

## **Annex I**

# **Standards for Safety Assessment of Foods and Food Additives Produced by Recombinant DNA Techniques**

## **Chapter 1: General Provisions**

### **Section 1: Objective**

The standards stated herein are requirements based on the announcement for “Procedure of Application for Safety Assessment of foods and food additives produced by recombinant DNA techniques”, with the objective of describing the assessment standards to confirm safety of such products.

### **Section 2: Scope of the Standard**

Safety assessment will be performed for foods or food additives produced by recombinant DNA techniques, aside from the case when host, vector and organisms are of the same variety (in other words, self-cloning) and when organisms possessing gene structure that is the same as that of natural organism (in other words, natural occurrence). For the mean while the target of the products can be defined as being equivalent to existing products and must be:

1. A seed plant produced by recombinant DNA techniques used as a food or;
2. Foods or food additives obtained through the use of non-pathogenic microorganisms (excluding when such microorganisms themselves are consumed) produced by recombinant DNA techniques.

### **Section 3: Observation Regarding Safety Assessment of Seed Plants Produced by Recombinant DNA Techniques**

- 1 The safety assessment of a seed plant produced by recombinant DNA techniques will be made in terms of all factors added to the seed plant that result in alteration. Namely, not only the added properties anticipated as a result of using recombinant DNA techniques, but any factors added by use of such techniques, or the possibility thereof will be evaluated for safety. Moreover, the method of use and processing of such seed plants will also be considered during the evaluation.
- 2 The scope of the safety assessment for such seed plants is to focus on assessment of new properties added to a seed plant that is considered as being substantially equivalent to existing foods. This is based on grounds that safety of existing

properties, aside from those that have been added by genetic engineering, have been widely accepted thus there is no need for further consideration; sufficient knowledge is readily available in the evaluation of such. Conversely, having substantially equivalence to existing products must not be translated as indicative of the inherent safety of the recombinant. A recombinant's safety must be compared with existing seed plants as well as through the necessary evaluation of each issue in the subject recombinant's safety assessment requirements.

- 3 Furthermore, in products such as rapeseed oil where only extracts from the recombinant are intended for consumption, measures of the safety assessment will be performed on recombinant itself as well as on products other than extracts from the recombinant.

#### **Section 4: Observation Regarding Safety in Manufacturing of Foods or Food Additives Utilizing Non-pathogenic Microorganisms Produced by Recombinant DNA Techniques**

- 1 The safety of foods or food additives manufactured through the use of microorganisms produced by recombinant DNA techniques will be evaluated through the assessment of all factors added to such a food product or food additive. Namely, it is necessary to evaluate concomitant issues introduced by use of recombinant DNA techniques. For instance, the recombinant itself, physiologically active substances produced by the recombinant, product contamination by cultured components and changes of ordinary constituents in a product must be evaluated. Furthermore, it is necessary to evaluate the manufacturing and purification process from a safety standpoint.
- 2 The scope of the safety assessment for foods or food additives manufactured through the use of microorganisms produced by recombinant DNA techniques is to focus on assessment of new properties added to the aforementioned food or food additive that are substantially equivalent to existing products. This is based on the grounds that the safety of existing foods or food additives, aside from those that have been added by recombinant DNA techniques, have been widely accepted and thus there is no need for further consideration; sufficient knowledge is readily available for the safety evaluation. However, being substantially equivalent to an existing product must not be directly translated as assuring the inherent safety of such foods or food additives. The safety of such foods and additives must be compared with existing foods or food additives as well as through the necessary evaluation of each issue in the subject's safety assessment requirements stated in Chapter 3.

## **Chapter 2: Standards for Safety Assessment of Seed Plants (Whole) Produced by Recombinant DNA Techniques or Food Products Utilizing a Portion of Such**

### **Section 1: Similarities between Produced Food and Existing Food**

An objective and comprehensive judgment of whether the food (seed plant produced by recombinant DNA techniques) can be considered as being substantially equivalent to the existing food must be made based on each of the four criteria listed hereafter.

Being considered as substantially equivalent to existing foods indicates the applicability of the concept that such seed plants can be compared with existing foods (seed plants) in the safety assessment process. By determining whether the food maintains the status of substantial equivalence upon comprehensive review of items 1 – 4 listed herein, the examination of the subject food through each criteria listed in Section 2 must be undertaken when comparing to existing foods.

