Application Note

Application Note No.4 (Lifescience)

Lifescience

Food Allergen Test
– Application of MultiNA –

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1. What are Allergies?

We have immune functions that protect our bodies by creating antibodies to substances entering the body (antigens) that are foreign and hostile to the body. This is known as the "antigen-antibody reaction" or "immune response." When the same antigen subsequently enters the body, the memorized antibody activates and binds to the antigen to render it harmless.
However, if the immune functions go out of control, excess antibodies can be created or harmful antibodies produced in the body. This imbalance causes allergic diseases. Typical allergic diseases include atopic dermatitis, allergic rhinitis (hay fever, etc.), allergic conjunctivitis, allergic gastroenteritis, asthma, childhood asthma, food allergy, drug allergy, and hives.

Immunity was originally intended to protect the body from harmful substances. However, for people with a certain disposition, the immune function can activate in response to foods, pollen, dust and other substances which are usually harmless. People predisposed to such symptoms are said to have an "allergic predisposition."
When specific substances enter the body of a person with an allergic predisposition, the antibodies act abnormally to cause specific symptoms.

2-1 What are Food Allergies?

A hypersensitive immune reaction resulting from eating specific foods is called a "food allergy." Food allergies can cause a diverse range of symptoms, including skin symptoms such as hives and eczemas; gastrointestinal symptoms such as diarrhea, vomiting, and stomachache; and respiratory symptoms such as coughing and breathing difficulties. In severe cases, food allergies can lead to systemic symptoms, such as anaphylactic shock.

The substances causing food allergies and the amounts required differ from person to person. The reaction also differs according to the person's physical condition. For children, in particular, food allergies are often caused by the so-called "three major allergens": eggs, milk, and wheat. Of these, chicken eggs are the major cause of food allergies. Other causes are fish (in particular, blue-backed fish), meat (in particular, pork), shellfish, shrimp, crab, soybeans, cereals, and buckwheat.

Food allergies are mainly caused by proteins derived from the ingredients in the food. The three major allergens – eggs, milk, and wheat – are all foods with a high protein content. Normally, the proteins in foods are broken down in the stomach and intestines and absorbed as amino acids and peptides (several amino acids linked together). These small molecules do not normally cause allergies.

However, when the digestive tract and its functions are immature during infancy, inadequately digested proteins (oligopeptides) are often absorbed and are said to cause many food allergies. When the digestive functions are suppressed during illness, in particular, more undigested substances than normal pass through the digestive tract. As a result, undigested substances are more frequently absorbed and the incidence of allergies also increases.

The 2005 Ministry of Health, Labour and Welfare Science Research Report (Fig. 1) of Japan lists the following incidences of food allergies:

1. Eggs (38 % of total)
2. Dairy products (16 % of total)
3. Wheat (8 % of total)
4. Fruit (6 % of total)
5. Buckwheat (5 % of total)
6. Shrimp (4 % of total)
7. Peanuts (3 % of total)

