

Procedure for Bovine Spongiform Encephalopathy (BSE) Screening Test

No.1: Treatment of prion materials in laboratories

- Work shall be conducted inside the safety cabinet in a special compartmented laboratory in principle.
- In order to prevent infection from cuts and contamination of eyes and mouths by droplets, workers shall wear latex or vinyl gloves, masks, preventive clothes and caps as well as protective glasses, etc. if necessary.
- Disposable working clothes, tools and equipment shall be used as much as possible.
- Test material shall be handled on a bench seat with caution not to generate droplets and aerosol.
- When the test material is spilled and when work has been completed, the surface of the worktable shall be cleaned with sodium hypochlorite solution.
- The bench seat used, disposable tools, etc. shall be placed in an autoclave bag for autoclave sterilization at 132 – 134°C for 60 minutes.
- Since scissors, tweezers, etc. are reused, wipe off the stains with tissue, etc. or cotton soaked in alcohol, and soak in 3 – 5% SDS to boil for 5 – 10 minutes (addition of sodium carbonate to 1% will prevent metal corrosion) or sterilize in an autoclave at 132 – 134°C for 60 minutes.
- Plastic tools that cannot be heated shall be soaked in sodium hypochlorite at 5% or higher or NaOH of normality 2 or higher for 2 hours or longer.
- Inflammables shall be put into an autoclave bag for autoclave sterilization at 132 – 134°C for 60 minutes.
- If an incinerator for medical waste is available, plastic tools and inflammables shall be placed in a biohazard bag together to be incinerated.
- If the outer surface of the centrifuge tube, etc. which needs to be kept operating, becomes contaminated, replace with an uncontaminated before continuing working.
- If only a normal autoclave is available, it is also valid to put the contaminated objects in an alkali-resistant container and soak in NaOH of normality 1 – 2 at 120°C for 30 minutes.
- When decontaminating tools, equipment, etc. that cannot be incinerated, wipe off with inflammables such as paper and cotton soaked in alcohol first and then conduct the sterilization process in principle since the amount of remaining prion will be large as a matter of course for highly contaminated cases and pieces of tissue, etc.

No.2: Collected parts

After separating the head from the dotted line in Figure 1, insert a spoon from the osculum and collect the specimen so that the Obex (shaded area in Figure 1) shown in Figures 1 and 2 is included. The shaded sections in black indicate the skull and cervical vertebra. Since lesion and accumulation of prions occur nearly symmetrically, divide into 2 at the mesial line to fix one with formalin buffer of 15 – 20% concentration as material for the histopathological test and the immunohistochemical test, and use the other as the material for the immunobiochemical test (ELISA method, western blot method, etc.).

Use a tissue flake from the immunobiochemical test material that cuts across so that Obex is included as the screening test specimen. Since prion is not always accumulated uniformly inside the Obex, it is necessary that the specimen be collected uniformly. Hence, the cut tissue flake shall be cut into small pieces with scissors to be mixed and balanced for fractionation of the necessary volume in preparing the specimen. However, this does not always apply if the specimen is collected according to methods other than cutting.

No.3: Procedure for using the bovine spongiform encephalopathy kit

Screening test shall be conducted using “Platelia BSE” or “Dynabott Enfer BSE” testing kit, and the procedure for using each is as provided in Appendixes 1-1 and 1-2.

No.4: Storage of test results

The raw data read by the micro plate reader shall be input in separate Form 1-1. Input the other items to be input on separate Form 1-1 and store the signed paper as well as its corresponding electronic data. In addition, affix the raw data read by the micro plate reader to the paper to be stored (if it uses sensitive paper, print it on normal paper). When sending the specimen for the confirmatory test, a copy of the stored data affixed with raw data shall be sent along.

No.5: Sending the specimen for the confirmatory test

For specimens judged positive¹ using the bovine spongiform encephalopathy kit, specimens shall be sent for the confirmatory test according to the following method except for cases in which the confirmatory test is implemented by the Prefecture, etc.:

1. Destination

Destination shall be as provided in the attachment to the notification No. 0407001 issued by the Inspection and Safety Division dated on April 7, 2004.

2. Method of communication for the confirmatory test results

It shall be informed to the municipality requesting the test by the Inspection and Safety Division.

Furthermore, the contact for questions on this issue is the Milk and Meat Safety Section, Inspection and Safety Division (Phone) 03-3595-2337.

3. Sent sections (as shown in Figure 2)

Divide the tissue including Obex at the mesial line, and

(1) Fix one with formalin buffer of 15 – 20% concentration and send at room temperature as material for the histopathological test and the immunohistochemical test. The size of the container for the specimen shall be 50 ml, and it shall be filled with formalin buffer.

(2) To use the other as the material for the immunobiochemical test (ELISA method, western blot method, etc.), send it frozen. Remaining sample from the specimen collection and ELISA method (homogenized emulsion sample, etc.) shall also be sent along frozen.

4. Method of communication in sending

When sending a specimen judged positive in the screening test, attach the test results in separate Form 1-1 and the specimen sending form in separate Form 1-2 and send with specified date and time of arrival (morning: 9:00 – 12:00 or afternoon: 13:00 – 16:00) to the testing institute.

Furthermore, send the test results in separate Form 1-1 and specimen sending form in separate Form 1-2 to the Inspection and Safety Division (FAX) 03-3503-7964 and call the section (phone) 03-3595-2337 in advance.

Emergency contact on holiday, etc. will be notified later.

5. Precautions in sending the specimen

Based on No.2 and No.3, Article 8 of the Postal Regulations (Ordinance of the Ministry of Communications, No.34, 1947), containers of United Nations standard shall be packed, etc. appropriately for sending.

