



VMRD, Inc.

BOVINE SPONGIFORM ENCEPHALOPATHY ANTIGEN TEST KIT, IMMUNOHISTOCHEMISTRY

For Veterinary Use Only
USDA Product Code 5430.40

General Description

This Bovine Spongiform Encephalopathy Antigen Test Kit, Immunohistochemistry uses monoclonal antibody immunohistochemistry to detect prion protein (PrP) in bovine brain samples.

Test Kit Components

- A. 10X Target Retrieval Solution, 50 ml, must be diluted before use.
- B. Proteinase K, 5 ml, ready to use.
- C. Monoclonal Antibody F99/97.6.1, 0.05 ml, must be diluted before use.
- D. Antibody Diluent, 5 ml, ready to use.
- E. Anti-Mouse IgG-Biotin, 5 ml, ready to use.
- F. Streptavidin-HRP, 5 ml, ready to use.
- G. AEC Substrate Chromogen, 15 ml, ready to use.

These instructions.

Materials Required But Not Included in the Test Kit

- Heat and formic acid-proof slide racks and containers.
- Coverplate holder (Shandon catalog no. 73310017).
- Coverplates (Shandon catalog no. 72110013).
- General laboratory containers.
- Ethanol, methanol, 30% stock hydrogen peroxide, xylene or xylene substitute, 95% formic acid solution, Tris base, NaCl, deionized or distilled water, nonalcoholic counterstain such as Mayer's hematoxylin, aqueous-based mounting medium, Tween, HCl, NaOH.

Storage and Stability

Store all reagents at refrigerator temperature (2-7°C; 35-45°F). **Do not freeze.** Reagents will remain stable until the expiration date when stored as instructed. **Do not use test kit past the expiration date printed on the box.**

Precautions

- Do not eat, drink or smoke where samples and kit reagents are handled.
- Do not use expired or contaminated reagents, or reagents from other kits.
- Do not pipette by mouth.
- Some reagents contain sodium azide, which may be harmful if ingested. If ingested, seek medical attention.

SAMPLING

Suitable samples for this test are transverse sections of the medulla at the level of the obex (Figures 1-4), no more than 7 mm thick, of cattle.

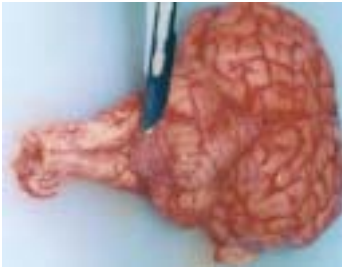


Figure 1. Scalpel pointing to the base of the cerebellum, just dorsal to the obex.



Figure 2. Cerebellum has been removed and the scalpel points to the obex area of the medulla.

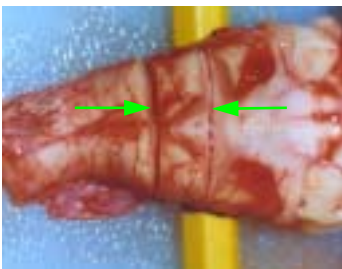


Figure 3. Arrows show proper incisions approximately 6 mm apart encompassing the obex.



Figure 4. Section of medulla removed with caudal portion facing up. Microtome sections should be taken from the caudal side.

TISSUE PREPARATION

Decontamination of Tissues:

Bovine spongiform encephalopathy is considered a zoonosis. The decontamination described in this protocol is based on the recommendations of the College of American Pathologists for decontamination of tissues from individuals with Creutzfeldt-Jakob disease and is consistent with the recommendations of the Centers for Disease Control, Atlanta, GA, USA, and the Advisory Committee on Dangerous Pathogens. Personnel wear protective clothing, gloves and safety glasses. Biosafety level 2 procedures are followed for all tissues. Disposal of formalin and formic acid is performed according to local regulations.

Formic acid decontamination of obex tissue is required for adequate test specificity. Formic acid treatment decontaminates prion infectivity, presumably by disrupting the beta sheet structure of the disease-associated form of the protein. Formic acid also increases the sensitivity of the immunostaining and reduces the rate of false positives due to detection of endogenous cellular PrP. However, formic acid antigen treatment alone (below) is not adequate for elimination of endogenous cellular PrP.

Caution: This protocol involves the use of formalin and formic acid. These agents are caustic and may cause sensitivity. Avoid contact with skin or eyes. Avoid breathing fumes. Handle only in fume hood. Transport in unbreakable containers. Label all secondary containers appropriately. Wear safety glasses at all times when handling or transporting formalin or formic acid.

