Chapter 7

Safety of Biological Material

Section 1 Introduction

The progress of biotechnology, specifically genetic manipulation, has opened wide research possibilities to investigate and manipulate biological materials from an engineering perspective. However, handling biological materials involves the potential for severe health hazards due to pathogenic microorganisms, toxins, and other hazardous substances, in addition to risks caused by genetically modified organisms and related products. Therefore, it is essential to follow the guidelines stipulated by law and ensure individual and collective safety while researching on biotechnology.

Section 2 Microbial Experiments

1 Laws and regulations related to pathogenic microorganisms

The possession and utilization of pathogenic microorganisms, including genetically modified microorganisms, accompanies risks of infection. Therefore, spread prevention and infection control measures are necessary. In particular, as a measure to counter biological terrorism, the law on infectious disease necessitates the proper management of specified pathogens that belong to Class I-IV, as listed in Figure 7-2-1. Every year, Nagaoka University of Technology (NUT) also reports annually to the government whether or not it owns specific pathogens and what they are.

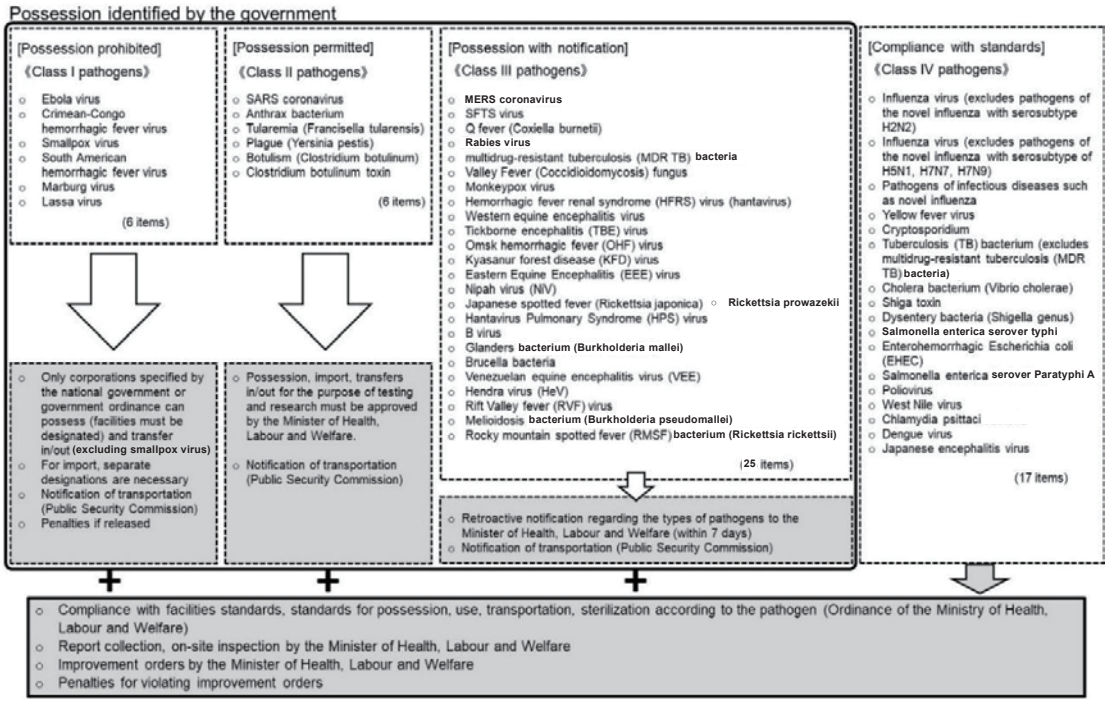

Figure 7-2-1. Diagram for the proper management of pathogens (cited from Japan Ministry of Health, Labour and Welfare website, www.mhlw.go.jp)

Class I specified pathogens cannot be possessed by the University. The possession and use of Class II specified pathogens require advance application and approval. Notification is required for the possession of Class III specified pathogens. The usage of specified pathogens requires specific infection control measures corresponding to each biosafety level (BSL) (See http://www.nite.go.jp/nbrc/list/risk/description.html#niid). Furthermore, it is necessary to ensure that the implemented measures are in accordance with the associated risk level, given in Table 7-2-1. Figure 7-2-2 shows an example of facility standards for laboratories that handle Class IV pathogens classified as BSL2 equivalent.

