(Separate Attachment 2-2)

Procedure for Immunohistochemical Examination

1. Preparation of Paraffin Block

<Materials>

Disposable bench protection sheet, cutting board, sectioning blade, tweezers, stainless steel tray, required number of plastic cassettes (numbered), blade disposal can, Kim Towels (absorbent paper wipes), 1N NaOH, container for formic acid disposal

<Operations>

1) Perform sectioning of the formalin-fixed tissue within a safety cabinet, wearing the prescribed garments (compliant with the garments for screening).
2) Wipe around the outside of the sample container with 1N NaOH and wipe again with water.
3) Section the formalin-fixed tissue.
   Spread the bench protection sheet and section on a plastic cutting board using a disposable blade. The obex must be sectioned in thickness 3 mm or less. Section a total of three slices, one from the obex and 2 from the region above it, and set them in the plastic cassettes.
   Fix by shaking in 15-20% formalin solution at 60°C for at least 1 hour (about 2-3 hours is advisable if sectioning raw tissue). If processing the following day, fix at 37°C up until 1.5 hours prior to embedding.
4) Treat with formic acid at room temperature for 1 hour.
   Place the plastic cassette containing the fixed brain tissue directly in 98% formic acid, and agitate for 1 hour at room temperature using a shaker. Rinse under running water for 30 minutes (to decrease infectivity).
5) Process, using a 4-hour protocol on a sealing type automatic embedder, or manually.
6) Paraffin embedding shall be performed on a dedicated machine using a dedicated mold.

<Post handling>

1) Discard the formalin in a dedicated tank. Incinerate at a later date.
2) Place the tweezers and blade used for sectioning in a stainless steel tray and soak in 1N NaOH at room temperature for 2 hours (or place in dedicated can and autoclave later). Wash with water. Dispose of the blade.
3) Cover the cutting board with a Kim Towel soaked with 1N NaOH and let stand for 2 hours at room temperature, then wash with water.
4) Replace any remaining brain tissue back into the fixation bottle for storage. When no longer required, autoclave (see below) and dispose of.

2. Sectioning

<Materials>

Bench protection sheet, microtome, water pail, paraffin stretching unit, humidifier, can for replacement blade disposal, glass slides (silane-coated), 1N NaOH, Kim Towels (absorbent paper wipes)

<Operation>

1) The operator shall wear gloves, mask with face shield, and gown. Use anti-cut gloves where necessary.
2) Spread the bench protection sheet, place the microtome on it and perform slicing. Using a dedicated paraffin stretching unit and water pail, place the sections on the silane-coated slides.
3) Vacuum the scrap from the tissue sections using a dedicated vacuum cleaner with HEPA filter. Incinerate or autoclave later.
4) Autoclave the knife holder at 135°C for 60 minutes, wash with water, and dry.
5) Soak the blade in 1 N NaOH for 2 hours at room temperature (or place in dedicated can and autoclave later).
6) Dry the sliced sections at 45°C.

<Post handling>

Vacuum all tissue scrap using the vacuum cleaner above.

3. HE Staining (Prepare a dedicated system of trays for deparaffinization & staining)

1) Deparaffinize, treat with ethanol, and wash with water.
2) Perform staining using Harris hematoxylin for 2 minutes at room temperature.
3) Allow the color to develop for 10 minutes in lukewarm water.
4) Perform eosin staining for 3 minutes at room temperature.
5) Differentiate, dehydrate, and clear.
6) Mount.

4. Immunohistochemistry

<Reagents, etc.>

Envision+ Kit (DAKO Japan Co., Ltd., for mouse and rabbit), Simple Stain DAB Solution (Histofine), 3% hydrogen peroxide solution, primary antibody, hematoxylin , PBS

Preparation of PB (0.1 M PB for immunostaining)

Na_2HPO_4 $12H_2O$	28.7 g
NaH_2PO_4 $2H_2O$	3.3 g
D.W. (distilled water)	1.0 L

Preparation of PBS (0.01 M PBS)

PB	100 mL
D.W. (distilled water)	900 mL

NaCl 8.5 g

<Operations>

1) Deparaffinize, treat with ethanol, and wash with water.
2) Place in dedicated autoclave in a stainless steel tray filled with distilled water, and autoclave at 121°C for 20 minutes. When cooled, remove and wash with PBS.
3) Treat with endogenous peroxidase (3% hydrogen peroxide solution, 5 minutes at room temperature).
4) Perform blocking (10% normal goat serum-PBS, 5 minutes at room temperature). (May be omitted)
5) Place primary antibody (see below) on top, and allow reacting for 30-40 minutes at room temperature.
6) Wash with PBS.
7) Allow to react with Envision+ Solution for 30 minutes at room temperature, and then wash with PBS.
8) Perform DAB staining reaction.
9) Wash with tap water, then allow to react with Meyer's hematoxylin for 30 seconds at room temperature.
10) Allow color to develop in lukewarm water, then dehydrate, clear, and mount.

