1. *Even if no Containment Level 2 measures were justified by the risk assessment, assignment of pathogen work to class 1 would be inappropriate and the activity must be notified to HSE as class 2.* If a GMM that is, or could be, pathogenic to humans or animals is assigned as a class 1 activity, then it is probable that the risk assessment is inadequate.

Remember that classification into a GM activity class does not necessarily mean that you will always have to apply all the measures from the associated containment level. If it is adequately justified by the risk assessment derogation may be sought from HSE to exclude unwarranted measures.

2.10 Poxviruses

Overview

1 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 2.10.1). Infection normally occurs via aerosol or direct contact and results in a vigorous immune response involving innate, humoural 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.

POXVIRUS	PRIMARY HOST	ALTERNATE HOST	DISEASE
Orthopoxviruses			
Variola virus	Humans	None	Smallpox
Vaccinia virus	Unknown	Humans, cows, rabbits	Localised epidermal lesions, eczema*, encephalitis*, vaccinia necrosum*
Cowpox virus	Rodents	Humans, cows, cats, foxes	Localised epidermal lesions
Monkeypox virus	Squirrels	Humans, monkeys	Smallpox-like
Camelpox virus	Camels	None	Smallpox-like (in camels)
Mousepox virus	Rodents	Laboratory mice	Infectious ectromelia in lab mice
Molluscipoxviruses			
Molluscum Contagiosum virus	Humans	None	Localised epidermal lesions
Parapoxviruses			
Orf virus	Ungulates	Humans, cats	Localised epidermal lesions
Yatapoxviruses			
Yaba monkey tumour virus/Tanapoxvirus	Unknown	Humans, monkeys	Localised epidermal lesions
Avipoxviruses			
Fowlpox/Canarypox	Birds	Humans as vaccine vector	Localised epidermal lesions in birds Diphtheric disease in birds
Leporipoxviruses			
Myxoma virus	Rabbits	None	Myxomatosis
Suipoxviruses			
Swinepox virus	Pigs	None	Epidermal lesions, acute but mild.
Capripoxviruses			
Lumpy Skin Disease virus	Cattle	None	Epidermal lesions. Occasionally fatal
Sheeppox/Goatpox virus	Sheep and goats	None	Lesions on mucous membranes and exposed skin, fever, paralysis.

Table 2.10.1 Host range of poxviruses and the typical symptomatic consequences of infection. *Less common adverse reactions to Vaccinia virus inoculation in humans

2 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 is determined by the expression of viral 'host range' genes in concert with host-cell characteristics.

3 Unusually for DNA viruses, replication 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.

Risk assessment for human health

Hazards associated with the recipient virus

4 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 (eg Western Reserve strain of VV is more virulent than Copenhagen strain) and the individual hazards associated with these strains should be carefully weighed.

Wild-type poxviruses fall into a range of ACDP hazard groups (see Table 2.10.2). An appropriate containment level should be adopted as a minimum requirement when handling wild-type viruses.

Wild-type viruses that are included in Defra's classification of animal pathogens, and which are specified animal pathogens, must be handled using the appropriate prescribed containment measures.

5 ***Disabled and attenuated vectors.*** 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 genetically modified vector derivatives. Modified Vaccinia virus Ankara (MVA) has been attenuated by serial passage in chicken embryo fibroblasts; approximately 31kb 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.

POXVIRUS	HAZARD GROUP
Vaccinia virus (wild-type strains)	ACDP Hazard Group 2
MVA	*ACDP Hazard Group 1*
NYVAC	*ACDP Hazard Group 1*
Cowpox virus	ACDP Hazard Group 2
Monkeypox virus	ACDP Hazard Group 3
Molluscum contagiosum virus	ACDP Hazard Group 2
Orf virus	ACDP Hazard Group 2
Yaba monkey tumour virus	ACDP Hazard Group 2
Tanapox virus	ACDP Hazard Group 2
Camelpox virus	ACDP Hazard Group 1
Sheeppox virus / Goatpox virus	Defra Group 3 (SAPO)
Lumpy Skin Disease virus	Defra Group 3 (SAPO)
Swinepox virus	ACDP Hazard Group 1
Myxoma virus	Controlled under Pests Act 1954

Table 2.10.2 Hazard Group classification of poxviruses

6 It is a requirement of the COSHH Regulations that a potentially harmful biological agent be substituted with an agent that is less hazardous or be eliminated entirely, if possible. Therefore, safer virus systems or less virulent strains should be employed wherever practicable. For instance, the use of attenuated Vaccinia strains (for example 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 Containment Level 1. 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.

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

7 ***Conditionally replicative vectors.*** 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 (*tk*) gene is required for nucleotide biosynthesis. Deletion of the *tk* 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 *tk* 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 workers have demonstrated that *tk*-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.

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

Hazards associated with genetic inserts

8 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 Section 2.2. In brief, factors to consider include the following.

9 ***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.

10 ***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 Section 2.11).

11 ***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 Enhanced Green Fluorescent Protein (EGFP) or Luciferase. Properties of the gene products with respect to individual cell types should also be considered.

Alteration of phenotype

12 ***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 2.10.3). 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.

POXVIRUS GENE	REQUIRED FOR GROWTH IN:
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

Table 2.10.3 Poxvirus host-range genes

13 ***Immunogenicity and pathogenicity.*** 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 2.10.4). 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.

POXVIRUS GENE	VIRAL PROTEIN	FUNCTION
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 β_2-Microglobulin
MCV MC159	FLIP (FLICE-like inhibitory protein)	Prevents Fas and TNF mediated apoptosis

Table 2.10.4 Examples of poxvirus immune-evasion genes and their function

14 Similarly, the insertion and expression of genes encoding immunomodulatory products might affect pathogenesis.

15 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.

16 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.

Recombination

17 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.

18 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 *tk* 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.

Risk assessment for the environment

Survivability and stability

19 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, genetically modified poxviruses might persist and could be transmitted to other humans or animal species.

20 Some poxviruses (Cowpox virus and avipoxviruses, but not Vaccinia virus) 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.

Hazards associated with genetic inserts

21 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. 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).

Alteration of phenotypic and pathogenic traits

22 While some poxviruses have a narrow host range and tissue tropism, others can productively infect other organisms (see Table 2.10.1). This is pertinent when evaluating genetically modified 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 (see Table 2.10.3), 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.

23 For example, poxviruses (eg 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.

Procedures and control measures

Operational considerations

24 Generally, genetic manipulation work on Poxviruses is undertaken in cell culture by homologous recombination between recipient virus and transfected plasmid DNA.

Contamination with parental virus is a feature of this system and therefore repeated purification steps and handling of high-titre virus is 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 all but eliminate contamination with parental virus and therefore reduce the risks posed by handling 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.

25 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 that no breakage occurs due to extreme temperature variation (eg 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.

26 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 (see Table 2.10.5). 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 waste container.

Control measures and monitoring procedures

27 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 classified as Containment Level 1 could, in principle if not in practice, be handled on the open bench. Other viruses must be handled under appropriate conditions and, if necessary handled within a microbiological safety cabinet to safeguard human health and to prevent environmental release.

