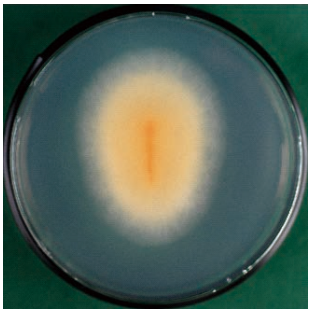
Application Note

Application Note No.5 (Lifescience)






Lifescience

Test of Food-Poisoning Bacteria and Molds in Foods – Application of MultiNA –

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1. Introduction

Food poisoning is a detrimental health effect caused by consuming a food containing harmful microorganisms or a chemical substance or a food with bacteria or a virus capable of causing food poisoning on it. Infectious diseases such as cholera or dysentery that can be passed from person to person can also be transmitted via foods and these are also handled as food poisoning.
A food with bacteria or a virus capable of causing food poisoning adhered to it does not change in taste or smell. Consequently, a food contaminated with microorganisms may be unknowingly consumed and cause food poisoning. Many food-poisoning bacteria cause gastrointestinal problems including stomachache, vomiting, and diarrhea.

In addition, some molds can also flourish on foods and produce mycotoxins that can cause food poisoning. Mycotoxins can cause cancer as well as food poisoning.
Such bacteria, viruses, and molds that can cause food poisoning must be strictly monitored and controlled in the food industry for hygiene management. All microorganisms, including food-poisoning causative agents, are taken extremely seriously by the cosmetics and pharmaceutical industries, as well as the food industry.

Applications Department Center, Analytical & Measuring Instruments Division

2. Types of Food Poisoning

Depending on what causes it, food poisoning can be classified as (1) bacterial food poisoning, (2) viral food poisoning, (3) naturally occurring food poisoning, (4) chemical food poisoning, and (5) other food poisoning (Table 1).

(1) Bacterial food poisoning is caused by bacteria adhering to food. Bacterial food poisoning is classified into two types: infectious food poisoning caused by bacteria entering the body, multiplying in the intestines and producing toxins; and toxic food poisoning caused by toxins produced by bacteria multiplying on a food that is ingested. Typical infectious types include food poisoning caused by Salmonella, Vibrio parahaemolyticus, Campylobacter, or enterohemorrhagic E. coli (O157, etc.). Typical bacteria causing toxic food poisoning include Staphylococcus aureus and Clostridium botulinum.

(2) Viral food poisoning is caused by viruses adhering to food. Norovirus is a typical virus causing food poisoning.

(3) Naturally occurring food poisoning is caused by harmful substances contained in plants or animals. Naturally occurring food poisoning is broadly classified into animal or plant food poisoning. Blowfish poison and shellfish poison are typical causes of animal food poisoning. Mycotoxins and poisonous mushrooms are typical causes of plant food poisoning.

(4) Chemical food poisoning is caused by harmful chemicals contained in foods. Typical causes are mercury, cadmium, and pesticides.

(5) Other food poisoning encompasses food poisoning caused by protozoa and parasites in foods such as meat and seafood.

Table 1. Types of Food Poisoning

Type	Cause	
Bacterial food poisoning	Infectious	Salmonella, Vibrio parahaemolyticus, Campylobacter, or enterohemorrhagic E. coli (O157, etc.), Clostridium perfringens, Shigella, etc.
	Toxic	Staphylococcus aureus, Clostridium botulinum, Bacillus cereus (*1) , etc.
Viral food poisoning	Norovirus, sapovirus, viral hepatitis (A-type, E-type), and rotavirus	
Naturally occurring food poisoning	Plant	Mycotoxins, poisonous mushrooms, poisonous plants (cowbane (cicuta virosa), potato shoots)
	Animal	Blowfish poison (*2), shellfish poison (*3), fish poison (*4), etc.
Chemical food poisoning	Mercury, cadmium, pesticides, lead, methanol, histamine, etc.	
Other food poisoning	Protozoa (cryptosporidium, cyclospora, toxoplasma, etc.)	
	Parasites (anisakis, spiruria, trichina worm)	

(*1) Food poisoning caused by Bacillus cereus can be categorized into two types: infectious type (diarrheal type) and toxic type (emetic type).
(*2) The poisonous component of blowfish poison is tetrodotoxin ($C_{11}H_{17}N_3O_8$) but this is not biosynthesized in the blowfish itself. Recent research suggests that the blowfish poison is generated by eubacteria, such as the genus Vibrio or Alteromonas, and accumulates in the blowfish body through the food-chain.
(*3) Toxins accumulated in bivalve shellfish through the ingestion of toxic plankton are known as shellfish poison.
(*4) Ciguatera toxins are a well-known fish poison. The poisonous component is ciguatoxin ($C_{59}H_{84}O_{19}$). It is said to be several tens of times more potent that blowfish poison. The ciguatera toxins are thought to be generated by toxic dinoflagellates. Similarly to blowfish poison, they accumulate in fish at the peak of the food-chain.