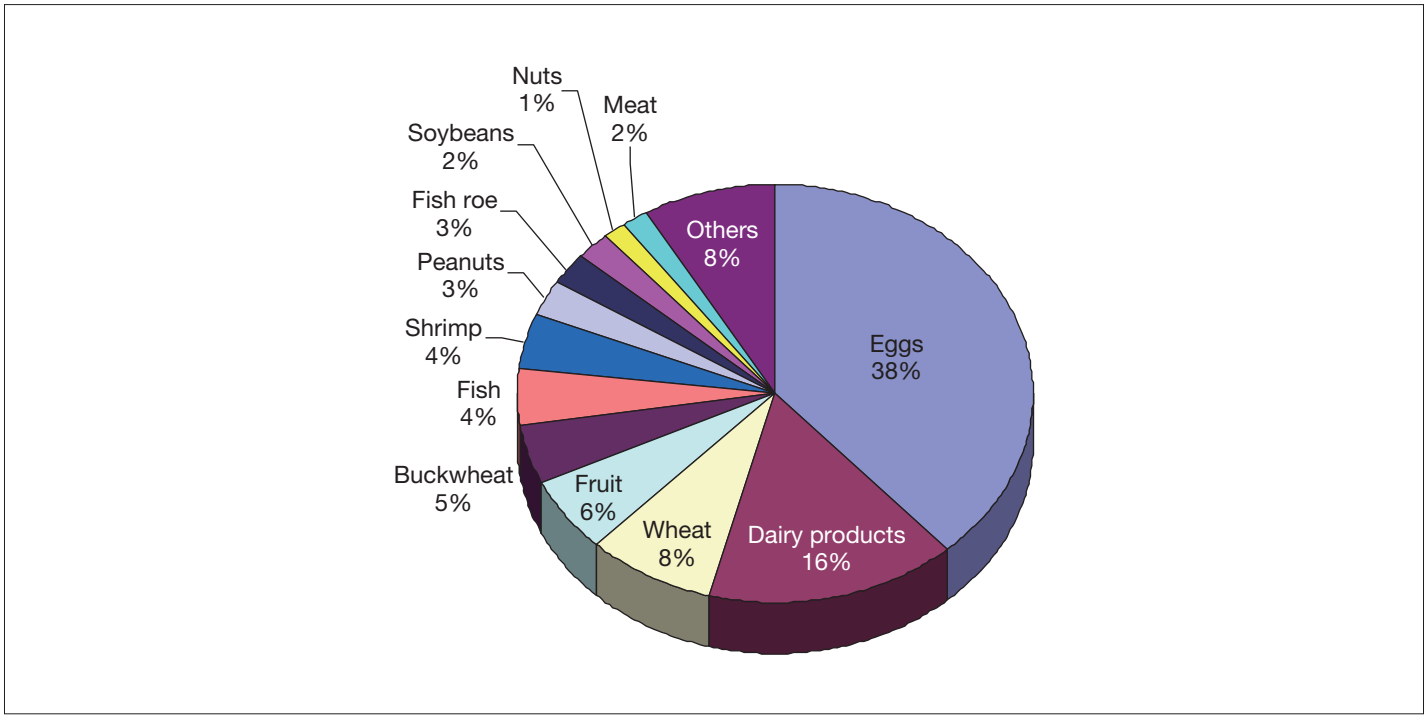
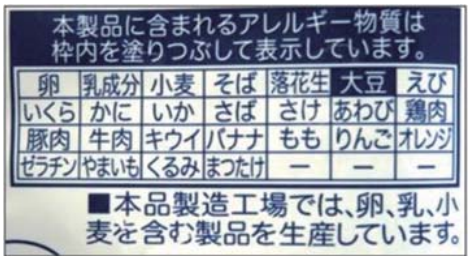


Fig. 1 Proportions of Foods Causing Allergies (source: 2005 Ministry of Health, Labour and Welfare Science Research Report of Japan)

3. Food Labeling

Japan was the world's earliest adopter of a labeling system for foods containing allergens (see Fig. 2).
The labeling of foods containing allergens is categorized into “Mandatory” (7 specified ingredients) and “Recommended” (18 specified ingredients).
Of the foods discovered to cause allergies in recent investigations, the five items with a high incidence or severity – eggs, milk, wheat, buckwheat, and peanuts – were prescribed as “specified ingredients” under the Japanese Ordinance for Enforcement of the Food Sanitation Act. Foods containing these ingredients were subject to mandatory labeling from April 2002. Two more items were added from June 2008: shrimp and crab. Labeling is required for foods containing 10 μg/g or higher of these seven specified ingredients, even if they are impurities mixed in during the manufacturing process. Labeling the possibility that the items could be included, such as "May contain xxx," is not permitted.
Eighteen other items for which labeling is recommended (items corresponding to specified ingredients) have been notified: abalone, squid, salmon roe, orange, kiwi fruit, beef, walnuts, salmon, mackerel, soybeans, chicken, banana, pork, matsutake mushroom, peach, yam, apple, and gelatin (Table 1). Labeling is intended to provide information to consumers to avoid health hazards due to allergies. Consequently, labeling must inform of even trace levels of specified ingredients contained in or mixed in a food product.
(*However, mandatory labeling of manufactured, processed, or imported food products was deferred to 3 June 2010.)

