(Separate Attachment 2)

Procedure for Confirmatory Testing of Transmissible Spongiform Encephalopathy (TSE) by Prefectural and City Governments

1. Confirmatory Testing Institutions

(1) The Inspection and Safety Division designates prefectural and city testing institutions which satisfy the requirements in (2) below as "Authorized TSE Confirmatory Testing Institutions."

(2) Requirements

a. The institution's registration must include an individual who has completed the technical training course on TSE confirmatory testing offered by the Inspection and Safety Division, or who possesses equivalent or higher capabilities as recognized by the Division.
b. The institution shall be fully equipped with the necessary instruments, etc. indicated in this Procedure for Confirmatory Testing.
c. The institution shall comply with the testing methods indicated in section 2 below.
d. The institution shall implement all required procedures for confirmation of testing technology, such as external accuracy control, to be announced separately.

2. Implementation of Confirmatory Testing

(1) Testing by Western blot shall be implemented according to Separate Attachment 2-1 "Procedure for Immunobiochemical Examination (Western Blot)." Western blot confirmatory testing by the prefectural or city government shall be performed once only.

If the test results are inconclusive, the sample shall be dispatched to the National Institute of Infectious Diseases, etc., for confirmatory testing.

A sample must be dispatched, in accordance with the established method for dispatching samples for confirmatory testing. As material for the immunobiochemical examination (ELISA, Western Blot), the frozen sample is forwarded together with the frozen remainders of the sample materials used in the previous immunobiolochemical examination.

(2) Immunohistochemical and histological examinations shall be performed by prefectural and city governments according to Separate Attachment 2-2 "Procedure for Immunohistochemical Examination," and in addition, for the time being, the sample shall also be dispatched to the National Institute of Infectious Diseases, etc., for confirmatory testing.

The material for dispatching, consisting of all regions including remainders of the sectioned regions A(A*) - C(C*), indicated in Figure 1 in Separate Attachment 2-2, "Procedure for Immunohistochemical Examination," shall be dispatched at room temperature in a 50 mL container filled with buffered formalin.

3. Definitive Diagnosis

1) The institution conducting the confirmatory test shall send the data of the confirmatory test in electronic media to the Inspection and Safety Division. If the confirmatory testing is implemented by a prefectural or city government, the relevant prefectural or city office shall send the confirmatory test data in electronic media to the Inspection and Safety Division.

2) The Inspection and Safety Division shall forward the confirmatory test data to the "Expert Committee on Bovine Spongiform Encephalopathy Testing" for definitive diagnosis.

3) The histopathologic specimen (stained specimen) shall be subjected to microscopic diagnosis by an expert as deemed necessary.

4) Results of the definitive diagnosis shall be notified to the prefectural or city government who performed the confirmatory test, by the Inspection and Safety Division.

(Separate Attachment 2-1)

Procedure for Immunobiochemical Examination (Western Blot)

1. Instruments

- Electrophoresis cell: XCell SureLock Mini-Cell Electrophoresis System (Invitrogen Japan K.K., EI0001)
- Blotting cell: Mini Trans-Blot Cell (Bio-Rad Laboratories, Inc., 170-3930)
- Power supply: Power Pack 200 (Bio-Rad Laboratories, Inc., 165-5052)
  PowerEase 500 Power Supply (Invitrogen Japan K.K. EI8600)
- Membrane roller: Membrane roller (Advantec (Toyo Roshi Kaisha, Ltd.), EBA-200)
- Ultrasonic homogenizer: With output of about 750 W or higher, or equivalent using a booster (Ex. Branson Ultrasonics Corporation Digital Sonifier S450D)
- Multi-Beads Shocker: Original product of Yasui Kikai Corporation
- Constant temperature bath (water bath): Adjustable to 37°C (preferably with cooling function)
- Balance: Minimum measuring unit of 10 mg or lower
- High-speed micro centrifuge with cooling: Capable of a rotational speed of 15000 rpm or higher