1. Tissue is fixed in 10% neutral buffered formalin. Transverse sections of the medulla at the level of the obex (Figures 1-4), no more than 7 mm thick, are fixed for at least 48 hours.
2. Samples of medulla are sliced to 2 mm thickness starting from the caudal surface, trimmed as needed and positioned in cassettes. Place cassettes in an acid-proof container.
3. Decontaminate tissue by incubating the cassettes in 95-98% formic acid for 60 minutes, using sufficient formic acid to cover the tissues.
4. Rinse the cassettes in a fume hood 3 times with 10 volumes of water each time. The container can now be moved to a sink; no formic acid odor should be detectable at this point.
5. Rinse the tissues in running distilled or deionized water for 10 minutes.
6. Transfer the cassettes to fresh 10% neutral-buffered formalin and hold for 24 hours to re-equilibrate.

Processing and Paraffin Embedding:

These steps are performed using conventional techniques.

Slide Preparation: Three to 5 micron sections are cut and placed on slides. Adherence of the sections to the slides can be a problem with some tissues. Charged slides (SuperFrost Plus or equivalent) have been used extensively; no gelatin or additives to the water bath are used and care is taken to remove any traces of detergent from the water bath after cleaning. Conventional frosted end slides (75 x 25 mm with a 19 x 25 mm frosted end) are acceptable.

Extended frosted end slides (75 x 25 mm with a 32 x 25 mm frosted end) cannot be used with this test kit.

Slides are baked at 56°C for a minimum of 2 hours and a maximum of 18 hours to remove excess paraffin.

Rehydration and Inactivation of Endogenous Peroxidase:

Typically sections are rehydrated by serial incubations in xylene or a xylene substitute (Clear-Rite) [two times, 5 minutes each], 100% ethanol [two times, 4 minutes each], 95% ethanol [1 time, 3 minutes], and 80% ethanol [1 time, 3 minutes].

At this stage endogenous peroxidase activity is inhibited by incubating the rack of slides in a container for 10 minutes in 200 ml of freshly prepared 3% hydrogen peroxide in methanol (prepared by diluting 20 ml of 30% hydrogen peroxide in 180 ml methanol).

The slides are then rinsed by dipping the rack into deionized or distilled water 10 times. The slides may be held in water for a few hours at this stage if needed.

Antigen Retrieval with Formic Acid and Heat:

1. Incubate slides in 95% formic acid for 5 minutes, using an acid-proof slide rack and container. Rinse and neutralize by putting slide rack through three changes (3 minutes, 2 minutes, 2 minutes) of 0.1 M Tris-HCl, pH 7.6. Formic acid can be used 5 times if held in an acid-proof container, then disposed of in accordance with local regulations.
2. Transfer slides to a heatproof vessel containing modified citrate buffer (1X Target Retrieval Solution, pH 6.1). Target Retrieval Solution must be held at 4°C until used. Standard formulations of citric acid buffers pH 6.1 do not produce adequate antigen retrieval. Only the proprietary Target Retrieval Solution is recommended. Target Retrieval Solution can be used 3 times if not diluted by steam from the autoclave or pressure cooker and held at 4°C between uses. Typical containers hold 200 ml of solution with

- racks that carry 20-25 slides. For smaller batches proportionally smaller containers with less solution should be used.
3. Transfer container of slides to an autoclave or medical pressure cooker. Incubate at 121°C for 20 minutes (timing begins when temperature reaches 121°C). Allow slides to cool to room temperature.
 4. Transfer slide rack to TBST (Tris Buffered Saline with Tween 20) for 10 minutes. Slides may be held in this buffer for a few hours if needed.

TEST PREPARATION

Prepare 1X Target Retrieval Solution: 50 ml of 10X Target Retrieval Solution (A) is diluted for use with 450 ml of double-distilled or deionized water. After dilution, pH should be between 6.0-6.2. If not, it may be adjusted down with 2N HCl or up with 2N NaOH. Store 1X Target Retrieval Solution at 2-7°C (35-45°F) between uses.

Prepare 1X Monoclonal Antibody: Monoclonal Antibody F99/97.6.1 (C) should be diluted on the day of use according to instructions on the label using Antibody Diluent (D). After dilution, antibody remains refrigerated (2-7°C; 35-45°F) or on ice until use. Storage of diluted antibody is not advised.

Prepare 0.1 M Tris-HCl Buffer: Dissolve 12.1 g of Tris Base (MW 121.1 g) in 800 ml double-distilled water. Adjust pH to 7.6 with concentrated HCl and dilute to 1 liter. This may be stored at room temperature.

Prepare TBST Buffer (50 mM Tris-HCl, 300 mM NaCl, 0.1% Tween 20): Dissolve 6.06 g Tris base (MW 121.1 g) and 17.5 g NaCl (MW 58.44) in 800 ml double-distilled water. Adjust pH to 7.6 with concentrated HCl. Dilute to 1 liter, mix well, then add 1 ml Tween 20 and mix gently to avoid foaming.

Prepare 37 mM Ammonium Hydroxide Water Solution: Add 2.5 ml of 15 N ammonium hydroxide to 1 liter of water. Hold at room temperature in capped bottle to prevent evaporation.