#### (1) Information on the genetic materials

1. Classification and origin of the host plant in which the gene is to be introduced
2. Classification and origin of the donor
3. Properties of the inserted gene

#### (2) Information on broad human consumption history

Availability of history related to broad human consumption of the host plant that was used in the development of the product under petition.

#### (3) Information on components of foods

1. Outline of substance/ingredients (protein, fat *etc.*) and their amounts in the host plant and recombinant.
2. Outline of types of toxic constituent(s) or anti-nutrient(s) such as trypsin inhibitor, phytic acid and their amounts in the host plant and recombinant.

#### (4) Information on differences in usage between the conventional variety and the new variety

1. Harvest schedule (level of maturity) and storage method
2. Consumption (edible) part
3. Consumption volume
4. Cooking and processing methods

## **Section 2: Purposes and usage of recombinants**

Purposes and usage of recombinants must be clarified.

## **Section 3: Host**

### 1. Taxonomy (scientific name, species and strain)

Scientific name, species and strain of the host must be clarified. Its history of safe consumption and wide acceptance among humans must be documented.

### 2. Genetic origin

It must be determined whether the genetic ancestry of the host is capable of generating harmful, physiologically active substances such as toxins and anti-nutrients.

### 3. Production of harmful physiologically active substances

The types, amount and effect of any harmful, physiologically active substances produced by the host organism must be determined.

### 4. Allergenicity

Any knowledge regarding the allergenicity of the host must be reported.

### 5. Capability of becoming parasitic and striking root

A host's capability of becoming parasitic and striking root in humans and other living organs must be clarified and if such capability exists, any adverse effect must be clarified.

(Note: In the case of seed plants, it might be sufficient to clarify item 1 of this section because they are generally non-parasitic and not striking root in humans and other living organisms (to be consumed as foods))

### 6. Foreign pathogenic factors (e.g. virus)

The presence of any known pathogens that are capable of infecting the host organism used in the development of the recombinant under review must be identified.

If such pathogens are known, the pathogens must not affect humans, or of pathogenic gene(s) must be absent.

### 7. Survival and propagation under experimental conditions simulating ordinary or natural environments

Reproductive and survival abilities (including the possibility of weeding) of the host organism utilized to produce a recombinant, in its place of origin and in Japan, require clarification. The host must lack strong weeding capability.

### 8. Sexual reproduction cycle and out crossing

The host's sexual reproductive cycle (lifecycle in the country of origin and in Japan)

and the possibility of cross breeding with other crops must be considered from a genetic dispersion perspective.

9. History of utilization as food

The host plant's history (dietary culture) of use as a food must be described.

10. Safe consumption

Any techniques utilized to ensure safe consumption of the host plant (e.g. In case of consuming beans that contains cyanogens, they have been treated to eliminate the cyanogens existing in them.) used in development of the recombinant must be identified.

11. Restrictive conditions on survival and proliferation abilities

The conditions for controlling survival and proliferation of the host plant as well as countermeasures in cases of weeding must be identified.

12. Pathogenicity and production of harmful physiologically active substances in ancestral or related species to the host.

If a host that has been utilized in producing the recombinant is closely related to a plant that is known to generate harmful substances, it must be determined whether physiologically active substances are reproduced in the recombinant as well. If harmful substances are reproduced in the recombinant, safety of such production must be proven based on the amounts of the recombinant product consumed.

**Section 4: Vector**

1. Name and Origin

- The name(s) and origin(s) of the vector(s), such as plasmid, used in gene transfer must be described.
- The vector(s) must lack adverse effects on humans.

2. Properties

(1) DNA molecular weight

The Molecular weight or the number of DNA base pairs must be indicated.

(2) Cleavage map using restriction enzymes

A cleavage map of the vector to be used to insert the gene into the plant cell must be prepared and the name of restriction enzymes used, the number of fragments, their size and electrophoretic mobility must be clarified.

(3) The presence of any potentially harmful nucleic acid sequence

Any DNA sequence that produces a currently known harmful protein must be absent.

3. Drug resistance

If a plasmid includes a drug-resistant gene, the properties of that drug-resistant gene must be described.

4. Transmission

The ability for a vector to move from the host to another independent organism (*i.e.* transmission) must be absent. If this ability exists, the types of organisms likely to be vulnerable to such transmission must be indicated.

5. Host dependency

The vector utilized in the recombinant must not multiply in other plants or humans. If such ability exists, the types of organisms that may be affected must be stated.

6. Expression vector preparation method

The expression vector preparation method must be identified.