3. Incidence of Food Poisoning

Bacterial food poisoning often occurs in warm periods from spring to summer (May to September); viral food poisoning often occurs in winter (November to March); while naturally occurring food poisoning and chemical food poisoning can occur at any time.
In 2008, 1,369 food poisoning outbreaks occurred in Japan, affecting 24,303 patients (*). Of these, viral food poisoning was the most common type, affecting 11,630 patients, or 47 % of the total (*).

This was followed by bacterial food poisoning, with 10,331 patients, representing 43 % of the total. (*) In addition, 2,342 patients suffered naturally occurring food poisoning and chemical food poisoning, which is 10 % of the total (*) (Fig. 1). The causative agent most responsible for food poisoning in Japan is Campylobacter. This food poisoning is caused by eating undercooked meat. Norovirus is the virus that causes the most viral food poisoning. This food poisoning is caused by eating raw or undercooked shellfish (particularly bivalves) which have the virus attached to their surface.

(*) Source: "Information on Food Poisoning," Japanese Ministry of Health, Labour and Welfare
http://www.mhlw.go.jp/topics/syokuchu/10hassei/xls/H20joukyou.xls

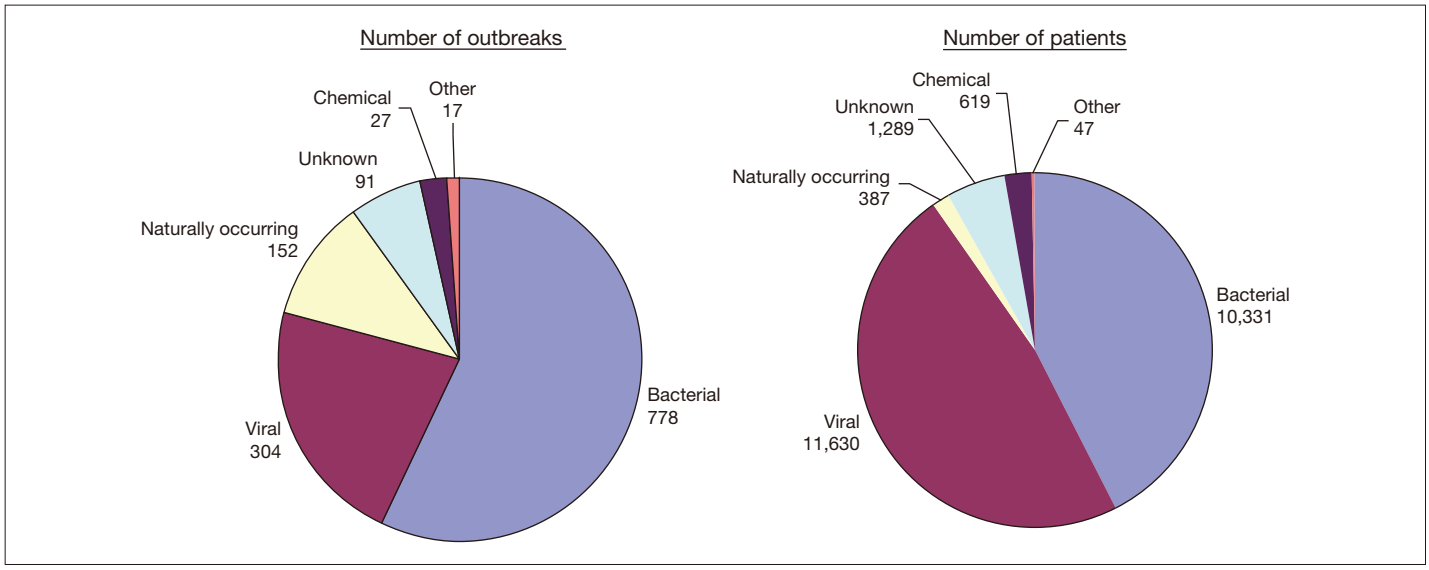


Fig. 1 Number of Food Poisoning Outbreaks and Patients in Japan in 2008, by Cause (*)