Most work with poxviruses will take place within a microbiological safety cabinet. It is acknowledged that this is to protect the purity of the culture and not to control aerosol dissemination. The use of a cabinet for these purposes will not in itself necessitate the assignment of the work to GM activity class 2 or higher.

However, where the risk assessment shows that exposure to airborne GM poxvirus represents a hazard, the use of a cabinet might be required as a control measure. These activities should be assigned to GM activity class 2 or higher and take place at an appropriate containment level, unless derogations are obtained from the competent authority.

28 The Smallpox vaccine should not be administered unless (i) a worker requests it (ii) if work involves Monkeypox virus or (iii) insofar as the risk assessment says it is required due to the GM virus representing specific hazard. Further guidelines can be found in the ACDP/ACGM joint guidance *Vaccination of laboratory workers handling vaccinia and related poxviruses infectious for humans* Guidance HSE Books 1991 ISBN 0 11 885450 X.

Health surveillance and staff training

29 It is an employer's responsibility to ensure that a worker's 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 workers 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.

30 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 (see Table 2.10.5). 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 HSE.

31 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 workers, 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 Vaccinia and other poxviruses should have suitable local rules to control these potential sources of infection, including suitable procedures for decontamination of equipment and surfaces.

32 As work with Class 2 organisms such as vaccinia virus requires restricted access, ideally only those who work with the virus should have access to the areas where the virus is used. Where vaccinia viruses are used in multi-user facilities, all users must be familiar with the risks associated with vaccinia and be trained to recognise the signs of vaccinia virus infection. Photographs of VV infections are available by searching for 'smallpox' at: http://phil.cdc.gov/phil/quicksearch.asp. Photographs of vaccinia virus infections can also be found in 'Vaccinia (Smallpox) Vaccine Recommendations of the Advisory Committee on Immunisation Practices (ACIP), 2001', available at: www.cdc.gov/mmwr/preview/mmwrhtml/rr5010a1.htm.

Risk awareness

33 Vaccinia virus is categorised by ACDP as a hazard group 2 biological agent in recognition that it 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 vaccinia 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 personnel who work with vaccinia virus should be:

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

COUNTRY	YEAR	COMMENT
US	1986	Needlestick during animal handling
UK	1991	Needlestick during animal handling
Canada	2003	No known incident, gloves not worn
Germany	2003	Immunomodulatory gene
US	2004	Coverslip cut
UK	2000	Autoinoculation of cosmetic piercing
UK	2003	No known incident
UK	2004	Needlestick during animal handling
UK	2004	No gloves, cut on hand
US	**2004**	**Ocular Infection**

Table 2.10.5 Laboratory-acquired vaccinia virus infections since 1986-2006

Class 1 activities are described in the Contained Use Regulations as being of 'no or negligible risk'. It is unlikely that any non-disabled human or animal pathogen could be deemed to be of 'no or negligible risk' (except where the host species is absent from the receiving environment) and such work will always be class 2 or higher. Since work with pathogens will almost invariably require at least some of the measures required at Containment Level 2 (eg an autoclave in the building; restriction of access) it would not normally be possible to assign the activity to class 1. *Even if no Containment Level 2 measures were justified by the risk assessment, assignment of pathogen work to class 1 would be inappropriate and the activity must be notified to HSE as class 2.* If a GMM that is, or could be, pathogenic to humans or animals is assigned as a class 1 activity, then it is probable that the risk assessment is inadequate.

Remember that classification into a GM activity class does not necessarily mean that you will always have to apply all the measures from the associated containment level. If it is adequately justified by the risk assessment derogation may be sought from HSE to exclude unwarranted measures.

2.11 Retroviruses

Overview

1 Retroviruses form a diverse and extensive family affecting both human and animal species. In humans, many retroviruses produce subclinical or benign infections, although some cause significant diseases, most of which result in haematopoietic disorders, although the range of manifested symptoms is broad (see Table 2.11.1). Several retroviruses are known to be oncogenic and cause malignant disease, either by insertional mutagenesis of the host chromosomes or as a result of acquiring host oncogenes. 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 or slowly developing infections. These viruses are strongly immunogenic, but host immunity usually suppresses replication rather than clearing the infection altogether.

RETROVIRUS	DISEASE
Alpharetroviruses	
Avian leukosis virus	Lymphoid leukaemia and wasting syndromes in chickens
Rous sarcoma virus	Sarcoma in chickens (encodes *v-src* oncogene)
Avian myeloblastoma virus	Myeloid leukaemia in chickens (defective - encodes *v-myb* oncogene)
Moloney Murine leukaemia virus	T-cell leukaemia in mice/rats
Moloney Murine sarcoma virus	Sarcoma in mice/rats (defective – encodes *v-mos* oncogene)
Betaretroviruses	
Mouse mammary tumour virus	Epithelial mammary tumours in mice
Gammaretroviruses	
Feline leukaemia virus	Feline immunodeficiency/Lymphoid Leukaemia in cats
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
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	
Human foamy virus	No pathogenicity definitely identified

Table 2.11.1 Typical diseases associated with commonly studied retroviruses

2 Retrovirus virions contain two copies of the RNA genome, encased within a capsid surrounded by a host-cell derived envelope. Cellular entry involves interaction between the Surface subunit (SU) 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 Transmembrane (TM) subunits of the virion envelope glycoprotein, resulting in delivery of the virus capsid into the target cell.

3 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 flanked by Long Terminal Repeats (LTRs) containing viral transcriptional promoter and enhancer regions (the U3 region) that regulate 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 replication-enhancing or modulatory proteins that might be involved in viral pathogenesis (see Figure 2.11.1).

Risk assessment for human health

Hazards associated with the recipient virus

4 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 are designed to give rise to replication-defective vectors. However, 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.

Wild-type retroviruses fall into a range of ACDP hazard groups (see Table 2.11.2). An appropriate containment level should be adopted as a minimum requirement when handling wild-type viruses.

Wild-type viruses included in the Defra classification of animal pathogens, and which are specified animal pathogens, must be handled using the appropriate prescribed containment measures.