<Post handling>

Discard the xylene for deparaffinization, the ethanol, and distilled water in separate dedicated containers and incinerate.
Subject the dedicated staining trays and staining baskets to treatment at 135°C for 1 hour, then wash with water.
Dispose of the staining solutions such as hematoxylin and eosin.

5. 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.

1. Schematic Overview of Paraffin Block Preparation (1): From Sectioning to Fixation/Embedding (Manual Procedure)

After sectioning, shake in 20% formalin at 60°C for 60 minutes to re-fix

⇩

Shake in 98% formic acid for 60 minutes

⇩

Rinse under running water for 30 minutes

⇩

Remove excess water with filter paper

⇩

80% A1 15 minutes
90% A1 15 minutes
100% A1 15 minutes
100% A1: Acetone
(1:1) 15 minutes
Acetone 15 minutes
Xylene 15 minutes
Xylene 15 minutes
Xylene 15 minutes
Paraffin 15 minutes
Paraffin 15 minutes
Paraffin 15 minutes
Embedding

Time required: 2 hours 45 minutes

(Note that intercellular gaps easily occur in the specimen.)

1. Schematic Overview of Paraffin Block Preparation (2): Durations of dehydration, clearing and paraffin soaking using automatic embedding system

Equipment: Sakura Finetek Japan Co., Ltd. Sealing Type Automatic Embedding System

	Time setting
80% Alcohol	10 minutes
90% Alcohol	10 minutes
95% Alcohol	10 minutes
99% Alcohol	20 minutes
100% Alcohol I	20 minutes
100% Alcohol II	30 minutes
100% Alcohol III	30 minutes
Xylene I	20 minutes
Xylene II	20 minutes
Xylene III	20 minutes
Paraffin I	10 minutes
Paraffin II	10 minutes
Paraffin III	10 minutes
Paraffin IV	20 minutes
Total	4 hours 30 minutes

Leave the vacuum switched ON at all times.

4. Schematic Overview of Immunohistochemistry: Procedure for Rapid Immunostaining in Confirmatory Testing

Deparaffinize	10 minutes
⇩	
Wash with water	5 minutes
⇩	
Autoclave （Soak in distilled water）	121°C, 20 minutes （Time: 1.5 hours）
⇩	
Drop 3% hydrogen peroxide solution	5 minutes
⇩	
Wash with PBS	5 minutes × 2-3 times
⇩	
Primary antibody reaction*	Room temperature, 30-40 minutes
⇩	
Wash with PBS	5 minutes × 3 times
⇩	
Secondary antibody reaction**	Room temperature, 30 minutes
⇩	
Wash with PBS	5 minutes × 3 times
⇩	
DAB coloration	7-10 minutes
⇩	
Wash under running water	5 minutes
⇩	
Staining of nuclei with hematoxylin	30 seconds
⇩	
Wash under running water (warm water)	5 minutes
⇩	
Dehydrate/ Mount	10 minutes

* Use PBS for diluting primary antibody.
The negative control is normal rabbit (mouse) serum (× 1000).

** Envision+ Polymer Reagent (DAKO Japan Co., Ltd.)

List of Reagents and Equipment

(A similar product of any arbitrary manufacturer may be used for general reagents and instruments)

	Reagent	Manufacturer	Specification	Unit
1	Sodium hydroxide	Wako Pure Chemical Industrials, Ltd.	197-02125	500 g
2	Formalin (37.5%)	Wako Pure Chemical Industrials, Ltd.	061-00411	3 L
3	Formic acid (99%)	Wako Pure Chemical Industrials, Ltd.	066-00466	500 mL
4	Paraffin	Wako Pure Chemical Industrials, Ltd.	164-13345	500 g
5	Alcohol	Sigma-Aldrich Co.		4 L
6	DAKO PEN	DAKO		1 unit
7	Harris hematoxylin	Muto Pure Chemicals Co., Ltd.	2002	500 mL
8	Meyer’s hematoxylin	Muto Pure Chemicals Co., Ltd.	3001	500 mL
9	Eosin	Muto Pure Chemicals Co., Ltd.		500 mL
10	B103 or 44B1 anti-PrP antibody	Fujirebio Inc. (44B1 is a distributed item)		
11	Envision+ Kit (for mouse or rabbit)	DAKO Japan Co., Ltd.		110 mL
12	Simple Stain DAB kit	Histofine (Nichirei Corporation)	415172	1 set
13	Hydrogen peroxide solution	Wako Pure Chemical Industrials, Ltd.	081-04215	500 mL
14	Normal rabbit serum	Arbitrary manufacturer		
15	Xylene for pathology use	Muto Pure Chemicals Co., Ltd.		15 kg
16	Mount-Quick (mounting medium)	Daido Sangyo Co., Ltd.		30 cc
17	Sodium dihydrogen phosphate (dehydrate)	Wako Pure Chemical Industrials, Ltd.	199-02825	500 g
18	Disodium hydrogen phosphate 12-water	Wako Pure Chemical Industrials, Ltd.	196-02835	500 g
19	Sodium chloride	Wako Pure Chemical Industrials, Ltd.	191-01665	500 g