4. Food Poisoning
4-1 Bacterial Food Poisoning

Currently, 16 types of bacteria are designated as food-poisoning causative agents in Japan (*1) (Table 2). The types that most commonly cause food poisoning in Japan are Campylobacter, Salmonella, Vibrio parahaemolyticus, and enterohemorrhagic E. coli (O157, etc.).
Annual trends of the incidence of bacterial food poisoning classified by cause indicate a decreasing incidence caused by Salmonella and Vibrio parahaemolyticus from about 1998 or 1999, while the number of outbreaks caused by Campylobacter has been increasing (*2) (Table 2).
In 2008, 778 bacterial food poisoning outbreaks occurred in Japan, affecting 10,331 patients (*3). The Campylobacter jejuni/coli strain resulted in the largest number of food poisoning patients: 3,071, or 30 % of all bacterial food poisoning cases (*3).
The next major cause was Salmonella, with 2,551 patients, representing 25 % of all cases (*3) (Table 3).
Campylobacter, which is the causative agent responsible for the largest number bacterial food poisoning cases in Japan, is widely dispersed in nature, including inside the intestines of cows, pigs, and chickens. The foods most often responsible for this food poisoning are meat (particularly chicken), drinking water, and milk.
Salmonella is widely distributed in rivers and sewers. It is carried by animals such as cows, pigs, and chickens, and is highly resistant to drying. The foods causing this type of food poisoning are mainly beef, pork, chicken meat and eggs. Vibrio parahaemolyticus exists in seawater and reproduces dramatically when the water temperature rises in summer. This food poisoning is mainly caused by seafood, such as fish and shellfish. Enterohemorrhagic E. coli (O157, etc.) lives in the intestines of cows and other animals. This type of food poisoning is caused by food or drink contaminated with excrement. As enterohemorrhagic E. coli produces verotoxin (VT), it is known as Verotoxin-producing E. coli (VTEC).
This food poisoning can be caused not only by foods or drinking water, but can also result from secondary infection via cooking utensils contaminated with the causative agent or by people carrying it.

(*1) Source: "Partial Amendments to the Enforcement Ordinance for the Food Sanitation Act," No. 1836, Environmental Health Division, December 28, 1999
http://www1.mhlw.go.jp/topics/syokueihou/tp1228-1_13.html
(*2) Source: "Information on Food Poisoning," Japanese Ministry of Health, Labour and Welfare
http://www.mhlw.go.jp/topics/syokuchu/xls/nenji.xls
(*3) Source: "Information on Food Poisoning," Japanese Ministry of Health, Labour and Welfare
http://www.mhlw.go.jp/topics/syokuchu/10hassei/xls/H20joukyou.xls

Table 2 Causative Agents of Bacterial Food Poisoning (*1)

1	Salmonella	9	Yersinia enterocolitica
2	Staphylococcus aureus	10	Campylobacter jejuni/coli
3	Clostridium botulinum	11	Nonagglutinable vibrios
4	Vibrio parahaemolyticus	12	Vibrio cholerae
5	Enterohemorrhagic E. coli	13	Shigella
6	Other pathogenic E. coli	14	Typhoid bacillus
7	Clostridium perfringens	15	Paratyphoid A
8	Bacillus cereus	16	Other bacteria

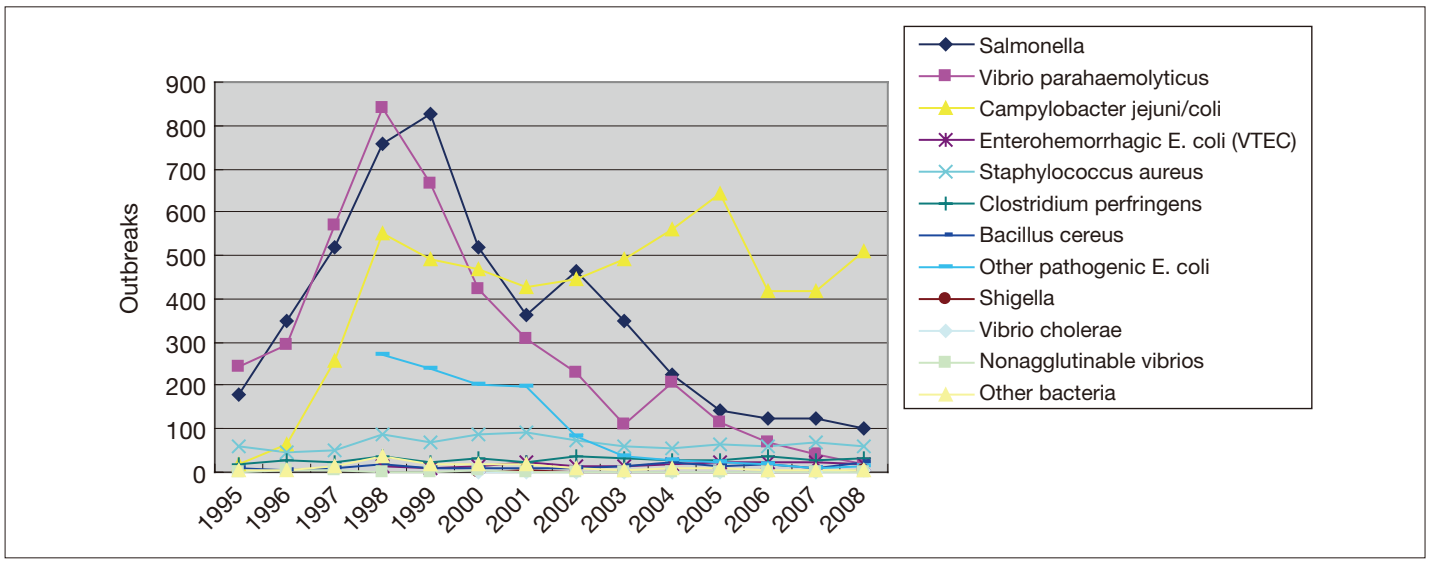


Fig. 2 Number of Bacterial Food Poisoning Outbreaks in Japan, by Cause (1995 to 2008) (*2)