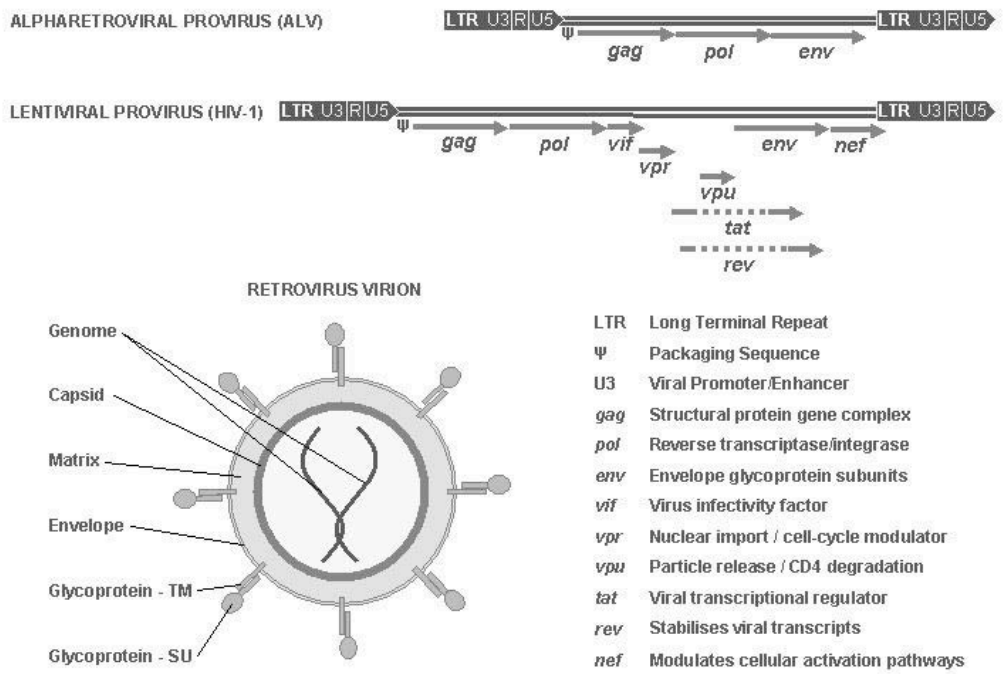

Figure 2.11.1 Representation of retroviral genomes and structure of a retrovirus particle

RETROVIRUS	HAZARD GROUP
Avian leukosis virus (ALV)	ACDP Hazard Group 1
Moloney Murine leukaemia virus (MoMLV)	ACDP Hazard Group 1
Mouse mammary tumour virus (MMTV)	ACDP Hazard Group 1
Feline leukaemia virus (FeLV)	ACDP Hazard Group 1
Bovine leukaemia virus (BLV)	ACDP Hazard Group 1/Defra Group 2 (SAPO)
Human T-cell lymphotrophic virus 1 and 2 (HTLV-1/-2)	ACDP Hazard Group 3
Human immunodeficiency virus 1 and 2 (HIV-1/-2)	ACDP Hazard Group 3
Simian immunodeficiency virus (SIV)	ACDP Hazard Group 3
Feline immunodefieciency virus	ACDP Hazard Group 1
Equine infectious anaemia virus (EIAV)	ACDP Hazard Group 1/Defra Group 3 (SAPO)
Visna/maedi virus (MVV)	ACDP Hazard Group 1
Human foamy virus (HFV)	ACDP Hazard Group 1

Table 2.11.2 Hazard group classification of commonly studied Retroviruses

Vector systems

5 Oncogenic retrovirus and lentivirus vector systems generally consist of two main components – a transfer vector and a packaging system. The transfer vector is usually a proviral cDNA from which virus coding sequences have been deleted or replaced with a genetic insert. The packaging system commonly consists of one or more helper constructs that express viral genes needed to generate infectious virus particles.

6 The generation of replication competent virus (RCV) and insertional mutagenesis as a result of provirus integration pose the major safety issues when handling retrovirus vectors. 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). Provirus 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). For example, provirus insertion within or close to the LMO2 oncogene appears to have contributed to the development of leukaemia in some children receiving haematopoietic stem cell gene therapy. Retrovirus vector systems have therefore been developed and refined in order to reduce the likelihood of RCV generation and provirus transactivation. Consequently, there is a range of systems that vary in their safety profile.

7 ***Oncogenic retrovirus vectors.*** The majority of these vectors have been derived from competent oncogenic retroviruses, such as ALV, MoMLV and FeLV, which efficiently infect actively dividing cells. ‘First Generation’ retrovirus vectors contain a packaging system that is essentially a retroviral cDNA, 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 is required to generate RCV. 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. 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 whenever possible. Additional biosafety can also be achieved by using self-inactivating (SIN) transfer vectors (see paragraph 27 of this section).

8 ***Lentivirus vectors.*** These have become widely used due to their ability to infect non-dividing cells, giving them an advantage over oncogenic retrovirus 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 some 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 currently not clear what features of the vector may contribute to this, or indeed whether or not this is due to vector activity.

9 'First Generation' lentivirus vectors resemble third generation oncogenic retrovirus systems, and are composed of a transfer vector containing all virus components except *gag*, *pol* and *env* which are provided *in trans* by two helper constructs. Several of the lentivirus 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 (see below).

10 ***Hybrid vectors (virus shuttle vectors).*** Another strategy employed is to use other virus vector systems to deliver retrovirus vector or packaging constructs to cells (for example, Vaccinia virus or Herpes simplex virus). These approaches are designed to improve the efficacy and scale-up potential of retrovirus 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 GMM, distinct from the intended retroviral vector. However, it should also be assessed as an integral part of the retrovirus system.

11 ***Vector choice.*** Clearly, there are 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 level of safety for the user. For example, third-generation lentivirus systems have a much-improved biosafety profile when compared to first- or second-generation oncogenic retrovirus systems. Safety versus functionality considerations should therefore be carefully weighed and the safest system possible should ultimately be employed.

Hazards associated with genetic inserts

12 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 Section 2.2 of the Compendium. In brief, factors to consider include the following:

13 ***Biological properties of the gene product.*** The expected activities or toxicity of the gene products should be assessed. For example, an oncogene or growth factor would represent greater risk of harm than a reporter gene such as Enhanced Green Fluorescent Protein (EGFP) or Luciferase. Properties of the gene products with respect to individual cell types should also be considered.

14 ***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 sequence 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 often 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 a vector is generated. Properties of the gene products with respect to cell types and tissues that could be affected should therefore be considered.

15 ***Provirus insertion.*** Integration of viral genomes into the host-cell chromatin is a characteristic of retrovirus biology and is a 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 sequences present in the provirus might activate genes adjacent to the integration site or, alternatively, insertion may disrupt genes and prevent their expression. Therefore, retrovirus infection might induce permanent changes in a cell, resulting in tumourigenesis. It is recognised that, in humans, this appears so far only to have occurred in the context of deliberate transduction of large numbers of stem cells with a retrovirus vector. Furthermore, the transferred gene enabled the cells to proliferate in response to cytokines, and the many ensuing cycles of cell replication may have allowed additional, co-operating events to occur. High-titre inoculations are required to establish a clinically significant level of infection or gene transfer, and accidental infections of this magnitude are unlikely during standard laboratory-based manipulations of retroviruses. Nevertheless, retrovirus vectors have been shown to have transforming properties *in vivo* and a cautious approach to handling them is advised.

16 ***Non-coding sequences.*** The potential effects of other exogenous non-coding sequences should be carefully assessed. For example, the lentivirus *rev* protein interacts with a *rev*-responsive element (RRE) in the lentivirus genome, enhancing and stabilising the export of viral mRNAs from the nucleus. In some vectors, this has been replaced by heterologous virus sequences with similar function, such as the woodchuck hepatitis B virus (WHV) regulatory element (WPRE), thus negating the need for *rev* in the packaging system. 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 account the possible harmful effects of this sequence. Vectors containing these forms of WPRE should be assigned to class 2 or higher. This highlights the need for rigorous scrutiny of the possible effects of regulatory sequences present in vectors.