2. Reagents

• Collagenase (for cell dispersion)	Wako Pure Chemical Industrials, Ltd.	100 mg, No. 038-10531
• Pefablock	Roche Diagnostics K.K.	500 mg, No. 1585916
• Proteinase K, PCR grade	Roche Diagnostics K.K.	5 mL, No. 1964372
• DNase I	Roche Diagnostics K.K.	100 mg, No. 104159
• N-Lauroylsarcosine (Sarkosyl)	Sigma-Aldrich Co.	100 g, No. L-5125
• Zwittergent 3-14	Calibiochem	5 g, No. 693017
• Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Co.	500 g, No. L-4509
• 2-mercaptoethanol	Sigma-Aldrich Co.	100 mL, M-6250
• Urea (special reagent grade)	Wako Pure Chemical Industrials, Ltd.	500 g, 217-00615
• 2-Butanol	Wako Pure Chemical Industrials, Ltd.	500 mL, 020-11215
• Tween 20	Wako Pure Chemical Industrials, Ltd.	500 mL, 167-11515
• Skim milk	Co-op, Meiji Dairies Corporation, Snow Brand Milk Products Co., Ltd., etc.	
• Fetal bovine serum (FBS)	Arbitrary brand	
• Immobilon-PVDF	Millipore Corporation	No. IPVH00010
• Filter paper	Bio-Rad Laboratories, Inc.	7.5 × 10 cm, No. 170-3932
	Advantec (Toyo Roshi Kaisha, Ltd.)	60 × 60 cm, No. 514A
• X-ray film (RX-U)	Fuji Photo Film Co., Ltd.	8 × 10", No. 03D051
• ECLWesten Blotting Detction Reagent	Amersham Pharmacia (General Electric Company)	No. RPN2209
• Anti-rabbit IgG HRP Label	Amersham Pharmacia (General Electric Company)	1 mL, NA 9340
• Anti-mouse IgG HRP Label	Amersham Pharmacia (General Electric Company)	1 mL, NA 9310
• 2 mL tube with O-ring (Not the tube for Multi-Beads Shocker)	Assist Co., Ltd.	No. 72.693S

3. Preparation of Reagents

- TN buffer: 100 mM NaCl, 50 mM Tris-HCl (pH 7.5)
- Detergent buffer: 4% Zwittergent 3-14, 1% Sarkosyl, 100 mM NaCl, 50 mM Tris-HCl (pH 7.5)
- Butanol-methanol solution: 2-butanol:methanol = 5 μ: 1
- Proteinase K: 1 mg/mL in 50 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, dispensed and stored frozen at -20°C
- Pefablock: 0.1 M in DDW, dispensed and stored frozen at -20°C
- Collagenase: 20 mg/mL in DDW, dispensed and stored frozen at -20°C
- DNase I: Dissolved in 50% glycerol, 10 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ at concentration of 10 mg/mL, and stored at -20°C.
- Sample buffer (×1): 62.5 mM Tris-HCl (pH 6.8), 5% glycerol, 3 mM EDTA, 5% SDS, 4M urea, 4% β-mercaptoethanol, 0.04% bromo phenol blue. Small quantities may be stored at room temperature during use. Long-term storage is advisable at 4°C (precipitation in urea and SDS may be dissolved by warming to about 50°C before use).

1 M Tris-HCl (pH 6.8)	1.25 mL
Glycerol	1 mL
0.5 M EDTA (pH 8.0)	120 μL
β-mercaptoethanol	800 μL
1% bromo phenol blue	800 μL
SDS	1 g
Urea	4.8 g
	Up to 20 mL

4. Preparation of Emulsion

a) Using the Multi-Beads Shocker (Yasui Kikai)
   1) Insert the metal cone (No. MC-01212PP) into its exclusive 2 mL tube with O-ring.
   2) Place 200 mg of brain tissue in the tube.
   3) Add 800 μL of TN buffer.
   4) Shake at 2000 rpm for 30 seconds using the Multi-Beads Shocker.
   5) Store the resulting 20% (W/W) brain emulsion in the tube with O-ring.

b) Using an ultrasonic homogenizer
   1) Cut 200 mg of brain tissue into small pieces on Parafilm and transfer into a 2 mL tube.
   2) Add 800 μL of TN buffer.
   3) Sonicate using a cup horn ultrasonic homogenizer until the tissue becomes a consistent emulsion.
   4) Store the resulting 20% (W/W) brain emulsion in the tube with O-ring.

c) Using the Enfer method stomacher homogenizer
   1) Place 500 ± 40 mg of brain tissue in the homogenizer bag.
   2) Add 7.5 mL of the Enfer Kit Reagent 1 (Enfer Buffer 1 (Bovine)).
   3) Homogenize for 2 minutes using the stomacher homogenizer at speed 'High.'
   4) Portion the resulting 6.25% (W/W) brain emulsion by 1 mL volumes and store.