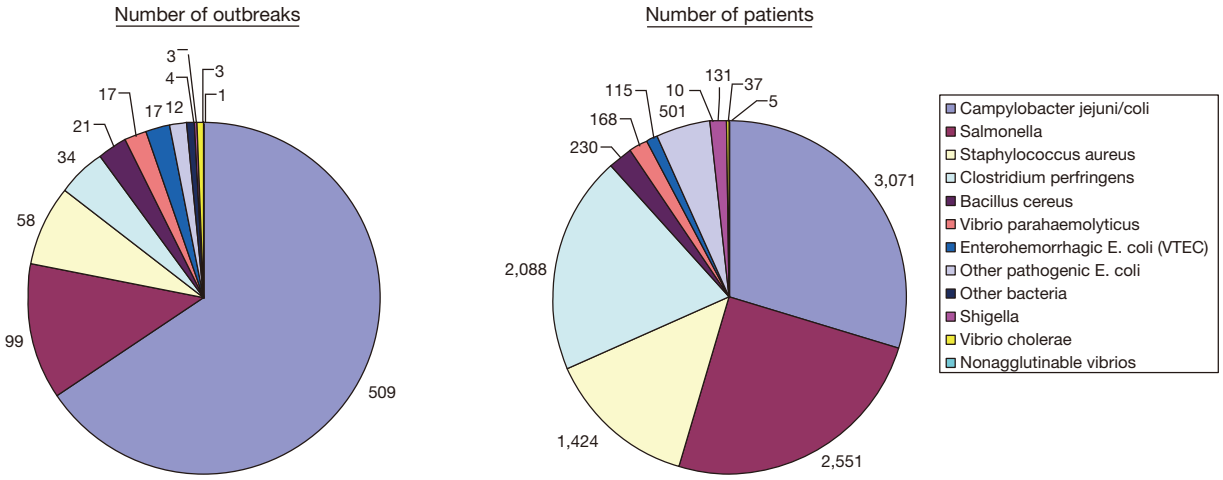


Fig. 3 Number of Bacterial Food Poisoning Outbreaks and Patients in Japan in 2008, by Cause (*3)

4-2 Viral Food Poisoning

Viral food poisoning can be caused by various viruses, including norovirus, rotavirus, sapovirus, adenovirus, and astrovirus. Viral food poisoning is the most common type of food poisoning in Japan, most of which is caused by norovirus. Of the 24,303 food poisoning patients in Japan in 2008, 11,618 patients contracted viral food poisoning due to norovirus, representing 47.8 % of the total (*).

Norovirus adheres to and is accumulated in bivalve shellfish, especially raw oysters. While bivalve shellfish (especially raw oysters) are the major food causing this food poisoning, it can also be contracted through foods (particularly uncooked foods) contaminated by a person carrying the virus. Therefore, many patients contract viral food poisoning due to norovirus via secondary infection from person to person.

(*) Source: "Information on Food Poisoning," Japanese Ministry of Health, Labour and Welfare
http://www.mhlw.go.jp/topics/syokuchu/10hassei/xls/H20joukyou.xls

5. Molds

Molds growing on foods produce toxins that can cause food poisoning. Mycotoxin is a generic term for secondary metabolite toxins produced by molds. Mold toxins related to food poisoning are called mycotoxins. Over 300 types of mycotoxin exist. The major mycotoxins are aflatoxins on peanuts and their processed products and on corn. Other types are the trichothecene mycotoxin deoxynivalenol that is produced by the mold that causes scab disease on cereals and patulin that occurs on rotten apples.
Many types of molds produce mycotoxins that cause food poisoning (Table 3).

Table 3 Molds Producing Mycotoxins

Mycotoxin	Major Mold
Aflatoxin	*Aspergillus flavus*
	Aspergillus parasiticus
	Aspergillus nomius
Trichothecene mycotoxin deoxynivalenol	*Fusarium graminearum*
	Fusarium culmorum
Patulin	*Penicillium expansum*
	Aspergillus clavatus
Ochratoxin	*Aspergillus ochraceus*
	Penicillium viridicatum
Citrinin	*Penicillium citrinum*
	Penicillium viridicatum

6. Analysis of Food-Poisoning Bacteria and Molds

To prevent the spread of food poisoning, it is necessary to identify the causative agent and take the appropriate measures. To ensure the safety of foods, tests must be performed accurately, easily, and rapidly at the food manufacturing stage. For norovirus, in particular, the 2008 "Hygiene Control Manual for Commercial Kitchens," No. 0618005, issued by the Department of Food Safety, Ministry of Health, Labour and Welfare of Japan prescribes a test for norovirus as part of the stool examinations for cooking staff at large facilities, where required.
Conventionally, various methods are used to identify the causative agents of food poisoning, depending on the type of causative agent, including genetic-level examinations and culturing on agar or ELISA (*1) to determine the biochemical status.
Recently, genetic-level examinations, such as PCR (*2), have become widely adopted (Fig. 4).
The inspection method by PCR for some food-poisoning bacteria is prescribed in the Food Hygiene Inspection Policies (Microorganism), edited by the Japanese Ministry of Health, Labor and Welfare.
In addition, it is not possible to determine visually (color, etc) or by sense of smell whether molds that cause food poisoning produce mycotoxins. Consequently, inspection methods are required for all molds that grow on foods.

