**Revised in January 2007 (3rd Edition)
*Revised in October 2005 (2nd Edition)

[For food inspection]

FASTKIT ELISA Ver. II Milk <<Instruction Manual>>

* Please read this manual carefully before using the kit.

Cat# NPH-999100424-EX

- Development history and characteristics -

With the amendment of Food Sanitation Law-related laws and regulations in April 2002, food manufacturers are now required to specify proteins derived from the designated five raw materials considered likely to induce food allergy (egg, milk, wheat, buckwheat, and peanut) on the product label for all food products containing at least a few μg/mL or μg/g of such proteins. To provide accurate protein information, it is important to perform an assay for the designated raw materials to confirm the content of protein and exclude possible raw material derived carry-over or unexpected contamination during the manufacturing process.

This product is an enzyme-linked immunosorbent assay (ELISA)-based measurement kit for milk protein in foods. It has been confirmed to meet the "Guidelines for Evaluating Test Methods for Food Products Containing Allergic Substances" as specified in the PFSB/DFS Notification No. 1106001 from the Director of the Dept. of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare dated November 6, 2002, “Test methods for food products containing allergic substances” (partially amended according to the PFSB/DFS Notification No. 1011002 dated October 11, 2005, PFSB/DFS Notification No. 0324001 dated March 24, 2006, and PFSB/DFS Notification No. 0622003 dated June 22, 2006 from the director of the same department)**.

[Characteristics]

1) This kit has high sensitivity in the measurement of milk proteins in a food product.
2) The kit can identify more than one milk protein in a food product at the same time.
3) The kit can be applied to a wide variety of samples from raw materials to processed foods.

- Contents of kit -

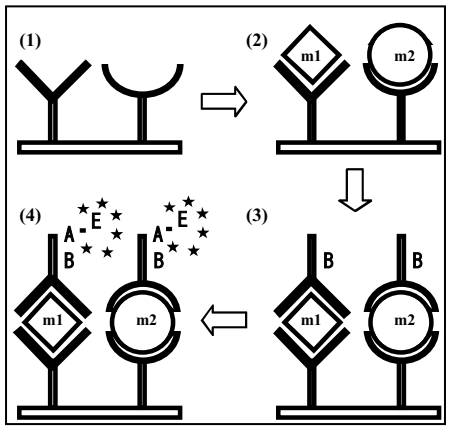
A: Antibody immobilized plate (with cover)	96 wells (8 wells × 12 strips) × 1
B: Standard solution (50 ng/mL) <manufactured by Nippon Gene Co., Ltd.>	1.8 mL × 1
C: Dilution buffer	100 mL × 1
D: Biotin-labeled antibody	150 μL × 1
E: Enzyme (peroxidase)-labeled streptavidin	150 μL × 1
F: Chromogenic substrate (TMB)	12 mL × 1
G: Stop solution (0.5 N H_2SO_4)	12 mL × 1
H: Concentrated washing solution (1/10 concentration)	100 mL × 1
I: Extraction reagent ① (1/20 concentration)	50 mL × 1
J: Extraction reagent ② (1/20 concentration)	50 mL × 1
K: Extraction reagent ③ (1/20 concentration)	50 mL × 1
L: Instruction manual	1

- Objective/performance -

- This kit is intended to measure milk proteins in food products.
- This kit can measure standard milk proteins in a solution at a concentration ranging from 0.78 to 50 ng/mL. *

- Measurement principle -

(1) An antibody to bind to more than one milk protein has been immobilized in the plate wells.
(2) The immobilized antibody captures multiple milk proteins (m1, m2…) in the measurement solution to form complexes.
(3) The Biotin-labeled antibody binds to the complexes to form sandwich complexes.
The Enzyme-labeled streptavidin then binds to the sandwich complexes through the reaction between biotin and streptavidin. The enzymatic substrate (TMB) is then added to produce a color.



- Necessary apparatuses -

[Preparation of reagents]

- Measuring cylinders, beakers, and micropipettes

[Preparation of measurement solution]

- Grinding machine (food cutter), balance, 50-mL plastic centrifugal tubes (with cap), shaking machine, centrifugal machine (A centrifugal machine of 3,000 × g or higher and with an ability of centrifugation at room temperature is recommended), filter paper, and funnel

[Measurement operation and data analysis]

- Micropipette, test tubes or micro tubes, blotting paper (paper towel), micro-plate reader (with filters with a wavelength of 450 nm and 600 to 650 nm), and analytical software (that can use the 4-parameter analysis)

- Preparation of reagents -

[Reagents to be used as supplied]

- Dilution buffer: Let it warm to room temperature (20 to 25°C) before use.
- Chromogenic substrate: Collect a necessary amount into a light-resistant container and let it warm to room temperature (20 to 25°C) before use.
- Stop solution: Let it warm to room temperature (20 to 25°C) before use.