Alteration of phenotype

17 ***Tissue tropism.*** Retroviruses 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 substitution of the *env* gene with the glycoprotein gene from another virus ('pseudotyping') or modification of the native *env* gene. 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).

18 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 be a major factor in determining the containment and control measures appropriate to the intended virus vector. It is also important to consider that the envelope glycoproteins of retroviruses are inherently biologically active. The TM subunit alone can mediate non-specific viral entry as well as causing cell fusion and syncytia formation. The effects of expressing these proteins should therefore be contemplated, especially if a packaging cell line is generated using a viable GM vector system.

19 Furthermore, it has been shown that pseudotyping viruses can alter the stability and potentially the transmission properties compared with the wild-type virus from which it is derived. For instance, some pseudotyped retrovirus and lentivirus vectors could be transmissible via aerosols as well as the recognised routes. The increased stability

resulting from pseudotyping can, in some instances, also permit vector concentration *eg* by centrifugation, which could increase the risk. 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 properties of transmission, and appropriate control measures employed (eg restricted access, the use of a safety cabinet).

20 ***Immunogenicity and pathogenicity.*** Deletions in the virus vector or the genetic insert may alter the immunogenic or pathogenic nature of the virus. This is particularly relevant to lentivirus vectors, where accessory genes with cytotoxic products are deleted in order to improve vector capacity and biosafety.

21 Retroviruses incorporate host cell-derived proteins into virions during packaging and these will be delivered to the target cells. These may be cellular or viral proteins expressed by the packaging system. For example, lentivirus proteins Nef, Vpr and Vif, as well as a number of cellular proteins, are incorporated into virus particles and may enhance the immunogenic nature of the vector. Furthermore, Nef and Vpr have cytotoxic properties that may affect target cells. The potential harmful effects of these proteins should therefore be considered, particularly with xenotropic/amphotropic vectors where the infected tissues could be very different from the cells in which the virus was generated.

If the risk assessment demonstrates they are adequately disabled, it might be possible to classify some activities with defective oncogenic retroviruses and lentiviruses as *class 1*. This would apply to vectors that have restricted tropisms (*ie* they are unlikely to infect human cells) and where the likelihood of RCV generation is low (for example, a third generation ecotropic retrovirus).

Pseudotyping may result in changes to both the stability of the construct and its properties of transmission. Some pseudotyped vectors are able to infect a range of human and animal cells and the potential for insertional mutagenesis remains a hazard, even when self-inactivating vectors (see paragraph 27 in this section) are employed. It is recognised that to date, tumourigenesis, apparently resulting from insertional mutagenesis, has only occurred in the context of transduction of a large number of stem cells with a retrovirus expressing a gene that favoured cell proliferation, and using a standard (non-self-inactivating) retrovirus design. If pseudotyping allows more cells to be transduced, the risk may be proportionately increased. Conversely, use of self-inactivating vectors, or vectors with a different integration site preference, may reduce the likelihood of oncogene activation at the site

of insertion. However, the theoretical risk remains, as provirus insertion is a fact of retrovirus/lentivirus biology. Therefore, control measures may be required to prevent exposure. In some cases, good microbiological practice (including restricting the use of sharps) may be sufficient and assignment to class 1 would be appropriate, for example, transient transfection to generate low titres of virus to make stable cell lines expressing the transgene for subsequent use. In other cases, specific control measures may be needed, for example, for work with viruses with potentially harmful transgenes, or factors that increase the likelihood of cell infection (eg by increasing titre, or pseudotyping), or where packaging cells with a higher chance of generating replication competent retrovirus (RCR) are used. The degree of control needed should be determined by the risk assessment on a case-by-case basis. If specific control measures are needed *to control worker exposure to the vector* (eg gloves, a microbiological safety cabinet or restricted access), then these activities must be assigned to class 2.

Hazards arising subsequently due to the insertion of sequences or other phenotypic alterations might also necessitate additional containment measures.

Risk assessment for the environment

Survivability and stability

22 Retroviruses 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 stability of the virus (for example, pseudotyping).

Hazards associated with genetic inserts

23 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, pseudotyped vectors as they are able to gain entry to many cell types across many species.

Phenotypic and pathogenic traits

24 While 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.

25 It is also important to pay particular attention to the potential environmental hazards when handling vectors derived from different species. For example, while a defective oncogenic retrovirus vector based on ALV or a lentivirus vector based upon 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 host.

Procedures and control measures

Vector system design

26 ***Sequence manipulation.*** 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

27 Further refinements of retrovirus vectors have involved the generation of self-inactivating (SIN) systems. This takes advantage of a feature of retrovirus replication whereby the 3' U3 region of the LTR (which contains the major viral promoters and enhancers) is copied to the 5' end of the provirus during reverse transcription. Thus, deletion of the 3' U3 region in the vector construct will result in a provirus that is entirely devoid of U3 sequences, therefore reducing the likelihood of *trans*-activation of cellular genes as a result of insertion. Furthermore, such vectors are not easily mobilisable as a result of superinfection with wild-type virus. While the effects of the viral LTRs are negated in these vectors, trans-activation by heterologous promoters used to drive expression of genetic inserts remains a possibility and the risks should be carefully considered.

28 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 defective but acutely transforming due to the expression of a cellular oncogene and therefore the possibility of this occurring must be considered. The mechanism for oncogene capture is thought to be transcriptional readthrough of virus genes generating a chimaeric RNA transcript that is subsequently packaged into a virion. Therefore, it is important that efficient termination and polyadenylation sequences are present in the vector sequence. This may necessitate the substitution of the natural virus sequences, as retrovirus termination/polyadenylation signals are frequently inefficient.

29 ***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 virus 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.

Control measures and monitoring procedures

30 The most likely route of accidental infection with a retrovirus will be via inadvertent percutaneous inoculation. Therefore, hollow needles and other sharps should be handled with extreme care and only used when necessary. Needles should never be resheathed, but rather disposed of directly into an appropriate container for autoclaving or incineration.

31 Some retrovirus 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.

32 If an RCV could be generated that is more hazardous than the intended vector, then it will be important to test vector stocks. This should be mandatory where the risk assessment and containment level are based on the assumption that RCV or wild-type viruses are not present. Direct plating of vector stock onto permissive cell lines and observing for indications of virus replication (for example cytopathic effect or syncitia formation) 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 virus protein expression could be detected by immunostaining or *gag*, *pol* or *env* DNA provirus sequences could be detected using PCR.