7. Insertion method and site of the expression vector insertion.

The insertion method and the site of expression vector insertion into the host genome must be stated.

## **Section 5: Inserted Gene and Its Gene product**

1. Donor

(1) Name, origin and taxonomy

Name, origin and taxonomy must be provided.

(2) Safety

- The donor of the inserted gene must not be pathogenic nor have the ability to produce toxins. Moreover, if there is a strain that is known to be pathogenic, such as *E. coli*, it must be stated that the donor originates from a non-pathogenic strain.
- If the inserted gene is known to be from a pathogenic organism or one that produces toxins, the inserted gene itself must be proven to lack association with pathogenesis or ability to produce toxins.
- The donor's history of safe consumption must be described.
- If the donor of the inserted gene is known to be allergenic, the likelihood that the introduced gene codes for an allergen must be considered.

2. Method of gene insertion

(1) Method of vector construction

The method of gene construction must be described. Specifically,

- The method used to create the DNA construct that will ultimately be introduced into the host plant

- The method used and the order in which the promoter, open reading frame and terminator were introduced into the vector.

(2) Method of gene insertion

The method of gene insertion into the host plant must be defined and the materials such as plasmid and DNA construct used to elicit gene expression must be specified. Specifically,

- The method used to introduce the gene into the host
- The method of selecting the transgenic plants
- The method of regeneration

3. Construction

(1) Promoter

The origin and properties of the promoter must be described.

(2) Terminator

The origin and properties of the termination sequence must be described.

(3) Hazardous DNA sequence

The sequence of all DNA inserted into the plant must be clarified and any DNA sequence encoding known harmful protein must not exist.

4. Properties

(1) Function of inserted DNA

The function of the inserted DNA requires clarification. Additionally, the characteristics of the protein(s) produced by the inserted DNA and its function must be described. Evidence that such proteins lack harmful effects must be presented.

(2) Cleavage map using restriction enzymes

A cleavage map of the inserted DNA must be drawn;

The name(s) of restriction enzyme(s) used for molecular analysis, the number of fragments, and their size and Southern Blotting pattern must be described.

(3) DNA molecular weights

The Molecular weight or numbers of base pairs comprising the inserted DNA must be described.

5. Purity

- The sequence, size and origin of the inserted DNA to be inserted must be stated.
- All DNA to be inserted must be purified and cloned to prevent contamination by any other unintended gene.

## 6. Stability

- The DNA sequence, size and origin of the inserted DNA must be stated.
- The stability of the inserted gene construct, its expression levels and sites must be confirmed by Southern Blotting and Western Blotting analysis in tests using adequate numbers of samples from several generations of the recombinant crop.
- When performing the above, the line and generation that was utilized for testing must be specified.
- The stability of the inserted gene(s) must be proved by showing that, the levels of expressed proteins, number of copies and gene constructions are unchanged over several generations.

## 7. Number of inserted gene copies

- The insertion and copy numbers (the structure of the insertion, single or tandem, duplication of the inserted DNA must be stated.
- The (plant genetic) DNA sequence that is adjacent to the inserted DNA must be described, thereby identifying the event that contains the inserted gene(s). That is the DNA sequence that enables identification of the event that the safety assessment is being conducted for.

## 8. Site, timing and level of gene expression

- The site, timing and level of gene expression must be described.
- The above items must be available for the recombinant, together with an explanation of the reasoning and evidence suggesting that the findings indicate that there are no safety concerns.

## 9. Safety of antibiotic-resistant marker gene

If an antibiotic-resistant marker gene is used, safety must be confirmed for items (1) and (2) listed below through comprehensive review, including observations of in vivo changes of the recombinant.

### (1) Properties of the gene and gene product

#### Structure and function

- The DNA sequence and the function of the protein (gene product) must be described.
- Potential harmful DNA sequences (excluding the inserted antibiotic resistant marker gene) must be absent.
- In a case where the gene product is enzyme, the substrate specificity must be determined
- The structure of the gene product must lack homology with currently known



allergens.

Mechanism of resistance, the method of use and related metabolites

- The method of antibiotic administration (oral, i.v., etc.) must be described.
- The mechanism of antibiotic resistance must be stated, if known.
- The safety of metabolites associated with the activity of the antibiotic resistance gene must be considered.

Method of identification and quantification

The method of identification used to assess the gene's identity or its homogeneity must be reported.

Changes accompanying cooking or processing

Sensitivity to physical processing procedures such as heating (*e.g.* enzyme inactivation) must be described.

