Developed: October 16, 2001
Latest Revision: September 20, 2005

Implementation Guideline for Transmissible Spongiform Encephalopathy (TSE) Testing

1 Purpose

This guideline aims to ensure the safety of meat, following the identification of Bovine Spongiform Encephalopathy (hereinafter referred to as "BSE") case in Japan. To relieve the public concerns, tests shall be conducted for Transmissible Spongiform Encephalopathy (hereinafter referred to as "TSE"), based on the Abattoirs Law (Law No. 114 in 1953; hereinafter referred to as "the Law"), to ensure that TSE-infected bovine, sheep and goat shall not be distributed for food, while contributing to the assessment of TSE infection status in Japan.

2 Necessity of TSE tests

To ensure safety of meat at abattoirs, it is necessary to exclude flesh, etc. derived from TSE-infected animals, which may be contaminated by high-density abnormal prion protein, in combination to adequate removal of specified risk materials, in which abnormal prion is accumulated. For this purpose, TSE tests shall be conducted in conformity of Article 14 of the Law.

3 Instructions concerning application for TSE tests, and notes on receiving the tests

(1) Applicants for carcass inspection shall be instructed beforehand on the following issues. Instructions shall be also given to related businesses as necessary.

a Applicants shall indicate the ages of bovines on a month basis, when submitting application for carcass inspection stipulated in Section 1, Article 15 of the Implementation Rules for the Abattoirs Law (hereinafter referred to as "Rules"). Applicants shall also attach documents identifying month ages of the bovines, such as copy of database for individual identification of cattle, in conformity with the Law Concerning Special Measures for the Management and Transmission of Information on Individual Identification of Cattle (Law No. 72 in 2003); calf registration certificate (issued by the Wagyu Registry Association), and/or pedigree registration certificate (issued by the Japan Holstein Registry Association) as necessary.

As for sheep and goat, it is desirable to attach documents identifying month ages of the sheep or goat, such as sheep/goat pedigree registration certificate (issued by the Japan Livestock Technology Association).

b Applicants shall also make prior confirmation on the information concerning names, addresses, etc. of senders/raisers of the cattle. This information is necessary for survey by the supervising livestock agencies, in case of TSE tests turn positive.

(2) Attention shall be paid on the following points, when receiving application for carcass inspection.

a Confirm the month ages of applied bovines, in both the "age" column of carcass inspection application and documents specified in (1) a.

As for the month ages of sheep/goat, confirm the documents as specified in (1) a, if such documents are attached.

b If information as specified (1) b is not clear, instruct the applicant to make necessary confirmations.

4 Ante-mortem inspection

(1) In the inspection based on provisions of Section 1, Article 14 of the Law, conduct the dentition examination for bovines, and make comprehensive judgment of month ages, referring to the documents stipulated in 3 (1) a, attached to the carcass inspection application. If the third incisor tooth is present, the bovine shall be judged 30 months or older, regardless of the information on carcass inspection application.

Conduct the dentition examination for sheep and goat as well. Eruption of the second permanent molars (in both upper and lower jaws) shall be an indicator for the judgment of 12 months old.

(2) Also check the existence of strange cries, turning around and other abnormal behaviors, ataxia and other nervous symptoms, combined with the results of gait checks.

Refer to the video concerning clinical symptoms of Transmissible Spongiform Encephalopathy, dispatched based on the Clerical Notice dated March 26, 2003, for the implementation of ante-mortem inspection.

5 Measures based on the results of ante-mortem inspection

(1) If the tested bovine, sheep or goat corresponds to 4 (2) as a result of ante-mortem inspection, and is suspected of TSE-infection ("suspected case" as stipulated in Article 2 of the Domestic Animal Infectious Diseases Control Law), slaughtering or dressing of such bovine/sheep/goat shall be prohibited in accordance with the provisions of Item 1, Article 16 of the Law, because slaughtering or dressing of such livestock may lead to contamination by the infectious agent (i.e. abnormal prion protein).

(2) If a meat inspection station takes the measure stipulated in (1), that station shall notify the fact to the applicant, the abattoir founder, and other related parties. The station shall also report to food sanitation departments of the relevant prefectures and municipalities operating health centers (hereinafter referred to as "prefectures"), which shall in turn report to livestock departments of the relevant prefectures. The information shall be also provided to food sanitation departments and livestock departments of the prefectures supervising the sender's location.

(3) Even if a meat inspection station takes the measure of prohibiting slaughtering or dressing in accordance with the provisions of Item 1, Article 16 of the Law, for bovine/sheep/goat indicating systemic symptoms in which TSE symptoms are not identified, but for sepsis, severe jaundice, or other reasons than the above, that station shall also notify the fact to the applicant, the abattoir founder, and other related parties.

The station shall also report to food sanitation departments of the relevant prefectures, which shall in turn report to livestock departments of the relevant prefectures. The information shall be also provided to food sanitation departments and livestock departments of the prefectures supervising the sender's location.

6 Post-mortem inspection

(1) Inspection for bovines, etc.

a As a rule, screening tests shall be conducted on the day of slaughter for bovines aged 21 months or older.

Screening tests may be conducted for bovines aged 20 months or younger, if it is considered necessary by livestock inspectors from disease identification point of view.

b Results of the screening and confirmatory testing shall be compiled in the following categories:

a) Bovine aged 24 months or older, indicating suspected symptoms including movement disorders, perception disorders, reflection/consciousness disorders and other nervous symptoms, and/or systemic symptoms in ante-mortem inspection;
b) Bovine aged 30 months or older; and
c) Other bovines.

(2) Tests for sheep and goat, etc.

a As a rule, screening tests shall be conducted on the day of slaughter for sheep/goat aged 12 months or older.

Screening tests may be conducted for sheep/goat aged 11 months or younger, if it is considered necessary by livestock inspectors from the standpoint of disease differentiation.

b Results of the screening and confirmatory testing shall be compiled.

(3) Sampling, etc.

For samples of bovine/sheep/goat, take medulla oblongata through the foramen magnum opening by using spatula technique and so on. Use part of one side of the medulla oblongata as sample for screening test, conducted in conformity with "Guideline for Screening Tests of Transmissible Spongiform Encephalopathy (TSE)" (Attachment 1). The remainder of medulla oblongata shall be frozen as sample to be sent.

The other side of medulla oblongata shall be fixed with 15 - 20% solution of buffer formalin, as sample to be sent. This fixation shall be performed promptly after sampling. If it is difficult, the fixation must be performed promptly after the results of ELISA test are given.

If medulla oblongata is damaged during the pithing operation, and the position for sampling is not clear, samples shall be taken from multiple locations.

(4) Measures to be taken in case the screening test turns positive

Following the conclusion of the screening test (i.e. end of the second ELISA test), and the test results turn out to be positive, then the meat inspection station shall immediately notify the fact to the applicant, the abattoir founder, and other related parties. The station shall also report to food sanitation departments of the relevant prefectures, which shall in turn report to livestock departments of the relevant prefectures. The information shall be also provided to food sanitation departments and livestock departments of the prefectures supervising the location of the sender of the relevant bovine/sheep/goat.

To conduct the confirmatory testing for the relevant bovine/sheep/goat, send the samples put aside in (3) to institutions designated in a separate notice. If the testing should be conducted by prefectures, the testing shall conform to Separate Attachment 2, "Procedure for Confirmatory Testing of Transmissible Spongiform Encephalopathy (TSE) by Prefecturral and City Governments."

(5) Notification on results of the confirmatory testing

Results of the confirmatory testing shall be notified to food sanitation departments of the relevant prefectures, by way of the Inspection and Safety Division, Department of Food Safety, Pharmaceutical and Food Safety Bureau, MHLW (hereinafter referred to as "Inspection and Safety Division"), from the institution stipulated in (4), which has conducted the confirmatory testing. If the testing has been conducted by prefectures, the results shall be notified to the Inspection and Safety Division by way of food sanitation departments of the relevant prefectures.

Food sanitation departments of the relevant prefectures shall notify the results of confirmatory testing immediately to the applicant, the abattoir founder, and other related parties. Food sanitation departments shall also provide information to livestock departments of the relevant prefectures, as well as food sanitation departments and livestock departments of the prefectures supervising the sender's location.

(6) Definitive diagnosis

If the results of screening and confirmatory testing turn out TSE-positive, the Expert Committee for the Testing of Bovine Spongiform Encephalopathy of the Ministry of Health, Labour and Welfare shall make the definitive diagnosis.

(7) Measures concerning carcass, etc. during screening and confirmatory testing

While screening and confirmatory testing are underway, dressed carcass, viscera, blood (to be reused only), bone, hide, heads, legs, tails, etc. of the corresponding bovine/sheep/goat, including the separated parts to be disposed, shall be stored so that identification should be available, and that the edible parts should not be contaminated by microbes, etc. If storage by individual carcass is difficult, several carcasses or a daily handling lot may be stored together. In case of TSE-positive, such lots may be treated (i.e. incinerated) together.

7 Measures based on the results of post-mortem inspection

The following measures shall be taken under the observance of livestock inspectors, etc., in conformity with Item 3 or 4, Article 16 of the Law:

(1) In case the screening or confirmatory testing turns positive

a Ensure the implementation of disinfection and other measures stipulated in 8, of facilities, equipment, machines, utensils, etc. that have, or might have, come into contact with bovine head (excluding tongue and cheek), spinal cord and distal ileum (two meters from connection to appendix); tonsil, spleen, small and large intestines (including lymph nodes attached to them) of sheep and goat aged 12 months or older; and head (excluding tongue, cheek and tonsil), spinal cord and placenta, as listed in Attached Table 1 of the Rules (hereinafter referred to as "parts listed in Attached Table 1"). As for facilities, equipment, machines, utensils, etc. that do not come into contact with the parts listed in Attached Table 1, thorough washing should be done.

b Incinerate as necessary part of dressed carcass, viscera, blood (to be reused only), bone, hide, heads, legs, tails, etc. of the corresponding bovine/sheep/goat, including the separated parts to be disposed, which have been stored as stipulated in 6 (7).

(2) In case the definitive diagnosis proves TSE

Prefectures receiving notification that the definitive diagnosis has proven TSE from the Inspection and Safety Division shall take the following measures:

a Ensure the implementation of disinfection and other measures stipulated in 8, of facilities, equipment, machines, utensils, etc. that have, or might have, come into contact with the parts listed in Attached Table 1, but have not been treated in (1) a. As for facilities, equipment, machines, utensils, etc. that do not come into contact with the parts listed in Attached Table 1 as well, thorough washing should be done.

b Incinerate dressed carcass, viscera, blood (to be reused only), bone, hide, heads, legs, tails, etc. of the corresponding bovine/sheep/goat, including the separated parts to be disposed, which have been stored as stipulated in 6 (7), but have not been incinerated in (1) b.

8 Disinfection methods for parts to be disposed, etc.

Inactivation methods for pathogens, in case TSE-infected bovine/sheep/goat has been identified in tests, stand as follows at present. Ensure the implementation of disinfection and other measures in conformity with the following:

(1) Complete incineration at Min. 800C° (of carcasses, rubber gloves, protective garments, etc.)

(2) Pressurized steam sterilization at 132 to 134C° for an hour (of utensils, etc.)

(3) Treatment in Min. 1-mol solution of sodium hydroxide, at 20C° for an hour (of facilities, dirt, etc.)