Class 1 activities are described in the Contained Use Regulations as being of 'no or negligible risk'. It is unlikely that any non-disabled human or animal pathogen could be deemed to be of 'no or negligible risk' (except where the host species is absent from the receiving environment) and such work will always be class 2 or higher. Since work with pathogens will almost invariably require at least some of the measures required at Containment Level 2 (eg an autoclave in the building; restriction of access) it would not normally be possible to assign the activity to class 1. *Even if no Containment Level 2 measures were justified by the risk assessment, assignment of pathogen work to class 1 would be inappropriate and the activity must be notified to HSE as class 2.* If a GMM that is, or could be, pathogenic to humans or animals is assigned as a class 1 activity, then it is probable that the risk assessment is inadequate.

Remember that classification into a GM activity class does not necessarily mean that you will always have to apply all the measures from the associated containment level. If it is adequately justified by the risk assessment derogation may be sought from HSE to exclude unwarranted measures.

2.12 Viral reverse genetics

Overview

1 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 (for example *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 (for example, Adenoviruses and Retroviruses) and aspects covered will be applicable to the manipulation of any viral system in this way, although specific guidance relating to the risk assessment of other virus systems can be found elsewhere in the compendium.

2 Reverse genetics approaches allow the generation of genetically modified 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 (for example cytokines, immunomodulators or antigens) for the purposes of improved vaccine performance and GM-virus-based therapy development.

Principles of reverse genetics of ssRNA viruses

3 The ssRNA viruses can be subdivided into two main groups on the basis that their genomes are either positive-strand (ie the genome in its native form is translatable) or negative-strand (ie 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 (for example

Influenza, which has 8 viral genome segments) or non-segmented (for example Measles, which has all its genes present on one RNA molecule). The genomes of Influenza and Measles viruses are shown diagrammatically in Figure 2.12.1 and a summary of different reverse-genetics approaches can be found in Figure 2.12.2.

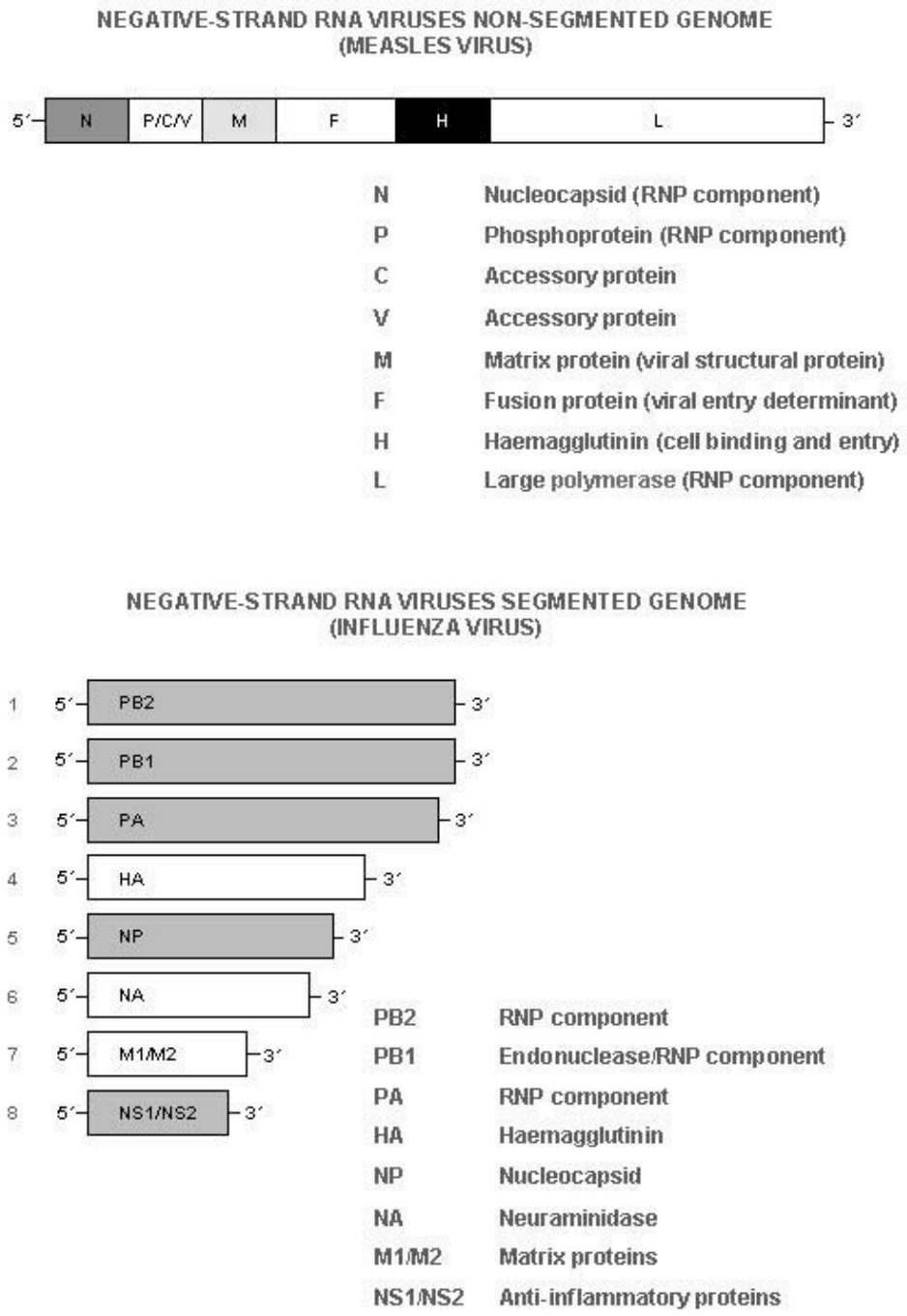

Figure 2.12.1 Diagram illustrating the representative segmented and non-segmented ssRNA(-) genomes (Measles and Influenza viruses)

4 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, for example 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.