	Instruments and Equipment	Manufacturer	Specification	Unit
1	Disposable bench protection sheet	Whatman Japan K.K.	40 × 57 cm	50 sheets
2	Sectioning blade replacement blade	Feather Safety Razor Co., Ltd.	No. 130	50 units
3	Plastic cassette	Tissue-Tek® (Sakura Finetek Japan Co., Ltd.)	Pro-Cassette	1000 units
4	Kim Towels	Crecia Corporation	J-120	24 packs
5	Container for disposing formic acid	Nalgene® (Nalge Nunc International)	2118-0032	units
6	Silane-coated slide	Muto Pure Chemicals Co., Ltd.	1106	100 units
7	Cover glass	Muto Pure Chemicals Co., Ltd.	24 × 36	1000 units
8	Microtome replacement blade	Feather Safety Razor Co., Ltd.	A35	50 units
9	Staining tray (20-unit)	Matsunami Glass Industries Ltd.		units
10	Staining basket (20-unit)	Matsunami Glass Industries Ltd.	B-20	units
11	Stainless steel tray		0.6 L	units
12	Wetting box	Cosmo Bio Co., Ltd.	Capacity: 20-unit	units
13	Latex gloves	Asahi Emers Co., Ltd.	DPG-350	Box (of 100)
14	Mask with face shield	Hogy Medical	FBM-281	50 units
15	Gown	Hogy Medical	MGM-14	30 units
16	Anti-cut gloves	As One Corporation (former Iuchi Seieido Co., Ltd.)	LA132	10 units
17	Safety cabinet	Labconco	LAD-1300XA	
18	Slide washer	Juji Field Inc.	SW-4	
19	Autoclave 135°C	Tomy Seiko Co., Ltd.	KS-323	
20	Hood with HEPA filter	Oriental Giken Inc.	Aura-700	
21	Vacuum cleaner with HEPA filter	Atomic	FC-111-A13	
22	Automatic embedding system	Sakura Finetek Japan Co., Ltd.	ETV-150CV	
23	Paraffin stretching unit	Sakura Finetek Japan Co., Ltd.	PS-53	
24	Humidifier	Sakura Finetek Japan Co., Ltd.	SMB-1	

(Supplementary) Pretreatment of Anti-PrP Antibody and Pathological Section

Presently, the anti-PrP peptide rabbit antibody and mouse monoclonal antibody are available for use in BSE confirmatory testing. B103 (Obihiro University of Agriculture and Veterinary Medicine) and T4 (National Institute of Infectious Diseases) are available for the former, and 44B1 and 43C5 (both Obihiro University), for the latter. It has been revealed that certain combinations in pretreatment of the antibody and section influence the results of the test. Presently, the advisable procedure is to autoclave in distilled water, followed by use of B103 as the main, and 44B1 as the alternative antibody.

1. Pretreatment of Section

The purpose of pretreatment on the deparaffinized histopathological section is to destruct the PrP^{C} and enhance reactivity towards PrP^{SC} (by recovering antigenicity, or increasing antigen exposure), therefore pretreatment is essential in BSE testing. The following two methods of pretreatment have been investigated so far.

1) Treatment in distilled water at 121°C for 20 minutes.

2) Treatment in 1 mM aqueous hydrochloric acid at 121°C for 20 minutes.

Formerly, proteinase treatment was performed in addition; however, it has become unnecessary with the current rapid tissue processing systems.
Autoclaving is performed under identical conditions in the two methods above, placing the staining baskets containing the sections in a 400 mL stainless steel tray with lid.