Changes in gastrointestinal tract environment

Instability must be determined through testing of resistance to degradation by artificial gastric and intestinal fluids. If stability exists, the safety assessment must be based on scientific rationale.

Allergenicity

Prior knowledge related to allergenicity must be described.

## (2) Consumption of genes and their products

Expected amounts of consumption

The amount of protein (gene product) likely to be consumed must be predicted based on the amount of expressed protein in the host plant.

Present usage of antibiotics associated with resistance

Conditions of use for antibiotics that are subject to resistance must be described.

Comparison with normally existing antibiotic-resistant microorganisms

The existence of antibiotic-resistant microorganisms possessing the same gene as the inserted antibiotic-resistant marker gene must be considered.

Estimated amount of antibiotics inactivated after oral administration and the possibility that inactivation may cause problems

Inactivation of an antibiotic can be assumed based on consumption volume of expressed protein (metabolic enzyme for the antibiotic), its decomposition volume in heat processing and gastrointestinal fluids treatment and its usage condition in medical areas *etc.* in order to prove that any problem probably would not be caused by the condition of inactivated antibiotic.

## 10. Presence or absence of exogenous open reading frames and the possibility of their

#### transcription and expression

- As a rule, the inserted gene must not include open reading frame(s) that express unintended protein (gene product). To confirm this, more than one ATG codon that functions as an initiating codon must not exist in the gene, and its inability to express multiple proteins (gene products) must be demonstrated by Northern Blotting RT-PCR or other relevant methods.
- If a gene(s) with the capability of expressing unintended proteins (gene product) is introduced, the subject gene(s) and the gene product(s) must not raise safety concerns.

### **Section 6: Recombinants**

#### 1. New properties acquired by recombinant DNA techniques

Acquired properties by the inserted DNA and its function(s) must be specified. And adverse effects on human and any effect it may have on other living organisms must be considered.

#### 2. Allergenicity of recombinant products

##### (1) Consumption history of the donor

- The donor must not be pathogenic nor have the ability to produce toxins. Moreover, if the donor is known to be pathogenic (such as *E. coli*), it must be demonstrated that the gene originates from a non-pathogenic variant.
- If the donor is known to be pathogenic or is able to produce toxins, it must be shown that the inserted gene itself has no association with pathogenesis and does not have the ability to produce toxins.
- Historical data regarding the consumption of the donor must be identified.
- If the donor is associated with allergenicity, knowledge about such allergenicity must be reviewed.

##### (2) Whether the gene product is known to be an allergen

Knowledge regarding allergenicity associated with the product of the inserted gene must be described.

##### (3) Sensitivity of the gene product to physicochemical treatment

Data regarding changes in molecular weight, enzyme activity and immunological reactivity must be described individually for each of the following three physicochemical treatments;

The gene product (protein) processed with acids and enzymes (pepsin) through an artificial gastric fluid;

The gene product (protein) processed with bases and enzymes (pancreatin) through an artificial intestinal fluid; and

The gene product (protein) processed with heat.

- Data regarding reductions in the molecular weight of the treated protein (gene product) must be compared with pre-treatment gene products by SDS polyacrylamide gel electrophoresis or other relevant methods.
- If a gene product is an enzyme, changes in activity of the treated protein must be compared with the activity of the pre-treatment gene product.
- Changes in reactivity of treated product with antibodies (polyclonal antibodies) must be compared with the reactivity of the pre-treated gene products, by Western Blotting or ELISA methods.

(4) Consumption volume of the gene product

It must be predicted whether the consumption volume of a gene product can be changed significantly. As a rule, the consumption volume must not be changed significantly.

(5) Structural homology of gene products with known food allergens

The search criteria used when attempting to identify structural homology between the gene products and currently known allergens' amino acid structures must be described. As a rule, the introduced gene's product must lack structural homology with such allergens.

(6) Whether the gene product constitutes a considerable part of the total protein intake per day

Whether the gene product constitutes a significant portion of daily protein intake in humans must be predicted. As a rule, protein intake derived from the gene product must not comprise a significant amount of total daily protein intake. If, in any case, the volume of protein intake derived from the gene product is considered to constitute a significant amount of the diet, the scientific rationale that such intake will not pose safety concerns must be presented.

\*Items (1) – (6) above could be exempted if adequate reasons for such exemption exist.