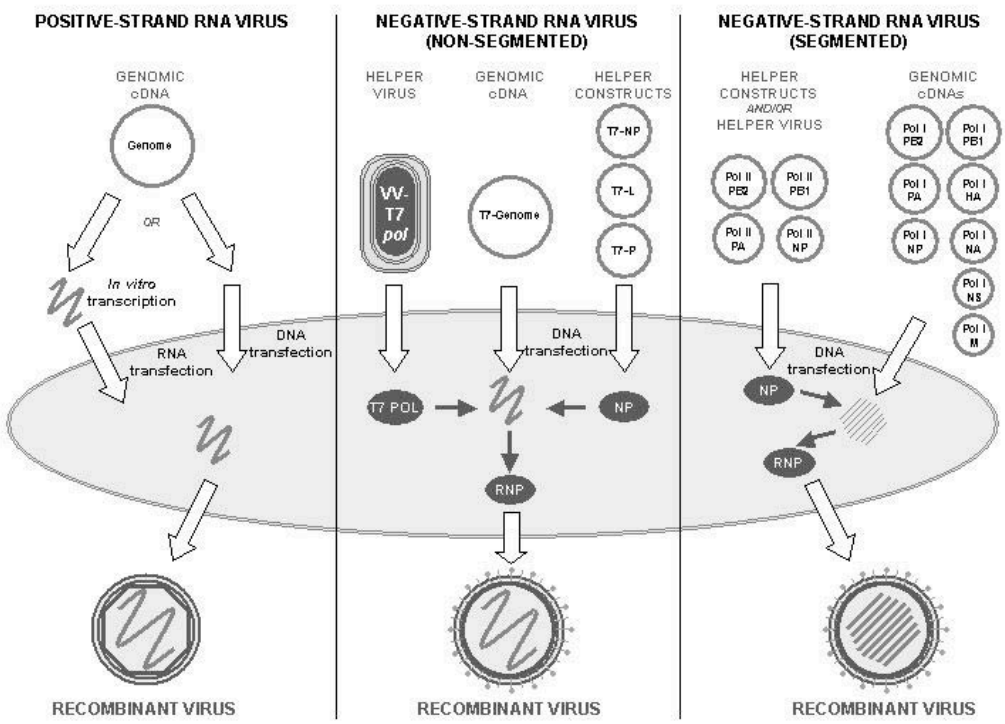

Figure 2.12.2 Examples of methods used to recover virus using reverse genetics. The recovery of segmented negative strand RNA virus from cloned DNA is exemplified using Influenza virus as a model. VV – Vaccinia virus; T7pol - bacteriophage T7 polymerase gene; T7 POL – T7 polymerase; NP- nucleoprotein; RNP – ribonucleoprotein; T7 – T7 polymerase-specific promoter; Pol I – RNA polymerase I promoter; Pol II – RNA polymerase II promoter

Replicons

5 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 (for example 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.

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

7 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 hitherto have been difficult to culture (for example Hepatitis C virus).

8 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 2.12.3).

Risk assessment for human health

Hazards associated with the recipient virus

9 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 ACDP or Defra hazard group and containment level appropriate to the wild type virus. A list of viruses that are commonly manipulated using reverse genetics methodology can be found in Table 2.12.1.

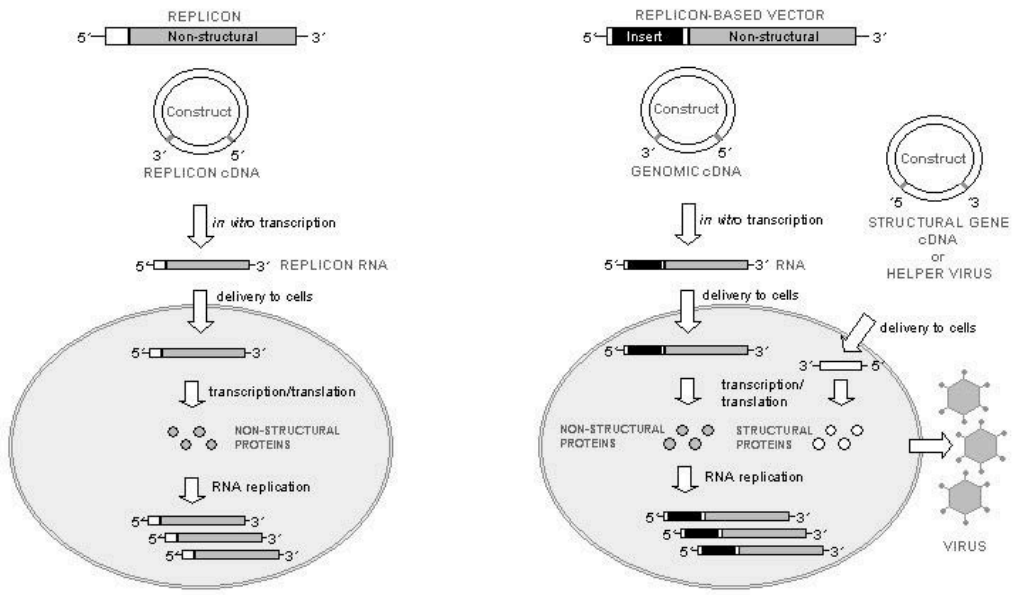

Figure 2.12.3 Schematic representation of Replicon and Replicon-based vector systems

FAMILY/GENUS	SPECIES	HAZARD GROUP	VACCINE
POSITIVE-STRAND RNA VIRUSES			
Togaviridae			
Alphaviruses	Sindbis virus	ACDP Hazard Group 2	
	Semliki Forest virus	ACDP Hazard Group 2	
	Venezuelan Equine Encephalitis	ACDP 3/Defra 3 (SAPO)	
Flaviviridae			
Flaviviruses	Yellow fever virus	ACDP Hazard Group 3	H
	Dengue 1, 2, 3, 4	ACDP Hazard Group 3	
Pestiviruses	Classical Swine Fever virus	Defra Group 3 (SAPO)	
Hepaciviruses	Hepatitis C virus	ACDP Hazard Group 3	
Picornaviridae			
Enteroviruses	Poliovirus	ACDP Hazard Group 2[4]	H
Rhinoviruses	Human Rhinovirus	ACDP Hazard Group 2	
Apthoviruses	Foot and Mouth Disease virus	Defra Group 4 (SAPO)	A
Coronaviridae			
Coronaviruses	Human Coronavirus	ACDP Hazard Group 2	
	SARS-Coronavirus	ACDP Hazard Group 3	
NEGATIVE-STRAND RNA VIRUSES			
NON-SEGMENTED GENOMES (*MONONEGAVIRALES*)			
Paramyxoviridae			
Morbilliviruses	Measles virus	ACDP Hazard Group 2	H
	Canine distemper virus	ACDP Hazard Group 1	

	Rinderpest virus	Defra Group 4 (SAPO)	
	Peste de petits ruminants virus	Defra Group 4 (SAPO)	
Rubulaviruses	Mumps virus	ACDP Hazard Group 2	H
	Newcastle Disease virus	ACDP 2/Defra 4 (SAPO) [1]	A
Respiroviruses	Sendai virus	ACDP Hazard Group 1	
Pneumoviruses	Human Respiratory Syncytial virus	ACDP Hazard Group 2	
	Bovine Respiratory Syncytial virus	ACDP Hazard Group 1	
Paramyxoviruses	Human parainfluenza virus 1, 2, 3, 4	ACDP Hazard Group 2	
Rhabdoviridae			
Lyssavirus	Rabies virus	ACDP 3/Defra 4 (SAPO)	H/A
Vesiculoviruses	Vesicular stomatitis virus	ACDP 2/ Defra 3 (SAPO)	
Bornaviridae			
Bornaviruses	Borna Disease virus	ACDP Hazard Group 3	
Filoviridae			
Filoviruses	Ebola virus	ACDP Hazard Group 4	
	Marburg virus	ACDP Hazard Group 4	
NEGATIVE-STRAND RNA VIRUSES			
SEGMENTED GENOMES			
Orthomyxoviridae			
Orthomyxoviruses	Influenza Type A, B, C	ACDP 2 /Defra 4 (SAPO) [2]	H/A[3]
Bunyaviridae			
Bunyaviruses	Bunyamwera	ACDP Hazard Group 2	
Arenaviridae			
Arenaviruses	Lassa fever	ACDP Hazard Group 4	
	Lymphocytic choriomeningitis virus	ACDP Hazard Group 3	

Table 2.12.1 Containment requirements and vaccine availability for viruses commonly manipulated using reverse genetics. ACDP – Advisory Committee for Dangerous Pathogens; Defra - Department for Environment, Food and Rural Affairs; SAPO – Specified Animal Pathogens Order; H – Human vaccine available; A- Animal vaccine available

[1]All strains of Newcastle disease virus that are specified animal pathogens are classified as Defra group 4 except Hitchener B1 and F strains, which are not specified animal pathogens.