Furthermore, the pickup and delivery post office that takes charge of the address (referred to as “the post office in charge” hereafter) shall provide information on the method of transporting the corresponding package .

¹ Refers to the case in which the result of re-testing in No.3 is concluded as positive.

(1) Package containing a specimen that is not transported by aircraft in the process of transporting

1) All necessary items shall be entered in the paper in the following form to be affixed on the package surface where it is easy to see.

Article name: Bovine tissue, etc. "Hazardous matter" (Note 1)

Sender:

Municipality name:

Test facility name:

Address:

Phone number:

Qualification: Slaughter test personnel (veterinarian)

Full name:

(Note 1) To be written in red.

(2) Package containing a specimen that is transported by aircraft in the process of transporting (Note 3)

1) All necessary items shall be entered in the paper in the following form to be affixed on the package surface where it is easy to see.

Article name: Bovine tissue, etc. "Hazardous matter" (Note 1)

UN Number:

Sender:

Municipality name:

Test facility name:

Address:

Phone number:

Qualification: Slaughter test personnel (veterinarian)

Full name:

Dry ice xxx kg contained (Note 2)

(Note 1) To be written in red.

(Note 2) To be written in red when sending with dry ice contained.

2) The container for specimen shall be a "container of United Nations standard."

- 3) Volume for 1 container shall be less than 1,000 ml for liquids and 50 g or less for solids.
- 4) Transport permissible object indication label (Class no.: 6.2) shall be affixed on the package surface where it is easy to see. (Note 4)
- 5) When dry ice is outside the United Nations standard container in cardboard package, etc., transport permissible object indication label (Class no.: 9) shall be affixed on the package surface where it is easy to see. (Note 4)
- 6) In the above case e, the post office personnel may request to open the cardboard box, etc. upon receiving the package to check if the specimen is contained in a United Nations standard container. This shall be granted.
- 7) Two copies of the hazardous matter application document shall be prepared to be submitted with the package. (Note 5)
Furthermore, an open envelope stating "containing hazardous matter application" shall be fixed to the package. The post office will check the information on hazardous matter application and return the document. Seal it in the corresponding envelope under the inspection of the post office personnel.

(Note 3) When transporting on aircraft, it is regulated by Article 86 of the Aviation Law, Article 194 of Aviation Law Enforcement Regulations and related notifications, etc.

(Note 4) The form of indication label shall be as shown in separate Form 1-3 (request the necessary quantity to the post office in charge).

(Note 5) The hazardous matter application document shall be as shown in separate Form 1-4 (furthermore, this is stipulated specially between the Postal Service Agency and airlines for transporting the specimens for this case, and it cannot be applied to others).

(Separate Form 1-1)

(Test / Re-test)
 Test date: Year month day
 Municipality name:
 Test institute name:
 Inspector name (signature):
 Cutoff value:
 -10% of the cutoff value:
 Number of specimens:
 (Number of positive/negative specimens)

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

	← Enter the sample name (code, etc. used for individual identification).
	← Enter the measurement value.

※1: The above table indicates the 96-hole plate. Enter values with correspondence to the wells used in actual testing.

※2: Draw a slant line on each unused well.

Appendix 1
(Separate Form 1-2)

Name of institute conducting confirmatory test:
Name of receiver:

Municipality name:
Person in charge:
Phone:

Bovine Spongiform Encephalopathy Confirmatory test Specimen Sending Form

Date sent	Source sending the specimen (name of test center)	Specimen no.	Specimen weight (g)	Date of specimen collection	Sex	Information on collected animal			Date of slaughter	Remarks
						Breed	Age in months	Clinical symptoms		
1						Shipped by				
						Address	Phone	Name		
						Address	Phone	Name		
2						Shipped by				
						Address	Phone	Name		
						Address	Phone	Name		
3						Shipped by				
						Address	Phone	Name		
						Address	Phone	Name		

Planned date and time of arrival at the test institute

(Separate Form 1-3)

1. Transport permissible object indication label (Class no.: 6.2)

INFECTIOUS SUBSTANCE
IN CASE OF DAMAGE OR LEAKAGE
IMMEDIATELY NOTIFY
PUBLIC HEALTH AUTHORITY

2. Transport permissible object indication label (Class no.: 9)

MISCELLANEOUS

(Separate Form 1-4)

(Transport by air)

Application on Hazardous Substance Contained in Mail (Bovine Tissue, etc.)

The article name, quantity, etc. for the postal package below are all precise, and they are contained in a United Nations standard container with packing, labeling, etc. according to the Aviation Law and its related regulations. This postal package falls within the range of the loading limit for aircrafts and it is in an appropriate condition for transport by aircraft.

Date of preparation of application		Year month day	
Article name		Bovine tissue, etc.	
UN2814 UN2900	Substances (liquid) that may transmit disease that is transmissible to humans and animals	(Note 1) ml	
UN2814 UN2900	Substances (solid) that may transmit disease that is transmissible to humans and animals	(Note 2) g	
UN1845	Dry ice	Kg	
Dry ice is put outside the United Nations standard container to be packed in another container, etc.			

Shipped by Municipality name: Test institute name: Address: Phone number: Name: Slaughter inspector (veterinarian)
Received by Institute name: Address: Phone number: Name:

Space for use by airline company

(Note 1) If the contained substance is liquid, the total volume that can be put inside one container is less than 1,000 ml.

(Note 2) If the contained substance is solid, the total weight that can be put inside one container is up to 50 g.

(Separate Form 1-4) (Sample)

(Transport by air)

Application on Hazardous Substance Contained in Mail (Bovine Tissue, etc.)

The article name, quantity, etc. for the postal package below are all precise, and they are contained in a United Nations standard container with packing, labeling, etc. according to the Aviation Law and its related regulations. This postal package falls within the range of the loading limit for aircrafts and it is in an appropriate condition for transport by aircraft.