2. Description of Antibodies

a) B103 rabbit antibody: Prepared using PrP protein N-terminal 103-121 peptide as the antigen (4.6 mg/mL)

b) T4 rabbit antibody: Prepared using PrP protein C-terminal 221-239 peptide as the antigen (0.6 mg/mL)

* Both of the rabbit antibodies above are affinity purified.

c) 44B1 mouse monoclonal antibody: Recognition of 155-231 (4 mg/mL)

d) 43C5 mouse monoclonal antibody: Recognition of 161-169 (4.6 mg/mL)

3. Antibody and Pretreatment

Antibody and Dilution Ratio	1) DDW 121°C, 20 minutes		2) 1 mM HCl 121°C, 20 minutes	
	Pos	Neg	Pos	Neg
B103 × 500	+/-	-/-	3+</+N	-/+D
T4 × 1000	2+/-	-/-	3+>/-	-/+-
44B1 × 500	+/-	-/-	2+</-	-/-
43C5 × 2000	2+/+D	-/+	3+/2+D	-/3+D

Pos: Positive control (Hokkaido Row 2), Neg: Negative control (previously showing non-specificity; B026), +/-: Signal positive (degree)/ Nonspecific reaction (degree)
Fujirebio Inc. antibody, having antibody concentration of 1 mg/mL, was used.

4. Comments

1) B103 antibody is a monospecific polyclonal antibody, believed to recognize multiple antigenic determinants. Normally under conditions of 1), it can detect PrP^{sc} without difficulty. However, its reactivity is rather low. At times, it exhibits a weak nonspecific reaction on the cell nucleus. Under conditions of 2), it shows strong nonspecific staining on the nucleus. Although best staining is achieved under the conditions of 2), as nonspecific reactions occur, currently 1) is the preferable method.

2) T4 antibody produces best results under conditions of 2). Findings of nonspecific staining have been observed in three cases in the past. Identical nonspecific positive reactions occurred using B103 and 43C5, however, no nonspecific findings were observed using 44B1. Regarding T4, there is no remaining stock for distribution.

3) 44B1 is characteristic in that it can be used under any of the conditions and shows no nonspecific reactions, but has somewhat low reaction intensity (signal intensity). Regarding potency of antibody-staining, it is lower compared to 43C5.

4) 43C5 shows nonspecific reaction, consisting of diffuse coloration of neural networks such as the olivary nucleus, in either method.

[Reference]

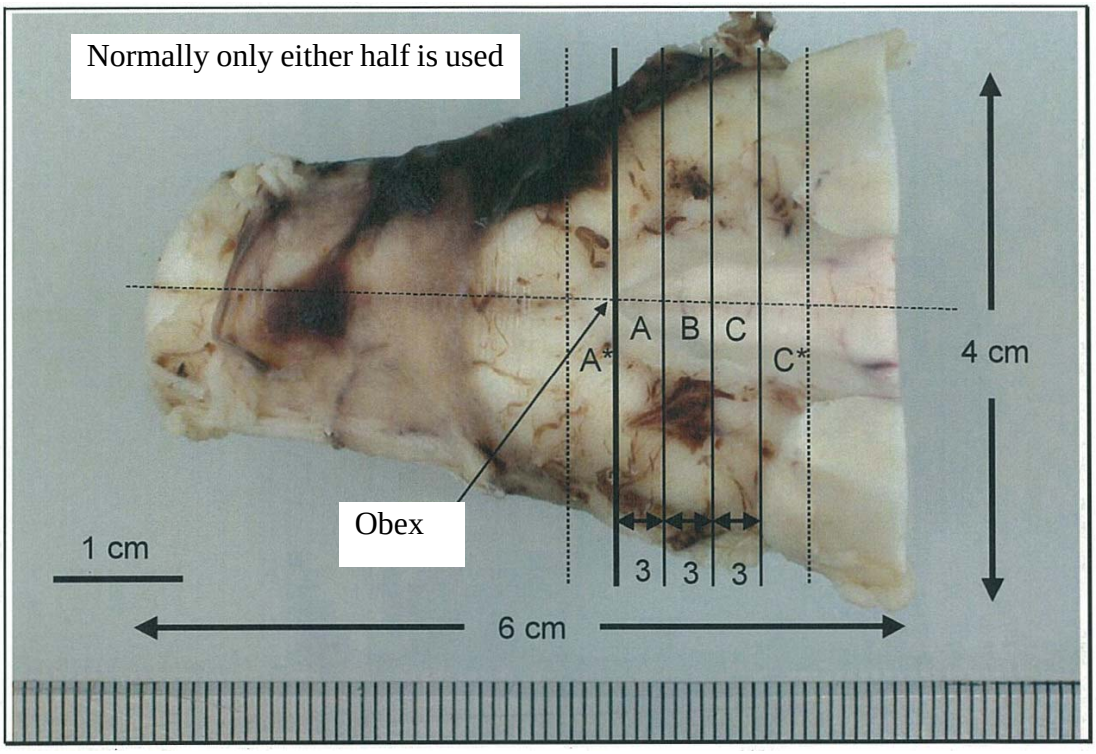

Figure 1. Normal bovine medulla oblongata

The regions to be sectioned are indicated. Normally, the obex (A) and the regions above the obex (B and C) are cut. The three regions A-C are sectioned at minimum. The upper region (right side) is thinly sliced.