[2]Uncharacterised or highly pathogenic avian influenza strains are classified as Defra group 4 specified animal pathogens.

[3]Influenza vaccines are specific for circulating strains and do not offer protection to newly emerging variants or strains.

[4]Poliovirus may be reclassified as ACDP Hazard Group 3/4 as part of the WHO eradication programme.

Single-stranded RNA viruses fall into a range of ACDP and/or Defra hazard groups. An appropriate containment level should be adopted as a minimum requirement when wild-type viruses will be handled.

Where wild-type viruses are included in Defra's classification of animal pathogens, they should be handled using the appropriate prescribed containment measures.

Organisms subject to licensing under SAPO must be handled in accordance with the licence conditions.

10 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 (eg alphavirus replicon-based vectors) or vaccine strains (eg 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.

11 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 (for example Vaccinia virus 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.

Vector systems

12 ***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 (see below).

13 ***Replicon-based vectors.*** 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; see Figure 2.12.4) 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 2.12.3.

14 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 (see below).

15 ***Negative-strand RNA virus vectors.*** SsRNA(-) viruses 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.

16 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.

17 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.

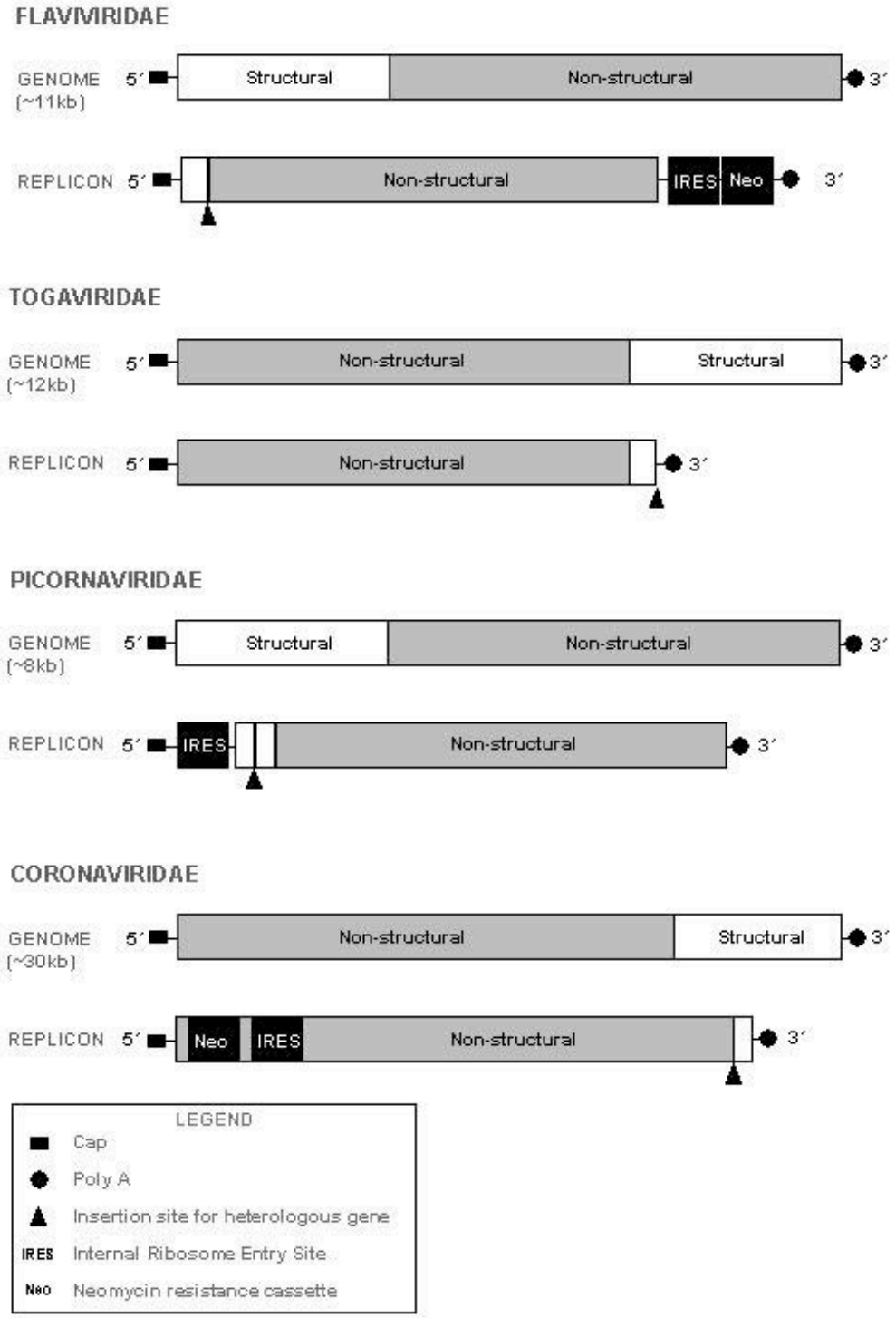

Figure 2.12.4 Diagram illustrating the construction of 'typical' *Alphavirus*, *Coronavirus*, *Flavivirus*, and *Picornavirus* Replicons. It should be noted that not all flavivirus genomes have endogenous poly A sequences

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.

Hazards associated with genetic inserts

18 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 Section 2.2. However, in brief, factors to consider include: aside from the following.

19 ***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.

20 ***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. 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.

21 ***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.

Alteration of phenotype

22 ***Tissue tropism.*** 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.

23 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.

24 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.

25 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 (for example, 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 (for example Morbillivirus P genes) and should also be carefully assessed.

26 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.

27 ***Pathogenicity.*** 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.

28 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 (for example 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.

29 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.

30 ***Immunogenicity.*** 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.

31 For example, the ability of influenza virus to spread and cause disease is, in part, due to the host immune system being naïve 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.

32 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.

Genetic stability

33 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.

34 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.

Recombination

35 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.

36 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.

37 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.

38 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.

Reassortment

39 Reassortment of genomic segments can take place in cells coinfected with different strains of ssRNA(-) viruses with segmented genomes (for example 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.

40 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 (see below).

Risk assessment for the environment

Survivability and stability

41 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.

42 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 (for example, 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.

43 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.

Hazards associated with genetic inserts

44 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.

Alteration of phenotypic and pathogenic traits

45 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 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.

46 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.

Procedures and control measures

Operational considerations

47 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 containment level 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.