\*If safety cannot be confirmed relative to items (1) – (6) above, safety must be assessed through data such as:

- 1) The binding ability of patient IgE antibody and the gene product relative to allergens with structural homologies to the gene product (e.g. the results of evaluations performed on IgE antibody binding ability in allergy patients by

Western Blot, ELISA or an equivalent method)

2) The binding ability of patient IgE antibody and the gene product relative to major allergens <see note> (e.g., the results of evaluations performed on IgE antibody binding ability in allergy patients by Western Blot, ELSIA or an equivalent method).

<Note: This would require using blood serum from patients with allergies toward items such as egg, milk, soy bean, rice, wheat, buckwheat, cod, shrimp and peanuts. >

3. Toxicity of recombinant products (other than allergenicity)

The criteria used when searching for structural homology of the introduced gene's product (protein) and currently known toxins' structure must be described. As a rule, the introduced gene's product must lack structural homologies with such toxins. If such structural homologies exist, the scientific rationale suggesting that such homologies will not pose safety concerns must be explained.

4. Effect of recombinant products on metabolic pathways

If the gene product is an enzyme, the substrate specificity must be determined. As a rule, the enzyme would be expected to have high substrate specificity. If the substrate specificity is low, its safety assessment must be based on scientific rationale. In addition, if the amount of any existing component on a metabolic pathway is changed as a result of the effect of the gene product, the scientific rationale that such changes will not pose safety concerns must be explained.

5. Difference from the host

Based on data for existing non-recombinant foods, including the host, it must be determined whether the recombinant is significantly different from the host in terms of nutrition, toxins and anti-nutrients. As a basic rule, significant difference must not exist, but if so, the scientific rationale that safety will not be affected by such must be explained. Furthermore, if there are differences between a host allergen's protein structure and that of the recombinant, the effects of such differences on allergenicity must be described.

6. Survival and proliferation in the external environment

Information on any difference between the host and the recombinant in terms of its survival and proliferation in an external environment must be stated. As a basic rule, such differences must not exist. If a difference exists, the scientific rationale that such differences will not pose safety concerns must be explained.

7. Restrictive conditions on survival and proliferation abilities of recombinants

Information on any difference between the host and the recombinant in terms of its

survival and reproductive limitations must be noted. As a basic rule, such differences must not exist. If a difference exists, the scientific rationale that such a difference will not affect safety must be described.

#### 8. Inactivation method of recombinants

Information on any difference between the host and the recombinant in terms of its method of inactivation must be stated. As a basic rule, such differences must not exist. If a difference exists, the scientific rationale that such a difference will not raise safety concerns must be explained.

#### 9. Approval and usage as food in other countries

Information on the approval of such recombinant foods by foreign governments must be described. Furthermore, information on whether the recombinant is utilized as a food product must be described.

#### 10. Methods of preparation, breeding and cultivation

- Information on any difference between the host and the recombinant in terms of harvesting, breeding and cultivation methods must be described. As a basic rule, such differences must not exist. If a difference exists, the scientific rationale that such a difference will not raise safety concerns must be discussed.
- Usage condition of the agricultural chemical's (pesticide) must be clarified.
- In cases where pesticide resistance is shown through metabolizing such pesticides, it must be identified what substances may be produced through the metabolism and the safety of the major metabolites must be proven.

#### 11. Methods of seed production and management

Information on any difference between the host and the recombinant in terms of its seed production and management method must be noted. As a basic rule, such differences must not exist. If a difference exists, the scientific rationale that such a difference will not affect safety must be explained. Furthermore, a host plant's seeds prior to recombination and seeds from each generation after its recombination must be stored.

### **Section 7: Matters related to assessment scores when safety cannot be confirmed based on sections 2 – 6**

In the case that the safety of the product (recombinant plant) is not well established based upon the items above, the safety of the product must be confirmed based on results of the following studies.

- (1) Acute toxicity study

- (2) Sub acute toxicity study
- (3) Chronic toxicity study
- (4) Reproduction study
- (5) Mutagenicity study
- (6) Carcinogenicity study
- (7) Other necessary studies (e.g. intestinal tract toxicity, immunological toxin, neurological toxicity, nutrition *etc.*)

(Note)

- 1) Studies, excluding nutritional studies, must be performed at a GLP (Good Laboratory Practice) compliant facility following GLP standards.
- 2) A portion or the entire set of studies can be exempted if adequate reasons for such exemption exist.