Date of preparation of application		October 30, 2002	
Article name		Bovine tissue, etc.	
	UN2814 UN2900	Substances (liquid) that may transmit disease that is transmissible to humans and animals	(Note 1) ml
✓	UN2814 UN2900	Substances (solid) that may transmit disease that is transmissible to humans and animals	(Note 2) 40 g
✓	UN1845	Dry ice	3 kg
✓	Dry ice is put outside the United Nations standard container to be packed in another container, etc.		

Shipped by Municipality name: xxx prefecture Test institute name: yyy meat sanitation test institute Address: 1-2-3 zzz, aaa city Phone number: xxxx-xxx-xxxx Name: Slaughter inspector (veterinarian) xx xx
Received by Institute name: yy Test Center Address: 〒000-0000 3-2-1 bbb city, ccc prefecture Phone number: xxxx-xxx-xxxx Name: xx xx

Space for use by airline company

(Note 1) If the contained substance is liquid, the total volume that can be put inside one container is less than 1,000 ml.

(Note 2) If the contained substance is solid, the total weight that can be put inside one container is up to 50 g.

(Reference Material)

Process for deactivating abnormal prion proteins completely

Table 1. Process for deactivating abnormal prion proteins completely²

Drug	Concentration	Process period	Temperature
Formic acid	≥ 60%	2 hours	Room temperature
Guanidine thiocyanate	≥ 4M	2 hours	Room temperature
Guanidine hydrochloride	≥ 7M	2 hours	Room temperature
Trichloroacetic acid	≥ 3M	2 hours	Room temperature
SDS	≥ 3%	5 minutes	100°C
Phenol	≥ 50%	2 hours	Room temperature

Table 2. Sterilization method for contaminated materials³

Drug, method, etc.	Temperature (°C)	Period (min)	Subject
Incineration	≥ 800	-	Viscera, inflammables, etc.
Autoclaving	134	60	Various equipment, devices, viscera, etc.
Soaking in 3% SDS	100	5	Various equipment, devices, etc.
Soaking in normality 2 NaOH	Room temperature	60	Various equipment, devices, etc.
Soaking in normality 1 NaOH	Room temperature	120	Various equipment, devices, etc.
Soaking in 1 – 5% sodium hypochlorite	Room temperature	120	Various equipment, devices, etc.

Cases should be dissected on a vinyl sheet spread out in an anatomy room, etc. and dissection should be limited to the minimum level necessary. When removing the head, contamination should be minimized by receiving the blood in a container. Take measures to place the separated head in a plastic bag and cover the cervical section with a plastic bag, etc. to prevent the spread of contamination.

²: Onodera Takashi, Kitamoto Tetsuyuki, Kurata Takeshi, Sato Takeshi, Tateishi Jun; Manual for Creutzfeldt-Jakob disease diagnosis, (edited by Disease Control Division, Health Service Bureau, Ministry of Health and Welfare), 18- 23, Shinkikaku Shuppannsya, Co., Ltd., Tokyo (1997).

³. Same as the above 2.

(Appendix 1-1)

Procedure for Using “Platelia BSE”

1. Sample refinement

(1) Preparation of reagents

To meet the number of specimens to be processed (see the table below), dilute proteinase K to 250-fold with the dissolving solution (Reagent A) for BSE refinement kit.

(The diluted proteinase K solution can be stored at room temperature for 4 hours.)

Number of specimens	Reagent A volume	Proteinase K volume
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) Refinement of abnormal prion peptides

- 1) Measure and take 350 ± 40 mg of the Obex section of the cattle.
- 2) Insert the measured specimen into a grinding tube.
- 3) Completely homogenize the sample in the grinding tube (the homogenized sample at this point can be stored for several weeks at -20°C . When storing the sample, freezing and defrosting shall be conducted only once).
- 4) Take 500µl and pour into a 2 ml microtube, etc. with caution not to contain solids from the homogenized sample (the sample can be stored for 8 hours at $2 - 8^{\circ}\text{C}$ and several weeks at -20°C).

- 5) Add 500µl of the proteinase K solution diluted in above 1. (1) and mix well. To unify the enzyme activation, add proteinase K solution and mix promptly within 5 minutes, or within 10 minutes when it is placed on ice.
- 6) When mixed well, promptly place in water bath, incubator, heat block, etc. for incubation at $37 \pm 1^\circ\text{C}$ for 10 ± 1 minutes. The period left between procedures 5) and 6) should not exceed 2 minutes.
- 7) Within 2 minutes after completion of incubation (or 10 minutes when tube is placed on ice), add 500µl of Reagent B and mix well until the entire solution turns blue (add Reagent B and mix promptly within 5 minutes, or within 10 minutes when it is placed on ice).
- 8) Centrifuge for 5 minutes at 20,000 x g or 7 minutes at 15,000 x g.
- 9) When centrifugal separation is finished, discard the supernatant within 5 minutes. To remove as much supernatant as possible, set the tube upside down and place it over paper for 5 minutes or suction dry with aspirator for 5 minutes.
- 10) When supernatant is discarded, add 50µl Reagent C1 to the microtube within 10 minutes. This must not be mixed using a vortex.
- 11) Promptly place in water bath, incubator, heat block, etc. for incubation at $100 \pm 1^\circ\text{C}$ for 5 ± 1 minutes. The period left between procedures 10) and 11) should not exceed 2 minutes.
- 12) Take out the microtube from incubator and mix well using a vortex (the sample can be stored for 5 hours at $2 - 8^\circ\text{C}$ or several weeks at -20°C at this point. When stored in either way, incubate at $100 \pm 1^\circ\text{C}$ for 5 ± 1 minutes and then mix well using a vortex).
- 13) Add 250µl of the diluting solution (R6) for BSE detection kit and mix (the sample can be stored for 5 hours at $2 - 8^\circ\text{C}$ at this point. When stored, mix well before moving on to the next procedure). When mixed, fractionate the sample to the well of detection kit microplate (continued to 2. (2) 2)).