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

49 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.

50 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.

51 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 microbiological safety cabinet or equivalent isolation equipment. Some viruses may be arthropod-borne (for example 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.

System design

52 ***Sequence manipulation.*** 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.

53 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.

54 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.

55 ***Use of helper viruses.*** Some reverse genetics systems have involved the use of helper viruses (for example Vaccinia Virus) to supply helper functions. COSHH regulations require that, 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.

Control measures and monitoring procedures

56 ***Vaccination.*** COSHH requires that if the risk assessment shows there to be a risk of exposure to biological agents for which effective vaccines exist, then these should be offered if the employee 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.

57 Therefore, vaccination could be offered to protect workers 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 worker 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 worker as a

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

58 ***Health surveillance.*** It is an employer's responsibility to ensure that a worker's 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. Periodic monitoring of immune status may be required and it may be necessary for workers 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.

59 ***Animal experimentation.*** Workers 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 able to be carried by humans or infect them subclinically. Therefore, workers 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 worker handling an animal could become infected. A 'cool-off' period should be implemented, whereby workers 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.

Class 1 activities are described in the Contained Use Regulations as being of 'no or negligible risk'. It is unlikely that any non-disabled human or animal pathogen could be deemed to be of 'no or negligible risk' (except where the host species is absent from the receiving environment) and such work will always be class 2 or higher. Since work with pathogens will almost invariably require at least some of the measures required at Containment Level 2 (eg an autoclave in the building; restriction of access) it would not normally be possible to assign the activity to class 1. *Even if no Containment Level 2 measures were justified by the risk assessment, assignment of pathogen work to class 1 would be inappropriate and the activity must be notified to HSE as class 2.* If a GMM that is, or could be, pathogenic to humans or animals is assigned as a class 1 activity, then it is probable that the risk assessment is inadequate.

Remember that classification into a GM activity class does not necessarily mean that you will always have to apply all the measures from the associated containment level. If it is adequately justified by the risk assessment derogation may be sought from HSE to exclude unwarranted measures.

Example GM 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 would need to be included to provide a comprehensive document that should enable a reviewer (GMSC or external) to determine whether the risk assessment is suitable and sufficient.

Example GM risk assessment: Construction of an Adenoviral vector with a modified tissue tropism

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.

Nature of the risks

The work involves the genetic modification of a human pathogen. Therefore, Risk Assessment for Human Health will take precedence.

Risk assessment for human health

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 an ACDP hazard group 2 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.

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 acknowledged. 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.

Containment level needed to sufficiently protect human health

The parental virus is ACDP Hazard Group 2. An important additional hazard that will arise as a result of the genetic modification is the possible alteration of tissue tropism. Therefore Containment Level 3 is needed to sufficiently safeguard human health.

This step will often involve considering the containment level 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.

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 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.

Likelihood that the GMM will be a risk to the environment

What is the likelihood that the hazard(s) will be manifested?

The likelihood of the GMM constituting a hazard to the environment is LOW.

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.

Containment level needed to protect the environment

CONTAINMENT LEVEL 2 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 EFFECTIVELY ZERO.

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 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 personnel 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 peroxygen disinfectant according to the manufacturer's instructions.

Activity class

Assign final GM activity class - 1, 2, 3 or 4

The measures required to prevent exposure or release indicate that CONTAINMENT LEVEL 3 is required. There is no case for seeking derogation on any of the measures that are specified.

The activity is therefore assigned to GM ACTIVITY CLASS 3.

Any additional measures or derogations from the standard Containment Level 3 conditions should be outlined here.

Example GM risk assessment: Development of an animal model for *Neisseria meningitidis* disease

Overview

The aim of the project is to develop a new animal model for the study of *N. 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.

Nature of the risks

The work involves the genetic modification of a human pathogen and will require the handling of that 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.

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?

Neisseria meningitidis is ACDP Hazard Group 2 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 CONTAINMENT LEVEL 2 is sufficient.

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.

Including 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 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, their 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 hazard(s) 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 NEGLIGIBLE.

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 level 2. 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 'LOW-MEDIUM'.

What will be the consequences if the hazard is realised?

The consequences of GMM dissemination in the environment could be MODEST-SEVERE.

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

CONTAINMENT LEVEL 2 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 below.

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 procedures used for handling *N. meningitidis* will also be appropriate for handling the GMM. Specific animal handling procedures will minimise the risk to personnel.

Any standard procedures or guidelines should be outlined here. For example, class II microbiological 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 in full 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 EFFECTIVELY ZERO.

Details of the waste disposal procedures and the effectiveness of inactivation methods would enhance this section.

Assign activity class

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 as CLASS 3 and will be handled at CONTAINMENT LEVEL 3.

Any additional measures or derogations from the standard Containment Level 3 conditions should be outlined here.

Example GM risk assessment: Analysis of helminth immune evasion genes by expression in *Leishmania*

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 genetically modified *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 (ie *L. major* is a more tractable system for modification and immune studies than helminths) and the nature of the work (ie laboratory manipulations and small animal experimentation) should also be outlined.

Nature of the risks

The work involves the genetic modification of a human pathogen and will require the handling of that pathogen. Therefore Risk Assessment for Human Health will take precedence.

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?

Leishmania major is ACDP Hazard Group 2 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 (ie phlebotomid sandflies).

What hazards does the inserted genetic material pose?

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 acknowledged here. 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 *Leishmania major.*

How severe might the consequences be?

It is possible that the genetic modification could suppress the normal immune response to *Leishmania* 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 CONTAINMENT LEVEL 2 is sufficient.

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.*

Including 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 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 the UK, 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 hazard(s) will be manifested?

Phlebotomid sandflies, the intermediate vector of *L. major*, is not present in the UK and therefore the likelihood of the hazards being manifested is NEGLIGIBLE.

What will be the consequences if the hazard is realised?

The consequences of environmental release is NEGLIGIBLE.

Containment level needed to protect the environment

The risk to the environment is EFFECTIVELY ZERO. CONTAINMENT LEVEL 1 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 SOPs 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 procedures or guidelines should be outlined here. For example, the use of sharps is minimised for all operations and specific SOPs for the injection of mice with *Leishmania* are in place and will be followed. All staff are trained in the SOPs for animal work with *Leishmania*. Records of training are kept. SOPs stipulate the wearing of double-gloves, laboratory coats and eye protection and that syringes are charged without the needle attached.. Contaminated syringes are disposed of in a sharps bin with the needle unsheathed.

Are the potential routes of environmental release known and managed?

Since the intermediate vector is not present in the UK, no transmission can occur. Thus, with the containment and waste disposal measures provisionally in place, the risk to the environment is EFFECTIVELY ZERO.

Activity class

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 as CLASS 2 and will be handled at CONTAINMENT LEVEL 2 for both laboratory work and animal work.

Any additional measures or derogations from the standard Containment Level 2 conditions should be outlined here.

Further information

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This document contains notes on good practice which are not compulsory but which you may find helpful in considering what you need to do.

This document is available web only at: www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp

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