5. Sample Preparation

1) Add 250 μL of detergent buffer to 250 μL of the 20%(W/W) brain emulsion in the 2 mL tube with O-ring and vortex (sonicate if necessary) (Note 1).
2) Add 12.5 μL of 20 mg/mL collagenase and vortex.
3) Digest at 37°C for 30 minutes (must be in a water bath).
4) Add 20 μL of 1 mg/mL PK and vortex.
5) Digest at 37°C for 30 minutes (must be in a water bath; vortex 1-2 times during digestion).
6) Add 10 μL of 0.1 M Pefablock and vortex.
7) Add 2 μL of the 10 mg/mL DNase and vortex, and let stand at room temperature for 5 minutes.
8) Add 250 μL of butanol-methanol solution and vortex.
9) Centrifuge for 10 minutes at 15000 rpm, 20°C.
10) Remove the supernatant and lightly dry the precipitate (Note 2).
11) Add 100 μL of the 1 × sample buffer and boil at 100°C for 5 minutes. Sonicate if the precipitate is not sufficiently dissolved.

a) Preparation of the 20% brain homogenate prepared using the BSE Purification Kit (Bio-Rad Laboratories, Inc.)
   1) Add 250 μL of detergent buffer to 250 μL of 20% brain homogenate. Vortex and sonicate (Note 1).
   2) Add 12.5 μL of 20 mg/mL collagenase and vortex.
   3) Digest at 37°C for 30 minutes.
   4) Add 20 μL of 1 mg/mL PK and vortex.
   5) Digest at 37°C for 30 minutes.
   6) Add 10 μL of Pefablock and vortex.
   7) Add 250 μL of butanol-methanol solution.
   8) Vortex.
   9) Centrifuge for 10 minutes at 15000 rpm, 20°C.
   10) Remove the supernatant and dry the precipitate (Note 2).
   11) Add 100 μL of the 1 × sample buffer and boil at 100°C for 5 minutes. Sonicate if the precipitate is not sufficiently dissolved.

   (Note 1) Clearer results may be obtained in the Western blot by adding 25 μL (5%) of 2-butanol and sonicating after adding detergent buffer, prior to enzymatic digestion, as it promotes the digestion of normal prion proteins and proteins with nonspecific antibody reactivity.
   (Note 2) As the centrifuged supernatant contains butanol, it must be disposed of as an organic solvent. Add 10-NaOH to it in a ratio of 1:10 and let stand for 2 hours or more to inactivate the prions, then neutralize.

b) Preparation of the 6.25% brain homogenate prepared using the Enfer method stomacher homogenizer
   1) Centrifuge 1 mL of the brain emulsion for 10 minutes at 15000 rpm, 20°C, and transfer 800 mL of the supernatant (50 mg tissue-equivalent) into a separate 2 mL tube.
   2) Add 20 μL of 20 mg/mL collagenase and vortex.
   3) Digest at 37°C for 30 minutes.

4) Add 20 μL of 19.2 mg/mL proteinase K and vortex.
5) Digest at 37°C for 30 minutes.
6) Add 16 μL of 0.1 M Pefablock and vortex.
7) Add 3.4 μL of 10 mg/mL DNase and vortex, and let stand at room temperature for 5 minutes.
8) Add 400 μL of 2-butanol and vortex.
9) Centrifuge for 10 minutes at 15000 rpm, 20°C.
10) Remove the supernatant, and then invert the sample tube on paper towel and let stand for 5 minutes to dry the precipitate.
11) Add 100 μL of the 1 × sample buffer and boil at 100°C for 5 minutes. Sonicate if the precipitate is not sufficiently dissolved.

c) Sample preparation using the 20 w/v% brain emulsion prepared using Frelisa BSE
These procedures basically conform to "Procedure for Immunobiochemical Examination (Western Blot)."
1) Collect 250 μL of 20 w/v% brain emulsion and transfer it to a separate 2 mL tube.
2) Add 250 μL of detergent buffer (may be replaced with the Surfactant Solution in the Freliza BSE Kit) and mix well by vortex (sonicate in addition if necessary).
3) Add 12.5 μL of 20 mg/mL collagenase and mix by vortex.
4) Digest at 37°C for 30 minutes (must be in a water bath).
5) Add 20 μL of 1 mg/mL proteinase K and mix by vortex.
6) Digest at 37°C for 30 minutes (must be in a water bath; mix by vortex 1-2 times during digestion).
7) Add 10 μL of 0.1 M Pefablock and mix by vortex.
8) Add 2 μL of 10 mg/mL DNase I and mix by vortex, then let stand at room temperature for 5 minutes.
9) Add 250 μL of butanol-methanol solution and mix by vortex.
10) Centrifuge for 10 minutes at 15000 rpm, 20°C.
11) Remove the supernatant and lightly dry the precipitate.
12) Add 100 μL of the 1 × sample buffer and boil at 100°C for 5 minutes (Do not mix after the sample buffer is added).
13) After boiling, mix by vortex (Sonicate if the precipitate is not sufficiently dissolved).