2. Sample detection

(1) Preparation of reagents

- 1) The reagents to be used and microplate for solid-phasing shall be taken out of the refrigerator to be set to room temperature ($20 \pm 5^\circ\text{C}$) before use.
- 2) Dilute the undiluted washing solution (R2) with purified water to 10-fold and mix well to prepare the washing solution (R2') (this can be stored at $2 - 8^\circ\text{C}$ for 2 weeks).

- 3) Lightly tap and open the bottle of positive control (R4) to add 2 ml purified water or dilution solution (R6). Leave for 1 minute and mix slowly to dissolve (this can be stored for 2 hours at 2 – 8°C or 6 months at -20°C after fractionating to appropriate volumes). When storing by freezing, subdivide into microtubes, etc. immediately after defrosting and store at -20°C.
- 4) Dilute the enzyme labeled antibody (R7) with the washing solution to 10-fold immediately before use and mix slowly to prepare the enzyme labeled antibody solution (R7'). One milliliter of enzyme labeled antibody solution (R7') will be required for each strip (this can be stored for 6 hours at 2 – 8°C).
- 5) Mix the substrate buffer (R8) and coloring solution (R9) at the ratio of 10:1 in a light-tight container shielded with aluminum foil, etc. to prepare the substrate coloring solution (R8 + R9). One milliliter of substrate coloring solution (R8 + R9) will be required for each strip (this can be stored for 6 hours at room temperature. However, it cannot be used if it has turned blue before use. Prepare a new solution again in such cases.)

(2) Detection of abnormal prions

- 1) Take out the required number of strips from the microplate rack (return the strips that are not to be used into the bag with desiccant and close the bag while squeezing out the air. It can be stored for 1 month at 2 – 8°C).
- 2) Dispense the negative control (R3), positive control (R4) and sample prepared with BSE refining kit into the microplate wells as shown below: When testing using several plates, place each control for each plate. When using 1 microplate in several tests, place each control for each measurement.
 - A1, B1, C1, D1: negative control (R3) 100µl
 - E1, F1: positive control (R4) 100µl
 - G1, H1: Sample 100µl
- 3) Cover with sealing film and incubate at $37 \pm 1^\circ\text{C}$ for 75 ± 15 minutes using a heat block (desired), incubator, etc.
- 4) Take off the sealing film and wash the plate with washing solution (18 – 22°C). When washing with an automatic microplate washer, use the overflow setting with 800µl per well with 3 washing cycles. To wash manually, repeat the process of removing the solution over wells and dispensing 350µl washing solution 3 – 6 times (adjust the number of washing cycles while observing the values). To remove the washing solution completely from the well after washing, tap out the solution on paper. Do not leave it in this condition for 5 minutes or

longer.

- 5) Dispense 100µl of the enzyme labeled antibody solution (R7) into each well.
- 6) Cover with sealing film and incubate for 60 ± 5 minutes at $2 - 8^{\circ}\text{C}$.
- 7) Take off the sealing film and wash the plate with washing solution ($18 - 22^{\circ}\text{C}$).
When washing with an automatic microplate washer, use the overflow setting with 800µl per well with 5 washing cycles. To wash manually, repeat the process of removing the solution over wells and dispensing 350µl washing solution 5 – 10 times (adjust the number of washing cycles while observing the values). To remove the washing solution completely from the well after washing, tap out the solution on paper. Do not leave it in this condition for 5 minutes or longer.
- 8) Dispense 100µl substrate coloring solution (R8 + R9) into the well and take measures to cover the plate with aluminum foil, etc. and incubate at room temperature ($18 - 22^{\circ}\text{C}$) for 30 minutes in light-tight dark room. Film must not be used in incubation.
- 9) Dispense 100µl of the reaction stopper solution (R10).
- 10) Within 30 minutes after adding the stopper solution, measure the OD at dominant wavelength 450 nm and subdominant wavelength¹ 620 nm using a microplate reader. Be sure to always shut off light until measurement is taken.

¹ The range of 600 – 700 nm for subdominant wavelength will not affect the judgment.

3. Judgment

Judgment shall use the cutoff value calculated as follows:

$$\text{Cutoff value} = (\text{average absorbance for 4 negative controls} + \text{constant } 0.210)$$

The constant is regularly reviewed. Use the value stated on the instruction manual for the kit.

Negative when OD value < -10% of the cutoff value

Re-testing required when OD value \geq -10% of the cutoff value

When re-testing is required by the above, use the 2 holes of the microplate and the stored sample in 1. (2) 4 (it is recommended that re-testing is conducted by a different inspector).

Furthermore, check the measurement system by checking that the absorbance values for negative and positive controls satisfy the following conditions:

i) Absorbance values for all 4 holes with negative controls < 0.150

ii) Absorbance values for both 2 holes with positive controls \geq 1.000

Judgment in re-testing shall be made as follows:

(1) OD value for either of the 2 holes is equal to or larger than the cutoff value, or the OD value for either of the 2 holes is within -10% of the cutoff value, it is judged positive.

(2) If OD values for both 2 holes are less than -10% of the cutoff value, it is judged negative.

