資料２

食安監発第１０２１００２号
１７消安第７５６６号
平成１７年１０月２１日

内閣府食品安全委員会事務局評価課長　殿

厚生労働省医薬食品局食品安全部監視安全課長
農林水産省消費・安全局畜水産安全管理課長
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（公　印　省　略）

食品健康影響評価に係る補足資料の提出について

平成１７年１０月１３日付け府食第１０１４号にて依頼のあった下記の補足資料について別添のとおり提出します。

記

1　米国及びカナダにおけるＢＳＥ確認検査結果の判定体制について（専門家会議の人数、専門家の専門分野、判定手順等）

2　米国及びカナダのパッカーにおける、食肉検査官によるせき髄除去の確認について

3　米国におけるホルスタイン用飼料の一般的成分として提示された、「プレミックス」の原料及び成分について

4　米国及びカナダにおける、牛由来の油脂（tallow、grease等）を介して牛のＳＲＭが牛用飼料（代用乳を含む）に入らないシステムの存在及びシステムの詳細について

5　米国及びカナダにおける、動物性油脂の生産量及び各国からの輸入量について

（参考資料）

1　米国食品医薬品局が平成１７年１０月６日に公表した飼料規則改正案について

2　米国の獣医官と食肉検査官の人数の内訳について（平成１７年９月１６日付け府食第９０８号で依頼のあった資料１に関する補足資料）

3　米国及び日本における飼料用動物性油脂の区分について

4　米国における代用乳の代表的な事例について

平成１７年１０月１３日付け府食第１０１４号で依頼のあった資料

1　米国及びカナダにおけるＢＳＥ確認検査結果の判定体制について

（専門家会議の人数、専門家の専門分野、判定手順等）

（米側回答）

USDA recognizes FSC's point regarding the expertise of U.S. personnel who reviewed information about confirmatory tests. However, it should be noted that a formal panel does not exist. The U.S. model does not mirror the Japanese model in this respect.

In accordance with normal testing/validation procedures, the Supervisory USDA Veterinarian/Microbiologist provides oversight and is responsible for validating test results. Confirmation of a positive test result is based on supervisory review. The supervisor reviews the laboratory technician's testing procedures and approves the results.

（仮訳）

確認検査のデータを検討する担当者の専門知識に関する食品安全委員会（FSC）の質問の要旨を USDA では充分に認識している。それでも、正式な委員会は存在しないことを指摘しておかなければならない。この点に関しては、米国のモデルは日本のモデルを反映しているわけではない。

通常の検査およびバリデーションの手順に基づき、USDA の獣医師または細菌学者が監督および検査結果の妥当性確認の責務を負っている。試験結果が陽性であった場合は監督者による検討の上で確定の判断をする。監督者は検査員の検査手順を検討して結果を承認する。

（カナダ側回答）

The following describes Canada's BSE diagnostic procedures.

All BSE sample submissions are initially screened with either the Prionics® Check Western blot (all samples are tested in duplicate) or the BioRad TeSeE elisa (samples are tested singularly), reflecting the individual preference and experience of the network screening laboratory. Any samples that generate inconclusive (non-negative) results are referred to the National BSE Reference Laboratory at the National Centre for Foreign Animal Disease (NCFAD) for confirmatory testing. The National BSE Reference Laboratory is the only facility in Canada authorized to diagnose BSE, and uses the immunohistochemistry procedure (IHC), designed to achieve the highest sensitivity possible, as the basis for confirmation of BSE status. The Canadian IHC test procedure employs at least 10 different monoclonal antibodies on approximately 30 to 40 tissue sections. Also, serial sections from 5 to 10 different levels of the obex (brain stem) are examined by histopathology. In recognition of the highest standards of diagnostic proficiency, the National BSE Reference Laboratory will soon be recognized by the World Organization for Animal Health (OIE) as a world BSE reference laboratory.