◎: Legal obligation/penalty ○: Improvement order

	Class I	Class II	Class III	Class IV
Minister designation required for possession/import	◎			
Permit required for possession/import		◎		
Notification required for possession/import			◎	
Creation of regulations to prevent infectious disease	◎	◎		
Appointment of pathogen biosafety officer	◎	◎		
Education and training	◎	◎		
Sterilization (if designation or permit is revoked)	◎	◎		
Bookkeeping obligation	◎	◎	◎	
Facility standards	◎/○	◎/○	○	○
Storage standards	○	○	○	○
Notification of transportation (to the Prefectural Public Safety Commission)	◎	◎	◎	
Notification of accidents	◎	◎	◎	◎
First aid in the event of disaster	◎	◎	◎	◎

Figure 7-2-1. Legal obligations and penalties for parties in possession of specified pathogens (cited from www.mhlw.go.jp).

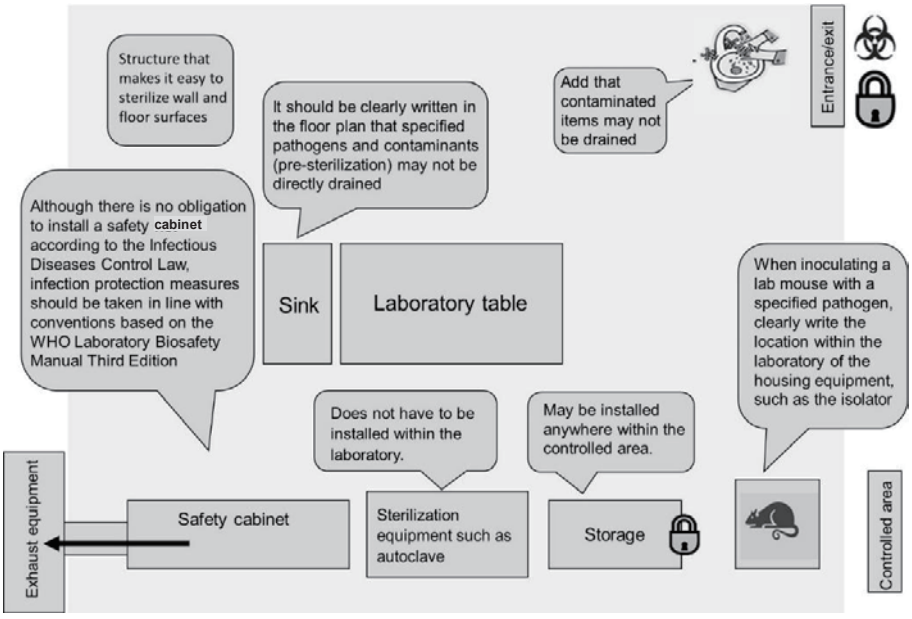

Figure 7-2-2. Facility standards for a laboratory that handles Class IV pathogens classified as BSL2 equivalent (cited from www.mhlw.go.jp)

In addition, the microorganisms isolated from the natural world must be treated appropriately if they are recognized as pathogenic microorganisms in their classification. If they are classified as a specified pathogen, a permit or notification must be obtained from the competent authority. If they correspond to a Class I specified pathogen, they must be discarded.

2 Handling of Microorganisms

Even non-pathogenic microorganisms may contaminate the research environment and hinder experiments by inviting the inclusion of other microorganisms. If one's personnel immune system is vulnerable due to a disease or medical treatment, an infection may occur from an opportunistic unlisted or unrated pathogenic microorganism. An unnecessary pathogenic microorganism may be contained within a group of microorganisms isolated from nature. Therefore, researchers must pay particular attention to the following points when conducting experiments using microorganisms:

(1) Wear work clothes, such as lab coats, during experiments to prevent the adhering of microorganisms.