The buffers and reagents to be used in the procedures above shall conform to indications in "Procedure for Confirmatory Testing of Transmissible Spongiform Encephalopathy (TSE): Procedure for Immunobiochemical Examination (Western Blot)."

* Decontamination of instruments, etc. used for sample preparation
- Scissors, tweezers, tips and tubes, etc. shall be placed in a pressure- and heat-resistant container filled with 150 mL of water, leaving the lid open, and autoclaved at 135°C for 30 minutes.
- Combustible materials shall also be autoclaved likewise to decontaminate.

6. SDS-PAGE

- Use the precast gel of Invitrogen Japan K.K. (former Novex electrophoresis GmbH)
- Gel: NuPAGE 12% Bis-Tris Gel, 1.0 mm, 12 well (Invitrogen Japan K.K. No. NP0342)
- Load 20 μL (10 mg tissue-equivalent) and 5 μL (2.5 mg tissue-equivalent) using the gel loading tip (Funakoshi Co., Ltd. SRPT-1381)
- Buffer: NuPAGE MOPS SDS Running Buffer (Invitrogen Japan K.K. No. NP0001). Add 500 μL of Antioxidant (Invitrogen Japan K.K. No. NP0005) to the cathode buffer chamber
- Migrate using 200 V constant voltages.

<Positive control for measurement of sensitivity>

Prepare a concentrate solution (4^0, 100 μg/10 μL tissue-equivalent) by diluting the positive control ($MoPrP^{Sc}$ lot 011209, 10 mg/mL tissue-equivalent) 10-fold using the sample buffer. During preparation of the concentrate solution (4^0), heat once to 100°C for 2 minutes. The concentrate solution is dispensed to about 50 μL per tube and stored at -20°C. Further, a four-level dilution array, consisting of 4^{-1}(25 μg/10 μL), 4^{-2}(6.25 μg/10 μL), 4^{-3}(1.6 μg/10 μL) and 4^{-4}(0.4 μg/10 μL), is prepared and stored dispensed at -20°C. The prepared dilution array is dissolved using warm water at 50°C before use. Do not reheat to 100°C. Load the positive controls 4^{-1} to 4^{-4} in volumes of 10 μL/lane (or 4^{-2} to 4^{-4} depending on the lanes used). As this positive control dilution array is required for sensitivity evaluation of the WB procedure, it must be subjected to electrophoretic migration in the gel together with the sample. If the PrP^{Sc} is detected up to the 4^{-3} dilution, the WB results are deemed valid.

The positive control is distributed by the National Institute of Infectious Diseases, etc.

7. Western Blot (WB)

- Blotting Cell: Bio-Rad Laboratories, Inc. Mini Trans-Blot Cell (170-3930)
- Transfer buffer

NuPAGE Transfer Buffer (Invitrogen Japan K.K. No. NP0006)	50 mL		
Antioxidant (Invitrogen Japan K.K. No. NP0005)	1 mL		
Methanol	200 mL	final	20%
20% SDS	0.5 mL	final	0.01%
	Up to 1 L		

- Cut the PVDF membrane (Immobilon-PVDF) to size 7.5 × 9 cm and soak in methanol for 1 minute to activate. Wash with DDW and leave soaked in transfer buffer.
- Soak the gel after electrophoretic migration is complete in the transfer buffer.
- Place the PVDF membrane on 2 sheets of filter paper (1 sheet if using Bio-Rad Laboratories, Inc. filter paper) wetted with the transfer buffer, and then place the gel on it. Be careful not to allow any air bubbles between the gel and PVDF membrane. Further add filter papers (2) wetted with transfer buffer on top of the gel.
- Sandwich the filter paper-PVDF-filter paper sandwich above once more with the blotting pad and set in the blotting system. As protein travels from the cathode to anode, make sure the PVDF membrane is positioned on the anode side of the gel.
- Conduct blotting under one of the conditions a)-c) below. a) or b) is recommended.

When speed is an issue, c) is acceptable but tends to cause higher background.

a) 30 V constant voltage for 6-15 hours
b) 60 V constant voltage for 2 hours
c) 80 V constant voltage for 1 hour

8. IHC Staining

Advisable to implement using 44B1 as the main, and B103 as the alternative antibody.