IMMUNOSTAINING

Complete all incubations at room temperature. Do not allow slides to dry at any time during the procedure. Note conditions for preparing and storing reagents (Solutions, Reagents and Supplies). Bring all reagents except AEC to room temperature before use.

1. **Place slides into cover plates:** Follow instructions supplied by Shandon.

2. **Rinse slide:** Fill the well in the coverplate with 2 ml of TBST Buffer (the well should be about $\frac{3}{4}$ full) and allow 5 minutes for the buffer to drain. Repeat twice more. If bubbles are trapped under the coverplate, disassemble, rinse with TBST, and reassemble the slide and coverplate.

NOTE: Bubbles trapped between the slide and the coverplate will result in poor staining. Check the slides at the end of each rinse and remove any large bubbles by disassembling the slide and coverplate, rinsing the slide and coverplate with buffer to remove the bubbles, and re-assembling the slide and coverplate.

3. **Proteinase K treatment:** Apply 100 μ l Proteinase K (B) for exactly 1.5 minutes. Promptly rinse as in step 2. (Use of other ready-to-use proteinase K solutions may result in overdigestion of the tissue.)
4. **Antibody staining:** Apply 100 μ l of 1X Monoclonal Antibody [freshly diluted 1:1000 to a final concentration of 1 μ g/ml in Antibody Diluent (D)]. Incubate for 10 minutes.
5. Rinse slide 3 times as described in step 2.
6. Apply 100 μ l of Anti-Mouse IgG-Biotin (E) and incubate for 10 minutes
7. Rinse slide 3 times as described in step 2.
8. Apply 100 μ l of Streptavidin-HRP (F) and incubate for 10 minutes.
9. Rinse slide 3 times as described in step 2.
10. Apply 100 μ l of AEC SubstrateChromogen (G) and incubate for 5 minutes, then add a second 100 μ l of AEC and incubate a second 5 minutes, lastly add a third 100 μ l of AEC and allow to incubate for 10 minutes. Each slide requires a total of 300 μ l AEC applied over 20 minutes.
11. Rinse slide 2 times as described in step 2.
12. Remove slides from the coverplate and place in slide rack.
13. Counterstain in Hematoxylin (Mayer's, Lillie's Modification). For brain tissue incubate for **10 minutes** in Hematoxylin bath.
14. Rinse gently in distilled water.
15. Dip slides 10 times into a bath of 37 mM ammonium hydroxide water solution.
16. Rinse slides in tap water for 2 to 5 minutes. Leave slides in water until ready to proceed to mounting and cover slip.
17. Coverslip using aqueous-based mounting medium. Xylene and alcohol dehydration or xylene-based mounting medium will remove the immunostain.

Histopathology

	+	-	Total
VMRD IHC	100	0	100
	0	400	400
Total	100	400	500

100% SENSITIVITY • 100 %SPECIFICITY

Samples were defined as negative or positive by histologic morphology per Manning, *et al.* ("Validation of an immunohistochemistry assay for the detection of PrP^{Sc} in Bovine Spongiform Encephalopathy: An international collaboration." *Abstracts, AAVLD 44th Annual Meeting, 2001*, p. 74).

SLIDE EXAMINATION by *Light Field Microscopy*

Figure 1. Positive reaction in obex.

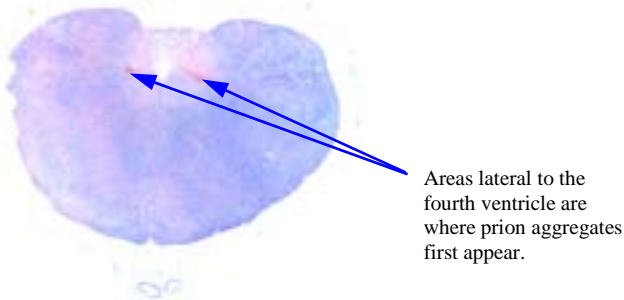


Figure 2. Strong positive 400X.

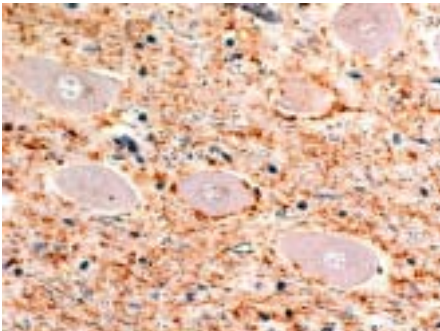
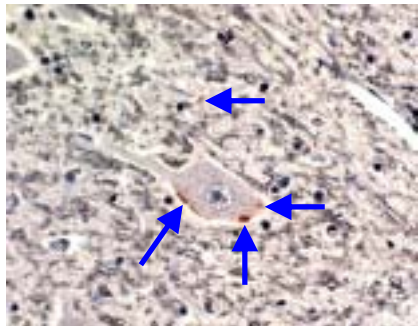


Figure 3. Weak positive 400X. Arrows point to positive reaction.



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