(2) To avoid contamination from non-target microorganisms, strictly enforce the sterilization of laboratory equipment and disinfection of laboratory tables and hands before starting an experiment.

(3) Avoid sucking the pipette using your mouth. Eating and drinking must be prohibited in the laboratory.

(4) Cultivated microbial samples must be sterilized or disinfected prior to disposal.

(5) Instruments used for cultivation must be sterilized or disinfected after each use.

(6) When using pathogenic microorganisms, strictly enforce:
the use of lab coats and safety pipette fillers,
the sterilization or disinfection of samples and instruments after use,
the sterilization or disinfection of laboratory tables,
and the disinfection of hands and fingers after conducting experiments to prevent infection and contamination.

If there are concerns of potential infection due to accidental ingestion, follow the necessary measures, such as administering antibiotics or neutralizing antibodies/serum.

Section 3 Animal Experiments

Animal experiments are necessary for the development in the fields of life sciences and medicine. However, it remains essential to give sufficient and ethical considerations for the animal welfare.

1 Rules and Regulations Regarding Animal Experiments

The Act on Welfare and Management of Animals was revised in 2005 due to the growing awareness of animal welfare, and the international principles of the Three Rs were enforced for experimental animal:

(1) Replacement
Avoid or replace the use of animals as much as possible.

(2) Reduction
Minimize the number of animals used for scientific experimentation purposes.

(3) Refinement

Minimize the pain, suffering, distress, or lasting harm that laboratory animals might experience.

The following regulations are established pertaining to the proper care and use of experimental animal, “Standards relating to the Care and Keeping and Reducing Pain of Experimental Animal” (Ministry of the Environment) and “Basic Guidelines to Conduct Animal Experiments at Research Institutes” (Ministry of Education, Culture, Sports, Science and Technology, hereafter referred “MEXT”). Based on these regulations, NUT has organized an Animal Care and Use Committee and established its own “Animal Care and Use Regulations".

Animal experiments must be only conducted at the laboratory that be applied for in advance. In addition, experiment researchers must participate in the animal experiment workshops held every year and acquire sufficient knowledge about experimental animal and experiments. Furthermore, they must consult the animal experiment manager or the NUT Animal Care and Use Committee regarding the experiment plan to ensure that safety regulations are met and to validate their experimental plan. Upon completion of the aforementioned steps, the plan of the animal experiment could be transferred to the President office for approval. They must perform periodic self-check of the animal experiments and report the implementation status to the President.

2 Handling of Experimental Animal

Researchers must strive for the appropriate use of experimental animal. They are required to ensure that they minimize pain and suffering of the experimental animal and use measures such as heat insulation. To minimize the distress and suffering of the experimental animal, it is necessary to refine and improve research techniques and set humanitarian endpoints (timing for euthanasia). If anesthesia is involved in the study, an appropriate anesthetic must be selected considering various viewpoints such as the type and age of the animal, type of pain, and stability associated with the surgical procedure.

Pathogens of major zoonotic diseases

Pathogen (disease that transmits to humans)	Infectable species
Hantavirus (hemorrhagic fever with renal syndrome, HFRS)	Rats
Lymphocytic Choriomeningitis Virus (LCMV)	Mice, hamsters
Salmonella (food poisoning)	Many animal species such as mice and rats
Dermatophyte (tinea capitis, tinea pedis/athlete’s foot)	Many animal species such as mice and rats

Experimental animal may carry zoonotic pathogens. Therefore, researchers are required to consider animal allergies and anaphylactic shocks caused by allergens such as the feces, urine, saliva, and blood of experimental animal. To ensure safety of researchers, the following precautions must be taken:

(1) Animals used in research experiments must be purposefully produced. Test results of genetic and

microbial monitoring must be always attached to experimental animal upon their reception.

