Containment measures for transgenic animals in accordance with the Cartagena Act

Nobukazu Tanaka (Hiroshima University),
Association for Promotion of Genetic Studies in Japan

1. What are containment measures?

Containment measures in the Cartagena Act* comprise of "Facilities standard (hardware requirements)" and "Procedure standard (software requirements)," which are described in (a) and (b) of each item in the appended Tables 2-5 in "The Ministerial Ordinance on Type 2 Use"**. "Hardware requirements" are the "physical containment" standards described in the preceding "Recombinant DNA Research Guidelines," which specify that facilities are to confine living modified organisms (LMOs) in laboratories/animal breeding rooms and to reduce the potential for exposure of the outer environment to LMOs. "Software requirements" specify the necessary procedures to avoid exposure of the outer environment to LMOs.

The idea of "physical containment" is first described during the Asilomar Conference on Recombinant DNA in 1975, held at Asilomar State Beach, California, USA, and is included in the above "Recombinant DNA Research Guidelines." P1, P2, P3, etc., indicate physical containment levels. The conference also suggested the use of "biological containments." An example of such biological containments is the use of hosts that are unable to survive in environments other than special culture conditions in combination with nontransmissible vectors that can replicate in only the specified hosts. The use of such host-vector systems prevents the spreading of modified nucleic acids even if the LMOs escape from laboratories into the outer environment; since they would soon die in the natural environment, they would not spread and distribute into wider areas. This is how biological containments maintain the biological safety of LMOs. Based on these ideas, appended Table 1 of "The Public Notice on Type 2 Use"*** describes the safe 11 host-vector combinations as authorized "B1" host-vector systems as well as host-vector systems of several *E. coli* strains (EK2), budding yeast (SC2), and *Bacillus subtilis* (BS2), which are also listed as particularly safe, as specified authorized "B2" host-vector systems. The use of authorized or specified authorized host-vector systems for the same donor DNA may reduce containment levels and make application for confirmation by ministers unnecessary.

When growing recombinant animals, said biological safety may sometimes present the wrong perception. For example, for animals that shed eggs and sperms into water, the eggs can be trapped by mesh filters, etc., but the sperms cannot be trapped owing to their small size. On the other hand, sperms lose motility and fertility shortly after spawning. Thus, some people may associate this with a "biological containment" in microorganisms, and it seems that this would hardly affect biodiversity in the natural environment. Consequently, some may consider that the sperms of such recombinant animals can be safely drained along with the used water. However, even if this is biologically safe, the Cartagena Act does not permit environmental release of the sperms of recombinant animals without inactivation. Sperm, a gamete, is subject to regulation similar to *E. coli* and yeast used for cloning genes with authorized host-vector systems, and draining without inactivation violates Article 12 of the Cartagena Act ("Implementation of Containment Measures Stipulated in Ordinance of the Competent Ministries").

2. Containment measures for recombinant animals

Containment measures for LMOs should be carried out to effectively prevent their spreading. Thus, hardware and software requirements for each species will of course be different. First of all, possible risks of release should be considered.

[1] Species: Virus, microorganisms (bacteria, yeast, and mold), fungi, animals, or plants?

[2] Ecology: Saprobic, parasitic (infectious), autonomous, or feeding?

[3] Habitat: terrestrial, aquatic, or soil?

[4] Means of release: Escaping (running, flying, swimming), clinging, or floating?

[5] Means of reproduction and fertilization: In water, on land, anemophilous, or entomophilous?

[6] Gametes and larvae: Sperms, eggs, larvae, spores, pollen, or seeds?

From here on, “animals” are specifically discussed.

Animals differ in size, means of escape, and other characteristics. Thus, it would be nonsensical to apply containment measures for recombinant mice onto recombinant drosophila. Also, one should consider how animal characteristics are changed by inserted genes when designing the structure and equipment of facilities and selecting proper procedures.

As already stated, appropriate containment measures are met only when both the hardware and software requirements are fulfilled. Fulfilling either of the two is not sufficient in and of itself. Thus, one should consider establishing facilities that meet hardware requirements first, and then ascertain implementation of software requirements as well.

[1] Hardware requirements

- Design and equipment of laboratories/animal breeding rooms
- Apparatus to prevent release and escape
- Apparatus for inactivation

[2] Software requirements

- Proper handling of hardware requirements
- Procedure to prevent release and escape
- Means of inactivation

3. Overview of hardware requirements

Hardware requirements assume the establishment of facilities that prevent LMOs from escaping from laboratories. However, excessive equipment is unnecessary; facilities simply need to be suitable for the characteristics of the animals of interest and the function of introduced genes. In short, facilities should be designed as to securely shut off possible routes to environmental release.

[1] Laboratory/animal breeding room

Housing equipment and structure are required to establish an animal breeding room to keep and feed the animals of interest. Twofold or threefold containment measures (e.g., keeping the animals in cages, tanks, or housing containers which may be placed into additional housing containers) are preferred.