(Appendix 1-2)

Procedure for Using “Dynabott Enfer BSE Test”

1. Kit components

(1) Components of Dynabott Enfer BSS Test

Reagent package (stored at 2 – 8°C)

Reagent	Volume	Storage condition	Reagent preparation method	Storage conditions after preparation/time limit for use
Reagent 3	20 mL x 1	2 – 30°C	N/A	N/A
Washing agent 1	100 g powder x 1 bottle	2 – 30°C	Add 50 g of washing agent 1 per 1L of purified water and dissolve.	6 months at 2 – 8°C
Goat serum	150µL x 1	2 – 8°C	See the section for anti-prion antibody.	N/A
Conjugate	Concentrated conjugate 100µL x 1	2 – 8°C	Dilute the conjugate to the specified scale with washing agent 2 solution for each lot.	To be used within 2 hours from preparation
Substrate A	10 mL x 1 bottle	2 – 8°C	Mix Substrates A and B in the same volumes.	Store in dark room and use on the day of preparation.
Substrate B	10 mL x 1 bottle	2 – 8°C		
Centrifuge plate	2 plates	2 – 30°C	N/A	N/A
Assay plate	1 plate	2 – 30°C	N/A	N/A
Positive control well	8 wells	2 – 8°C	N/A	N/A
Blank control	30 mL x 1	2 – 30°C	N/A	N/A

Antibody package (stored at -25 – -15°C)

Reagent	Volume	Storage condition	Reagent preparation method	Storage conditions after preparation/time limit for use
Reagent 2	3 mL x 1	-25 – -15°C	N/A	N/A
Anti-prion antibody (rabbit serum)	Concentrated antibody 50µL x 1 bottle	-25 – -15°C	Dilute the anti-prion antibody to 500-fold using washing agent 2 solution and dilute the goat serum to the specified scale for each lot.	Use on the same day of preparation.

Buffer/wash package (stored at 10 – 30°C)

Reagent	Volume	Storage condition	Reagent preparation method	Storage conditions after preparation/time limit for use
Reagent 1	1L x 1	10 – 30°C	N/A	N/A
Washing agent 2	10-fold concentrated solution 500 mL x 1 bottle	10 – 30°C	Add 100 mL washing agent per 900 mL purified water and mix.	2 weeks at 10 – 30°C 1 month at 2 – 8°C

(2) Ingredients and volumes

Component reagent	Ingredient	Content (in 100 mL)
Reagent 1	Methanol	16 mL
	Sodium lauryl sulfate (SDS)	15 g
Reagent 2	Proteinase K	0.2 g
Reagent 3	Guanidine hydrochloride salt	28.659 g
Washing agent 1	Sodium chloride	100 g ^{*1}
Washing agent 2	Lauromacrogol	0.5 mL
Anti-prion antibody	Rabbit anti-prion serum	100 mL
Conjugate	Horseshoe peroxidase labeled anti-rabbit immunoglobulin (goat)	100 mL
Goat serum	Normal goat serum	100 mL
Positive control well	Synthetic prion peptides	2.4 ng ^{*2}
Substrate A	Substrate A (oxygenated water)	100 mL
Substrate B	Substrate B (3-aminophthalic hydrazide solution)	100 mL
Blank control	Methanol	16 mL
	Sodium lauryl sulfate	15 g
Assay plate	96-hole microplate	1 plate ^{*3}
Centrifuge plate	96-hole microplate	2 plates ^{*3}

^{*1}: Per 1 bottle

^{*2}: Per 1 well

^{*3}: Number of plates

2. Necessary tools and reagents

Materials contained in kit

- Reagents sufficient for testing 45 specimens are contained in the kit.

Materials not contained in kit

- High-grade deionized water, distilled water or reverse osmosis water shall be used (abbreviated as purified water hereafter).
- Stomacher Biomaster 80 (manufactured by Seward) homogenizer*
- Homogenizer bag (with filter) (manufactured by Interscience)

- Two units of Skatron SkanwasherR 300 (manufactured by Skatron) microplate washer*
 - iEMS incubator/shaker (manufactured by Thermo LabSystems)*
 - Luminoscan Ascent (manufactured by Thermo LabSystems) chemiluminescence measuring instrument*
 - Microplate centrifuge (2750G or higher)
 - Seal for microplate
 - Pipettes, etc.
 - Tools for specimen collection
 - Containers for diluting anti-prion antibody and conjugate
 - Glass or polypropylene containers for diluting other reagents
 - Negative control from tissue (see the section for preparation of tissue control.)
- * indicates specified devices required for this testing.

3. System parameter settings

The following provides the parameter information set for the recommended devices:
(It is not necessary for the user to set up.)

Washer

- This test requires 2 units of washers.
- For both Washing Protocols 1 and 2,
- Air pressure: 0.25 atm
- Volume/flow rate, adjustment offset >> ov: 1.00
- Aspirating position (normally 3.00 – 4.00 mm)
- Dispensing position: 0.00 mm

Washing Protocol 1*			Washing Protocol 2		
(Using Washing Agent Solution 1)			(Using Washing Agent Solution 2)		
Step:			Step:		
#1	Aspirate	6 sec	#1	Aspirate	4 sec
#2	Dispense	300µL	#2	Wash	3 sec
#3	Soak	5 sec	#3	Soak	5 sec
#4	Aspirate	4 sec	#4	Aspirate	2 sec
#5	Wash	5 sec	#5	Wash	3 sec
#6	Soak	5 sec	#6	Soak	5 sec
#7	Aspirate	3 sec	#7	Aspirate	2 sec
#8	Wash	2.5 sec	#8	Wash	3 sec
#9	Soak	5 sec	#9	Soak	5 sec
#10	Aspirate	2 sec	#10	Aspirate	2 sec
#11	Wash	2 sec	#11	Wash	2 sec
#12	Soak	5 sec	#12	Soak	5 sec
#13	Aspirate	5 sec	#13	Aspirate	4 sec
#14	End Wash		#14	End Wash	

* Operation of Washing Protocol 1 shall be conducted within a bio-safety cabinet.