[Reagents to be prepared before use]

- Sample extracting medium: Prepare a sample extracting medium by thoroughly shaking a mixture consisting of the extraction reagents ①, ②, and ③ and purified water at a ratio of 1:1:1:17 before use.
- Diluent for reference standard: Prepare a 1/20 dilution of the sample extracting medium with the Dilution buffer.
- Standard solution: Dilute the standard solution with the diluent for the reference standard with reference to the following dilution examples.

<Example of preparing sample extracting medium: preparation for 24 samples>

Extraction reagent ① (1/20 concentration)	: 25 mL
Extraction reagent ② (1/20 concentration)	: 25 mL
Extraction reagent ③ (1/20 concentration)	: 25 mL
Purified water	: 425 mL
Total	: 500 mL

＜Examples of dilution of standard solution＞

Concentration of reference standard (ng/mL)	50	25	12.5	6.25	3.125	1.5625	0.78125	0
Amount of standard solution to be added (μL)	800	400	400	400	400	400	400	0
Amount of diluent for reference standard to be added (μL)	0	400	400	400	400	400	400	400

- Concentrated washing solution: Prepare a 1/10 dilution of the concentrated washing solution using purified water.
- Biotin-labeled antibody: Prepare a 1/100 dilution of the Biotin-labeled antibody using the Dilution buffer that was let warm to room temperature beforehand. Use the dilution within 15 minutes.
- Enzyme-labeled streptavidin: Prepare a 1/100 dilution of the Enzyme-labeled streptavidin using the Dilution buffer that was let warm to room temperature beforehand. Use the dilution within 15 minutes.

＜Diluents to be used and objects to be diluted＞

Objects to be diluted	Standard solution	Supernatant from extraction or filtrate	Biotin-labeled antibody	Enzyme-labeled streptavidin
Dilution buffer		○	○	○
Diluent for reference standard	○			

Note 1) Keep the standard solution, Biotin-labeled antibody, and Enzyme-labeled streptavidin in a refrigerator until immediately before and immediately after use.
Note 2) Do not mix or replace the same reagent from different lots.
Note 3) Do not use the reagents for which the designated measurement parameter is specified on the label (Antibody immobilized plate, standard solution, Biotin-labeled antibody, and Enzyme-labeled streptavidin) for other measurement parameters. Reagents without designated measurement parameter may be used for all measurement parameters.
Note 4) Accurately perform pipetting including dispensing and diluting because it greatly influences measurement accuracy. Replace the micropipette tip at every dispensing or diluting operation.
Note 5) Although the extraction reagent ② may become cloudy and form precipitates during storage or distribution, the changes do not indicate any loss of performance. Warm it by putting it in warm water before use to melt the precipitates.

- Extraction operation (Example of operation for a general food product) -

1) Uniformly crush a package unit of a food sample (test sample) using a Grinding machine to a prepareed sample (Note 1).
2) Weigh one gram of the prepared sample into a plastic centrifugal tube. Add 19 mL of the sample extracting medium into the tube. Mix them well to prepare a uniform dispersion (Note 2).
3) Set the centrifugal tube horizontally in a shaking machine and shake it overnight (at least 12 hours) at room temperature to obtain an extract (Notes 3 and 4).
4) Centrifuge the extract at 3,000 × g for 20 minutes to collect the supernatant. If no sediment occurs, pass the centrifuged extract through a filter (Notes 5 and 6).
5) Prepare a 1/20 dilution of the collected supernatant or filtrate using the Dilution buffer and use it as a measurement solution.

<Example of extraction operation>

Food sample
↓ Uniformly crush using grinding machine
1 g of prepared sample
↓ Mix with 19 mL of prepared sample extracting medium
Shake overnight (at least 12 hours) at room temperature
Extraction sample
↓ Centrifuge at 3,000 × g at room temperature (for 20 minutes)
Supernatant from centrifugation
↓ Pass through filter, as required
Supernatant or filtrate
↓ Prepare 1/20 dilution using buffer solution for dilution
Measurement solution