### **Chapter 3: Standards for Safety Assessment of Food Products or Food Additives through the use of microorganisms produced by Recombinant DNA Techniques**

#### **Section 1: Similarities between Produced Food and Existing Food**

An objective and comprehensive judgment, of whether food or food additive through the use of microorganisms produced by recombinant DNA techniques can be considered as being substantially equivalent to existing products, must be made, based on information regarding composition, properties and method of use related to the aforementioned food or food additive, and to existing food products or food additives.

Determination that the new substances is substantially equivalent to existing substances indicates the applicability of the concept such that the food or food additive can be compared with existing food or food additive in safety assessment process. By determining whether the product maintains the status of substantial equivalence upon comprehensive review of its composition, properties and method of use, the examination of the subject product or additive through each criteria listed in Section 2 becomes feasible in comparing it to existing additives.

#### **Section 2: Recombinants**

1. Confirmation that a non-pathogenic recombinant can be utilized in GILSP (Good Industrial Large-Scale Practice) or Category 1 manufacturing

The recombinant must be used at the working level of GILSP or the characteristics of the recombinant must satisfy Category 1 criteria.

Furthermore, judgement that the recombinant complies with the level of a GILSP or Category 1 recombinant must be based upon the definition set forth in Article 2, Clauses 9 and 11 of “Standard for Manufacturing of Foods and Food Additives Produced by Recombinant DNA Technique” (The Announcement of the Ministry of Health and Welfare, No. 234 - 2000).

## 2. Purposes and usage of recombinants

The purposes and usage of the recombinant(s) must be stated.

### 3. Host

#### (1) Taxonomy (scientific name, strain name)

The scientific or strain name of the host microorganisms must be given, and the general human exposure to the microorganism must be determined.

#### (2) Production of pathogens or harmful, physiologically active substances (confirmation of being non-pathogenic)

Microorganisms utilized in the recombinant process must be non-pathogenic, and the type, amount and effect of any harmful, physiologically active substances produced by the host organism must be determined.

#### (3) Parasiticity

The capability of the host microorganisms to become parasitic and fix in humans or other living organisms must be determined for microorganisms utilized in the recombinant process. If such capability exists, it must be clarified whether it may have adverse effects on humans or other organisms.

#### (4) Foreign pathogenic factors (e.g. virus)

The host microorganism used in the development of the subject recombinant must not be contaminated by foreign pathogens.

#### (5) Survival and proliferation abilities under experimental conditions simulating ordinary or natural environments

The reproductive and survival abilities of microorganisms used in the development of the subject recombinant must be considered.

#### (6) Sexual or asexual reproduction cycle and cross reactivity on sexual life cycle.

The sexual or asexual reproductive cycle of microorganisms used in the development of the recombinant and capacity for out crossing must be examined from a genetic dispersion perspective.

#### (7) History of utilization as food

The history (dietary culture) of the host microorganism's use as a food or food additive must be described.

#### (8) Restrictive conditions on survival and proliferation abilities

The conditions for controlling the survival and reproduction of the host microorganisms must be stated.

(9) Pathogenicity and production of harmful physiologically active substances of host-related strains

If the host microorganism used on a process for producing recombinants is closely related to a microorganism known to be pathogenic or capable of generating harmful physiologically active substance, the recombinant microorganism must lack pathogenicity or the ability to produce harmful physiologically active substance.

4. Vector

(1) Name and Origin

- The name(s) and origin(s) of vector(s) (e.g. a plasmid) utilized in gene expression must be stated.
- The vector(s) must lack adverse effect on humans.

(2) Properties

DNA molecular weight

The molecular weight or numbers of base pairs of the DNA must be stated.

Cleavage map using restriction enzymes

A cleavage map of the vector to be used to insert the gene into the microorganism must be prepared.

The name of the restriction enzyme, the number of fragments, and their size and electrophoretic profile must be determined.

Presence of any potentially harmful nucleic acid sequence

The recombinant must lack a DNA sequence that produces currently known hazardous protein.

(3) Drug resistance

If a plasmid includes a drug-resistant gene, the properties of such a drug-resistant gene must be described.

(4) Transmission to other species

The ability for a vector to move from the host to another independent microorganisms (*i.e.* transmission) must be determined. If this ability exists, the types of microorganisms likely to be vulnerable to such transmission must be determined.

(5) Host dependency

The vector utilized in genetic engineering must not multiply in other microorganisms or humans. If such ability exists, the types of hosts that may be



affected must be identified.

(6) Preparation method of Expression vector

The preparation method of expression vector must be stated.