Shaking incubator

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

Chemiluminescence measuring instrument

- Plate acceleration: 10, Settle delay: 100, Filter: none, Measurement type: single,

Integration time: 300, Lag time: 30 sec, Measurement count: 1, Photomultiplier (PMT) voltage: default value, Plate type: 96 wells, Scale factor: - 8

4. Preparation of reagents

Prepared reagents should be at room temperature at the time of use.

(1) Washing Agent Solution 1

Add 1 L purified water to 50 g of Washing Agent 1 (Enfer Wash 1) powder to prepare Washing Agent Solution 1. Shake until it dissolves (or set in rotary bottle shake for 10 minutes) and check that it is dissolved before using.

(Prepared Washing Agent Solution 1 can be stored for 6 months at 2 – 8°C.)

(2) Washing Agent Solution 2

Dilute the Washing Agent 2 (Enfer Wash 2) undiluted solution with purified water to 10-fold to prepare Washing Agent Solution 2.

(Prepared Washing Agent Solution 2 can be stored for 2 weeks at 10 – 30°C or 1 month at 2 – 8°C.)

(3) Anti-prion antibody + goat serum solution

Dilute the anti-prion antibody (Anti-PrP-1° Ab (Rabbit)) and goat serum (Normal Goat Serum (Goat)) with Washing Agent Solution 2 and reverse and mix to prepare anti-prion antibody + goat serum solution.

Since the dilution scale differs by the lot, dilute according to the instructions on the bottle label.

(Use up the anti-prion antibody + goat serum solution within the day of preparation.)

(4) Conjugate solution

Dilute the conjugate (Enzyme-conjugate-2° Ab (goat anti-rabbit)) with Washing Agent Solution 2 and reverse and mix to prepare the conjugate solution. Since the dilution scale differs by the lot, dilute according to the instructions on the bottle label.

(Store the prepared conjugate solution in a dark room and use up within 2 hours after preparation.)

(5) Substrate solution

Add Substrate A (Substrate Solution A) to Substrate B (Substrate Solution B) in equal volume.

Prepare the substrate solution at least 1 hour before use so that it is at room temperature when it is used.

(Store the prepared substrate solution in a dark room and use up within the day

of preparation.)

5. Preparation of specimen

- 1) Prepare the homogenate using 500 ± 40 mg of the collected bovine medulla (specimen).
- 2) Insert the specimen into a homogenizer bag (this bag is partitioned with a filter inside) in front of the filter and check that the specimen is pressed into the bottom of the bag. Mush the specimen with fingers to facilitate homogenizing.
- 3) Add 7.5 mL of Reagent 1 (Enfer Buffer 1 (Bovine)) into the deeper side of the filter in the homogenizer bag. Although there is no stipulation regarding the period until homogenizing after Reagent 1 is added, consideration should be given so that it is put through the immunoassay step smoothly.
- 4) Set the speed of Stomacher homogenizer to "high" to homogenize the specimen for 2 minutes. Since emulsion is prepared using a homogenizer bag with a filter, unnecessary parts such as membrane will be removed.

Note: Put the homogenized specimen through the immunoassay step immediately after homogenizing.

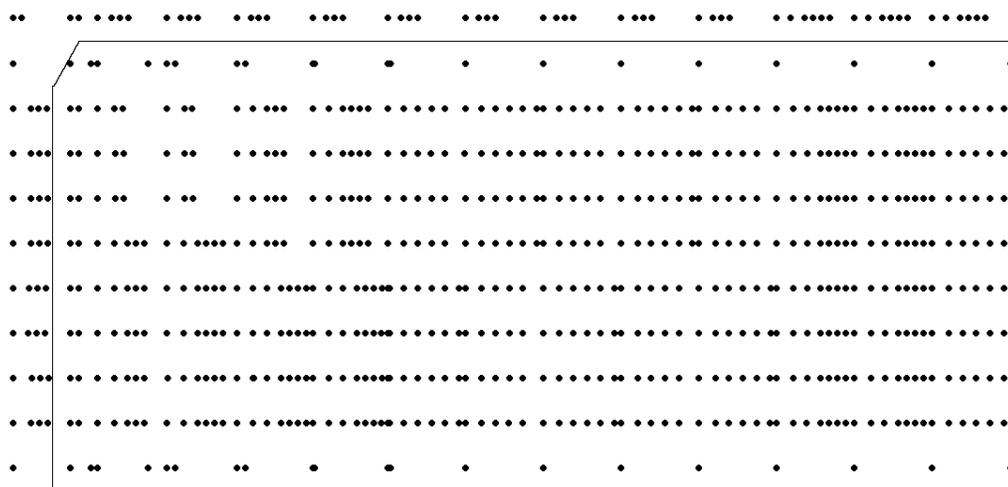
Store the residue from the emulsion used in testing in the homogenizer bag at room temperature until the first test result is obtained. Do not refrigerate since it will cause crystallization.