(4) Treatment in sodium hypochlorite solution of Min. 2% effective chlorine concentration, for an hour (of facilities, dirt, etc.)

9 Instructions to founders, managers, slaughterers and their employees

(1) The parts listed in Attached Table 1 shall be removed in the treatment process, and incinerated following the confirmation of livestock inspectors.

(2) If the storage cooling equipment is too small for the storage of carcasses during TSE tests, solve the problem by using the existing facilities effectively, such as separation of storage refrigerators, so as the carcasses shall not contaminate others.

(3) This Guideline stipulates necessary measures with regard to TSE tests. If necessity arises to restrict the use of abattoirs, as a result of implementing this guideline, that restriction shall be regarded as for "due reasons," stipulated in Article 11 of the Law.

The number of animals processed shall be reviewed as necessary.

If TSE-positive bovine, sheep or goat is identified, the relevant carcass, etc. must be incinerated. Therefore, available incinerating facilities must be designated beforehand.

10 Reporting of test results to the Inspection and Safety Division, and their publication

(1) If food sanitation departments of prefectures take the measure of prohibiting slaughtering or dressing as stipulated in 5 (1), the relevant sanitation departments shall report the fact to the Inspection and Safety Division immediately, using Attached Form 1.

(2) As for screening, results from the previous Sunday through to Saturday shall be reported by 6:00 p.m. of Monday next week (If holiday falls on Monday, the day following the holiday(s)). Use the Meat Inspection Support System for bovine, and report by Attached Form 2 for sheep/goat.

If the Meat Inspection Support System is out of order, or if tests are not conducted for bovines aged 20 months or younger, report those facts to the Inspection and Safety Division in Attached Form 3.

(3) The Inspection and Safety Division discloses TSE-infected cases as soon as the determinant diagnoses are given, and publishes every week the total number of animals proving negative in screening and confirmatory testing. There shall be no omissions in the result reports.

11 Others

In the case of screening or confirmatory testing for bovines without disorders, aged 20 months or younger, etc., which are not required by the Abattoirs Law, this Implementation Guideline shall be also applied to the handling of dressed carcass, viscera, etc. derived from tested bovines.

(Revision history)

October 18, 2001; partial revision: Addition to implementation guideline
November 5, 2001; partial revision: Change to handling of sample mailing
January 23, 2002; partial revision: Revision from daily to weekly reporting of screening
November 11, 2002; partial revision: Change to handling of sample mailing
February 28, 2003; partial revision: Addition to implementation guideline for confirmatory testing
July 1, 2003; partial revision: Addition of BSE diagnostic kit, etc.
April 13, 2004; partial revision: Addition of video concerning ante-mortem inspection, etc.
August 4, 2004; partial revision: Addition of screening kit, etc.
July 1, 2005; partial revision: Revision of month ages covered by the tests, etc.

(Attached Form 1)

Attn: Inspection and Safety Division,
Department of Food Safety,
Pharmaceutical and Food Safety Bureau,
Ministry of Health, Labour and Welfare

FAX: 03-3503-7964

Report on Identification of TSE-suspected Bovine, Sheep and Goat

1	Municipality			Abattoir name			
2	Date & time of slaughter prohibition		Date: Time:				
	Reason for the slaughter prohibition						
3	Relevant animal	Type Species		Sex	M / F	Age	month(s) old
4	Findings from ante-mortem inspection						
5	Remarks						
	No. of ear tags, etc.						

Attached Form 2

Weekly Results of Screening Test for Transmissible Spongiform Encephalopathy (TSE) of Sheep and Goat
Brought in : Month Date - Month Date

Municipality

	Sheep and goat indicating symptoms*1			Other sheep and goat			Total			No. of sheep and goat below 12 months old, for which tests were not conducted
	Negative	Positive	Total	Negative	Positive	Total	Negative	Positive	Total	
Month - Date										
Month - Date										
Month - Date										
Month - Date										
Month - Date										
Month - Date										
Month - Date										
Month - Date										
Month - Date										
Month - Date										
Month - Date										
Total										

*1 Sheep and goat indicating suspected symptoms including movement disorders, perception disorders, reflection/consciousness disorders and other nervous symptoms, and/or systemic symptoms in ante-mortem inspection

No. of incidents in which slaughter was prohibited due to suspected TSE between Month Date and Month Date : (incidents)

Attached Form 3

Weekly Results of Screening Test for Bovine Spongiform Encephalopathy (BSE)
Brought in : Month Date - Month Date

Municipality

	Bovine indicating symptoms *1			Bovine aged 30 months or older			Other bovine			Total			No. of bovine below 21 months old, for which tests were not conducted
	Negative	Positive	Total	Negative	Positive	Total	Negative	Positive	Total	Negative	Positive	Total	
Month - Date													
Month - Date													
Month - Date													
Month - Date													
Month - Date													
Month - Date													
Month - Date													
Month - Date													
Month - Date													
Month - Date													
Month - Date													
Total													

*1 Bovine aged 24 months or older, indicating suspected symptoms including movement disorders, perception disorders, reflection/consciousness disorders and other nervous symptoms, and/or systemic symptoms in ante-mortem inspection

No. of incidents in which slaughter was prohibited due to suspected BSE between Month Date and Month Date : (incidents)

(Separate Attachment 1)

Procedure for Screening of Transmissible Spongiform Encephalopathy (TSE)

Part 1. Handling of Prion Materials within the Laboratory

- Operations shall be performed in a sectioned exclusive laboratory and, as a general rule, within a safety cabinet.
- The operator shall wear latex or vinyl gloves, a mask, protective clothing and cap, as well as protective eyeglasses, etc. where necessary, in order to avoid contamination through wounds or droplet contamination through eyes or mouth.
- Disposable products shall be used where possible, including work clothing and various instruments and equipment.
- Handle test materials on a bench protection sheet, taking care to avoid generating droplets and aerosols.
- Upon spilling of any test materials or at the end of operation, wipe the work surface clean with sodium hypochlorite solution.
- Place used bench protection sheets and disposable instruments in an autoclave bag and sterilize by steam under pressure at 132-134°C for 1 hour.
- Apparatus such as scissors and tweezers, etc. which are to be reused must be wiped clean with paper such as tissue paper or alcohol-soaked cotton and soaked in 3-5% SDS and boiled for 5-10 minutes (metal corrosion may be controlled by adding sodium carbonate in the amount of 1%), or sterilized by steam under pressure at 132-134°C for 1 hour.
- Soak plastic instruments which can not be heated in sodium hypochlorite of 5% or higher, or in NaOH of 2-molar or higher, for 2 hours or longer.
- Place combustible materials in an autoclave bag and sterilize by steam under pressure at 132-134°C for 1 hour.
- If a medical waste incinerator is available for use, place plastic instruments and combustibles in biohazard bags and incinerate.
- If the exterior of a centrifuge tube which is required in subsequent procedures becomes contaminated, transfer the contents into a fresh tube and continue operations.
- In cases where only a regular autoclave is available, an effective means of sterilization is to place contaminated items in an alkali-proof container and soak in 1- to 2-molar NaOH and treat at 121°C for 30 minutes.
- As a general rule when decontaminating equipment and instruments which can not be incinerated, wipe highly contaminated items or masses of tissue having a high residual prion level first with combustibles such as paper or alcohol-soaked cotton to remove contaminants as much as possible before sterilizing.

Part 2. Sample Region

The head is removed along the dotted line in the figure. A spoon or spatula is inserted from the foramen magnum to collect a sample including the obex shown in Figure 1 (the region colored in black) and Figure 2. The other black regions are the skull and cervical vertebra. As lesions and prion accumulation occur roughly symmetrically, sample collection is divided along the midline. The sample from one side is fixed in 15-20% concentration buffered formalin as material for the histopathological and immunohistochemical examinations, while the remaining side is used as material for the immunobiochemical examination (ELISA, Western blot, etc.).

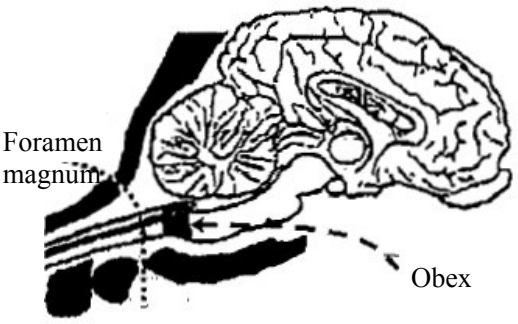


Figure 1. Bovine brain cross-section

Using the material for the immunobiochemical examination, the tissue is sectioned in cross sections containing the obex to serve as the samples for screening. As prion is not accumulated consistently throughout the obex, the sample needs to be balanced out. Therefore, in sample preparation, the tissue slices are roughly cut up into small pieces using scissors and averaged out before collecting the required amount. However, this method does not apply in cases where sampling is conducted using methods other than sectioning.

Part 3. Bovine Spongiform Encephalopathy (BSE) Test Kit Procedures

The screening test is to be performed using one of the following test kits: Platelia BSE, Dynabott Enfer BSE Test, or Frelisa BSE. The procedures for these kits are described in Separate Attachments 1-1, 1-2 and 1-3 respectively.

Part 4. Storage of Test Results

The raw data read by the microplate reader is to be entered in Separate Form 1-1. Once the remaining items in Separate Form 1-1 are filled in, the signed sheet and electronic data shall be stored. The sheet shall be stored pasted with the raw data read by the microplate reader (data on photosensitive paper shall be copied onto plain paper). If the sample is to be dispatched for confirmatory testing, a copy of the stored data pasted with raw data is to be attached.

Part 5. Dispatching Sample for Confirmatory Testing

Samples which tested positive[1] using a BSE test kit shall be dispatched for confirmatory testing according to the procedures indicated below, with the exception where confirmatory testing is to be conducted by the prefectural or city government.

[1] Refers to cases tested positive in the third repeat test.

1. Delivery address

The delivery address is indicated in the Notification No. 0407001 of the Inspection and Safety Division, Department of Food Safety of the Pharmaceutical and Food Safety Bureau (PFSB), Ministry of Health, Labour and Welfare, dated April 7, 2004, attached separately.

2. Method of notification of confirmatory test results

The Inspection and Safety Division shall inform the local authority who requested the confirmatory test.

Inquiries regarding the confirmatory test should be addressed to the Dairy and Meat Safety Section of the Inspection and Safety Division, at phone number 03-3595-2337.

3. Material for dispatching (As shown in Figure 2)

The obex and surrounding tissue is divided into two along the midline.

(1) One side constitutes material for histopathological and immunohistochemical examinations, fixed in buffered formalin of 15-20% concentration and shipped at room temperature. The sample shall be placed in a 50 mL container filled with buffered formalin.

(2) The remainder constitutes material for the immunobiochemical examination (ELISA, Western blot, etc.), to be shipped frozen. Any materials remaining after sample collection or after ELISA use (homogenized emulsion samples, etc.) are to be shipped frozen as well.

4. Method of dispatch notification

When dispatching samples which tested positive in the screening, a Table of Test Results (Separate Form 1-1) and a Notice of Sample Dispatch (Separate Form 1-2) shall be attached, and the date and time (9-12 am or 1-4 pm) of delivery to the testing laboratory shall be specified.