[2] Containment measures

In the cases of flying animals, doors, windows, and vent holes of the laboratory/animal breeding room must be made escape-proof by the use of proper equipment. In addition, equipment to prevent entry of insects and rodents must be in place. Of course, as with the common requirement for all species, doors and windows should not be opened during experimentation and when keeping any sort of animals. Appropriate traps (e.g., rodent traps with guards or nets high enough to prevent the animals from escaping) or anterooms should be appropriately placed to prevent any animal from escaping when doors are being opened. Also, doors should not directly lead to outside. For aquatic animals, the risk of draining the animals along with water must be noted; drains should be made escape-proof, by putting lids on drains, placing tanks away from the drains, and placing mesh in sink drains. In addition, gametes are classified under the Cartagena Act as living organisms, and draining fertile sperms and eggs regardless of fertilization status along with water to the outer environment is strictly prohibited. Eggs can be trapped by placing appropriately sized filters over drains, but it is virtually impossible to trap sperms by filters, so proper inactivation procedure should be taken prior to draining.

[3] Biosafety cabinet (BSC)

BSC is required when living modified microorganisms of P2 or above are used; however, its use is limited to procedures that are likely to produce aerosol. However, "The Ministerial Ordinance Providing Containment Measures to Be Taken in Type 2 Use of Living Modified Organisms for Research and Development" does not specify such procedures, making it difficult to determine whether or not to use BSC. Aerosol is generated by centrifugation, grinding, crushing, vigorous shaking/mixing, sonication, and flame sterilization of platinum-inoculating loops as well as by pipetting. Thus, most of the usual experimental procedures do generate aerosol. It may be easier to consider procedures that do NOT generate aerosol. To confirm that aerosol is not produced by a procedure, the procedure should be sufficiently examined with extra caution, and supporting scientific evidence is required. Therefore, in a practical way, it can be concluded that experiments using recombinant viruses, etc., of P2 or above as well as animal experiments inoculating such microorganisms almost always require BSC. On the other hand, for experiments involving recombinant viruses, etc., of P1 level, aerosol production should be kept at a minimum. If the viruses used have the risk of infecting humans, their handling should be done in BSC.

[4] Inactivation equipment

Inactivation equipment include autoclave, incinerator, hot air oven, freezer, and decontamination tank; and appropriate equipment should be selected according to the animal species used. However, for equipment other than autoclave and incinerator, the conditions and duration of the inactivation procedures for secure inactivation should be confirmed beforehand.

Animals inoculated with recombinant viruses, etc., should be autoclaved or incinerated. P2 level requires that autoclaves should be placed within the same buildings where the laboratories/animal breeding rooms are located. The description regarding the placing of autoclaves in "The Ministerial Ordinance Providing Containment Measures to Be Taken in Type 2 Use of Living Modified Organisms for Research and Development" suggests that incinerators should also be placed in the same building, but no explicit statement can be found in the ordinance. In most cases, incinerators are placed in different buildings from the incineration facility, and transporting materials from laboratories to such facilities requires transport taking place outside of authorized areas. To securely inactivate recombinant viruses, etc., by incineration, rules to prevent their release during transport should be established in each institute. This will be discussed in more detail in the section concerning software requirements.

[5] Waste collection/disposal

If recombinant viruses, etc., are excreted, a special facility to collect and dispose the excreta is required. If this is not the case, waste collection/disposal should be carried out in the same manner as non-infected animals of the same species. For aquatic animals, excreta collection/disposal facilities are not expected, since the excreta are released into tank water and cannot be recovered alone. However, if recombinant viruses, etc., are excreted into water along with feces/urine, appropriate inactivation procedure must be carried out. Thus, facilities/equipment to treat water are required.

4. Overview of software requirements

In order to prevent release of recombinant animals, software requirements specified in (b) of each appended Tables 1-4 in "The Ministerial Ordinance Providing Containment Measures to Be Taken in Type 2 Use of Living Modified Organisms for Research and Development" must be followed. Characteristics of the species of interest should be fully understood. Measures should then be taken to prevent recombinant animals from escaping during experimentation/housing, and disposal should always follow secure inactivation procedure. There are additional requirements that should be closely adhered to as well.

[1] Means of inactivation

Inactivation of recombinant animals should be achieved according to the characteristics of each animal. It is generally believed that inactivation of LMO means complete destruction, but it is not explicitly stated in "the Ministerial Ordinance Providing Containment Measures to Be Taken in Type 2 Use of Living Modified Organisms for Research and Development" so that "inactivation" is not clearly defined. On the other hand, the Cartagena Act defines "living organism" as "any biological entity capable of transferring or replicating genetic material, including sterile organisms, viruses and viroids," meaning that cells which do not have the ability to transfer or replicate genetic material are not considered living organisms. If a living organism loses the ability to transfer or replicate genetic material, it is unlikely that such organism would affect biodiversity in the outer environment; establishment of inactivation procedure may be based upon this for the time being. Of course, recent development of new molecular biological methods indicates that interpretation of inactivation should be reviewed regularly.