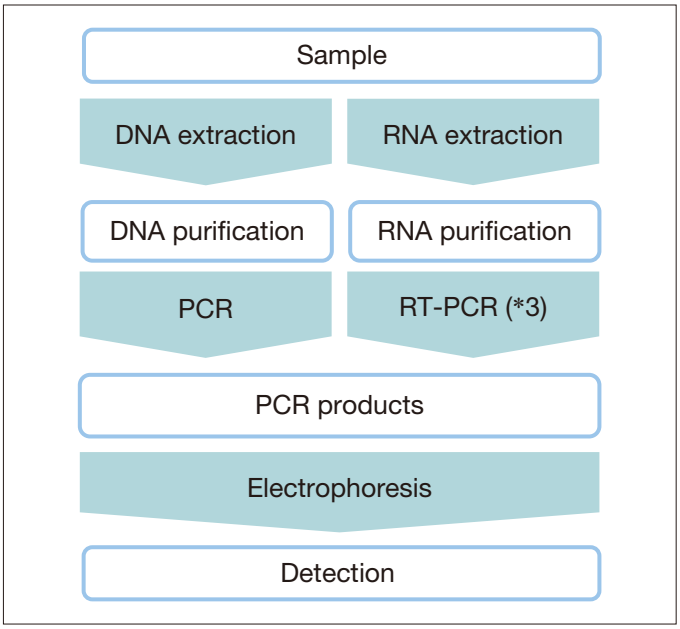


Fig. 4 Example of Detection by PCR

(*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) 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 repeatedly 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.

(*3) Reverse Transcription – Polymerase Chain Reaction (RT-PCR)
This is a method of gene amplification that combines reverse transcription (to synthesize DNA from RNA) and PCR to detect RNA.

7. 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. 5 MultiNA Microchip

Fig. 6 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. 7 MultiNA Operation Screen

8. Ampdirect® Plus DNA Amplification Reagent Enzyme Set

As analysis of the genome sequence information progresses for humans and other organisms, the sequence information is starting to become applied in a diverse range of fields, including medical science and medical treatment. The polymerase chain reaction (PCR), which is a DNA amplification technology, is an essential basic technology for such gene examinations.

Normally, the PCR amplification of genes requires the extraction and purification of template DNA. However, the revolutionary Ampdirect® Plus DNA Amplification Reagent developed by Shimadzu Corporation eliminates or simplifies this DNA extraction and purification process to accelerate, simplify, and reduce the cost of gene detection.