Recently, more and more food companies are producing products free of egg, milk, wheat, buckwheat, peanuts and other allergens. They implement strict product development, ingredient selection, production line cleaning (and subsequent checks), and inspections of individual production lots (according to the official method prescribed by the Japanese Ministry of Health, Labour and Welfare).



This product contains the allergens in the highlighted frames below.

Egg	Milk	Wheat	Buckwheat	Peanuts	Soybeans	Shrimp
Salmon roe	Crab	Squid	Mackerel	Salmon	Abalone	Chicken
Pork	Beef	Kiwi fruit	Banana	Peach	Apple	Orange
Gelatin	Yam	Walnuts	Matsutake mushroom	–	–	–

■The factory that produced this product manufactures products containing egg, milk, and wheat.

Fig. 2 Example of Labeling

Table 1 Items Labeled as Allergens
(Source: March 2009 revision of "Handbook for Labeling of Processed Foods Containing Allergens," Japanese Ministry of Health, Labour and Welfare)

Labelling	Term	Name
Mandatory	Specified ingredients (7 items)	Egg, milk, wheat, buckwheat, peanuts, shrimp, crab
Recommended	Items pursuant to specified ingredients (18 items)	Abalone, squid, salmon roe, orange, kiwi fruit, beef, walnuts, salmon, mackerel, soybeans, chicken, banana, pork, matsutake mushroom, peach, yam, apple, and gelatin

*The scope of the specified ingredients is basically the range designated by the numbers in the Japan Standard Commodity Classification (JSCC). (For more details, see the March 2009 revision of "Handbook for Labeling of Processed Foods Containing Allergens," Japanese Ministry of Health, Labour and Welfare.)

4. Analysis of Allergenic Substances

Test methods have been established for 20 items: egg, milk, wheat, buckwheat, peanuts, shrimp, crab, abalone, squid, kiwi fruit, beef, walnuts, salmon, mackerel, soybeans, chicken, pork, yam, apple, and banana.
These test methods are included in the Japanese Ministry of Health, Labour and Welfare Notification "Regarding the testing method for foods containing allergenic substances," No. 0622003 issued by the Dept. of Food Safety, June 22, 2006.
Test methods for proteins derived from specified ingredients in foods include the ELISA method (*1) based on antigen-antibody reactions for quantitative analysis, Western blotting method (*2) for qualitative analysis, and PCR method (*3) (Table 2).
The ELISA quantitative test method is used for the screening of the seven specified ingredients – egg, milk, wheat, buckwheat, peanuts, shrimp, crab – as well as soybeans, which are listed as items corresponding to specified ingredients.
Western blotting method is generally used for the qualitative analysis of egg and milk.
The polymerase chain reaction (PCR) method uses specific sequences for the confirmation testing of the specified ingredients wheat, buckwheat, peanuts, shrimp, and crab (excluding egg and milk) and for soybeans, beef, pork, chicken, salmon, mackerel, abalone, squid, kiwi fruit, walnuts, yam, apple, and banana that are items corresponding to specified ingredients.

Table 2 Test Methods of Allergen

Test Method	Application
ELISA	Screening (quantitative)
Western blotting	Confirmation testing (qualitative)
PCR	Confirmation testing (qualitative)

Table 3 summarizes the test methods applicable for each item.

Table 3 Test Methods for Each Item

	Item	Test Method
Specified Ingredients Mandatory Labeling	Egg	ELISA, Western blotting
	Milk	ELISA, Western blotting
	Wheat	ELISA, PCR
	Buckwheat	ELISA, PCR
	Peanuts	ELISA, PCR
	Shrimp	ELISA, PCR
	Crab	ELISA, PCR
Items Corresponding to Specified Ingredients Recommended Labeling	Soybeans	ELISA, PCR
	Beef	PCR
	Pork	PCR
	Chicken	PCR
	Salmon	PCR
	Mackerel	PCR
	Abalone	PCR
	Squid	PCR
	Kiwi fruit	PCR
	Walnuts	PCR
	Yam	PCR
	Apple	PCR
	Banana	PCR

(*1) ELISA (Enzyme-Linked ImmunoSorbent Assay) Method
The Enzyme-Linked ImmunoSorbent Assay is an analysis method that combines an immunoreaction (antigen-antibody reaction) and an enzyme-substrate reaction. This method is used to detect and quantify the concentration of antibodies and antigens contained in the sample. This method is known as ELISA.