The Table of Test Results (Separate Form 1-1) and the Notice of Sample Dispatch (Separate Form 1-2) shall be faxed in advance to the Dairy and Meat Safety Section of the Inspection and Safety Division (Fax: 03-3503-7964), together with notification to the same by telephone (Phone: 03-3595-2337).

An emergency number for contact during non-working days, etc., will be provided at a later date.

5. Notes on sample dispatching

The samples shall be dispatched packaged in containers complying with UN specifications, based on Nos. 2 and 3, Article 8 of the Postal Regulations (Notification No. 34 of the Ministry of Communications, 1947).

Additional information regarding the sample shipping container as well as notes on packaging are to be announced separately.

Prior to depositing the package, an inquiry shall be made at the post office in charge of collection and delivery in your district (referred to hereinafter as the "district post office") regarding the mode of transportation of the postal package. Depending on the mode of transportation, measures shall made, as indicated below, before depositing the package at the post office.

(1) Sample-containing postal packages whose shipping process does not include air transport

A label in the following format with all necessary items filled shall be pasted on the postal package surface where clearly visible.

Item: Bovine tissue, etc. "Hazardous Substance" (Note 1) Sender: Name of Local Authority: Name of Laboratory: Address: Phone No.: Qualification: Livestock Inspector (Veterinarian) Name:

Note 1: Marked in red

(2) Sample-containing postal packages whose shipping process includes air transport (Note 3)

1) A label in the following format with all necessary items filled shall be pasted on the postal package surface where clearly visible.

Item: Bovine tissue, etc. "Hazardous Substance" (Note 1) UN No.: Sender: Name of Local Authority: Name of Laboratory: Address: Phone No.: Qualification: Livestock Inspector (Veterinarian) Name: Contains xx kg dry ice (Note 2)

Note 1: Marked in red
Note 2: To be marked in red if packaged in dry ice

2) Samples shall be stored in containers complying with UN specifications.

3) The contents of one container shall be limited to less than 1000 mL for liquids and 50 g for solids.

4) A Transportation Permit Label (Classification No.: 6.2) shall be pasted on the

postal package surface where clearly visible (Note 4).

5) If the container complying with UN specifications is surrounded by dry ice and packaged in a carton box, a Transportation Permit Label (Classification No.: 9) shall be pasted on the postal package surface where clearly visible (Note 4).

6) In the case of 5) above, the post office personnel may request opening of the external carton box when accepting the postal package in order to confirm that the sample is stored in a container complying with UN specifications, in which case the request should be catered to.

7) Two copies of the Hazardous Substance Declaration Form shall be prepared and presented with the package (Note 5).

   An open envelope stating "Contains Hazardous Substance Declaration Form" shall be pasted on the package. At the post office, the Hazardous Substance Declaration Form, after being reviewed and returned, it is to be enclosed in the pasted envelope in the presence of the post office personnel.

Note 3: Transportation by air is regulated by Article 86 of the Aviation Law, Article 194 of the Enforcement Regulations for the Aviation Law, and other relevant notifications.

Note 4: The format for the label is shown in Separate Form 1-3. (Request the required number of labels from the local post office.)

Note 5: The Hazardous Substance Declaration Form is shown in Separate Form 1-4. (This Form has been established by the Postal Service and various air carriers exclusively for the purpose of transporting the samples of this operation, and is not for use otherwise.)

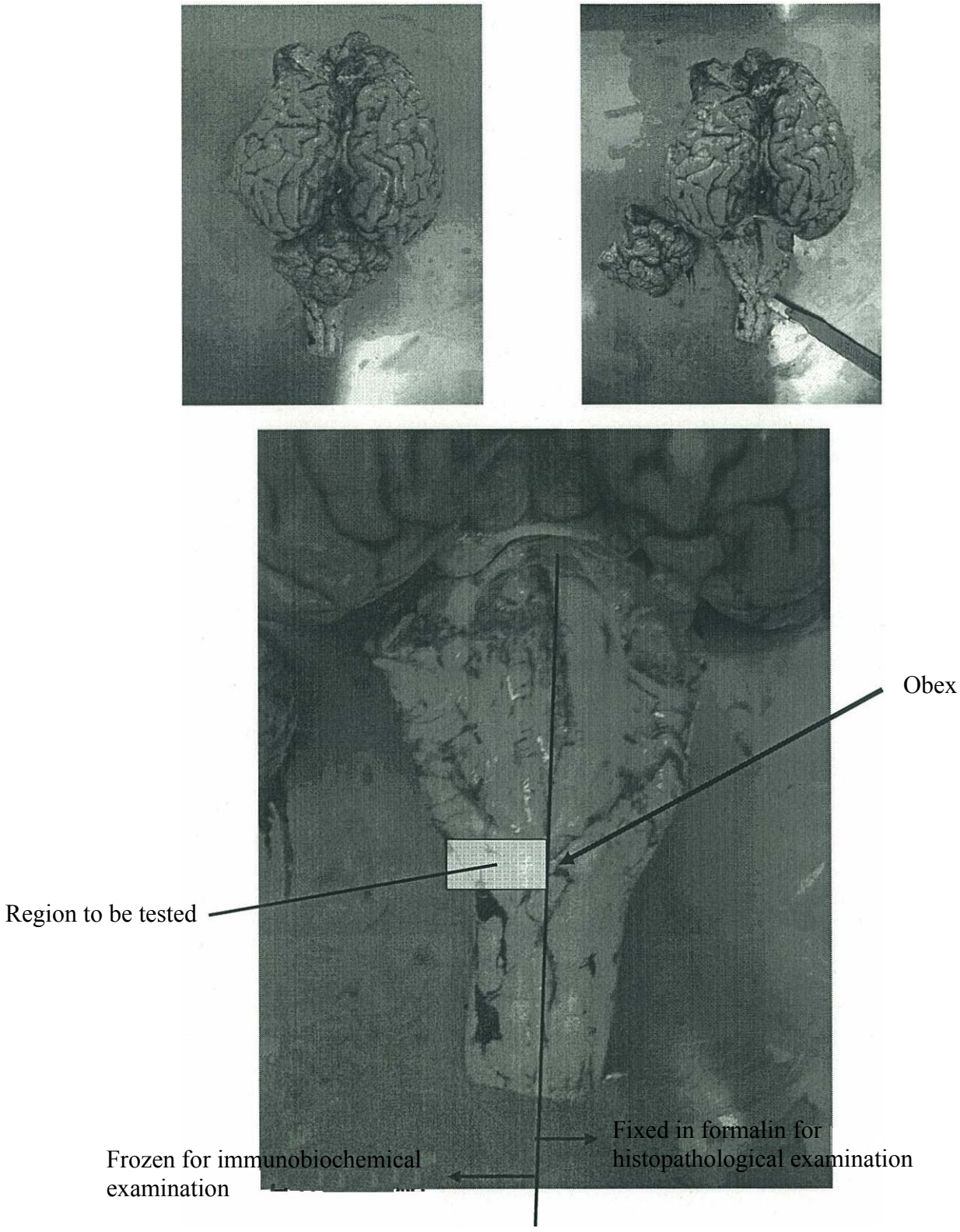


Figure 2. Sampling region for BSE screening

(Reference Material)

Treatment to Completely Inactivate Abnormal Prion Protein

Table 1. Treatment to completely inactivate abnormal prion protein[2]

Chemical Agent	Concentration	Treatment Time	Temperature
Formic acid	≥60%	2 hours	Room temp.
Guanidine thiocyanate	≥4 M	2 hours	Room temp.
Guanidine hydrochloride	≥7 M	2 hours	Room temp.
Trichloroacetic acid	≥3 M	2 hours	Room temp.
SDS	≥3%	5 min.	100°C
Phenol	≥50%	2 hours	Room temp.

Table 2. Methods for sterilizing contaminated materials[3]

Chemical Agent/ Method, etc.	Temperature (°C)	Time (min.)	Applicable items
Incineration	≥800	-	Organs, combustibles, etc.
Autoclave	134	60	Instruments, equipment, organs, etc.
Soaking in 3% SDS**	100	5	Instruments, equipment, etc.
Soaking in 2-molar NaOH	Room temp.	60	Instruments, equipment, etc.
Soaking in 1-molar NaOH	Room temp.	120	Instruments, equipment, etc.
Soaking in 1-5% sodium hypochlorite	Room temp.	120	Instruments, equipment, etc.

Each case is autopsied on a plastic sheet spread in an autopsy room, etc., and only minimal dissection shall be performed. When removing the head, blood is received in a container to minimize contamination. The removed head is placed in a plastic bag, and measures such as covering the neck with a plastic bag are taken to control the spread of contamination.

2 Onodera, T., T. Kitamoto, T. Kurata, T. Sato, J. Tateishi. *Creutzfeldt-Jakob Disease Treatment Manual* (Under editorship of Disease control Division, Health Service Bureau, Ministry of Health and Welfare). Tokyo: Shinkikaku Publishing Co., Ltd., 1997, pp18-23.

3 See 2 above

(Separate Form 1-1)

(Test / Repeat test)

Test date	D/M/Y:
Controlling local authority	
Laboratory name	
Analyst name (Signature)	
Cutoff value	
-10% from cutoff value	
Number of samples	
(No. of positive samples, no. of negative samples)	

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	← Record name of sample (Identification code, etc.)
	← Record measured value

*1 The table above represents a 96-well plate. Record data corresponding to the actual wells used in the test.

*2 Mark a diagonal slash in unused well positions.

(Separate Format 1-1) (Example)

(Test / Retest)

Test date	D/M/Y: 18/10/2001
Controlling local authority	×× City
Laboratory name	×× Meat Inspection Laboratory
Analyst name (Signature)	John Doe
Cutoff value	0.251
-10% from cutoff value	0.2259
Number of samples	18
(No. of positive samples, no. of negative samples)	(18, 0)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative control	bovine-3	bovine-11									
	0.041	0.172	0.104									
B	Negative control	bovine-4	bovine-12									
	0.040	0.132	0.082									
C	Negative control	bovine-5	bovine-13									
	0.042	0.156	0.101									
D	Negative control	bovine-6	bovine-14									
	0.041	0.122	0.166									
E	Positive control	bovine-7	bovine-15									
	2.53	0.098	0.145									
F	Positive control	bovine-8	bovine-16									
	2.45	0.087	0.191									
G	bovine-1	bovine-9	bovine-17									
	0.083	0.115	0.072									
H	bovine-2	bovine-10	bovine-18									
	0.106	0.095	0.126									

	← Record name of sample (Identification code, etc.)
	← Record measured value

*1 The table above represents a 96-well plate. Record data corresponding to the actual wells used in the test.

*2 Mark a diagonal slash in unused well positions.

(Separate Form 1-2)

Confirmatory Testing Laboratory: _____
Recipient: _____

Name of Local Authority: _____
Person in Charge: _____
Phone No.: _____

Notice of Dispatch: Sample for Transmissible Spongiform Encephalopathy Confirmatory Test

	Dispatch Date	Dispatching Institution (Laboratory name)	Sample No.	Sample Weight (g)	Date of Sampling (D/M/Y)	Data Regarding Animal of Sample Origin					Remarks
						Breed/ Variety	Sex	Age in Months	Clinical Symptoms	Slaughter Date (D/M/Y)	
1				Emulsion for ELISA							
				For WB							
				For pathological examination							
	Shipper					Breeder					
	Name	Address			Phone No.	Name	Address			Phone No.	
	Dispatch Date	Dispatching Institution (Laboratory name)	Sample No.	Sample Weight (g)	Date of Sampling (D/M/Y)	Data Regarding Animal of Sample Origin					Remarks
						Breed/ Variety	Sex	Age in Months	Clinical Symptoms	Slaughter Date (D/M/Y)	
2				Emulsion for ELISA							
				For WB							
				For pathological examination							
	Shipper					Breeder					
	Name	Address			Phone No.	Name	Address			Phone No.	