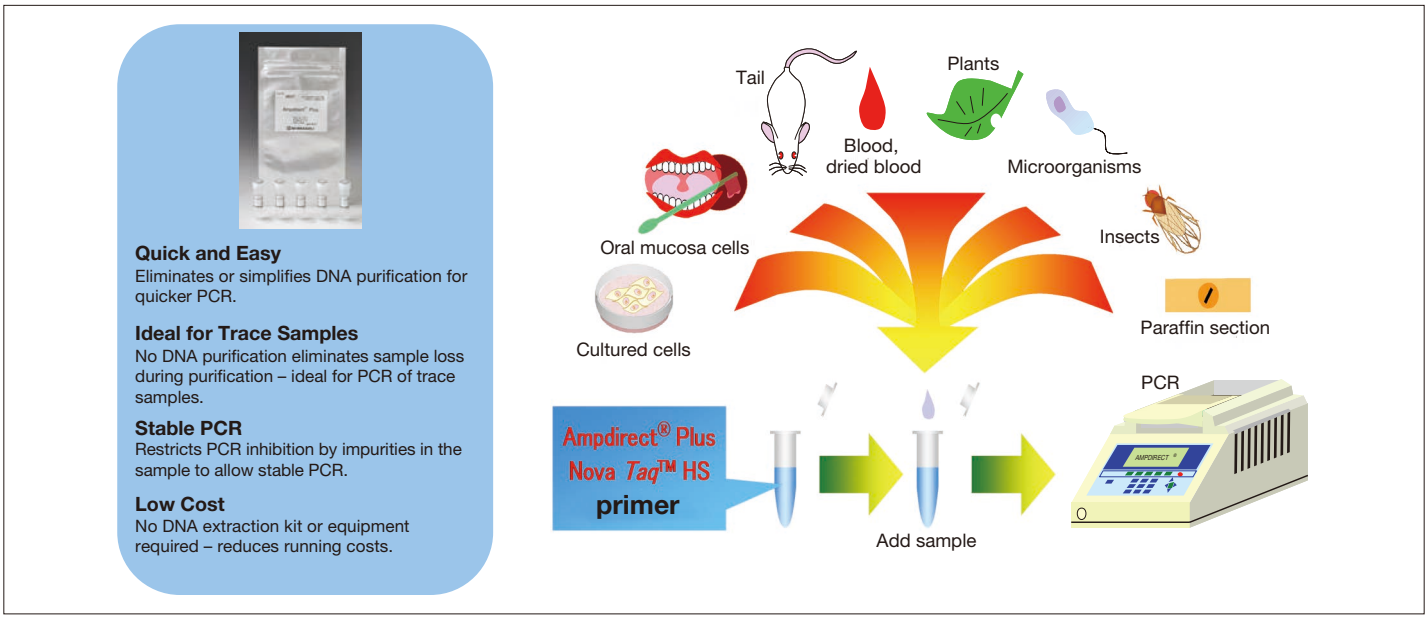


Fig. 8 Ampdirect® Plus DNA Amplification Reagent

9. Norovirus G1/G2 Detection Reagent Kit

As norovirus cannot be cultured, PCR amplification is widely used for virus detection. However, as biosamples contain large amounts of enzymes that degrade genes and substances that inhibit PCR amplification, it is necessary to separate the virus from the biosample and then extract and purify the genes it contains.

The Norovirus Detection Reagent Kits restrict gene-degrading enzymes and PCR amplification inhibitors in biosamples to permit the direct amplification and detection of norovirus genes from a biosample without gene purification.

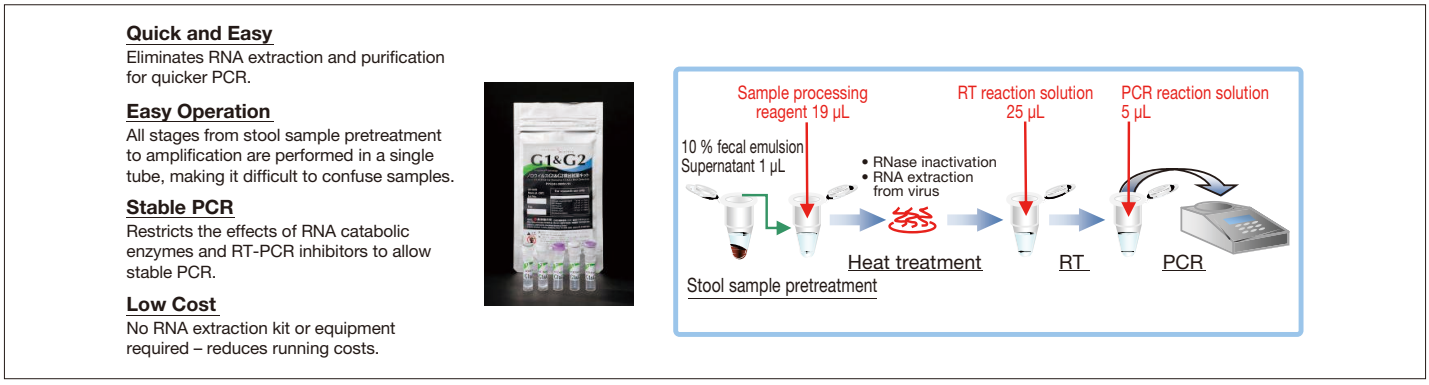
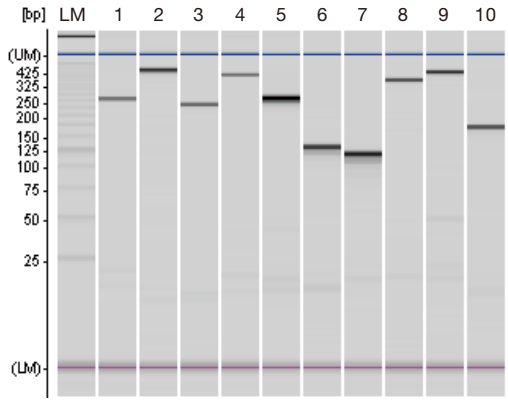