6. Procedure for immunoassay

- 1) Leave Positions A1 and A2 on centrifuge plates as positive control wells (Peptide Indicator Wells).
Dispense blank control (Blank Control Reagent (Bovine)) in 4 wells from Position B1 and 2 wells for each specimen at the volume of 180 μ L each.
- 2) Cover the centrifuge plate with a seal.
- 3) Centrifuge the centrifuge plate for 5 minutes at 2750 G.
- 4) Dispense 20 μ L of Reagent 2 (Enfer Buffer 2) into the bottom of each well of the assay plate (Enfer Test Plate) to be used in measurement.
- 5) Remove the seal from the centrifuge plate for which centrifuge has been completed to collect the 100 μ L supernatant for each specimen and blank control and transfer to the assay plate with Reagent 2.
- 6) Cover the assay plate with a seal.
- 7) Shake the assay plate for 60 minutes at 34°C.
- 8) Remove the seal and wash the assay plate using Washing Agent Solution 1 and

- (Washing Protocol 1).
- 9) Turn over the assay plate on soft paper and tap well to remove the remaining liquid.
 - 10) Add 150 μ L of Reagent 3 (Enfer Buffer 3) on each well.
 - 11) Cover the assay plate with a seal.
 - 12) Shake the assay plate for 15 minutes at 34°C.
 - 13) Remove the seal and wash the assay plate using Washing Agent Solution 2 and (Washing Protocol 2).
 - 14) Turn over the assay plate on soft paper and tap well to remove the remaining liquid.
 - 15) Remove Wells A1 and A2 from the assay plate to replace with the positive control well.
 - 16) Dispense 150 μ L of the prepared anti-prion antibody + goat serum solution into each well.
 - 17) Cover the assay plate with a seal.
 - 18) Shake the assay plate for 40 minutes at 34°C.
 - 19) Remove the seal and wash the assay plate using Washing Agent Solution 2 and (Washing Protocol 2).
 - 20) Turn over the assay plate on soft paper and tap well to remove the remaining liquid.
 - 21) Dispense 150 μ L of the prepared conjugate solution into each well on the assay plate.
 - 22) Cover the assay plate with a seal.
 - 23) Shake the assay plate for 30 minutes at 34°C.
 - 24) Remove the seal and wash the assay plate using Washing Agent Solution 2 and (Washing Protocol 2).
 - 25) Turn over the assay plate on soft paper and tap well to remove the remaining liquid.
 - 26) Dispense 150 μ L of the prepared substrate solution into each well on the assay plate.
 - 27) Cover the assay plate with a seal.
 - 28) Shake the assay plate for 10 minutes at 34°C.
 - 29) Remove the seal and read the luminescence strength using the chemiluminescence-measuring instrument.

Example of assay plate (Enfer Test Plate) arrangement



P: Positive control wells (Peptide Indicator Wells)

B: Blank control (Blank Control Reagent (Bovine))

S1 – S45: Samples

Flow of Measurement Procedure

Step	Process	Period/ temperature	Device/equipment used	Preparation for the next process	Precautions
Collection of specimen/preparation process					
Collection of specimen/weighing	Weighing the specimen: 500 ± 40 mg		Balance		
Homogenizing	Add 7.5 mL Reagent 1 per 500 ± 40 mg tissue	2 min (Speed "High")	Stomacher 80 homogenizer		Insert the tissue sample into the bottom of filter bag. Let it stand at room temperature until bubbles disappear from the sample when finished.
Transferring the homogenate	Transfer the homogenate to centrifuge plate: 180µL		Pipette	Transfer 180µL of the blank control to plate as well.	Cover the plate with seal
Centrifuge	Centrifuge: 2,750 G	5 min Room temperature	Centrifuge	Check that the incubator temperature is 34°C.	Ensure balance before centrifuging. Centrifuge at 2 – 8 °C is strictly prohibited.
Addition of Reagent 2 (PK)	Add into assay plate: 20µL		(8-gang) pipette		Add to the corner of well bottom (check addition of Reagent 2 visually at the end).
Dispensing the specimen into plate	Add centrifuge supernatant to plate: 100µL		(8-gang) pipette		Collect supernatant with caution on precipitate.
Incubation 1	Incubation	60 min at 34°C	LabSystem iEMS incubator		
Washing 1	Wash with Washing Agent 1 under Protocol 1.		Skanswasher 300 washer		After washing, turn over and tap several times over paper towel to

Appendix 1

					remove remaining liquid.
Addition of Reagent 3	Add 150µL of Reagent 3.		(8-gang) pipette		
Incubation 2	Incubation	15 min at 34°C	LabSystem iEMS incubator	Preparation of the first antibody solution (dilute anti-prion antibody and goat serum with prepared Washing Agent Solution 2.) Preparation of conjugate (second antibody) solution (dilute the conjugate with prepared Washing Agent Solution 2.) Also, prepare the substrate solution.	
Washing 2	Wash with Washing Agent solution 2 under Protocol 2.		Skanswasher 300 washer	After washing, break off Wells A1 and A2 and set the positive control wells.	After washing, turn over and tap several times over paper towel to remove remaining liquid.
ELISA process					
First antibody	Addition of the first antibody: 150µL		(8-gang) pipette		Check that the positive control is placed at Positions A1 and A2 and then add the first antibody.
Incubation 3	Incubation	40 min at 34°C	LabSystem iEMS incubator		
Washing 3	Wash with Washing Agent solution 2 under Protocol 2.		Skanswasher 300 washer		After washing, turn over and tap several times over paper towel to

					remove remaining liquid.
Conjugate	Addition of conjugate: 150µL		(8-gang) pipette		
Incubation 4	Incubation	30 min at 34°C	LabSystem iEMS incubator		
Washing 4	Wash with Washing Agent solution 2 under Protocol 2.		Skawasher 300 washer		After washing, turn over and tap several times over paper towel to remove remaining liquid.
Substrate	Addition of substrate: 150µL		(8-gang) pipette		
Incubation 5	Incubation	10 min at 34°C	LabSystem iEMS incubator		
Measurement	Measurement of chemiluminescence		Luminometer		

7. Judgment method

(1) Verification of test performance

It is necessary that the control results be verified before judging the specimen results. Average luminescence strength values for the blank control and positive control wells should be obtained. If the following standards are not satisfied, assay results are invalid. Conduct re-testing from the process of bovine medulla (specimen) collection described in "5. Preparation of specimen." In this case, measurement results are judged using the 2 wells as well.