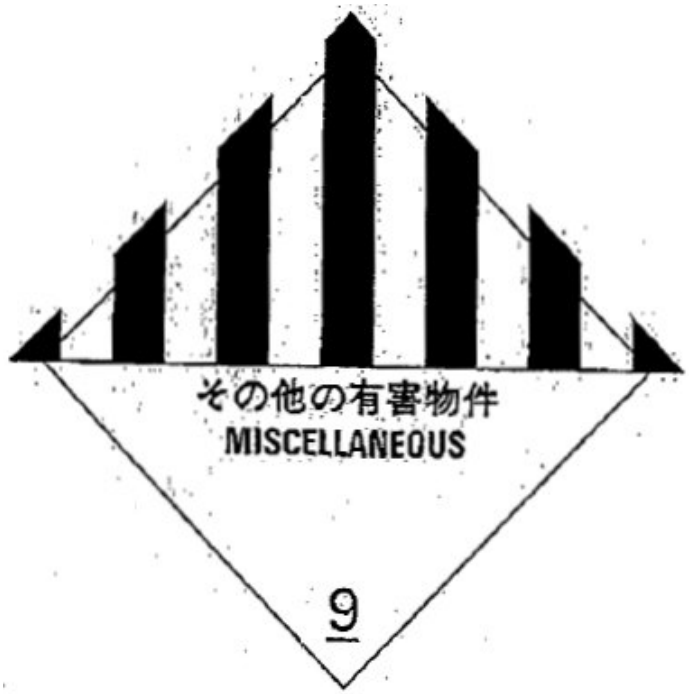
Expected date and time of arrival of sample at confirmatory testing laboratory
Date: , Between and AM
Between and PM

Screening Kit Type/ Lot No.:

(Separate Form 1-3)

1. Transportation Permit Label (Classification No.: 6.2)

2. Transportation Permit Label (Classification No.: 9)



(Separate Form 1-4)

(Air Transportation)

Declaration of Hazardous Substance in Postal Matter (Bovine tissue, etc.)

The item name and quantities, etc. described below regarding this postal package are entirely accurate. The material is stored in a container complying with UN specifications, and packaged, labeled, etc. according to the Aviation Law and its relevant regulations. This postal package is within the carrying limits of aircraft and is in suitable condition for air transport.

Date of Form Filling		D/M/Y:	
Item		Bovine tissue, etc.	
	UN2814 UN2900	Highly infectious substance transmissible to humans and animals (Liquid)	(Note 1) mL
	UN2814 UN2900	Highly infectious substance transmissible to humans and animals (Solid)	(Note 2) g
	UN1845	Dry ice	kg
	Stored in a container complying with UN specifications, packed with dry ice and further packaged in another container or such.		

Sender
Local Authority:
Laboratory :
Address:
Phone No.:
Name: Livestock Inspector (Veterinarian)

Recipient
Name of Laboratory:
Address:
Phone No.:
Name:

Margin for air carrier use

Note 1: The limit to contents stored within a single container is less than 1000 mL for liquids.
Note 2: The limit to contents stored within a single container is less than 50 g for solids.

(Separate Form 1-4)
(Example)

(Air Transportation)

Declaration of Hazardous Substance in Postal Matter (Bovine tissue, etc.)

The item name and quantities, etc. described below regarding this postal package are entirely accurate. The material is stored in a container complying with UN specifications, and packaged, labeled, etc. according to the Aviation Law and its relevant regulations. This postal package is within the carrying limits of aircraft and is in suitable condition for air transport.

Date of Form Filling		D/M/Y: 30/10/2002	
Item		Bovine tissue, etc.	
	UN2814 UN2900	Highly infectious substance transmissible to humans and animals (Liquid)	(Note 1) mL
√	UN2814 UN2900	Highly infectious substance transmissible to humans and animals (Solid)	(Note 2) 40 g
√	UN1845	Dry ice	3 kg
√	Stored in a container complying with UN specifications, packed with dry ice and further packaged in another container or such.		

Sender
Local Authority: XX Prefecture
Laboratory : XXXXX Meat Inspection Station
Address: 1-2-3, XXXX, XXX-city
Phone No.: XXX-XXX-XXXX
Name: Livestock Inspector (Veterinarian)
XXXXXXX

Recipient
Name of Laboratory: XXX Laboratory
Address: 3-2-1 XXX, XXX-city, XXX Prefecture, Postal code: XXX-XXXX
Phone No.: XXX-XXX-XXXX
Name: XXXXXXX

Margin for air carrier use

Note 1: The limit to contents stored within a single container is less than 1000 mL for liquids.
Note 2: The limit to contents stored within a single container is less than 50 g for solids.

(Separate Attachment 1-1)

Procedures for Platelia BSE

1. Sample Purification

(1) Preparation of reagents

Dilute the Proteinase K by 250-fold using the **Dissolver Solution** (**Reagent A**) in the BSE Purification Kit, in the required amounts depending on the number of samples to be treated (see table below).

(The diluted Proteinase K Solution may be stored for 4 hours at room temperature.)

No. of Samples	Volume of Reagent A	Volume of Proteinase K
2	2 mL	8 μL
10	6 mL	24 μL
18	10 mL	40 μL
26	14 mL	56 μL
34	18 mL	72 μL
42	22 mL	88 μL
50	26 mL	104 μL
58	30 mL	120 μL
66	34 mL	136 μL
74	38 mL	152 μL
82	42 mL	168 μL
90	46 mL	184 μL

(2) Purification of abnormal prion peptides

1) Collect and weigh **350 ± 40 mg** of the bovine obex region.
2) Place the weighed sample in the grinding tube.
3) Completely homogenize the sample in the grinding tube. (The sample thus homogenized may be stored for several weeks at -20°C, during which time it may be frozen/thawed only once.)
4) Collect 500 μL of the homogenated sample, taking care not to include solids, and transfer into a **2 mL microtube**, etc. (This sample may be stored for 8 hours at 2-8°C, or for several weeks at -20°C.)
5) Add 500 μL of the diluted **Proteinase K Solution** prepared in procedure 1. (1) above and mix well. In order to allow for uniform enzymatic activity, the procedure of adding the Proteinase K Solution must be completed swiftly within 5 minutes, or within 10 minutes if placed on ice.
6) When mixed well, immediately place in a hot water bath, incubator, or heat block, etc., and incubate for **10 ± 1 minutes at 37 ± 1°C**. The time interval between procedures 5) and 6) above shall be **less than 2 minutes**.
7) When incubation is complete, add 500 μL of **Reagent B** within 2 minutes (within 10 minutes if the tube is placed on ice) and mix the solution until it becomes a blue color overall. (The procedure for adding Reagent B shall be completed swiftly **within 5 minutes**, or within 10 minutes if placed on ice.)
8) Centrifuge the solution at **20000 × g for 5 minutes** or at **15000 × g for 7 minutes**.

9) After centrifuging, discard the supernatant **within 5 minutes**. In order to remove as much of the supernatant as possible, set the tube upside down on paper for 5 minutes, or dehydrate by aspiration for 5 minutes using an aspirator.
10) After discarding the supernatant, add 50 μL of **Reagent C1** to the microtube **within 10 minutes**. Do not mix by vortex.
11) Immediately place in hot water bath, incubator or heat block, and incubate for **5 ± 1 minutes at 100 ± 1°C**. The time interval between procedures 10) and 11) above shall be **less than 2 minutes**.
12) Remove the microtube from the incubator and mix well by vortex. (This sample may be stored for 5 hours at 2-8°C, or for several weeks at -20°C. Following any storage, it should be incubated for **5 ± 1 minutes at 100 ± 1°C** and mixed by vortex.)
13) Add 250 μL of the **Diluent** (**R6**) in the BSE Detection Kit and mix. (This sample may be stored for 5 hours at 2-8°C. After storing, mix well, then perform the following procedures.) After mixing, dispense the sample in the wells of the microplate of the Detection Kit. (To be continued in procedure 2. (2) 2).)

2. Sample Detection

(1) Preparation of reagents

1) Remove the reagents and solidifying microplate from the refrigerator prior to use and allow them to return to room temperature (20 ± 5°C).
2) Dilute the **Wash Concentrate** (**R2**) 10-fold using purified water and mix to make the **Wash Solution** (**R2'**). (May be stored for 2 weeks at 2-8°C)
3) Lightly tap the **Positive Control** (**R4**) bottle before opening, and then add 2 mL of purified water or Diluent (R6). Let stand for 1 minute, then mix gently to dissolve. (May be stored for 2 hours at 2-8°C, or for 6 months at -20°C after dispensing into adequate portions) When freezing, immediately dispense into microtubes, etc. after dissolving, and store at -20°C.
4) Immediately before use, dilute the **Enzyme-Labeled Antibody** (**R7**) 10-fold using the Wash Solution, and mix gently, to make the **Enzyme-Labeled Antibody Solution** (**R7'**). 1 mL of Enzyme-Labeled Antibody Solution (R7') is required per strip. (May be stored for 6 hours at 2-8°C)
5) In a container light-shielded with aluminum foil, etc., mix the **Substrate Buffer** (**R8**) and **Chromogen Liquid** (**R9**) in a ratio of 10:1, to make the **Chromogenic Substrate Solution** (**R8+R9**). 1 mL of Chromogenic Substrate Solution (R8+R9) is required per strip. (May be stored for 6 hours at room temperature. However, if it shows a blue color at the time of use, discard and prepare a fresh solution.)

(2) Detection of abnormal prions

1) Take the required number of strips from the microplate rack. (Return unused strips to the bag with the desiccant, and thoroughly expel any remaining air before resealing the bag. May be stored for 1 month at 2-8°C.)
2) Dispense the **Negative Control** (**R3**), **Positive Control** (**R4**) and the **sample** prepared using the BSE Purification Kit into the wells of the microplate as described below. A control must be provided on each plate when using multiple plates for screening, or for each run when using a single microplate for multiple

runs.