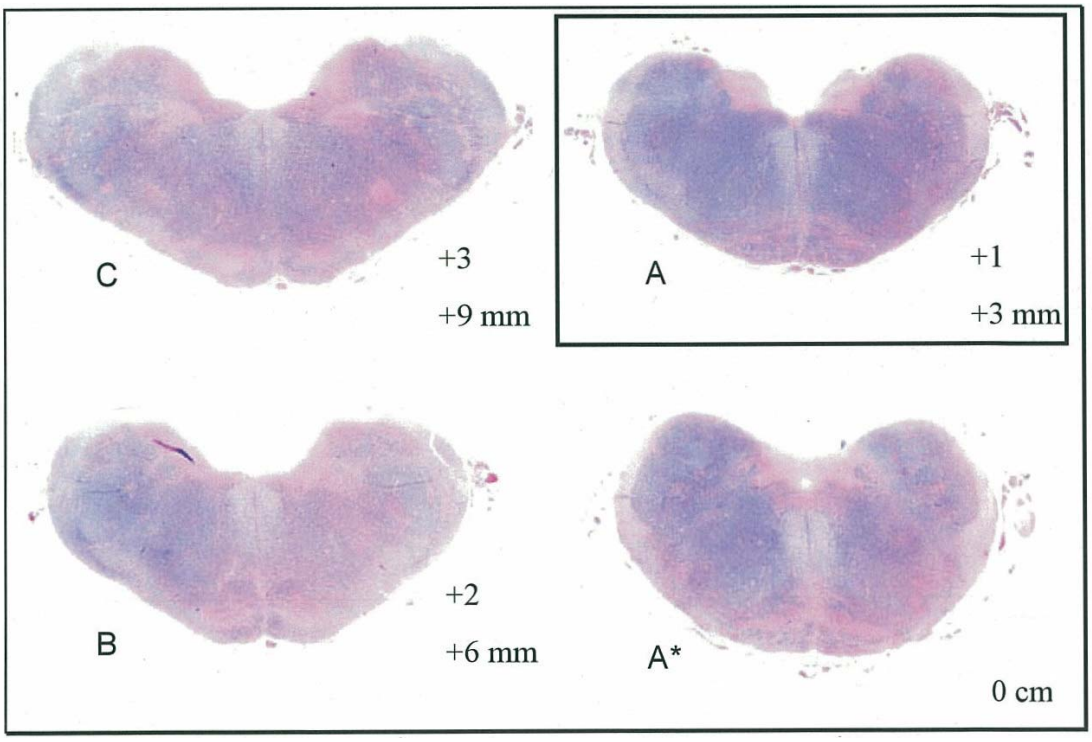

Figure 2. HE-stained specimens of the planes A-C and A*

The numerical values to the right are best at the position directly above the obex (A, +1 = +3 mm). See the next figure for the distribution of nerve nuclei in this region of the medulla oblongata.