1) Blank control

The median value for measurement using the 4 blank control wells must be less than 4.0LU. The median value is calculated by averaging the 2 measurement values that are not the largest or smallest for the 4 wells.

2) Positive control well

After subtracting the median value for the blank control, check that the average value of positive control wells stays inside the control range for the positive control wells from the lot used (stated on the label for the positive control wells).

The individual measurement values for positive control wells must not exceed $\pm 30\%$ from the average value of the positive control wells.

- (2) The cutoff value for this kit is 5.5LU. In addition, measurement values for all samples shall be used for judgment after subtracting the median value for blank controls.

When the measurement values for the 2 wells are both 5.5LU or smaller, it is judged as negative according to this kit. On the other hand, if a value exceeding 5.5LU is obtained in at least 1 of the 2 wells, the specimen needs to be re-tested with a test using 2 wells from the process of bovine medulla (specimen) collection described in “5. Preparation of specimen.”

If at least one of the measurement values using 2 wells exceeds 5.5LU as the result of re-testing, it is considered positive according to this kit, and a confirmatory test is required since it is suspected to be positive. If both of the measurement values using 2 wells in re-testing are 5.5LU or smaller, it is considered negative according to this kit.

Specimen measurement results (n = 2)



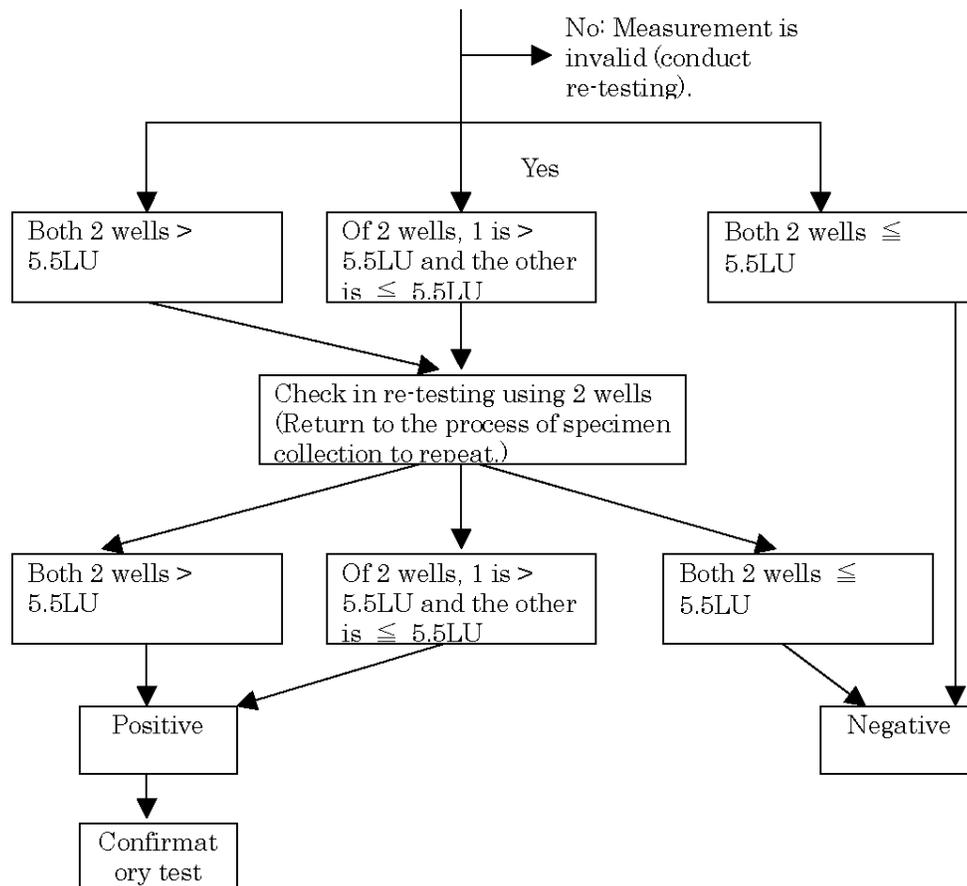
Blank control

- The median value for quadruple blank control measurement must be less than 4.0LU. The median value is calculated by averaging the 2 measurement values that are not the largest or smallest for the 4 wells.

Positive control wells

(Applied only when positive control wells accessory to this kit are used.)

- After subtracting the median value for the blank control, check that the average value of positive control wells stays inside the control range for the positive control wells from the lot used.
- The individual measurement values for positive control wells must not exceed $\pm 30\%$ from the average value of the positive control wells.



8. Dividing the kit

If the number of specimens is small, it is possible to divide the kit into 4 different tests. The minimum number of specimens in this case is 1.

9. Treatment in relation to specimen judged as positive in this test method

If re-testing is required as the result of judgment, move the emulsion that is supposed to be stored in the homogenizer bag at room temperature until judgment is made to a 15 ml plastic centrifuge tube for cultivation and store by freezing.

If it is positive as the result of re-testing, move the emulsion used in re-testing to a 15 ml plastic centrifuge tube and freeze for use in the confirmatory test along with the stored frozen emulsion from the first test.

If transport is necessary in implementing the confirmatory test, fix the lid of the 15 ml plastic centrifuge tubes for cultivation that contain the emulsion with Parafilm and wrap the entire tube with tissue, etc. as absorbing material in case the plastic centrifuge tubes are broken or the cap is removed and as cushioning material for shock. Then include in a biohazard can, etc. to ship as a specimen to be sent for western blot testing.

If both wells are negative as the result of re-testing, the stored frozen emulsion from the initial test should be discarded.