Note 1) To prevent contamination by experimental apparatuses, use only well-cleaned or disposable apparatuses. Thoroughly clean the Grinding machine or homogenizer every time it is used (clean it using a neutral detergent and immerse it in an alkaline detergent solution overnight or ultrasonically clean it in an alkaline detergent solution for at least 30 minutes).
Note 2) Fully disperse the sample using a vortex mixer, taking care to prevent bubbling.
Note 3) Set the shaking cycle at 90 to 110 cycles per minute and the shaking width at about three centimeters so that the solution in the tube will hit both ends of the tube during shaking. Occasionally turn the centrifugal tube upside down to prevent the sample from adhering to the tube at the fluid level.
Note 4) Determine pH of the extract and, if necessary, adjust it to a neutral area (pH 6.0 to 8.0).
Note 5) Keep the collection volume of the supernatant as constant as possible. Remove the oil layer, if possible. It is recommended that filtration should be performed to obtain accurate results.
Note 6) Keep the collected supernatant or filtrate at 4°C and use it for measurement within one week. It is recommended that the supernatant or filtrate should be cryopreserved if it is to be kept for one week or longer. The allowable storage period under refrigeration or cryopreservation depends on the ingredients of the food product.*

- Measurement operation -

1) Let the Antibody immobilized plate warm to room temperature in the original aluminum pouch. Remove it from the pouch immediately before use (Note 1).
2) Add 100 µL each of the diluted standard and measurement solutions into the wells (Note 2).
3) Lightly agitate the solution in the wells and allow the plate to stand for one hour at room temperature (20 to 25°C) for reaction.
4) After the reaction is completed, discard the standard and measurement solutions. Add 250 µL of the diluted washing solution to each well and discard it, and repeat this procedure five times (Note 3).
5) Add 100 µL of the prepared Biotin-labeled antibody solution to each well.
6) Lightly agitate the solution in the wells and allow the plate to stand for one hour at room temperature (20 to 25°C) for reaction.
7) After the reaction is completed, discard the Biotin-labeled antibody solution and wash the plate as described in the above Step 4.
8) Add 100 µL each of the prepared Enzyme-labeled streptavidin solution to each well.
9) Lightly agitate the solution in the wells and allow the plate to stand for 30 minutes at room temperature (20 to 25°C) for reaction.
10) After the reaction is completed, discard the Enzyme-labeled streptavidin solution and wash the plate as described in the above Step 4.
11) Let the Chromogenic substrate warm to room temperature beforehand and add 100 µL of the reagent to each well.
12) Lightly agitate the reagent in the wells and allow the plate to stand for 20 minutes at room temperature (20 to 25°C) to make the reagent produce a color. It is recommended that this step should be performed under a light-shielded condition to increase data reproducibility.
13) Let the stop solution warm to room temperature beforehand and add 100 µL of the solution to each well. Lightly agitate the solution to stop color development (Note 4).
14) After agitation, measure the absorbance at the primary wavelength of 450 nm and secondary wavelength of 600 to 650 nm using a plate reader.

<Example of measurement operation>

Antibody solid-phased plate
↓ Add 100 µL of diluted standard and measurement solutions to each well
Agitate and allow to stand at room temperature (for one hour)
Washing
↓ Add 100 µL of biotin-bound antibody solution to each well
Agitate and allow to stand at room temperature (for one hour)
Washing
↓ Add 100 µL of Enzyme-labeled streptavidin solution to each well
Agitate and allow to stand at room temperature (for 30 minutes)
Washing
↓ Add 100 µL of Chromogenic substrate to each well
Agitate and allow to stand at room temperature (for 20 minutes)
↓ Add 100 µL of stop solution to each well
Agitate and measure absorbance (450 nm/600 to 650 nm)

Note 1) When a part of the plate is used, return the unused strips into the aluminum pouch with a desiccant and keep the pouch in a refrigerator.
Note 2) It is recommended that three wells each should be used for the measurement of the standard and measurement solutions. Make sure to prepare a standard curve based on the measurements of the standard solution for every measurement.
Note 3) The cleaning operation is critical for accurate measurement. Completely remove the solution and air bubbles in the wells by holding the plate upside down and strongly tapping it onto a power towel several times, and then add the next reagent immediately.
Note 4) Handle the stop solution carefully because it contains 0.5 N sulfuric acid.

- Data analysis -

1) Prepare a standard curve graph from the absorbance values of the standard solution using the 4-parameter analysis (Note 1).
2) Read the milk protein concentration in the measurement solution (ng/mL) from the standard curve.
3) Multiply the milk protein concentration by the dilution factor during the extraction operation (400) to calculate the milk protein concentration in the food product.

Note 1) The measurement results may vary when the standard curve graph is prepared using an analytical technique other than the the 4-parameter analysis.