(*2) Western Blotting Method
After separating a sample by electrophoresis, it is transferred and bound to a membrane. It is reacted with an antibody (primary antibody) for the protein of interest. A secondary enzyme-marked antibody is reacted with the primary antibody and the target substance is detected through luminescence or fluorescence.

(*3) Polymerase Chain Reaction (PCR) Method
This method selectively amplifies part of the DNA, using the sample DNA as a template. Cycle reactions (separation of double-stranded DNA → primer binding → DNA synthesis) are performed using a primer (short sequence-specific single-stranded DNA with each end of the region to be amplified) and DNA polymerase to amplify the required DNA region. In principle, even a single DNA molecule can be amplified in multiples of the number of reaction cycles. The presence of the substance of interest can be evaluated from whether the regions straddling the primer are amplified.

5. Analysis by PCR

1) Extracting and Purifying DNA from Food Samples
The extraction and purification of DNA can be performed by the cetyltrimethylammonium bromide surfactant (CTAB) method or methods using a silica gel membrane or ion-exchange resin. Each method has its own characteristics. The CTAB method makes it difficult for PCR inhibitors to remain in the food. Commercial kits are available for extraction and purification methods using a silica gel membrane or ion-exchange resin, making them relatively simple to perform.
The CTAB method is applicable to test samples with a low degree of processing, such as wheat flour or buckwheat flour. Methods using a silica gel membrane or ion-exchange resin are applicable to test samples subjected to a high degree of processing, including sweetening, oil treatment, hot mixing, or fermentation.

2) Confirming DNA Purification and Quantitation
The extracted and purified DNA sample solution is diluted ten times and the absorbance measured at 230 nm, 260 nm, and 280 nm. In principle, the DNA sample solution is prepared at 20 ng/μL concentration.

3) PCR
The base sequence region of interest contained in the extracted and purified DNA is amplified by performing polymerase chain reaction (PCR) using the appropriate organism-specific primer (Table 4). These amplification products are separated and detected by electrophoresis to determine the absence or presence of the specified ingredient in the inspected sample. Fig. 3 shows the detection procedure.

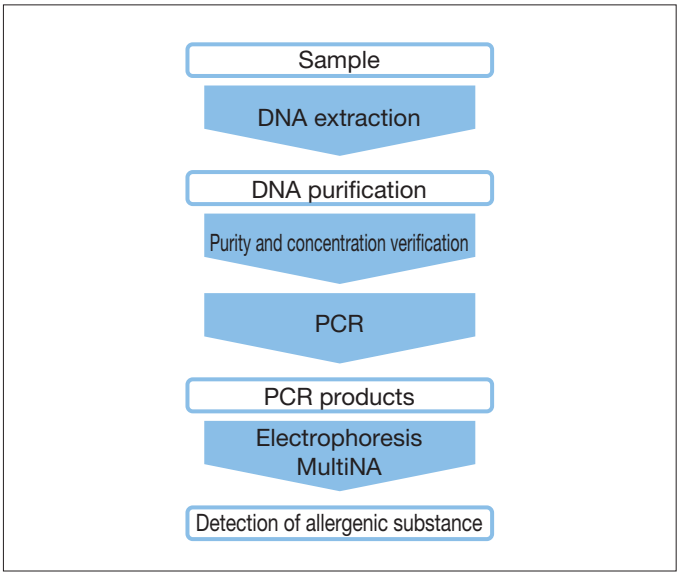


Fig. 3 Experimental Procedure for Detection of Allergenic Substances

Table 4 Primers for Enzyme Detection
Japanese Ministry of Health, Labour and Welfare Notification "Regarding the testing method for foods containing allergenic substances," No. 0724, Publication No. 1 issued by the Dept. of Food Safety, July 24, 2009 (See Note)

	Plant DNA	Animal DNA	Wheat	Buckwheat	Peanuts	Shrimp	Crab
PCR Amplification Product Size (bp)	124	370-470	141	127	95	187	62