[1] Using 44B1 monoclonal antibody(Note 3) (Note 4)

1) Blocking: 5% skim milk, 5% FBS in PBST (0.1% Tween 20). Always warm the skim milk to dissolve (about 80°C). After cooling in cooling water, add FBS to a final concentration of 5% (FBS is added for a blocking effect, as the background of the PVDF membrane tends to rise with 44B1).
2) Let stand for 1 hour on membrane roller (Advantec (Toyo Roshi Kaisha, Ltd.), No. EBA-200).
3) Primary antibody: Dilute using 1% skim milk and 1% FBS in PBST. Use at concentration of roughly 0.1-0.2 μg/mL.
4) Let stand for 1 hour on membrane roller.
5) Wash for 20 minutes using PBST. Replace the PBST 5 times.
6) Secondary antibody (Amersham Pharmacia (General Electric Company) NA9310): Dilute to 1:2500 using 1% skim milk and 1% FBS in PBST.
7) Let stand for 45 minutes on membrane roller.
8) Wash for 20 minutes using PBST. Replace the PBST 5 times.
9) Induce luminescence using ECL Western Blotting Detection Reagent.
10) Expose to X-ray film for 2 minutes and develop.
11) While developing, expose the next X-ray film.
12) After 30 minutes, develop (make two X-ray films, of exposures 2 and 30 minutes)(Note 5).
13) If necessary, further expose overnight.
    Developing solution: HI Rendol
    Stop solution: 3% acetic acid
    Fixing solution: Super Fuji Fix

[2] Using B103 affinity-purified polyclonal antibody(Note 6)
(Differs from [1] Using 44B1 in steps 1), 3) and 6).)

1) Blocking: 5% skim milk in PBST (0.1% Tween 20). Always warm the skim milk to dissolve (about 80°C).
2) Let stand for 1 hour on membrane roller (Advantec (Toyo Roshi Kaisha, Ltd.), No. EBA-200).
3) Primary antibody: Dilute using 1% skim milk in PBST. Use at concentration of roughly 1 μg/mL.
4) Let stand for 1 hour on membrane roller.
5) Wash for 20 minutes using PBST. Replace the PBST 5 times.
6) Secondary antibody (Amersham Pharmacia (General Electric Company) NA9340): Dilute to 1:2500 using 1% skim milk in PBST.
7) Let stand for 45 minutes on membrane roller.
8) Wash for 20 minutes using PBST. Replace the PBST 5 times.
9) Induce luminescence using ECL Western Blotting Detection Reagent.
10) Expose to X-ray film for 2 minutes and develop.

11) While developing, expose the next X-ray film.
12) After 30 minutes, develop (make two X-ray films, of exposures 2 and 30 minutes)(Note 5).
13) If necessary, further expose overnight.
Developing solution: HI Rendol
Stop solution: 3% acetic acid
Fixing solution: Super Fuji Fix

(Note 3) The 44B1 antibody is to be distributed by National Institute of Infectious Diseases, etc. The current lot is 02011, 6.5 mg/mL.
(Note 4) If, upon conducting the operations above, the background of PVDF is high and the prescribed sensitivity (see p18) is not obtained, sensitivity may be improved through the following methods.
(1) Change the blocking solution to 5% skim milk in 50 mM Tris-HCl (0.1% Tween 20), and the antibody reaction solution to 1% skim milk in 50 mM Tris-HCl (0.1% Tween 20).
(2) Change the membrane wash process following the antibody reaction to 5 minutes × 6 times using 0.1% Tween 20 in PBS (50 mL).
(3) Transfer at 20V for 1 hour using Invitrogen Japan K.K. transfer unit (XCell II Blot Module E19051), transfer buffer (NuPAGE Transfer Buffer: NP0006, NP0006-1), and PVDF membrane (LC2005).
(Note 5) The exposure time of 30 minutes is merely a rough guide, thus it should be adjusted case-by-case, based on the results of the 2-minute exposure.
(Note 6) The B103 antibody is distributed by Fujirebio Inc. The current lot is SB21103, 1 mg/mL.

For Reference: Examples of execution

[Example 1]
Day 1: Sample preparation (2 hours)
Day 2: PAGE (1.5 hours) → WB (2 hours) → Staining (4 hours)

[Example 2]
Day 1: Sample preparation (2 hours) → PAGE (1.5 hours) → WB (12 hours max.)
Day 2: Staining (4 hours)

[Example 3]
Day 1: Sample preparation (2 hours) → PAGE (1.5 hours) → WB (2 hours) → Staining (4 hours)

9. Accuracy Control

1) Internal accuracy control shall be implemented at least once a month, using the positive control distributed by the National Institute of Infectious Diseases, etc. and a sample confirmed to be negative in the screening test.
2) The external accuracy control measures, to be announced separately, shall be implemented.