A1, B1, C1, D1: **Negative Control** (**R3**) 100 μL
E1, F1: **Positive Control** (**R4**) 100 μL
G1, H1: **Sample** 100 μL

3) Cover the microplate with sealing film and incubate for 75 ± 15 minutes at 37 ± 1°C using a heat block (preferable) or incubator.
4) Remove the sealing film and wash the plate with the Wash Solution (18-22°C). If using an automatic microplate washer, set an overflowing volume at 800 μL per well and run 3 wash cycles. When washing manually, remove the solution in the well, then fill with 350 μL of Wash Solution and remove, and repeat these steps 3-6 times (adjust the number of wash repetitions while observing the value). When washing is complete, completely remove the washing solution from the well by tapping it out on paper. Do not leave in this position for 5 minutes or longer.
5) Dispense 100 μL of the **Enzyme-Labeled Antibody Solution** (**R7'**) into each well.
6) Cover with sealing film, and incubate for 60 ± 5 minutes at 2-8°C.
7) Remove the sealing film and wash the plate with the Wash Solution (18-22°C). If using an automatic microplate washer, set an overflowing volume at 800 μL per well and run 5 wash cycles. When washing manually, remove the solution in the well, then fill with 350 μL of Wash Solution and remove, and repeat these steps 5-10 times (adjust the number of wash repetitions while observing the value). When washing is complete, completely remove the washing solution from the well by tapping it out on paper. Do not leave in this position for 5 minutes or longer.
8) Dispense 100 μL of the Chromogenic Substrate Solution (R8+R9) into the well, and after taking measures such as covering the plate with aluminum foil, incubate for 30 minutes at room temperature (18-22°C) in the dark, protected from light. Do not use film for incubation.
9) Dispense 100 μL of the **Reaction Stopper** (**R10**) into the wells.
10) Within 30 minutes after adding the Reaction Stopper, measure the OD using a microplate reader, at **main wavelength 450 nm** and **reference wavelength 620 nm**[1]. Avoid all light exposure prior to making the measurement.

[1] Any reference wavelength within the range of 600-700 nm will yield consistent results.

3. Interpretation

A cutoff value determined as follows is used for interpretation.

Cutoff value = (Mean absorption of 4 negative controls + Constant 0.210)
As the constant is periodically updated, the value indicated in the instructions of the Kit is to be used.
When **OD value < -10% from the cutoff value: Negative**
When **OD value ≥ -10% from the cutoff value: Repeat test necessary**

When a **repeat test** is required according to the criteria above, use the sample stored in procedure 1. (2) 4), using duplicate wells on the microplate. (The repeat test is preferably conducted by another independent laboratory technician.).

The measurement system is validated by seeing that the absorptions of the negative and positive controls satisfy the criteria below:

(1) **Absorption of all 4 negative control wells < 0.150**
(2) **Absorption of both positive control wells ≥ 1.000**

The following criteria are used to interpret results of the **repeat test**:

(1) **Positive** when **either one of the two wells has an OD value at or above the cutoff value**, or is **within -10% from the cutoff**.
(2) **Negative** when the **OD values of both wells are less than -10% from the cutoff**.

(Separate Attachment 1-2)

Procedures for Dynabott Enfer BSE Test

1. Kit Components

(1) Dynabott Enfer BSE Test components

Reagent Pack (Store at 2-8°C)

Reagent	Content	Storage Conditions	Method of Preparation	Post-Preparation Storage Conditions and Expiry Date
Reagent 3	20 mL × 1 unit	2 - 30°C	Not applicable	Not applicable
Washing Agent 1	100 g powder × 1 bottle	2 - 30°C	Add 50 g of Washing Agent 1 per 1 L of purified water and dissolve	6 months at 2-8°C
Goat Serum	150 µL × 1 unit	2 - 8°C	See section on Anti-PrP Antibody	Not applicable
Conjugate	Concentrated conjugate 100 µL × 1 unit	2 - 8°C	Use Wash Solution 2 to dilute Conjugate according to the dilution ratio specified per lot	Use within 2 hours of preparation
Substrate A	10 mL × 1 bottle	2 - 8°C	Mix equal amounts of Substrates A and B	Store in the dark and use on day of preparation
Substrate B	10 mL × 1 bottle	2 - 8°C		
Centrifuge Plate	2 plates	2 - 30°C	Not applicable	Not applicable
Assay Plate	1 plate	2 - 30°C	Not applicable	Not applicable
Positive Control Well	8 wells	2 - 8°C	Not applicable	Not applicable
Blank Control	30 mL × 1 unit	2 - 30°C	Not applicable	Not applicable

Antibody Pack (Store at -25 - -15°C)

Reagent	Content	Storage Conditions	Method of Preparation	Post-Preparation Storage Conditions and Expiry Date
Reagent 2	3 mL × 1 unit	-25 - -15°C	Not applicable	Not applicable
Anti-PrP Antibody (Rabbit serum)	Concentrated antibody 50 µL ×1 bottle	-25 - -15°C	Use Wash Solution 2 to dilute the Anti-PrP Antibody 500-fold, and the Goat Serum to the dilution ratio specified per lot	Use on day of preparation

Buffer/ Wash Pack (Store at 10-30°C)

Reagent	Content	Storage Conditions	Method of Preparation	Post-Preparation Storage Conditions and Expiry Date
Reagent 1	1 L × 1 unit	10 - 30°C	Not applicable	Not applicable
Washing Agent 2	10x concentrate 500 mL × 1 bottle	10 - 30°C	Add 100 mL of Washing Agent 2 per 900 mL purified water and mix	2 weeks at 10-30°C 1 month at 2-8°C

(2) Ingredients and contents

Reagent	Ingredient	Content (per 100 mL)
Reagent 1	Methanol	16 mL
	Sodium lauryl sulfate (SDS)	15 g
Reagent 2	Proteinase K	0.2 g
Reagent 3	Guanidine hydrochloride	28.659 g
Washing Agent 1	Sodium chloride	100 g*1
Washing Agent 2	Lauromacrogol	0.5 mL
Anti-PrP Antibody	Rabbit anti-PrP serum	100 mL
Conjugate	Horseradish peroxidase-labeled anti-rabbit immunoglobulin (goat)	100 mL
Goat Serum	Normal goat serum	100 mL
Positive Control Well	Synthetic prion peptide	2.4 ng*2
Substrate A	Substrate A (hydrogen peroxide solution)	100 mL
Substrate B	Substrate B (3-aminophthalhydrazide solution)	100 mL
Blank Control	Methanol	16 mL
	Sodium lauryl sulfate	15 g
Assay Plate	96-well microplate	1 plate*3
Centrifuge Plate	96-well microplate	2 plates*3

*1: Per bottle
*2: Per well
*3: No. of plates

2. Required Instruments and Materials

Materials included in Kit

This Kit includes sufficient reagents to assay 45 samples.

Materials not included in Kit

- High quality deionised, distilled or reverse osmosis water must be used (hereinafter referred to simply as purified water)
- Stomacher Biomaster 80 (Seward Ltd.) Homogeniser*
- Homogeniser bags (with filter) (Interscience)
- 2 Skatron Skanwasher® 300 microplate washers (Skatron Instruments AS)*
- iEMS incubator/shaker (Thermo LabSystems)*
- Luminoskan Ascent chemiluminescence reader (Thermo LabSystems)*
- Microplate centrifuge (2750 G or higher)
- Microplate sealers
- Pipettes
- Apparatus for sample collection
- Containers for dilution of the Anti-PrP Antibody and the Conjugate
- Glass or polypropylene containers for dilution of other reagents
- Negative tissue controls (See section on Preparation of Tissue Controls)

* Specific instrument requirements in this assay

3. Instrument Parameter Settings

The pre-set parameter settings in the recommended instruments are shown below. (No setting of parameters is required on the part of the user)

Washer

- 2 separate washers are required for this assay.
- Settings for Wash Protocols 1 and 2:
  - Air pressure: 0.25 atm
  - Volume/ flow rate, adjustment offset >> σv : 1.00
  - Aspirate position (usually 3.00-4.00 mm)
  - Dispense position: 0.00 mm

Wash Protocol 1* (Use Wash Solution 1) Steps:			Wash Protocol 2 (Use Wash Solution 2) Steps:		
# 1	Aspirate	6 seconds	# 1	Aspirate	4 seconds
# 2	Dispense	300 μL	# 2	Wash	3 seconds
# 3	Soak	5 seconds	# 3	Soak	5 seconds
# 4	Aspirate	4 seconds	# 4	Aspirate	2 seconds
# 5	Wash	5 seconds	# 5	Wash	3 seconds
# 6	Soak	5 seconds	# 6	Soak	5 seconds
# 7	Aspirate	3 seconds	# 7	Aspirate	2 seconds
# 8	Wash	2.5 seconds	# 8	Wash	3 seconds
# 9	Soak	5 seconds	# 9	Soak	5 seconds
# 10	Aspirate	2 seconds	# 10	Aspirate	2 seconds
# 11	Wash	2 seconds	# 11	Wash	2 seconds
# 12	Soak	5 seconds	# 12	Soak	5 seconds
# 13	Aspirate	5 seconds	# 13	Aspirate	4 seconds
# 14	End Wash		# 14	End Wash	

* Procedures of Wash Protocol 1 must be performed within a biosafety cabinet.

Shaking incubator

- Shake value: 5 (1400 rpm), Temperature: 34°C

Chemiluminometer

- Plate acceleration: 10, Settle delay: 100, Filter: none, Measurement type: single, Integration time: 300, Lag time: 30 seconds, Measurement count: 1, Photomultiplier (PMT) voltage: default voltage, Plate type: 96 well, Scale factor: up to 8 times

4. Preparation of Reagents

Prepared reagents must be allowed to come to the temperature of the room prior to use.

(1) Wash Solution 1

Prepare Wash Solution 1 by adding 1 liter of purified water to 50 g of powder **Washing**

Agent 1 (**Enfer Wash 1**). Shake until dissolved (or subject to a rotating bottle shaker for 10 minutes), and confirm dissolution before use.
(The prepared Wash Solution 1 may be stored for 6 months at 2-8°C.)

(2) Wash Solution 2

Prepare Wash Solution 2 by diluting the **Washing Agent 2** (**Enfer Wash 2**) concentrate 10-fold using purified water.
(The prepared Wash Solution 2 may be stored for 2 weeks at 10-30°C or for 1 month at 2-8°C.)

(3) Anti-PrP Antibody + Goat Serum Solution

Prepare the Anti-PrP Antibody + Goat Serum Solution by diluting the **Anti-PrP Antibody (Anti-PrP- 1° Ab (Rabbit))** and **Goat Serum (Normal goat Serum (Goat))** using Wash Solution 2 and mixing by inversion. Dilute according to the directions on the labels, as dilution ratios differ per lot.
(The Anti-PrP Antibody + Goat Serum Solution must be used on day of preparation.)

(4) Conjugate Solution

Prepare the Conjugate Solution by diluting the **Conjugate (Enzyme-conjugate- 2° Ab (goat anti-rabbit))** using Wash Solution 2 and mixing by inversion. Dilute according to the directions on the label, as dilution ratio differs per lot.
(The prepared Conjugate Solution must be stored in the dark and used within 2 hours of preparation.)

(5) Substrate Solution

Add an equal volume of **Substrate A** (**Substrate Solution A**) to **Substrate B** (**Substrate Solution B**).
The Substrate Solution must be prepared at least 1 hour before use to allow it to come to the temperature of the room.
(The prepared Substrate Solution must be stored in the dark and used on the day of preparation.)

5. Sample Preparation

1) Prepare a homogenate using **500 ± 40 mg** of the collected bovine medulla oblongata (sample).

2) Place the sample in front of the filter in the homogeniser bag (the bag is compartmented by an internal filter), and confirm that the sample is pushed to the bottom of the bag. Squash the sample between thumb and forefinger to aid subsequent homogenization.

3) Add 7.5 mL of **Reagent 1** (**Enfer Buffer 1 (Bovine)**) into the far side of the filter in the homogeniser bag. Although no particular limit is specified regarding the time interval to homogenization after adding **Reagent 1**, care should be taken to proceed smoothly to the immunoassay.