	F-Primer	R-Prime
Plant DNA	CP03-5' : 5'-CGG ACG AGA ATA AAG ATA GAG T-3'	CP03-3' : 5'-TTT TGG GGA TAG AGG GAC TTG A-3'
Animal DNA	AN1-5': 5'-TGA CCG TGC GAA GGT AGC-3' AN2-5': 5'-TAA CTG TGC TAA GGT AGC-3' Use 1:1 mixture of AN1-5' and AN2-5'.	AN-3' : 5'-CTT AAT TCA ACA TCG AGG TC-3'
Wheat	Wtr01-5' : 5'-CAT CAC AAT CAA CTT ATG GTG G-3'	Wtr10-3' : 5'-TTT GGG AGT TGA GAC GGG TTA-3'
Buckwheat	FAG19-5' : 5'-AAC GCC ATA ACC AGC CCG ATT-3'	FAG22-3' : 5'-CCT CCT GCC TCC CAT TCT TC-3'
Peanuts	agg04-5' : 5'-CGA AGG AAA CCC CGC AAT AAA T-3	agg05-3' : 5'-CGA CGC TAT TTA CCT TGT TGA G-3'
Shrimp	ShH12-05' : 5'-TTA TAT AAA GTC TRG CCT GCC-3' ShH12-05' is synthesized as A and G mixed bases (R) to the 8th base from the 3' terminal.	ShH13-03'-1: 5'-GTC CCT CTA GAA CAT TTA AGC CTT TTC-3' ShH13-03'-2: 5'-GTC CCT TTA TAC TAT TTA AGC CTT TTC-3' ShH13-03'-3: 5'-GTC CCC CCA AAT TAT TTA AGC CTT TTC-3 Use a 1:1:1 mixture of ShH13-03'-1, ShH13-03'-2, and ShH13-03'-3.
Crab	CrH16-05'-1: 5'-GCG TTA TTT TTT TTG AGA GTT CWT ATC GTA-3' CrH16-05'-2: 5'-GCG TAA TTT TTT CTG AGA GTT CTT ATC ATA-3' CrH16-05'-3: 5'-GCG TTA TTT TTT TTA AGA GTA CWT ATC GTA-3' CrH16-05'-4: 5'-GCG TTA TTT CTT TTG AGA GCT CAT ATC GTA-3' CrH16-05'-1 and CrH16-05'-3 are synthesized as A and T mixed bases (W) to the 8th base from the 3' terminal. Use a 10:1:6:3 mixture of CrH16-05'-1, CrH16-05'-2, CrH16-05'-3, and CrH16-05'-4.	CrH11-03' : 5'-TTT AAT TCA ACA TCG AGG TCG CAA AGT-3'

* The amplification products of Shanghai hairy crab, Dungeness crab, giant spider crab, red queen crab, deep sea red crab, or swimming crab may be detected by PCR for shrimp. If it is unknown whether the amplification products obtained are derived from shrimp or crab, they can be identified by performing restriction enzyme digestion on the PCR products. For details, see the Japanese Ministry of Health, Labour and Welfare Notification "Regarding the testing method for foods containing allergenic substances."
Note) See rear cover for details.

6. MCE-202 MultiNA Microchip Electrophoresis System

The long series of operations required for agarose gel electrophoresis – reagent preparation, gel preparation, electrophoresis, acquiring result images, and clean-up – requires a lot of time and effort. Moreover, the data obtained is objectively poor in terms of sensitivity, separation, reproducibility, and quantitativeness.

The MCE-202 MultiNA Microchip Electrophoresis System overcomes the problems with agarose gel electrophoresis.

Fig. 4 MultiNA Microchip

Fig. 5 MultiNA Regent Kit

Features of MultiNA

- Microchip electrophoresis by MultiNA offers superior sensitivity, separation, reproducibility, and quantitativeness to agarose gel electrophoresis.
- Simply load the samples and reagents for automated, unmanned analysis of up to 120 samples. Pretreatment and electrophoresis proceed in parallel to achieve an analysis time of just 80 s (*) per sample.
- MultiNA offers extremely easy analysis operation. Once the analysis schedule is created, simply load the samples and reagents and click the Start button.
- Reusable high-performance microchip achieves running costs equal to or lower than agarose gel electrophoresis.