(2) Strive to prevent stabs and wounds caused by laboratory equipment, and bites and scratches caused by animals. Wear thick gloves when handling animals larger than rats.

(3) Avoid eating, drinking, smoking, or applying makeup in animal laboratories to prevent oral infection.

(4) Wash and disinfect hands, and wear gloves and masks before and after animal experiments.

(5) Keep the animal laboratory clean and organized.

(6) Injection needles, scalpels, contaminated equipment, and other contaminants used in the experiments should be sterilized and then disposed in appropriate containers. These are to be considered as essential measures to prevent infections or incidents with the cleaning agents and personnel.

(7) In the event of an accident, take measures to minimize the hazards from contamination and infection. Notify the experiment manager immediately.

Section 4 Recombinant DNA Experiments

1 Regulation on Recombinant DNA Experiments

Genetic recombination allows researchers to utilize the structure and function of organism's genes. Nonetheless, it has become an indispensable, important technology applied in a wide range of studies and industries in the field of life sciences. Its application includes the elucidation of cancer and other intractable diseases, mass production of rare drugs such as insulin and interferon, and breeding of useful microorganisms and crops.

As this new technology adds properties that did not originally exist in living organisms, genetic recombination can create unpredictable effects. Thus, researchers are urged to take careful measures while handling the recombinant DNA technology. It is worth mentioning that many arguments existed to question the safety of genetic recombination and oppose the use of genetically modified foods (GMOs) and recombinant DNA technology. In 1979, the Prime Minister passed the Guidelines for Recombinant DNA Experiments for researchers to ensure the safety of recombinant DNA technology and its appropriate use. Since then, studies on the recombinant DNA have elucidated that an individual organism consists of an exquisite and harmonious combination of many genes, and that it is impossible to create a new organism by merely inserting some genes. Thus, the fear of creating organisms that have an unexpected and significant impact on human society and the global environment that arose in the early stage is nothing more than a fantasy. It also became clear that genetic recombination occurs frequently in nature due to genetic exchange between microorganisms. Such knowledge led to the establishment of a method to evaluate the harmfulness (pathogenicity, toxicity) in relation to the recipient organism (host) and the inserted recombinant gene (foreign DNA), and thus evaluate the safety of the resulting living modified organism (LMO). Moreover, if the DNA of living organisms that frequently exchange genes in the natural world is inserted into a host of the same species, it would not be employed in recombinant DNA experiments.

Meanwhile, LMOs, developed via recombinant DNA technology, have been used in many fields such as

the cultivation of genetically modified plants and breeding of genetically modified animals. The cultivation and import/export of genetically modified crops raise concerns over the impairment of wildlife's diversity in the natural environment. Consequently, the Cartagena Protocol on Biosafety has been adopted as an international measure. Domestically, the aforementioned guidelines were removed and replaced with the Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Act) passed in 2004, and it is used until the present day.

2 Mechanism of the Regulation (Cartagena Act)

The Cartagena Act aims to ensure biodiversity through containment measures by regulating the use of LMOs and fused-cell organisms. The regulation applies to organisms that are obtained through genetic recombination between the organisms of different species or cell fusion between the organisms of different families. Humans are not regulated by the Cartagena Act, but by another law. When researchers plan to create or use LMOs, an application form that contains LMO information and creation/usage plan (a "protocol" in research and development) must be submitted in advance to the safety committee of the institution. The application could either receive a positive decision (Institutional Approval) on the verified safety and containment measures if they comply with the regulations set by the competent authority (MEXT for universities). If the application deviates from the provisions stipulated above, it will be transferred to the competent authority, and the genetic recombination committee will decide on the verified safety and containment measures (Minister Approval). Although the regulation scope does not include animal and plant cultured cells that do not grow outside the laboratory, the genetically modified organisms of animal and plant cultured cells must be handled in accordance with the conventional guidelines. (NUT considers such experiments subject to notification requirements.)