Fig. 9 Norovirus G1/G2 Detection Reagent Kit

10. Example of Detection of Food Poisoning-Related Genes Using MCE-202 MultiNA Microchip Electrophoresis System

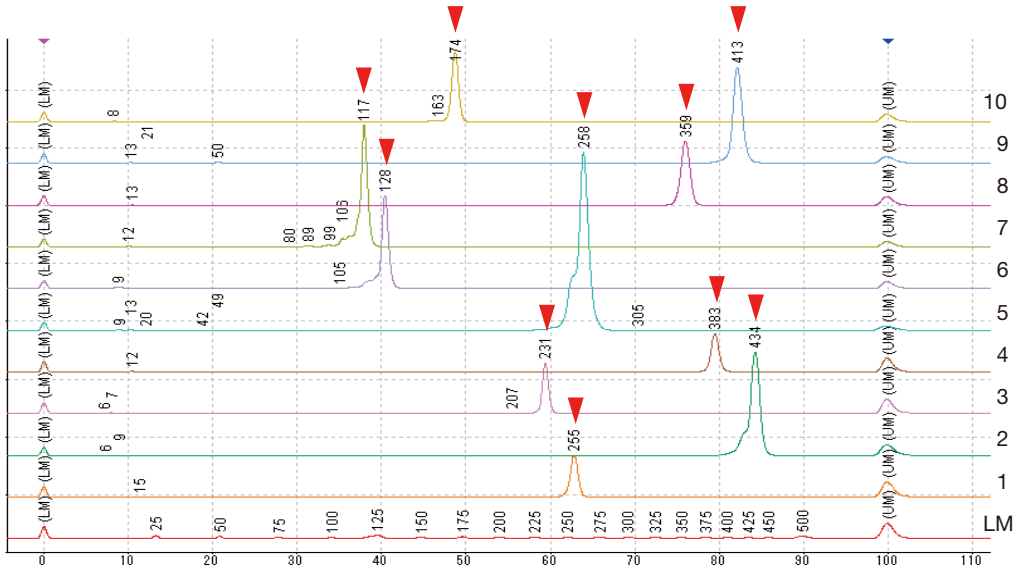
The samples were DNA extracted and purified from the microorganism strain.
PCR was performed using Shimadzu Ampdirect® Plus DNA Amplification Reagent, and the PCR amplification products obtained were analyzed using MultiNA. The results are shown in Fig. 10.

MultiNA was able to clearly detect the PCR amplification products in each area of interest. (The estimated size values in the diagram were obtained from this test.)
MultiNA provides conclusive data, as it obtains both a gel image (Fig. 10-a) and electropherogram (Fig. 10-b). MultiNA achieves superior resolution and sensitivity to agarose gel electrophoresis, it offers distinct detection of the gel image and electropherogram.
The primers used for PCR are commercially available from Takara Bio Inc.



(a) Gel Images

LM: Ladder Marker (25 bp DNA Ladder)
Lane 1 : Vibrio parahaemolyticus, heat-resistant hemolytic toxin gene (trh1&2) (250 bp)
Lane 2 : Staphylococcus aureus enterotoxin A gene (423 bp)
Lane 3 : Staphylococcus aureus toxic shock syndrome gene (228 bp)
Lane 4 : Salmonella invA gene (378 bp)
Lane 5 : Enterotoxigenic E. coli LT gene (263 bp)
Lane 6 : Enterotoxigenic E. coli STh gene (131 bp)
Lane 7 : Enterotoxigenic E. coli STp gene (123 bp)
Lane 8 : Enterohemorrhagic E. coli VT1 gene (349 bp)
Lane 9 : Enterohemorrhagic E. coli VT2 gene (404 bp)
Lane 10: Enterohemorrhagic E. coli VT1, VT2 gene (171 bp)



(b) Electropherogram

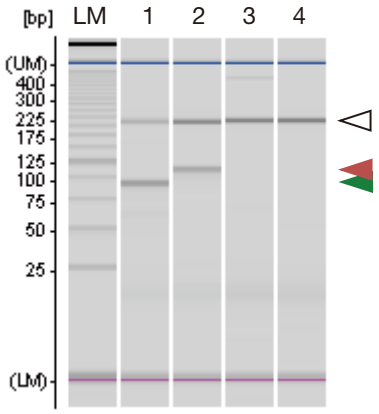
Fig. 10 Analytical Results of Food Poisoning-Related Genes

Reference:
Shimadzu Application News No. B22 "Detection of Food Poisoning-Related Genes with MCE-202 MultiNA

11. Example of Detection of Norovirus G1/G2 Genes Using MCE-202 MultiNA Microchip Electrophoresis System

The samples were norovirus G1/G2 positive samples and negative samples from stool samples.
All stages from stool sample pretreatment to RT-PCR were performed according to the instruction manual supplied with the Shimadzu Norovirus G1/G2 Detection Reagent Kit. Fig. 11 shows the results of MultiNA analysis on the RT-PCR amplification products obtained.

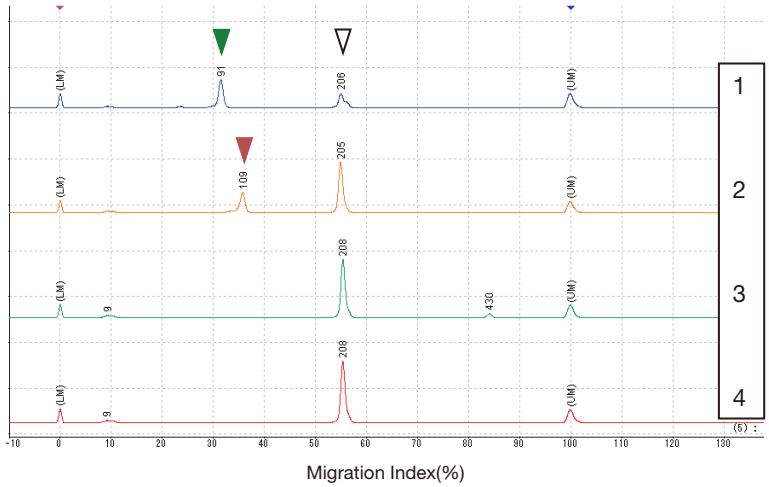
Specific amplification products were clearly detected for the norovirus G1 positive fecal sample and G2 positive fecal sample.
(The estimated size values in the electropherogram (Fig. 11-b) were obtained from this test. Using the Norovirus G1/G2 Detection Reagent Kit produced 85 bp amplification products for norovirus G1, 101 bp amplification products for norovirus G2, and 204 bp to 211 bp amplification products for the internal control.)