- Validation results ** -

[Samples]

- White rice porridge, *oshiruko* (*azuki* bean soup with *mochi*), *kamaboko* (steamed fish paste), meatballs, coffee jelly, and *miso* soup: Each sample contained milk primary standard powder (Nippon Gene Co., Ltd.) at a protein concentration of 10 µg/g.

[Participating institutions]

13 institutions

- Osaka Prefectural Institute of Public Health
- Kanagawa Prefectural Institute of Public Health
- San-Ei Gen F.F.I., Inc.
- Japan Food Research Laboratories
- Yokohama City Institute of Health
- Oriental Yeast Co., Ltd. Nagahama Biological Research Center
- Kawasaki City Institute of Public Health
- Japan Inspection Association of Food and Food Industry Environment
- Japanese Consumers' Cooperative Union Product Inspection Center
- Kagome Co., Ltd. Research Institute
- Saitama Institute of Public Health
- Life Quality Science Laboratory Inc.
- Mizkan Group Co., Ltd. headquarters

(Arranged according to the Japanese syllabary)

[Validation procedure]

The document containing the extraction method, kit operation method, and report form and six samples were sent to each participating institution where each sample was extracted and measured for milk proteins in duplicate. Three wells were measured for the measurement of each extract. The calibration curves of eight concentrations (including a blank) were determined on the same plate and the results obtained were sent back to the Nippon Meat Packers, Inc. R&D Center.

The Nippon Meat Packers, Inc. R&D Center examined the data from each institution with the Cochran and Grubbs tests to exclude outliers according to the procedure of JIS Z8402-2 (significance level of 1.0% for both tests) and then determined the mean, repeatability, and reproducibility.

[Validation results]

The recovery, repeatability (RSD_r), and reproducibility (RSD_R) obtained from the validation of the kit are shown in the table below. Both recovery rate and reproducibility (RSD_R) met the acceptance criteria of assay tests (recovery rate: 50% or higher and 150% or lower, reproducibility: 25% or lower) as specified in the notification (PFSB/DFS Notification No. 1106001from the Director of the Dept. of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare dated November 6, 2002 "Test methods of food products containing allergic substances").

Table: FASTKIT ELISA Ver. II Milk validation results

Sample	Number of participating institutions	Recovery rate	Repeatability (RSD_r)	Reproducibility (RSD_R)
White rice porridge	12	89.2%	3.4%	4.4%
Oshiruko	12	100.3%	3.4%	5.6%
Kamaboko	11	74.4%	3.7%	4.0%
Meatball	13	80.8%	3.2%	8.3%
Coffee jelly	12	96.7%	4.1%	4.5%
Miso soup	13	73.6%	4.0%	9.9%

- False positive/false negative -

1) No cross reactivity has been observed with the designated raw materials except for milk (egg, wheat, buckwheat, and peanut).
2) The test at our laboratory confirmed that the kit produced a false positive result for the following substances: goat milk, ewe milk, and *funori* seaweed. For the other false positive results, refer to the "List of food products producing false positive or false negative results" on the homepage of Nippon Meat Packers, Inc. R&D Center on the web.**
3) The kit may cause a non-specific reaction under the presence of a very high concentration of a protein. In such a case, appropriately dilute the sample and repeat the test. Always use the diluent for the reference standard when a 1/20 dilution of a sample is further diluted.
4) Lactose may produce a false negative result because it contains a limited amount of milk protein.

- Precautions for use or handling -

[General precautions]

1) Read this instruction manual carefully and observe the operation procedures.
2) Do not use the kit or its component after its expiration date. The expiration date is indicated on the outer package and each component's label.
3) This kit is intended for measuring the designated raw materials in food products and not for making a diagnosis of food allergy. The correlation between the measurement results of the kit and development of allergic symptoms has not been established.
4) Make a comprehensive decision on the presence/absence of the designated raw materials by examining not only the result of the present kit, but also other data including raw materials and manufacturing records.
5) For how to use the machines and apparatuses used with the kit, refer to the instruction manuals provided by their manufacturers/distributors.
6) The present instruction manual complies with the notification from the Director of the the Dept. of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare "Test methods of food products containing allergic substances"* and is intended to serve as guidelines for those in charge of food inspection. Verify the validity of the application of each inspection step to each food product.
7) The specifications of this kit are subject to change without prior notice.**

[Safety precautions]

1) Take care to prevent the exposure of the skin, mucosal membrane, or clothes to the reagents of the kit.
2) When any of the reagents accidentally enters the eye or mouth, immediately take appropriate first-aid treatment, such as rinsing with tap water, and receive medical attention.