In the Cartagena Act, the creation and use of LMOs is classified into two types. (1) Type 1—LMO is used in environments without containment measures, such as cultivation in outdoor fields and utilizing for feeding. (2) Type 2—LMO is used in the environment with containment measures, such as laboratory, fermentation device, and breeding area. Type 1 use is rarely implemented at NUT; hence, we focus only on Type 2 use hereafter.

In accordance with the regulations, NUT has established the Nagaoka University of Technology Regulations for the Safe Management of Recombinant DNA Experiments and the Recombinant DNA Safety Committee to oversee the institutional safety. At the start of an experiment, the experiment manager (faculty) is required to specifically follow procedure (1) or (2), which are discussed below. While conducting the experiment, the experiment manager and all experiment personnel must comply with procedures (3)–(7).

(1) The experiment manager must contact the recombinant DNA safety officer before starting the experiment (during the planning process), and create a protocol in the prescribed format. The protocol must receive written feedback on containment measures from the safety officer and signature stamps from both the safety officer and department head, after which it can be submitted to the Section of Research Support of Division of Industry-Academia Cooperation and

Research Promotion (hereafter referred “Section of RS”) (Figure 1). The Section of RS compiles the application forms submitted by the faculty member, and submits them to the Recombinant DNA Safety Committee. After the Section of RS receives safety confirmation from the committee, they notify each experiment manager of the institutional approval.

(2) A safety confirmation from the Minister of Education is required for experimental approach that deviate from the scope of containment measures stipulated by the MEXT Ordinance. If the experimental procedures do not comply with the containment measures, the protocol must be rewritten at the direction of the safety officer to be applied for ministerial confirmation and then submitted to the Section of RS. The Section of RS submits the protocol to the MEXT following confirmation by the safety committee. MEXT's Expert Committee on Recombinant DNA Technology verifies the effective containment measures and notifies the university of the decision on the Minister’s confirmation. The Section of RS notifies the results to the experiment managers.

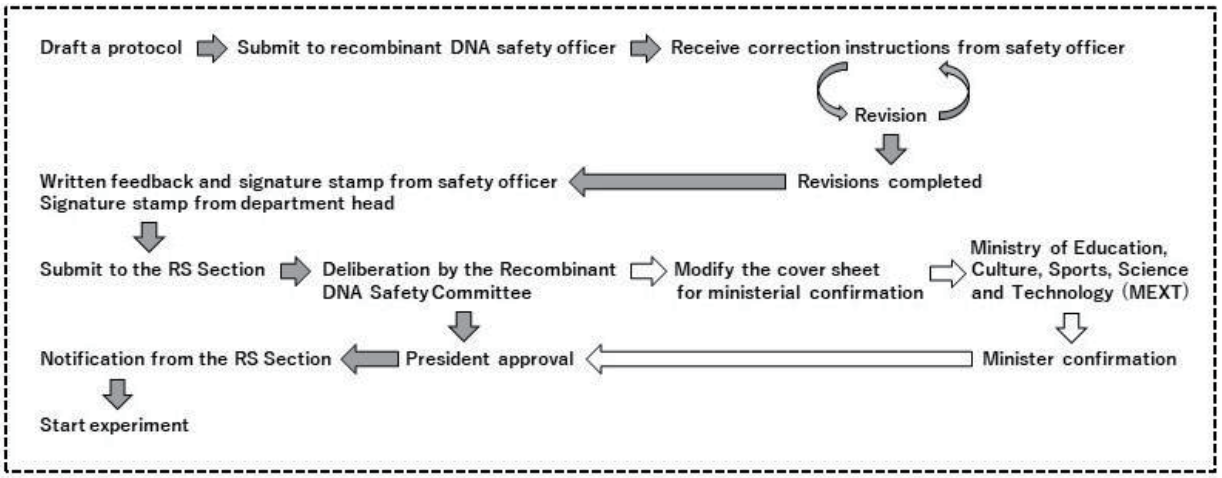

Figure 1 Application Procedure for genetic engineering (recombinant DNA) experiments

(3) When performing the experiment, the experiment’s manager must take the necessary containment measures including appropriate equipment, methods, disposal and treatment processes, storage, management, and labeling. In addition, the experiment’s manager must inform all who work on the experiment of the protocol details and necessary containment measures.