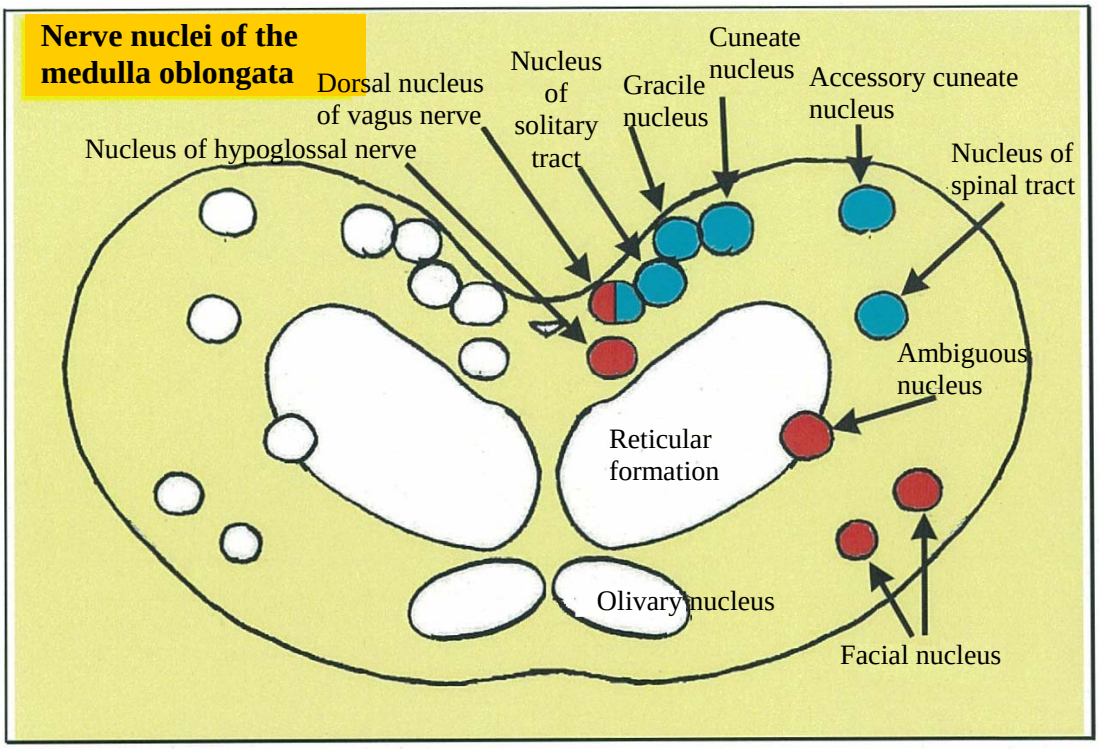

Figure 3. Nerve nuclei of the medulla oblongata

Figure 4.

The actual specimen is divided into two along the major axis of the medulla oblongata. Confirm the position of the obex (1), and section the specimens. The sectioned slice (2; of a separate specimen). Section at 3 mm intervals as if slicing off the surface from the head-side, then place in plastic cassettes (3). Pay attention to the order and the surface of sectioning.

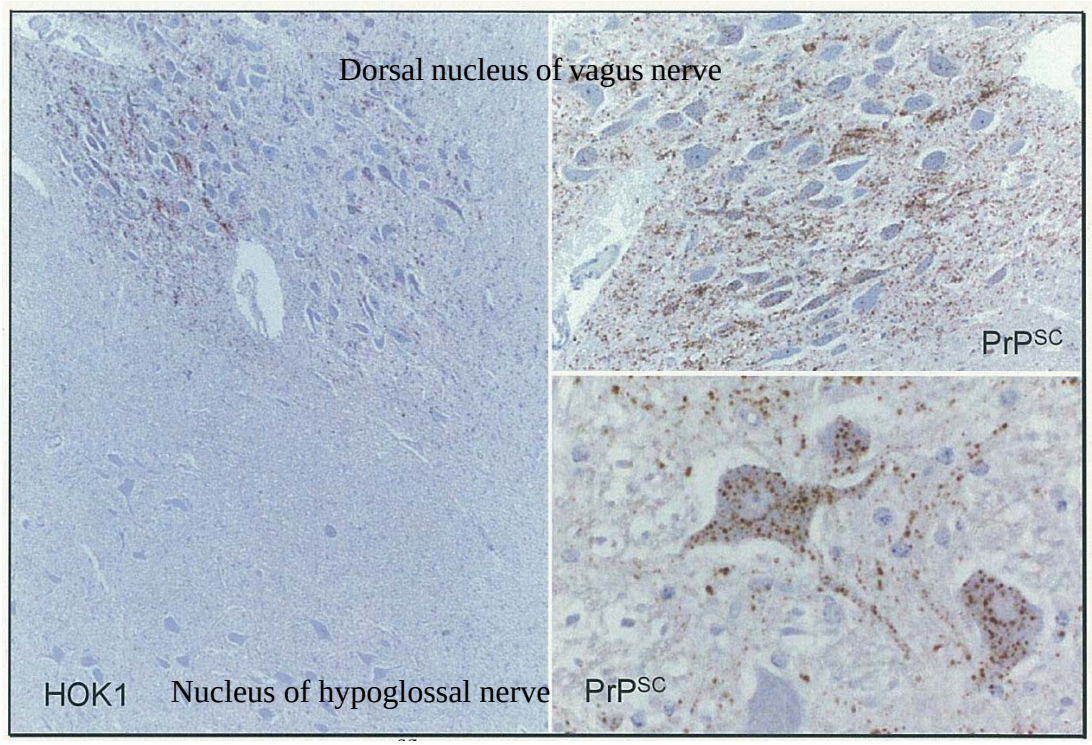

Figure 5. Positive image of Hokkaido Row 1

Note the PrP^{SC} positive findings. Granular prion is detected.