(7) Insertion method and site of the expression vector

The method used to insert the expression vector into the host and the site of the expression vector that has been inserted into the host must be stated.

5. Inserted Gene and Its Gene product

(1) Name, origin and taxonomy of donor

The name, origin and taxonomy of the DNA donor must be given.

(2) Method of gene insertion

Method of vector construction

The method of gene construction and its insertion into the vector must be described. Specifically,

- The methods used to create the DNA construct that will ultimately be introduced into a microorganism.
- The method and the order in which the promoter, open reading frame and terminator were introduced into the vector.

Method of gene introduction

The method of gene introduction into the host microorganisms must be defined, and materials such as the plasmid and DNA construct used in gene expression must be given. Specifically,

- The method for introducing the gene into a host
- The method of selection of the transgenic microorganism
- The method for regenerating the microorganisms

(3) Construction

Promoter

The origin and properties of the utilized promoter must be stated.

Terminator

The origin and properties of the utilized terminator must be stated.

Hazardous DNA sequence

The sequence of all DNA inserted into the microorganism must be specified. Any DNA sequence encoding for a known harmful protein must not be included.

(4) Properties

Function of inserted DNA

The function of the inserted DNA must be described. Additionally, the characteristics of the proteins produced by the inserted DNA, and their function

must be given. Such proteins must not have harmful effects.

Cleavage map using restriction enzymes

A cleavage map of the inserted DNA must be drawn;

The names of the restriction enzymes used for molecular analysis, the number of fragments, and their size and southern blotting pattern must be provided.

DNA molecular weights

The molecular weight or numbers of base pairs of the inserted DNA must be given.

(5) Purity

- The DNA sequence, size and origin of the inserted DNA must be described.
- All DNA to be inserted must be purified and cloned to exclude contamination by other unintended genes.

(6) Safety of antibiotic-resistant markers

If an antibiotic-resistant marker gene is used, the construction and function of the inserted marker gene or its products must be described. Furthermore, if antibiotic resistance potential with the marker gene or its byproducts during the manufacturing process is not sufficiently reduced in the recombinant, their safety must be confirmed for items 1 and 2 listed below through comprehensive review, including observations in vivo. .

Properties of the gene and gene product

- Structure and function
  - \*The DNA sequence and protein (gene product) functions must be provided.
  - \*Harmful DNA sequences other than the inserted antibiotic-resistant marker gene must be absent.
  - \*In the case of a gene product that is enzyme, substrate specificity must be determined.
  - \*The structure of gene product must lack similarity with currently known allergens.
- Mechanism of expression resistance, the method of use and related metabolites.
  - \*The method of likely antibiotic administration (oral, i.v. *etc.*) must be stated.
  - \*The mechanism of antibiotic resistance must be described.
  - \*The safety of metabolites associated with resistance must be evaluated.
- Method of identification and quantification

The method of identification used to determine the gene's identity, and its homogeneity must be described.

- Changes accompanying cooking or processing  
The sensitivity of the gene products to physical processing such as heating and enzyme inactivation must be determined.
  - Changes in gastrointestinal tract environment  
Stability /Instability of the gene product must be determined by testing for resistance to degradation by use of artificial gastric and intestinal fluids. If stability exists, the safety of such a protein must be based on scientific rationale.
  - Allergenicity  
Prior knowledge related to allergenicity must be provided.
- Consumption of genes and their products
- Estimated amounts of consumption  
The intake amount of protein (gene product) must be estimated/calculated based on the amount of expressed protein.
  - Present usage of antibiotics associated with resistance  
Conditions of use for antibiotics that are subject to resistance must be described (directions for use, amounts, purpose *etc.*).
  - Comparison with existing antibiotic resistance  
The origin of the antibiotic marker gene inserted into the microorganism must be similar to antibiotic-resistance genes existing in natural antibiotic-resistant microorganisms.
  - Estimated amount of antibiotics inactivated after oral administration and the possibility that inactivation may cause problems  
Inactivation of an antibiotic can be assumed based on consumption volume of expressed protein (metabolic enzyme for the antibiotic), its decomposition volume in heat processing and gastrointestinal fluids treatment and its usage condition in medical areas *etc.* in order to prove that any problem probably would not be caused by the condition of inactivated antibiotic.
- (7) Presence or absence of exogenous open reading frames and the possibility of their transcription and expression
- As a rule, the inserted gene must not include open reading frame(s) that express unintended protein (gene products). To confirm this, more than one ATG codon that function as an initiating codon must not exist in the gene, and its inability to express multiple proteins (gene products), must be demonstrated by Northern Blotting, RT-PCR or other relevant methods.
  - If there is a gene which may express unintended proteins, the gene (s) or the

gene products must not raise safety concerns.