4) Set the speed setting of the Stomacher homogeniser to 'high', and homogenize the sample for 2 minutes. As the emulsion is prepared using a homogeniser bag with filter, unnecessary matter such as membranes are removed.

Note: After preparation, the homogenised samples must be started immediately on the immunoassay procedure.
The emulsion remaining from the assay is to be stored in the homogeniser bag at room temperature until the results of the first test run are known. Refrigeration must be avoided as it causes crystallization.

6. Immunoassay Procedure

1) The positions A1 and A2 on the **Centrifuge Plate** (**Centrifuge Plate**) are to be reserved for the **Positive Control Wells** (**Peptide Indicator Wells**).
Dispense 180 µL of the **Blank Control** (**Blank Control Reagent (Bovine)**) into each of 4 wells starting at B1, and dispense 180 µL of sample into each of 2 wells per sample.

2) Cover the centrifuge plate with a plate sealer.

3) Centrifuge the plate for 5 minutes at 2750 G.

4) Pipette 20 µL of **Reagent 2** (**Enfer Buffer 2**) directly to the bottom of all wells to be used on the **Assay Plate** (**Enfer Test Plate**).

5) Remove the plate sealer from the centrifuged plate. Collect 100 µL each from the supernatant of the samples and Blank Controls, and transfer to the Assay Plate containing **Reagent 2**.

6) Cover the Assay Plate with a plate sealer.

7) Shake the Assay Plate for 60 minutes at 34°C.

8) Remove the plate sealer and wash the Assay Plate using Wash Solution 1 and <Wash Protocol 1>.

9) Invert the Assay Plate on soft paper and tap well to remove any remaining liquid.

10) Add 150 µL of **Reagent 3** (**Enfer Buffer 3**) to all the wells.

11) Cover the Assay Plate with a plate sealer.

12) Shake the Assay Plate for 15 minutes at 34°C.

13) Remove the plate sealer and wash the Assay Plate using Wash Solution 2 and <Wash Protocol 2>.

14) Invert the Assay Plate on soft paper and tap well to remove any remaining liquid.

15) Remove the wells in positions A1 and A2 from the Assay Plate and replace them with the **Positive Control Wells**.

16) Dispense 150 μL of the prepared Anti-PrP Antibody + Goat Serum Solution into each well.

17) Cover the Assay Plate with a plate sealer.

18) Shake the Assay Plate for 40 minutes at 34°C.

19) Remove the plate sealer and wash the Assay Plate using Wash Solution 2 and <Wash Protocol 2>.

20) Invert the Assay Plate on soft paper and tap well to remove any remaining liquid.

21) Dispense the prepared Conjugate Solution by 150 μL volumes into the Assay Plate.

22) Cover the Assay Plate with a plate sealer.

23) Shake the Assay Plate for 30 minutes at 34°C.

24) Remove the plate sealer and wash the Assay Plate using Wash Solution 2 and <Wash Protocol 2>.

25) Invert the Assay Plate on soft paper and tap well to remove any remaining liquid.

26) Dispense the prepared Substrate Solution by 150 μL volumes into the Assay Plate.

27) Cover the Assay Plate with a plate sealer.

28) Shake the Assay Plate for 10 minutes at 34°C.

29) Remove the plate sealer and use the chemiluminometer to read the luminescence.

A Example of an Assay Plate (Enfer Test Plate) Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	P	P	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
B	B	B	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
C	B	B	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
D	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
E	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
F	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
G	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44
H	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37	S45	S45

P: Positive Control Well (Peptide Indicator Well)
B: Blank Control (Blank Control Reagent (Bovine))
S1-S45: Samples

Work Flow of Assay

Step	Operation	Time/Temp.	Instrument/ Equipment	Preparation for Subsequent Operations	Precautions
Sample Collection/ Sample Preparation Process					
Collect sample/ Weigh	Sample weight: 500±40 mg		Balance		
Homogenise	Add 7.5 mL of Reagent 1 per 500±40 mg of tissue	2 min. (Speed "High")	Stomacher 80 Homogeniser		Place tissue sample at bottom of filter bag. Following homogenization, let stand at room temperature for a few minutes to allow bubbles to subside.
Transfer homogenate	Transfer homogenate into Centrifuge Plate: 180 µL		Pipette	Likewise transfer 180 µL of the Blank Control into plate	Cover plate with sealer.
Centrifuge	Centrifuge: 2750 G	5 min., Room temp.	Centrifuge	Confirm that incubator temperature is 34°C	Check balance before centrifuging. DO NOT centrifuge at 2-8°C.
Add Reagent 2 (PK)	Add to Assay Plate: 20 µL		Pipette (8-channel)		Add to corner at bottom of well. (Visually confirm addition afterwards)
Dispense sample into plate	Dispense centrifuged supernatant into plate: 100 µL		Pipette (8-channel)		Remove supernatant, watching out for any precipitate.
Incubation 1	Incubate	60 min. at 34°C	LabSystems iEMS Incubator		
Wash 1	Wash using Washing Agent 1 and Protocol 1		Skanwasher 300 microplate washer		After washing, invert and tap several times on paper towel to remove any remaining liquid.
Add Reagent 3	Add 150 µL of Reagent 3		Pipette (8-channel)		

Incubation 2	Incubate	15 min. at 34°C	LabSystems iEMS Incubator	Prepare primary antibody solution (dilute Anti-PrP Antibody+ Goat Serum with prepared Washing Agent 2) Prepare Conjugate (secondary antibody) Solution (dilute Conjugate with prepared Washing Agent 2) Prepare Substrate Solution	
Wash 2	Wash using Washing Agent 2 and Protocol 2		Skanwasher 300 microplate washer	After washing, remove the wells in positions A1 and A2 and set the Positive Control Wells in these positions	After washing, invert and tap several times on paper towel to remove any remaining liquid.
ELISA Process					
Primary antibody	Add primary antibody: 150 µL		Pipette (8-channel)		Confirm that Positive Control Wells are in positions A1 and A2 before adding primary antibody.
Incubation 3	Incubate	40 min. at 34°C	LabSystems iEMS Incubator		
Wash 3	Wash using Washing Agent 2 and Protocol 2		Skanwasher 300 microplate washer		After washing, invert and tap several times on paper towel to remove any remaining liquid.
Conjugate	Add Conjugate: 150 µL		Pipette (8-channel)		
Incubation 4	Incubate	30 min. at 34°C	LabSystems iEMS Incubator		
Wash 4	Wash using Washing Agent 2 and Protocol 2		Skanwasher 300 microplate washer		After washing, invert and tap several times on paper towel to remove any remaining liquid.
Substrate	Add Substrate: 150 µL		Pipette (8-channel)		
Incubation 5	Incubate	10 min. at 34°C	LabSystems iEMS Incubator		
Measure	Measure chemiluminescence		Chemiluminometer		

7. Interpretation

(1) Validation of Test Performance

The control results must be validated before the sample results can be interpreted. Determine the mean luminescence values of the Blank Control and Positive Control Wells. If the following criteria are not met, the assay is invalid, thus a repeat test must be conducted starting from the process of collecting the bovine medulla oblongata (sample) in Section 5. Sample Preparation. Results in the repeat test are evaluated using duplicate wells as well.

1) Blank Control

The median value of the 4 wells of Blank Control must be below 4.0 LU. The median is determined as the mean of the middle 2 values, excluding the maximum and minimum values, for the 4 wells.

2) Positive Control Well

The mean of the Positive Control Wells after subtracting the median of the Blank Control must be confirmed to be within the control range of the lot of Positive Control Wells used (indicated on the Positive Control Well label).

The individual measurements for the Positive Control Wells must not be outside of the mean value of all Positive Control Wells ±30%.

(2) Interpretation of Results

The cutoff value for this Kit is 5.5 LU. The measurements for all samples are interpreted after subtracting the mean of the Blank Control.

If the measurements of both the duplicate wells are 5.5 LU or lower, the sample is interpreted as being negative. Meanwhile, if at least one of the two wells assayed produces a value exceeding 5.5 LU, a complete repeat test using duplicate wells must be conducted for confirmation, starting from the process of collecting the bovine medulla oblongata (sample) in Section 5. Sample Preparation.

In the repeat test results, if at least one of the two wells measured exceeds 5.5 LU, the interpretation of this Kit is positive. As the sample is potentially positive, it requires confirmatory testing. If the result of the repeat test is 5.5 LU or lower for both wells measured, this Kit interprets the sample as negative.

Assay Result of Sample (n=2)

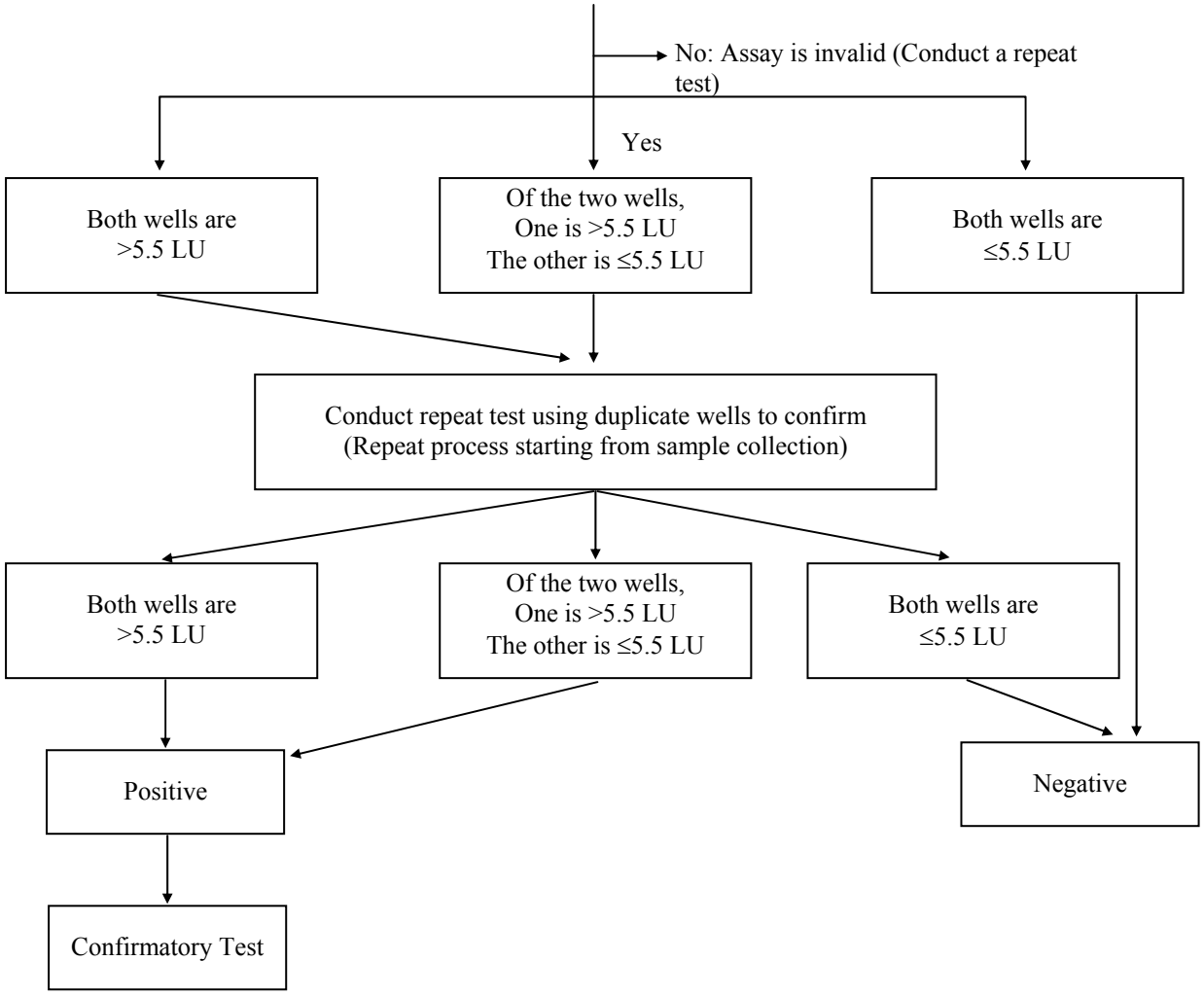
↓

Blank Control

- The median value of the quadruplicate Blank Control measurements must be below 4.0 LU. The median is determined as the mean of the 2 middle values, excluding the maximum and minimum values, for the quadruplicate wells.