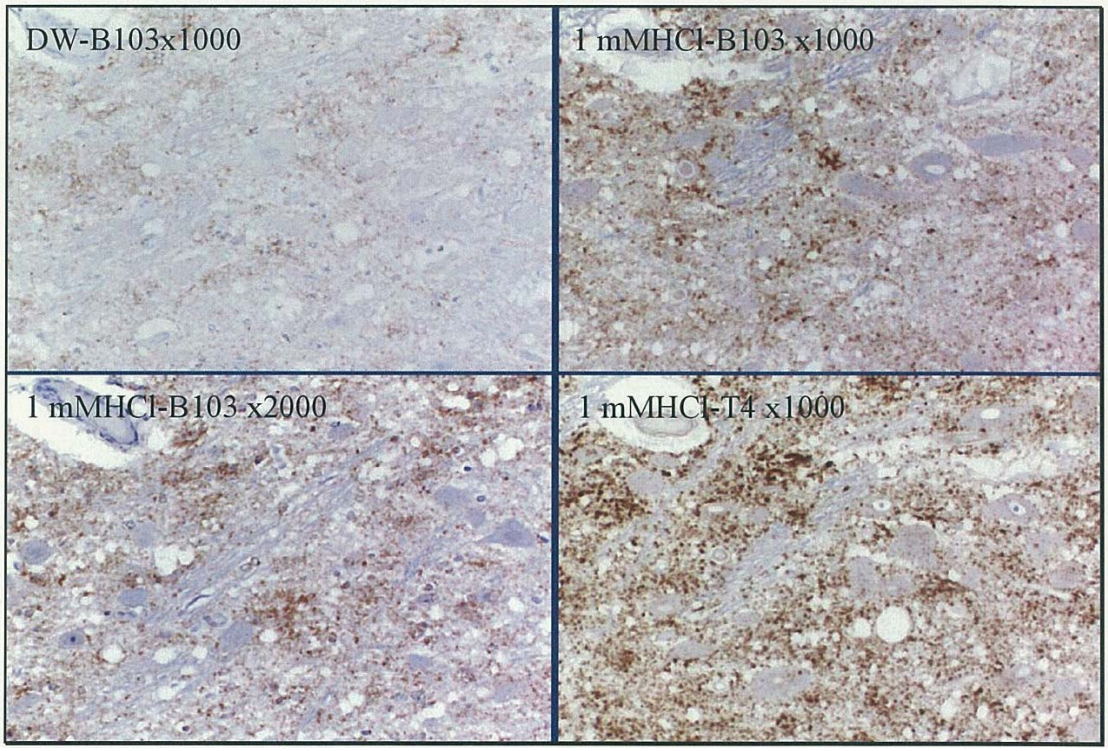

Figure 6. IHC staining using B103, and effect of pretreatment of section by hydrochloric acid (Saitama example)

Normally, autoclave treatment of 20 minutes at 121°C is performed using distilled water. Treatment by hydrochloric acid intensifies the prion positive reaction, but causes nonspecific staining in the nucleus. Hydrochloric acid treatment is used on occasion for confirmation purposes when necessary.