(*) DNA standard analysis (DNA-100 kit/Pre-Mix mode) using four microchips.
However, this time does not include the times for initial and subsequent rinsing or the time for initial analysis.

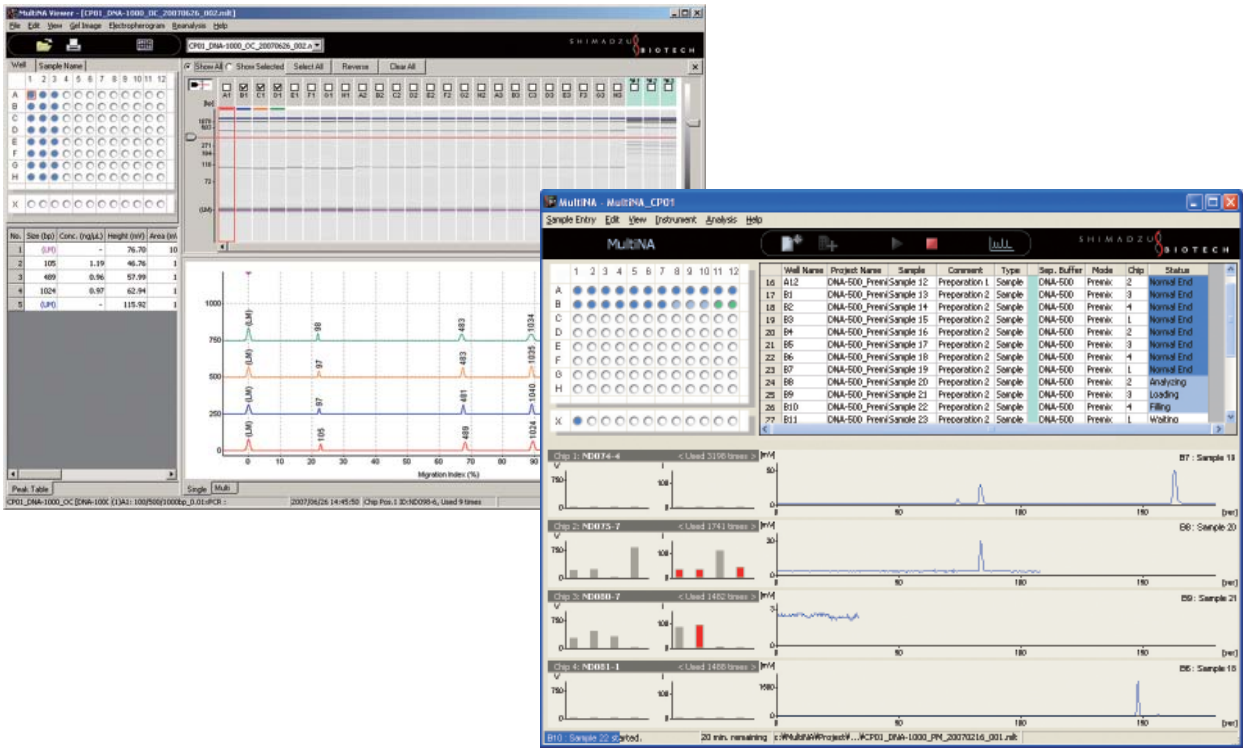


Fig. 6 MultiNA Operation Screen

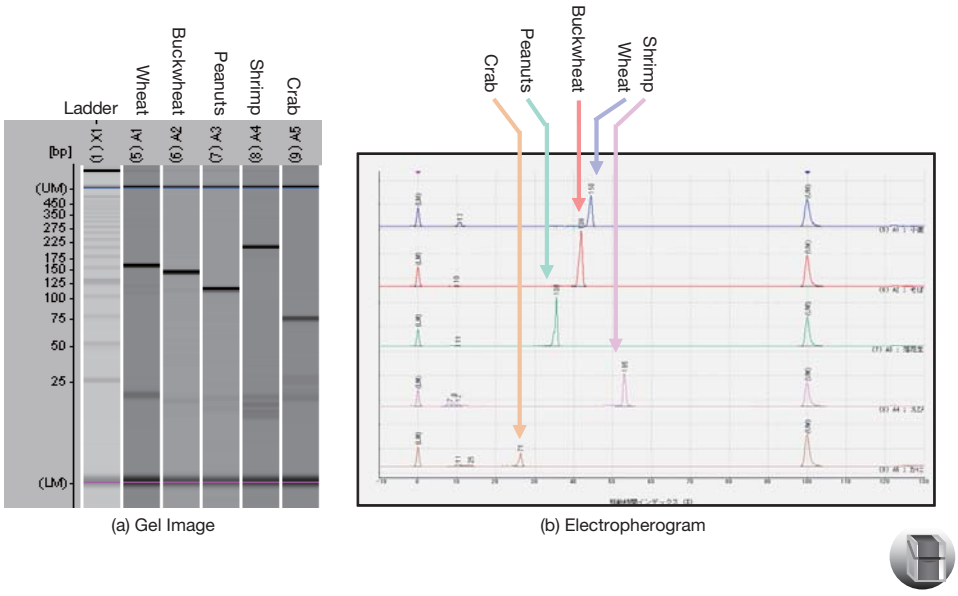
7. Detection of Allergenic Substances Using MCE-202 MultiNA Microchip Electrophoresis System

7. Detection of Allergenic Substances Using MCE-202 MultiNA Microchip Electrophoresis System

The results of analysis of the PCR amplification products of DNA derived from wheat, buckwheat, peanuts, shrimp and crab, respectively, using the MultiNA are shown in Fig. 7. The PCR amplification products derived from the wheat, buckwheat, peanuts, shrimp and crab substances were all clearly detected using the MultiNA. (The estimated sizes shown in the figure were obtained in this experiment.)

The results of analysis by agarose gel electrophoresis of the same PCR amplification products are shown in Fig.8 [Reference]. The sizes of the PCR amplification products are imprecise, resulting in the lack of objectivity in interpreting the gel electrophoresis. However, the results obtained using the MultiNA consist of an electropherogram (Fig. 7-b) in addition to a gel image (Fig. 7-a), ensuring a high level of accuracy. Despite the proximity of the wheat and buckwheat amplification products, they could be separated. Compared to agarose gel electrophoresis, the MultiNA's excellent resolution and sensitivity allow these to be clearly detected.