(a) Gel Images

▶ : Norovirus G1 (85 bp)
▶ : Norovirus G2 (101 bp)
▷ : Internal control (204 bp to 211 bp)

LM: Ladder Marker (25 bp DNA Ladder)
Lane 1: Norovirus G1 positive fecal sample
Lane 2: Norovirus G2 positive fecal sample
Lane 3: Norovirus negative fecal sample
Lane 4: Distilled water



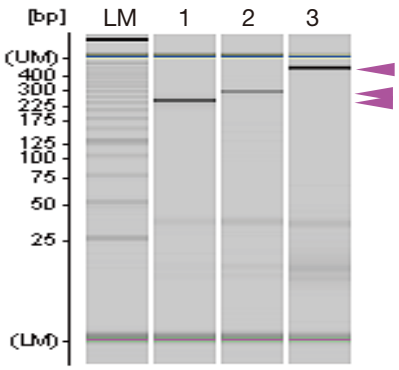
(b) Electropherogram

Fig. 11 Analysis Results of Norovirus G1/G2 Genes

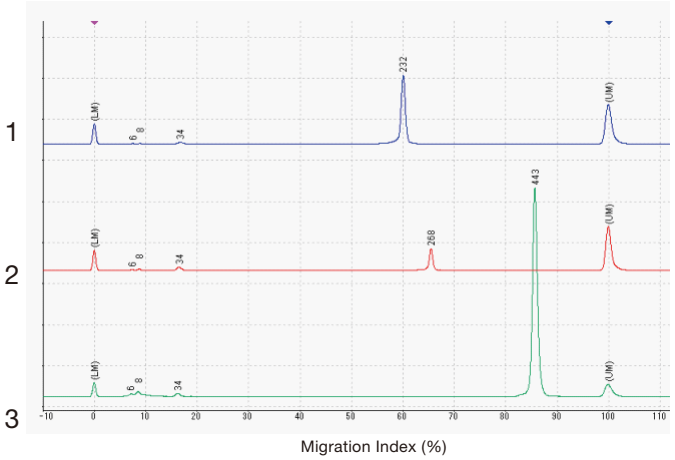
12. Example of Detection of Mold and Yeast Genes Using the MCE-202 MultiNA

Samples consisted of two types of mold and one type of yeast.
Eurotium is a type of mold which grows in dried foods, such as dried goods, bread, filled buns, and jam, etc. Penicillium, also referred to as "blue mold," is a genus of mold that occurs in many types of food, such as citrus fruits, grains, and dairy products. There are various types of Penicillium, ranging from beneficial varieties that are used in foods such as in the production of cheese, to harmful types such as toxic mold. Saccharomyces cerevisiae is budding yeast, and includes such varieties as baker's, wine, and sake yeast.

For the PCR reaction reagent, we used Shimadzu's "Ampdirect® Plus DNA Amplification Reagent Enzyme Set," and the PCR reaction conditions used are listed in the included instruction manual.
The mold and yeast that had been cultured in agar medium were attached to a micropipette tip, then suspended in the PCR reaction solution, and PCR was conducted.
Primers (ITS primers, for fungi, designed for quick identification of microorganisms by genetic analysis as described in the Japan pharmacopeia (*2)) were used for detection of ITS regions (*1) when conducting PCR.



(a) Gel Image



(b) Electropherogram

LM : Ladder Marker (25bp DNA Ladder)
1 : Eurotium chevalieri (Product Length 229bp)
2 : Penicillium digitatum (Product Length 261bp)
3 : Saccharomyces cerevisiae (Product Length 448bp)

Fig. 12 Analytical Results of Mold and Yeast Genes

(*1) The Internal Transcribed Spacer (ITS) regions refer to the 2 regions between the 3 ribosomal RNA genes (rDNA), 18 S, 5.8 S and 28 S, (ITS 1 between 18 S and 5.8 S, ITS 2 between 5.8 S and 28 S). It is known that there are differences in the base sequences in these ITS regions among different kinds of bacteria.
(*2) The Japanese Pharmacopeia is book containing standard criteria for pharmaceutical products intended to provide guidelines for establishing standard properties and quality of pharmaceutical products.

Reference:
Shimadzu Application News No. B27 "Detection of Mold and Yeast Genes with MCE-202 "MultiNA""

* 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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