[Precautions for disposal]**

1) Observe local regulations to dispose of the kit, samples, and measurement solution, etc. with full attention to the possible effect on sanitation and environment.

- Storage and expiration date -

1) Storage: Keep this kit under a refrigerated (2 to 8°C) and light-shielded condition. Avoid freezing the kit.
2) Expiration date: Six months from the date of manufacture: The expiration date is indicated on the outer package and each component's label.

- References -

1) PFSB/DFS Notification No. 1106001 from the Director of the Dept. of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, "Test methodss for food products containing allergic substances" (partially amended according to the PFSB/DFS Notification No. 1011002, PFSB/DFS Notification No. 0324001, and PFSB/DFS Notification No. 0622003)**
2) Marui E.: Food Sanitation Research, Vol. 52 (5), 25-31, 2002
3) Akiyama H. and Toyoda M.: Food Sanitation Research Vol. 52 (6), 65-73, 2002

Manufactured by Nippon Meat Packers, Inc. R&D Center

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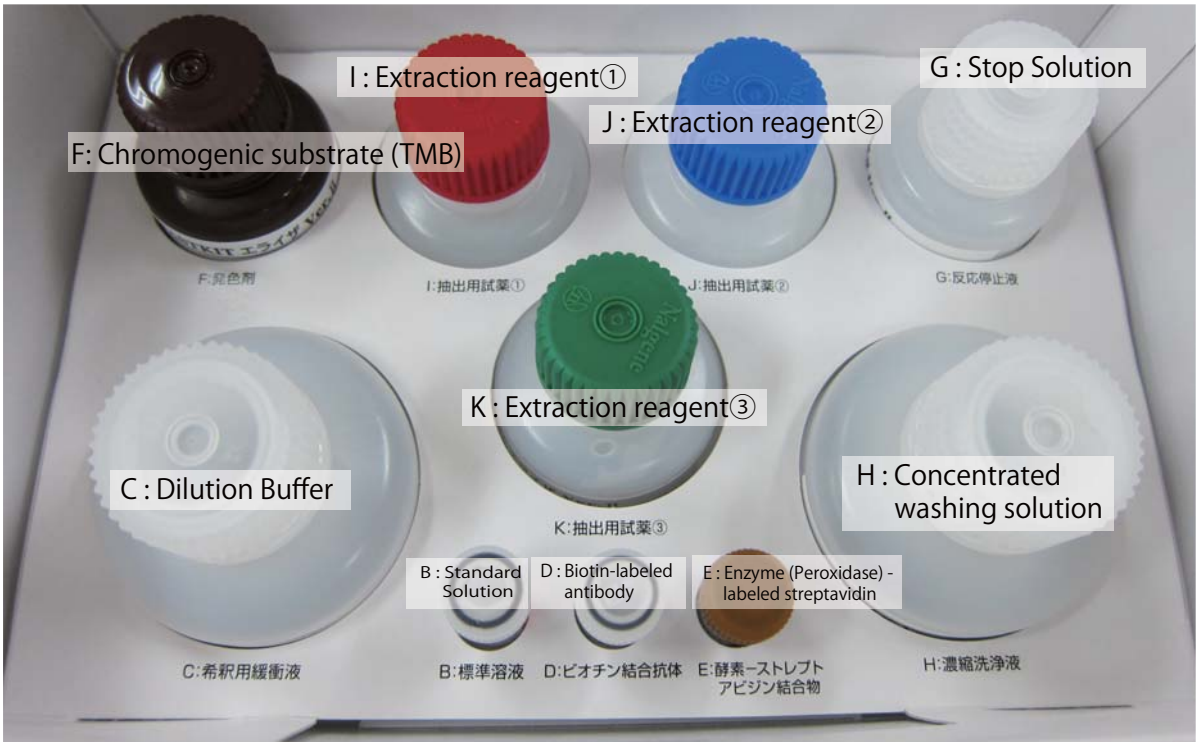
I : Extraction reagent①
G : Stop Solution
J : Extraction reagent②
F: Chromogenic substrate (TMB)
F:発色剤
I:抽出用試薬①
J:抽出用試薬②
G:反応停止液
K : Extraction reagent③
C : Dilution Buffer
H : Concentrated washing solution
K:抽出用試薬③
B : Standard Solution
D : Biotin-labeled antibody
E : Enzyme (Peroxidase) - labeled streptavidin
C:希釈用緩衝液
B:標準溶液
D:ビオチン結合抗体
E:酵素−ストレプトアビジン結合物
H:濃縮洗浄液