Positive Control Well
(Applicable only when the Positive Control Wells provided in this Kit are used.)

- The mean of the Positive Control Wells after subtracting the median of the Blank Control must be confirmed to be within the control range of the lot of Positive Control Wells used.
- The individual measurements for the Positive Control Wells must not be outside of the mean value of all Positive Control Wells ±30%.



8. Partial Use of the Kit

This Kit may be used in portions over four separate runs to test small numbers of samples. The minimum number of samples required for a run in this case is 1.

9. Handling of Samples Interpreted as Positive in this Assay

If the results of the assay make it necessary to conduct a repeat test (if both or either one of the wells are positive), the emulsion which had been stored at room temperature in the homogeniser bag awaiting these results is to be transferred to a 15 mL plastic centrifuge tube for cultivation and stored frozen.

If the result of the repeat test is positive, the emulsion used in the repeat test is also transferred to a 15 mL plastic centrifuge tube for cultivation and frozen, for use in the confirmatory test together with the frozen emulsion from the first test.

If transportation is required for conduct of the confirmatory test, the lid of the 15 mL plastic centrifuge tube for cultivation containing the emulsion is fixed using Parafilm. As a sample for Western Blot, the tube is dispatched wrapped in tissue paper, etc.-- to absorb contents in case the plastic tube breaks or the cap comes loose and as a buffer against shock -- and further packaged in a biohazard can, etc.

Meanwhile if both wells are negative in the repeat test results, the emulsion stored frozen following the first test is disposed of.

(Separate Attachment 1-3)

Procedures for Frelisa® BSE

1. Kit Components

(1) Components of Frelisa® BSE

Frelisa® BSE is a BSE screening reagent kit consisting of 3 reagent sets (Extraction Reagent Set A, Extraction Reagent Set B, and Detection Reagent Set), comprising a total of 17 reagents as shown below.

	No.	Component Reagent	Form	Content per Unit	No. of Units
Extraction Reagent Set A	1	DNase I Solution	Frozen	0.3 mL	1
(Store at -30- -10°C)	2	Collagenase Solution	Frozen	1.8 mL	1
	3	Proteinase K Solution	Frozen	1 mL	2
	4	PK Reaction Stopper	Frozen	1.5 mL	1
Extraction Reagent Set B	5	Homogenizing Solution	Liquid	45 mL	2
(Store at 2-10°C)	6	Surfactant Solution	Liquid	35 mL	1
	7	Concentrating Solution	Liquid	12 mL	1
	8	Solubilizer	Liquid	7 mL	1
	9	Sample Diluent	Liquid	25 mL	1
Detection Reagent Set	10	Antibody-Binding Plate	Well	96 wells	1
(Store at 2-10°C)	11	Enzyme-Labeled Antibody	Liquid	0.6 mL	1
	12	Labeled Antibody Diluent	Liquid	6 mL	1
	13	Negative Control	Liquid	1.5 mL	2
	14	Positive Control	Liquid	1.5 mL	1
	15	Substrate Solution	Liquid	12 mL	1
	16	Wash Solution	Liquid	50 mL	2
	17	Reaction Stopper	Liquid	12 mL	1

(2) Preparation of reagents

The reagents included in the kit must be allowed to return to room temperature before use, and are prepared according to the table below. The detail procedures for preparing Reagents 1, 2 and 3 are given below.

No.	Component Reagent	Form	Method of Preparation
1	DNase I Solution	Frozen	Dilute to 126 times of Surfactant Solution (Prepared Reagent 1)
2	Collagenase Solution	Frozen	Dilute to 21 times against Surfactant Solution (Prepared Reagent 1)
3	Proteinase K Solution	Frozen	Dilute 6-fold using Surfactant Solution (Prepared Reagent 2)
4	PK Reaction Stopper	Frozen	Dilute 31-fold using Concentrating Solution (Prepared Reagent 3)
5	Homogenizing Solution	Liquid	Use as is
6	Surfactant Solution	Liquid	Use as is
7	Concentrating Solution	Liquid	Use as is
8	Solubilizer	Liquid	Use as is
9	Sample Diluent	Liquid	Use as is
10	Antibody-Binding Plate	Well	Use as is
11	Enzyme-Labeled Antibody	Liquid	Dilute 11-fold using Labeled Antibody Diluent
12	Labeled Antibody Diluent	Liquid	Use as is
13	Negative Control	Liquid	Use as is
14	Positive Control	Liquid	Use as is
15	Substrate Solution	Liquid	Use as is
16	Wash Solution	Liquid	Dilute 20-fold using purified water
17	Reaction Stopper	Liquid	Use as is

Prepared Reagent 1: Prepare by diluting the DNase I Solution to 126 times and the Collagenase Solution to 21 times of the Surfactant Solution. A guideline of the volumes to be used per the number of samples to be assayed is given in the table below.

No. of Samples	DNase I Solution (μL)	Collagenase Solution (μL)	Surfactant Solution (mL)
5	12	75	1.5
10	24	150	3.0
20	48	300	6.0
40	84	525	10.5
60	108	675	13.5
80	144	900	18.0
100	180	1125	22.5

Prepared Reagent 2: Prepare by diluting the Proteinase K Solution 6-fold using the Surfactant Solution. A guideline of the volumes to be used per the number of samples to be assayed is given in the table below.

No. of Samples	Proteinase K Solution (μL)	Surfactant Solution (mL)
5	200	1.0
10	300	1.5
20	500	2.5
40	900	4.5
60	1200	6.0
80	1600	8.0
100	2000	10.0

Prepared Reagent 3: Prepare by diluting the PK Reaction Stopper 31-fold using the Concentrating Solution. A guideline of the volumes to be used per the number of samples to be assayed is given in the table below.

No. of Samples	PK Reaction Stopper (μL)	Concentrating Solution (mL)
5	40	1.2
10	60	1.8
20	100	3.0
40	170	5.1
60	250	7.5
80	320	9.6
100	400	12.0

2. Required Instruments and Reagents

(1) Instruments

Electronic balance:	Master Balance LA120S (Sartorius K.K.) or equivalent (reading limit: 0.1 mg, maximum: 10 g or higher, with hood)
Homogenizer:	FastPrep (Qbiogene, Inc.) or Multi-Beads Shocker (Yasui Kikai Corporation)
Constant temperature bath (1):	Constant temperature bath adjustable to 37°C, or a dry block heater
Constant temperature bath (2):	Constant temperature bath adjustable to 100°C, or a dry block heater
Centrifuge:	High-Speed Micro Centrifuge himac CF15R (Hitachi High-Technologies Corporation) or equivalent (main unit capable of 15000 G, with rotor)
Incubator:	Incubator adjustable to 37 °C or plate incubator for ELISA
Microplate washer:	PW-40 (Bio-Rad Fujirebio Inc.) or equivalent
Microplate reader:	Microplate reader Model 550 (Bio-Rad Laboratories, Inc.) or equivalent (with adjustable main/reference wavelengths)
Micropipettes:	200 μL, 1000 μL, 5000 μL, etc.

(2) Consumables

Sample collection apparatus:	Sample collection set (Fujirebio Inc.) or sampling syringe
Homogenizing tube:	2 mL freezing tube (Assist Co., Ltd.)* or grinding tube (Yasui Kikai Corporation)
Metal cone or ceramic beads:	Magnetic metal cone (Yasui Kikai Corporation) or YTZ Balls (Nikkato Corporation)*
Sample tube:	2 mL sampling tube or 2 mL freezing tube (Assist Co., Ltd.)
Micropipette tip:	Various types

* A 2 mL freezing tube (Assist Co., Ltd.) containing 0.5 g of YTZ Balls (Nikkato Corporation) is planned to be released from Fujirebio Inc. as the “Homogenate Tube FR” (tentative).

(3) Instrument Parameters

Microplate washer: The procedures for setting the parameters of PW40 are shown below.

Press IN and OUT simultaneously in the SELECT: RUN screen
↓
PRG: ADD YES
↓
ADD: KIT YES
↓
NAME: Enter a suitable name
↓
Enter, referring to MAIN PARAMETERS and METHOD 1
↓
END OF KIT: NO YES
↓
MET.INTER: OMN OS
↓
Enter, referring to METHOD 2
↓
END OF KIT: YES YES
↓
Nr OF KITS: 1 YES

End

METHOD 1	METHOD 2	MAIN PARAMETERS
MODE: STRIP	MODE: STRIP	PLATE: Flat 01
CROSW ASP.:NO	CROSW ASP.:NO	MANIFOLD: 8
ASP.TIME: 0.1 S	ASP.TIME: 0.1 S	STRIP: -
VOLUME: 800 μL	VOLUME: 800 μL	Nr OF KITS: 1
OVERFLOW: 2.5 mm	OVERFLOW: 2.5 mm	
LIQUID: WASH R1 (W1)	LIQUID: WASH R1 (W1)	
FLOW: 0	FLOW: 0	
Nr OF CYCLES: 1	BOT.ASP.NUMBER: 1	
SOAKING: 0.0 S	Nr OF CYCLES: 4	
MET. INTER: 0MN 0 S	SOAKING: 0.0 S	

Microplate reader: Set the parameters as follows

Blank: Air blank
Main wavelength: 450 nm
Reference wavelength: 600-630 nm

3. Assay Method

(1) Preparation of emulsion

Either a metal cone or ceramic beads may be used as the means for grinding, to be decided by the user.

(1)-1 Add 800 μL of the Homogenizing Solution to the homogenizing tube containing the ceramic beads. (If using a metal cone, first collect the obex of the bovine medulla oblongata into the homogenizing tube, then insert the metal cone and finally add the Homogenizing Solution.)

(1)-2 Collect 200 ± 20 mg of the obex of the bovine medulla oblongata and transfer it into the homogenizing tube.

(1)-3 Seal the lid of the homogenizing tube and set it in the homogenizer.
Recommended homogenizing parameters:
[For ceramic beads]
FastPrep: Speed 6.5, time 45 seconds
Multi-beads Shocker: Speed 3000 rpm, time 1 minute
[For metal cone]
FastPrep: Speed 4.0, time 45 seconds
Multi-beads Shocker: Speed 2000 rpm, time 1 minute

(1)-4 The resulting emulsion is the 20 w/v% emulsion (if lumps are clearly visible, repeat agitation process).