Fig. 9 shows a photograph of the MCE-202 MultiNA Microchip Electrophoresis System.



(a) Gel Image (b) Electropherogram

Fig. 7 Analytical Results for PCR Products from Allergenic Substances

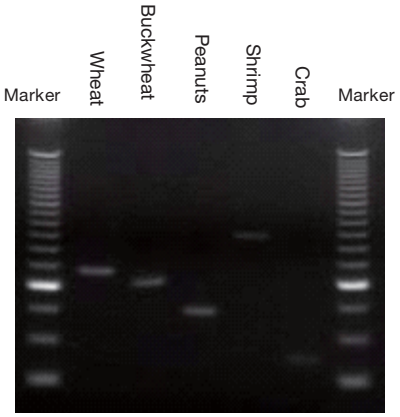


Fig. 8 [Reference] Agarose Gel Electrophoresis of PCR Products from Allergenic Substances

Fig. 9 MCE-202 MultiNA Microchip Electrophoresis System

References:

"Regarding the testing method for foods containing allergenic substances," No. 0724, Publication No. 1, the Dept. of Food Safety, Ministry of Health, Labour and Welfare of Japan, July 24, 2009
"Handbook for Labeling of Processed Foods Containing Allergens," Japanese Ministry of Health, Labour and Welfare, March 2009 revision
"What You Need to Know About Food Labeling," Japanese Ministry of Health, Labour and Welfare, Japanese Ministry of Agriculture, Forestry and Fisheries, Japan Fair Trade Commission, March 2009

Note)
Separate arrangements are required for contract testing using the primers described above for commercial applications on behalf of analytical laboratories, with the exception of public institutions.
Contact the appropriate company below. The synthesis and application of these primers for research applications is unrestricted.

- Animal: Nissin Food Products Co., Ltd.
- Wheat, buckwheat, soybeans: Nisshin Seifun Group Inc.
- Shrimp, crab: House Foods Corporation

* MCE®-202 MultiNA is not available in the United States.
* This document is based on information valid at the time of publication. It may be changed without notice.

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