(4) The experimentalists must understand and follow the containment measures necessary to conduct the experiment.

(5) Experimentalists must undergo training on the recombinant DNA experiment procedures and health examinations (may be substituted with general health examination).

(6) The experiment manager must submit a list of the experimentalists for health examination at the beginning of the year and a progress report of the experiment at the end of the year to the Section of RS. As specified in the protocol, the experiment manager must submit a final report of the experiment when the experimental period expires and a cancellation report if the experiment had to be stopped. In addition, the experiment manager must implement measures for the storage and disposal of

genetically modified organisms and manage records.

(7) Researchers must provide necessary containment measures, display, and information provision during the storage, transportation, and transfer in/out of LMOs.

3 Determination of Containment Measures

Containment measures for Type 2 LMO are implemented by combining physical and biological containment measures for the safety of the experiment. Physical containment is prioritized, but employing biological containment may mitigate the required extend of physical containment use.

Physical containment aims to prevent the spread of LMO into the environment by confining them to the facility or equipment. Physical containment consists of two factors—requirements of the containment facilities and compliance items for conducting the experiments. Three levels—P1, P2, and P3—are established for experiments using microorganisms based on the level of containment. "P" denotes the first letter of the word "physical," and higher numbers correspond to higher degrees of containment. Similar to the P1/P2/P3 scale used in the experiments with microorganisms, large-scale culturing experiments employ an LSC/LS1/LS2 scale, animal experiments use a P1A/P2A/P3A scale, and experiments on plants use a P1P/P2P/P3P scale.

Biological containment uses a safe host that cannot survive in the environment (certified host) or a "host vector system" that combines a safe host that cannot survive in the environment with a vector (specified certified host). The aim of biological containment is to prevent the spread of LMOs to the environment. The level of containment is divided into the following two sub-levels based on the safety level of the host vector system—certified (B1) and specified certified host vector systems (B2). "B" denotes the first letter of the word "biological," and higher numbers correspond to higher degrees of containment and safety.

The level of physical containment is determined based on the safety of the gene to be inserted (supplied nucleic acid), classification of the organism from which it is derived (nucleic acid donor), and classification of the organism receiving the gene (host) (Table 1). The list of organisms contains viruses; when a foreign gene is introduced into the virus, the virus itself becomes the host, rather than the cell that receives the virus. The specific experimental classification is based on the MEXT public notice, "Establishment of certified host vector systems based on the provisions of the Ministerial Ordinance for containment measures in the use of Type 2 Living Modified Organisms related to research and development." Organisms of Class 2 or higher are listed in Appendix Table 2; unlisted organisms fall under Class 1. Animals and plants fall under Class 1. The certified and specified certified host vector systems are listed in Appendix Table 1 of the MEXT notice. Because MEXT revises the public notice according to new scientific discoveries, it is desirable to download it from the website (listed in the reference at the end) at the time of use.

Table 1. Basis for experimental classification

Experimental category	Class 1	Class 2	Class 3	Class 4
Pathogenicity (1)	None	Low	High	High
Propagability (2)	—	—	Low	High

(1) Nature and extent to which it causes diseases to animals that belong to class Mammalia and Aves.
(2) Nature and degree at which nucleic acid transfers from one organism to another.

Table 2 shows the mechanism of determination considering the classification and safety of the supplied nucleic acid. For example, a P1 level is assigned when a gene derived from a Class 1 nucleic acid donor is introduced into a Class 1 host vector system. A P2 level is assigned when various genes derived from a Class 2 nucleic acid donor are introduced into a Class 1 host vector system without identification. However, a P1 level is assigned if the insertion is of genes derived from a Class 2 nucleic acid donor who is verified to be safe (identified/ safe nucleic acid). Further, a P2 level is assigned regardless of the safety of the gene, when a gene derived from a nucleic acid donor of Class 2 or lower is introduced into a Class 2 host vector system. When introducing various nucleic acids extracted from the environment into a Class 1 host vector system without identification, the highest experimental classification of the pathogenic microorganisms assumed in that environment is applied. However, a P1 level is assigned when the nucleic acid insertion is specifically amplified using the PCR (Polymerase Chain Reaction) method and identified as safe. The safety of the gene is determined through its lack of involvement in pathogenicity or toxin production.