• Animals without recombinant viruses, etc.

The inactivation procedure according to each recombinant animal should be undertaken. For example, mammals, birds, and reptiles specified as experimental animals in the "Act on Welfare and Management of Animals" and "Standards Relating to the Care and Management, etc., of Experimental Animals" should be euthanized based on the act and the standards by anesthetics, etc. Amphibians, fish, and certain invertebrates (with sophisticated nervous systems such as cephalopods that allow the animals to recognize pain) should also be euthanized by anesthetics. Insects and aquatic animals should be destroyed using appropriate measures such as with autoclaves, hot air ovens, freezing, and chemical treatments. For procedures other than with autoclaves and hot air ovens, conditions for complete destruction of the animals of interest, for example, temperature and time for freezing as well as concentration and time for chemical treatments, should be first assessed to provide scientific basis, and the actual procedures should last longer to secure sufficient inactivation.

- Animals inoculated with recombinant viruses, etc.

Generally, autoclaves are used to inactivate recombinant viruses, etc. However, autoclaving is virtually impossible for large animals, so incineration is required. Most of the time, the incinerator is off-site, and transfer of the animals to the incineration site requires measures to prevent the release of recombinant viruses, etc. Thus, it is recommended that each institute establishes a rule for incineration based on the Cartagena Act. For example, the incineration facility may be included as part of the laboratory, and additional personnel responsible for incineration may be assigned as laboratory workers to make incineration a method of inactivation as part of experimentation. In this case, animals inoculated with recombinant viruses, etc. are euthanized, put into a double-bag, tightly sealed, and then removed from the laboratory. Needless to say, the incineration facility should pursue proper containment measures, and if possible, incineration records should be kept.

- Water

The most difficult issue in recombinant aquatic animals is the decontamination of gametes, particularly sperms. Owing to its size, sperm cannot be trapped by filters placed over drain. Draining water without treatment results in the release of sperms derived from recombinant animals into the natural environment, which violates the Cartagena Act. Thus, sperm should be inactivated before water drainage. Inactivation of water may be carried out by autoclaving, but a large amount of water would complicate this method. Chemical treatment such as the addition of sodium hypochlorite may be possible, but its impact on the natural environment after water disposal should be considered. Usually, a sperm loses motility and fertility shortly after spawning. If a sperm completely loses motility and fertility, it will no longer be regarded as "a biological entity capable of transferring or replicating genetic material" according to the Cartagena Act and thus be excluded from legislation. By measuring the time for a sperm to completely lose motility and fertility and providing this data as a scientific basis, water can be drained without special treatment after the stipulated time has passed. In practice, water should be drained after substantial time (e.g., several fold time) has passed from the required time for complete loss of motility and fertility.

[2] Opening/closing doors

Animal escapes are most likely to occur when the doors of animal breeding rooms are open. Doors should be opened and closed as promptly as possible and never be left open.

[3] Identifying and counting recombinant animals

In mice and rats, recombinant animals are usually identified and counted: the same procedure should apply to birds and other animals. For frogs, counting tadpoles may be difficult, but once they turn to frogs, identifying and counting them are necessary. For small fish and insects, if their size does not permit individual marking and counting, their containers should be marked and counted. In either case, all tanks/housing containers must be appropriately labeled to identify the nature of the recombinant animals contained therein.

[4] Warning sign

Signs stating “Modified animal being bred” or “Modified animal being bred (P2)” must be posted on the doors of the laboratories/animal breeding rooms for P1A or P2A experiments, respectively.

5. Overview of storage

If recombinant animals can be stored in the form of gametes, put them in appropriate leakproof containers and store them in appropriate locations with labels indicating that LMOs are stored inside. If recombinant animals cannot be stored in gametes, the animals should be kept and maintained. In such cases, maintaining strains are in themselves regarded as animal development experiments and protocols should be submitted to obtain institutional approval.

6. Overview of transport

When transporting recombinant animals, they should be contained in escape-free containers, which can be placed within other containers to employ double or triple containment measures. The outermost container should be labeled for careful handling. If recombinant animals are transported to different institutes, supplementary information must be included.

7. Experiments requiring confirmation by ministers

The most common application requiring confirmation by ministers regarding experiments with recombinant animals is “inoculation of recombinant viruses, etc. which replicate throughout experiment.” Characteristics of recombinant viruses being used and the animals being inoculated should be considered to establish appropriate containment measures before submitting the application. One should be aware that inoculation of donor DNAs to animal hosts, resulting in expression of the receptors used by pathogens to infect mammals, etc., (e.g., expressing receptors, which are used by pathogens to infect humans, insects, or birds) requires confirmation by ministers via an application.

[*] Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms

[**] “The Ministerial Ordinance Providing Containment Measures to Be Taken in Type 2 Use of Living Modified Organisms for Research and Development”

[***]The Public Notice to Specify Certificated Host-Vector Systems in accordance with the Provisions of the Ministerial Ordinance stipulating Containment Measures to be taken in Type2 Use of Living Modified Organisms for Research and Development