(2) Extraction procedure

(2)-1 Make the Prepared Reagent 1 in the amount required according to the number of samples to be assayed.

(2)-2 Transfer 250 μL of the 20 w/v% emulsion into a 2 mL sample tube and add 200 μL of Prepared Reagent 1 and mix, and incubate for 30 minutes at 37°C.

(2)-3 Make the Prepared Reagent 2 while incubating the above.

(2)-4 When the reaction above is complete, immediately add 100 μL of Prepared Reagent 2 and mix, and incubate for 30 minutes at 37°C.

(2)-5 Make the Prepared Reagent 3 while incubating the above.

(2)-6 When the reaction above is complete, add 100 μL of Prepared Reagent 3 and mix.

(2)-7 Using a high-speed cooling centrifuge, centrifuge at 15000 G for 10 minutes (25-30°C).

(2)-8 After centrifuging, thoroughly remove the supernatant (following decantation, use a micropipette to extract the solution remaining at the bottom of the tube, or let stand inverted for 5 minutes).

(2)-9 Add 50 μL of the Solubilizer to the precipitate obtained from centrifugation, and subject to heat treatment at 100°C for 5 minutes. (Do not mix as it may cause the precipitate to stick to the sides of the tube and not be fully immersed in the Solubilizer.)

(2)-10 Following heat treatment, mix thoroughly to suspend.

(2)-11 After cooling, add 100 μL of the Sample Diluent and mix, to obtain the treated sample. (Sonicate if necessary.)

(3) Detection (ELISA) procedure

(3)-1 Dilute the Enzyme-Labeled Antibody 11-fold using the Enzyme-Labeled Antibody Diluent, according to the number of samples.

(3)-2 Prepare the Wash Solution according to the number of samples (make 500 mL (25 mL Wash Solution + 475 mL distilled water) for up to 45 samples).

(3)-3 Dispense the treated sample (1 well), Negative Control (2 wells) and Positive Control (1 well) by 100 μL each into the wells of the Antibody-Binding Plate. After dispensing, immediately add 50 μL of the diluted Enzyme-Labeled Antibody Solution into each well. Cover the plate with a plate sealer, gently mix, and allow reacting at 37°C for 1 hour.

(3)-4 When the reaction is complete, remove the plate sealer and wash using the diluted Wash Solution (800 μL × 5 times). After washing, invert the plate on paper towel, etc. and tap lightly to remove any remaining Wash Solution in the wells. (For the washing process, the mode must be set individually according to the model of automatic washer used.)

(3)-5 Add 100 μL of Substrate Solution to each well and gently mix, shield from light, and allow to react for 30 minutes at room temperature (20-30°C).

(3)-6 Add 100 μL of the Reaction Stopper to each well and gently mix.

(3)-7 Using a microplate reader, take measurements at main wavelength 450 nm and reference wavelength 600-630 nm.

Example of Assay Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	S-6	S-14	S-22	S-30	S-38	S-46	S-54	S-62	S-70	S-78	S-86
B	NC	S-7	S-15	S-23	S-31	S-39	S-47	S-55	S-63	S-71	S-79	S-87
C	PC	S-8	S-16	S-24	S-32	S-40	S-48	S-56	S-64	S-72	S-80	S-88
D	S-1	S-9	S-17	S-25	S-33	S-41	S-49	S-57	S-65	S-73	S-81	S-89
E	S-2	S-10	S-18	S-26	S-34	S-42	S-50	S-58	S-66	S-74	S-82	S-90
F	S-3	S-11	S-19	S-27	S-35	S-43	S-51	S-59	S-67	S-75	S-83	S-91
G	S-4	S-12	S-20	S-28	S-36	S-44	S-52	S-60	S-68	S-76	S-84	S-92
H	S-5	S-13	S-21	S-29	S-37	S-45	S-53	S-61	S-69	S-77	S-85	S-93

S1-S93: Sample
NC: Negative Control
PC: Positive Control

4. Interpretation

(1) Cutoff value determination

The cutoff value is defined as the mean of the 2 Negative Control wells (assayed together) plus 0.150.

Cutoff Value = Mean of Negative Controls + 0.150

(2) Validation of the assay system

The mean of the Negative Control absorptions is 0.1 or lower

The absorption of the Positive Control is 1.0 or higher

Sound execution of the assay is confirmed if the reactions of the Positive and Negative Controls satisfy the criteria above. Once the execution of the assay is deemed sound, the results are interpreted according to (3) Interpretation of results below. However, if the criteria above are not met, there may have been a problem in the operations, thus a repeat test must be conducted.

(3) Interpretation of results

If the absorption of the assayed sample is at or above the cutoff value, the sample is interpreted as positive, while if it is less than the cutoff value, the sample is interpreted as negative.

However, samples interpreted as positive using this kit must be subjected to other tests such as immunological, histopathological, or immunohistochemical examinations for confirmation before a final verdict is reached.

(4) Handling of positive cases

A repeat test shall be conducted on samples interpreted as positive in the first test or those showing an absorption only slightly lower than the cutoff (within -10%). The repeat test shall be started from (2) Extraction procedure, using duplicate wells for each sample. If at least one of the wells shows absorption at or above the cutoff in the repeat test, the sample is interpreted as positive.

5. Precautions

- Temperature control must be implemented carefully. If a reaction temperature of 37°C is not maintained during the extraction procedure, enzymatic treatment may not be fully carried out, leading to false negative or false positive results.
- Final mixing or sonication of the emulsion must be thoroughly performed. If large amounts of precipitate are remaining in the treated sample, the washing process in the detection procedure may become insufficient, possibly leading to occurrence of nonspecific reactions.
- Light-shielding must be thorough, as insufficient light-shielding during the enzymatic reaction of the detection (ELISA) procedures may cause the background to rise.

Work Flow

Procedure	Operation	Temp./ Time	Instrument/ Equipment	Preparation for Subsequent Procedures	Precautions
Preparation of Emulsion					
Sample collection			Sampling apparatus		Operations from sample collection to weighing must be performed within safety cabinet
Weighing	200±20 mg		Balance		
Homogenization		Speed: 6.5 Time: 45 sec.	Homogenate tube FR	Make Prepared Reagent 1	If using ceramic beads and FastPrep
Extraction Procedure					
Sampling	Collect emulsion: 250 µL		Micropipette		Operations from sampling to dilution must be performed within safety cabinet
Enzymatic treatment-1	Add Prepared Reagent 1: 200 µL	37°C, 30 min.	Repeating dispenser/ Water bath/ Dry block heater	Make Prepared Reagent 2	
Enzymatic treatment-2	Add Prepared Reagent 2: 100 µL	37°C, 30 min.	Repeating dispenser/ Water bath/ Dry block heater	Make Prepared Reagent 3	
Concentration	Add Prepared Reagent 3: 100 µL / Centrifuge	15000 G, 10 min (25-30°C)	Repeating dispenser/ High-speed centrifuge		Pool and sterilize waste solution before disposing
Solubilization	Add Solubilizer: 50 µL	100°C, 5 min.	Repeating dispenser/ Water bath/ Dry block heater		
Dilution	Add Sample Diluent: 100 µL				
Detection (ELISA) Procedure					
Preparation of Labeled Antibody	Dilute Enzyme-Labeled Antibody: × 11				
Preparation of Wash Solution	Dilute Wash Solution: × 20				
Primary reaction	Sample: 100 µL NC: 100 µL × 2 PC: 100 µL + Labeled Antibody Solution: 50 µL	37°C, 1 hour	Micropipette/ Repeating dispenser/ Incubator		Carefully prevent droplets from entering nearby wells when dispensing the Labeled Antibody
Washing	0.8 mL × 5 times		Microplate washer		Sterilize waste solution before disposing
Enzymatic reaction	Add Substrate Solution: 100 µL	Room temp., 30 min.	Repeating dispenser/ Incubator		Carefully light shield
Reaction stop	Add Reaction Stopper: 100 µL		Repeating dispenser		Remove bubbles before measuring
Measurement of absorption			Microplate reader		Discard used plates as contaminated items

6. Precautions on Handling and Usage

[General]

1) The kit contents must be used strictly according to the specified doses and methods of use.
2) Do not use on samples other than bovine medulla oblongata.
3) Samples interpreted as positive using this kit must be subject to other tests such as immunological (Western Blot), histopathological, or immunohistochemical examinations for confirmation.
4) Diagnosis of bovine spongiform encephalopathy (BSE) shall be conducted according to the scheme established by the Japanese government.

[To the User]

1) All procedures for extracting prion protein from bovine medulla oblongata shall be performed within a safety cabinet as a general rule, and care must be taken in handling to avoid generating droplets and aerosols.
2) The operator shall wear rubber gloves or protective gloves, mask, protective eyeglasses, and other protective clothing when operating.

[Usage]

1) A sample stored at 2-10°C must be used within 24 hours. For longer periods of time, the sample must be stored frozen.
2) Disposable products shall be used where possible for work clothes and instruments.
3) Care must be taken to avoid cross-contamination between samples during sample extraction and detection procedures.
4) The Prepared Reagents prepared using DNase I Solution, Collagenase Solution and Proteinase K Solution must be used within 4 hours.
5) Reagents of differing lot numbers are not to be mixed for use.
6) Extraction Reagent Set A must be mixed after thawing. Extraction Reagent Set B and the Detection Reagent Set must be returned to room temperature (20-30°C) prior to use.
7) Use a fresh sample tip for collection of each sample.
8) The Antibody-Binding Plate must be washed the specified number of times, and confirmed to be thoroughly clean.
9) Dispense the substrate without delay upon completing washing of the Antibody-Binding Plate.
10) After dispensing the Substrate, shield from light during the reaction.
11) Measurements must be taken within 10 minutes after dispensing the Reaction Stopper.
12) The Wash Solution may show a crystalline precipitate when stored at 4°C, in which case the precipitate must be dissolved at 37°C before use.
13) As the Concentrating Solution consists of 2-butanol, a hazardous substance, it should not be used near fire.

[Handling]

1) Perform all testing in a clean environment. When reusing reagents, take great care to avoid contamination by bacteria.
2) Do not use expired kits.
3) Do not use components that are abnormal in appearance or content.
4) Do not freeze/thaw the Extraction Reagent Set A for 12 cycles or more.
5) Remove only the required number of strips. Return unnecessary strips to the bag with the desiccant and seal and store at 2-10°C.
6) Only the required amounts of reagents are taken out and brought to room temperature prior to use. Any reagents removed but not to be used must be returned swiftly to the storage temperature for storage.
7) If any assay materials are spilled, wipe clean with hypochlorous acid solution (2% effective concentration) and soak in the same for 120 minutes or longer.
8) Contaminated materials such as samples and used instruments must be sterilized according to one of the methods below before being disposed of.
   - Autoclave sterilization (134-138°C, 3 atm, 20 minutes or longer)
   - Soak in 3% SDS solution at 100°C for 5 minutes or longer
   - Soak in 3% SDS solution and autoclave (120°C, 10 minutes or longer)
   - Soak in hypochlorous acid solution (2% effective concentration) for 120 minutes or longer.
   - Saturate in 1 mol/L sodium hydroxide solution for 60 minutes or longer.

[Storage]

1) Store out of reach of children.
2) Avoid storage in direct sunlight or a humidified environment, etc. as it will cause deterioration.