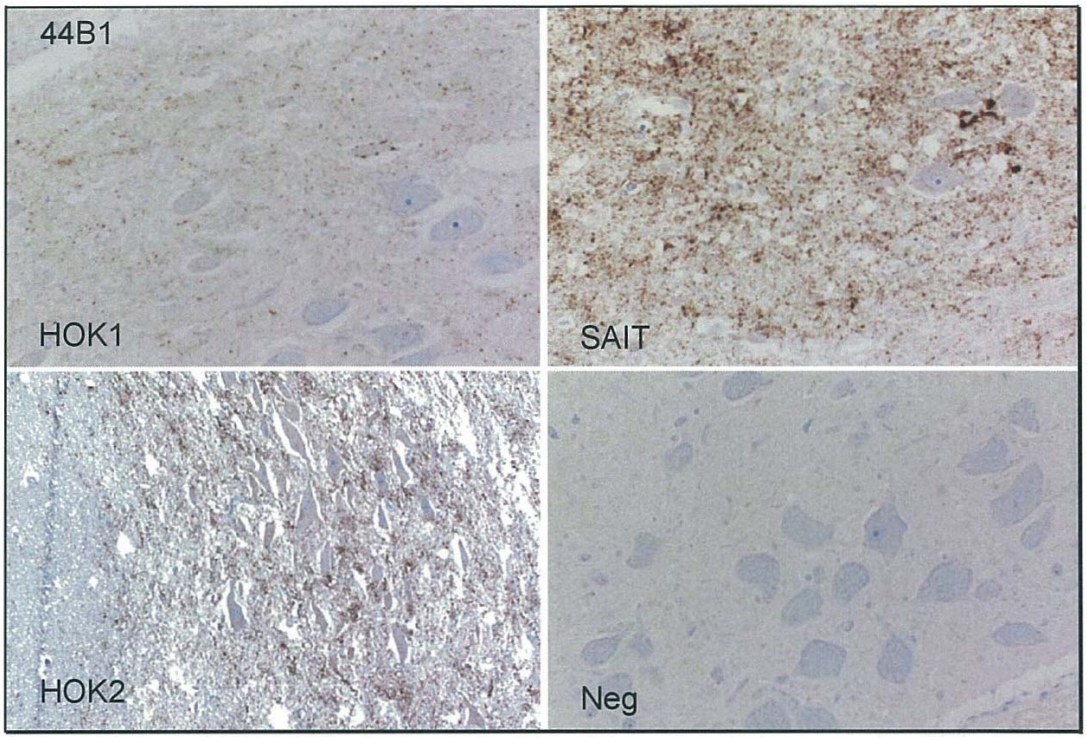

Figure 7. IHC staining image using 44B1

HOK2 represents the positive image using rabbit anti-PrP polyclonal antibody (T4). SAIT includes spongiform change.

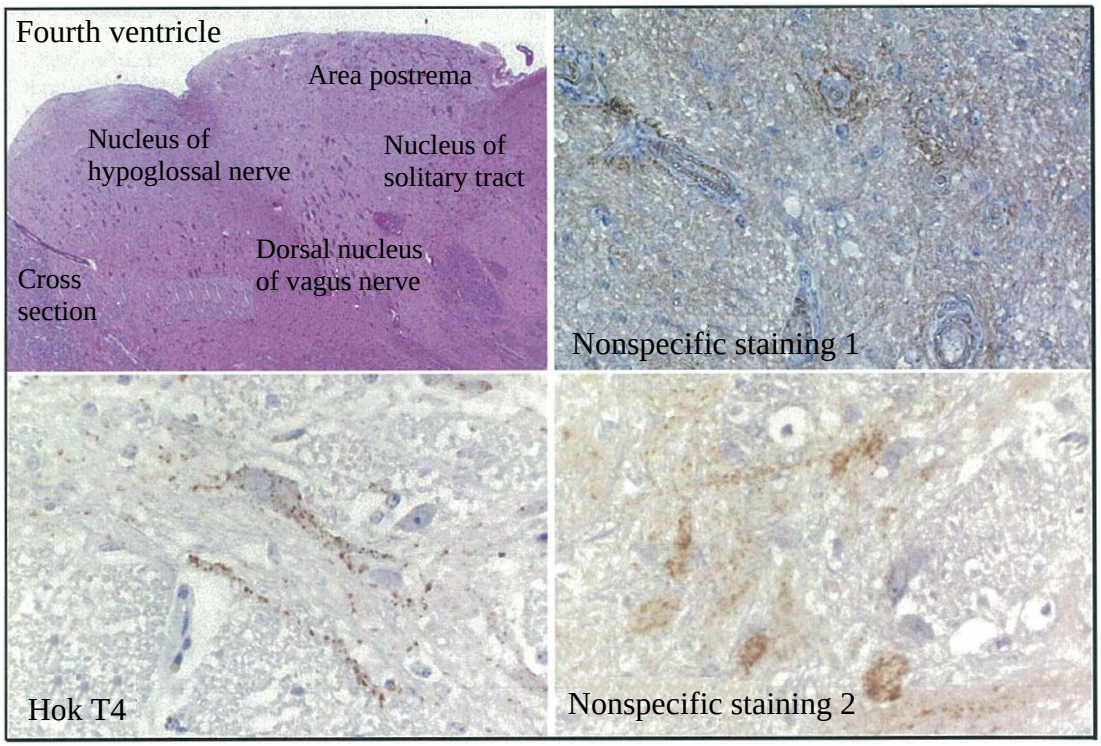

Figure 8.

The area postrema appears in the cross section of A. In this region, nonspecific staining is sometimes observed surrounding the small blood vessels when using a polyclonal antibody (T4 or B103). In addition, on occasion, findings such as the Nonspecific staining 2 are obtained. The stained findings of Nonspecific staining 2 are clearly dissimilar from the positive findings of Hok T4. There have been no incidences of such nonspecific staining up to present using the 44B1 monoclonal antibody.