Table 2. Determine of Containment Measures

Host	nucleic acid donor	supplied nucleic acid	Containment level	Determination mechanism
Certified host	1	Identified/safe	P1	Identified -> match host level
1	1	Identified/safe	P1	
1	2	Identified/safe	P1	
2	1	Identified/safe	P2	
3	1	Identified/safe	P3	Minister confirmation
1	1	Unidentified/unknown	P1	Unidentified -> match higher level
1	2	Unidentified/unknown	P2	
2	1	Unidentified/unknown	P2	
Specified certified host	2	Unidentified/unknown	P1	Eased containment level based on host
3	1	Unidentified/unknown	P3	Minister confirmation
1	1	Identified/pathogenic etc.	P2	Pathogenicity-> increase by one level
1	2	Identified/pathogenic etc.	P3	
	2	Identified/pathogenic	P1	Eased containment level based on host

4 Realities of Physical Containment

To specifically explain physical containment, the details of physical containment at P1 and P2 levels are shown below:

(1) Physical containment at the P1 level necessitates the following requirements and compliance items:

Requirements for P1 level facilities

Details of containment measures		✓
1	The laboratory possesses a structure and equipment of a normal biological laboratory	

Compliance items for conducting recombinant DNA experiments

Details of containment measures		✓
1	For LMO-containing waste (including liquid waste), take measures to inactivate the LMO prior to disposal	
2	If LMOs are attached to equipment, devices, and tools, take appropriate measures to inactivate the LMO before disposal or reuse (clean equipment first if necessary)	
3	For the laboratory table, take measures to inactivate the LMO after completion of the experiment on the same day, and also immediately after LMOs attach	
4	Keep the laboratory door closed (except when entering and exiting the laboratory)	
5	Take important measures such as closing the laboratory windows to prevent entrance of insects.	
6	Minimize the generation of aerosols during all operations	
7	When taking LMOs out of the laboratory in the course of an experiment, ex. to inactivate LMOs outside the laboratory, the LMOs must be placed into a container with a leak-proof structure for physical containment	
8	Take necessary measures to prevent LMO attachment or infection, such as hand washing after handling	
9	Take measures to prevent the unnecessary entrance of visitors into the laboratory who are not familiar with the experiment details	

(2) Physical containment at the P2 level necessitates the following requirements and compliance items, in addition to those required in the P1 level:

Requirements for P2 level facilities

Details of containment measures		✓
2	ThThe laboratory is equipped with a biological safety cabinet for research (only when performing operations prone to release aerosols)	
3	A high-pressure sterilizer must be installed in the building where the laboratory is located, if it will be used to inactivate LMOs	

DCompliance items for conducting recombinant DNA experiments

Details of containment measures		✓
10	A biological safety cabinet should be used for operations that are prone to releasing aerosol, and measures must be taken to inactivate LMOs attached to the safety cabinet following the end of the experiment on the same day and immediately after adherence of LMOs	
11	Indicate "P2 level experiment in progress" on the entrance of the laboratory and on equipment used to store LMOs while the experiment is in progress	
12	When experiments with containment levels of P1, P1A, or P1P are performed simultaneously in the same laboratory, the experiment areas should be clearly set. Otherwise, containment measures should be taken corresponding to containment levels P2, P2A, and P2P, respectively	

Reference

- Life Sciences Square of MEXT; Bioethics and Safety Initiatives: Genetic Modification Experiments http://www.lifescience.mext.go.jp/bioethics/anzen.html#kumikae