## 6. Recombinants

- (1) New properties acquired by use of recombinant DNA techniques (must be non-pathogenic)

Expression mechanisms of the inserted gene must be clearly described. It should be also clarified that recombinant does not acquire pathogenicity originating from recombinant DNA techniques. Furthermore, properties or functions of proteins newly derived from recombinant must be specified, and the proteins must not have any adverse effects on humans.

- (2) Survival and proliferation in the external environments

Information on any difference between the host root and the recombinant in terms of survival and proliferation in an external environment must be stated. As a basic rule, such differences must not exist. If a difference exists, scientific rationale that such a difference will not pose safety concerns must be explained.

- (3) Restrictive conditions on survival and proliferation abilities

Information on any difference between the root utilized in the production of recombinant and the recombinant itself in terms of its survival and reproductive limitations must be stated. If the product is for industrial use, it must be as safe in the industrial setting as the host organism, with limited survival and reproductive ability and without adverse influence on the environment.

- (4) Inactivation method of recombinants

Information on any difference between the host root and the recombinant in terms of its method of inactivation must be stated. As a basic rule, such differences must not exist. If a difference exists, the scientific rationale that such a difference will not raise safety concerns must be explained. Furthermore, the survival rate of a recombinant utilizing such method of inactivation must be examined.

- (5) Difference from the host

Any dissimilarity between the host used in the production of the recombinant and the recombinant itself in terms of being non-pathogenic or its capacity to produce substances causing hazardous physiological activation must be described. Moreover, safety of the product must not be affected by such difference.

### **Section 3: Miscellaneous Ingredients and Manufacturing Apparatus other than the Recombinant**

1. The history of actual use as a manufacturing apparatus on manufacturing process or as a raw ingredient for foods or food additives must be described.
2. Existing knowledge regarding its safety in use as a manufacturing apparatus or ingredients in food products or food additives must be presented.
3. Studies must be available to demonstrate the level of safety for use as a manufacturing apparatus or ingredients in food products or additives if the background cannot be confirmed by item 1 and 2, noted above.

### **Section 4: Products**

1. Absence of contamination by recombinants

Absence of contamination by recombinants must be proven through appropriate procedures utilizing testing materials based on suitable testing methods such as the dot-blot hybridization method.

2. Purity and amounts of impurities

The amount of impurity in food products or food additives derived from recombinants must not be significantly increased compared to that in existing food products or food additives. Additionally, impurities not found in existing products or additives must not be found in the subject. In all other cases not listed herein, safety of the matter must be based on scientific rationale.

3. Purifying process

The method used in the purifying process of the product must be described. In addition, the amount of harmful substances possibly introduced as contaminants during the manufacturing process must be predicted, and the substances must not raise safety concerns based on scientific rationale.

4. Changes in levels of normal ingredients which may be harmful

Changes in the levels of normal ingredients that may elicit problems must not exceed the range known to be safe in existing food products or food additives. If such a change occurs, the subject amount of concentration must not raise safety concerns based on scientific rationale.

5. Approval and usage as food or food additives in other countries

Information on the approval of such recombinant foods or food additives by foreign governments must be described. Furthermore, information on whether the recombinant is utilized as a food product or a food additive must be described.

**Section 5: Matters related to testing scores when safety cannot be confirmed based on sections 1– 4**

In the case that safety of the product (recombinant plant) is not well established based upon the items above, the safety of the product must be confirmed based on results of the following studies.

- (1) Acute toxicity study
- (2) Subacute toxicity study
- (3) Chronic toxicity study
- (4) Reproduction study
- (5) Mutagenicity study
- (6) Carcinogenicity study
- (7) Other necessary studies (e.g., intestinal tract toxicity, immunity toxin, neurological toxicity, nutrition *etc.*)

(Note)

1. Studies, excluding nutritional studies, must be performed at a GLP (Good Laboratory Practice) compliant facility following GLP standards and key results of the score must have been publicly disclosed.
2. A portion or an entire set of studies can be exempted if adequate reasoning for such exemption exists.

Note: This English version of the Notice is translated to meet the need of the non-Japanese speaking people. In the case of any discrepancy between the Japanese original and the English translation, the former will take priority.