There are two situations in which the OIE SAF Western blot procedure would be routinely incorporated into the testing regime. Firstly, it is important to recognize that the immunohistochemistry procedure requires that anatomical landmarks, within the brain stem, be recognizable in order that the correct part of the brain stem is examined. In rare instances the quality of the sample submission may be such that it can be confirmed to be brain stem tissue, but specific anatomical landmarks cannot be identified, and in such cases confirmatory testing is done with the OIE SAF Western blot procedure. Secondly, Canada's experience to date has reinforced the sensitivity and specificity of the rapid tests currently in use and therefore, should there be a discrepancy between the results of the rapid test evaluation and the immunohistochemistry evaluation, the OIE SAF Western blot procedure is performed to provide further information relative to the true status of the sample. It is important to note that both the immunohistochemistry procedure and the OIE SAF Western blot procedure are recognized internationally as confirmatory tests and positive results by either test procedure would constitute confirmation of disease.

The Prionics® Check Western test is also used in the confirmatory work up. Although not considered to be a confirmatory test, it is used to deliver a rapid test result against which the preliminary result of the screening laboratory is compared and provides an early opportunity to examine the banding pattern of the protein for evidence of atypical BSE forms.

The following table summarizes the confirmatory testing protocol currently used in Canada.

BSE Diagnostic Regime		
Screening Tests Available	**Confirmatory Tests**	**Ancillary**
Prionics Check Western[1]	**Immunohistochemistry[2]** -if brainstem landmarks unconfirmed / or if result disagrees with screening test then; **OIE SAF Western blot**	Histopathology & Prionics Check Western
BioRad TeSeE[1]		
Enfer Elisa		
Prionics PrioStrip		
IDEXX		

[1] Rapid tests currently used in screening laboratories

[2] Immunohistochemistry utilizes multiple monclonal Ab's, on 30 - 40 tissue sections.

Unlike in Japan, confirmation of BSE in Canada does not require assessment by an expert panel. The authority and responsibility for the diagnosis of BSE in Canada resides solely within the expertise at the National BSE Reference Laboratory, and ultimately with the Head of the laboratory.

The following three tables provide details of the individual diagnostic work up used in the confirmation of BSE in the three cases detected by Canada, beginning May 2003. In addition to the summaries below, digital images of the diagnostic immunohistochemistry, demonstrating the positive findings, have been attached. It is important to note that Canada did not incorporate rapid BSE screening tests in the evaluation of BSE surveillance samples until after the first case of BSE was detected. Prior to the introduction of rapid test technologies, BSE surveillance samples were routinely screened for the presence of abnormal prion protein using the immunohistochemistry procedure. It is also worthy of mention that the two subsequent cases of BSE that were confirmed were initially evaluated with the BioRad TeSeE elisa rapid test.

Note as well that the elisa test results frequently refer to an optical density of "****". This reflects that the reading exceeded the diagnostic software calibration limits. The tables also identify the antibodies used in the immunohistochemistry procedure and a number of ancillary diagnostic tests that were performed, once the sample was confirmed to be positive, as part of the laboratory's ongoing research and development functions (including technological transfer, test method validation and quality assurance).

As was mentioned previously, for each of the positive cases described below, the National BSE Reference Laboratory has provided images of the immunohistochemistry slides used to confirm the diagnosis. They are labelled as follows:

a) T03 - 387 corresponds to the BSE case confirmed in May 2003.

b) T04 - 1266 corresponds to the BSE case confirmed January 2, 2005.

c) T05 - 45 corresponds to the BSE case confirmed January 11, 2005.

Pathology No.	Results
T03 - 387	**Positive** **by confirmatory test (May 2003):** IHC: 6H4, F89, Pullman cocktail, F99, DF7, KG9 by H & E <u>by rapid molecular tests (after confirmation):</u> Prionics Check Western (for test validation) Prionics Check Priostrip (for test validation) BioRad TeSeE (for test validation) Enfer ELISA (for test validation) IDEXX HerdCheck BSE ELISA (for test validation) FYI: at the NCFAD T03-387 obex - original OD - **** - diluted 1:100 - OD value 1.874

Pathology No.	Results
T04 - 1266	**Positive** **by confirmatory test (January 2005):** IHC: 6H4, F89, F99, BG4, DF7, FH11, KG9 not by H & E: Severe freezing artefacts excluded histopathological diagnosis by rapid molecular tests (prior to confirmation): Prionics - Check Western by molecular tests (after confirmation): SAF/OIE Immunoblot (for technology transfer) Hybrid Western Blot (for technology transfer) BioRad Confirmatory WB (for technology transfer) BioRad Sheep & Goat WB (for technology transfer) BioRad TeSeE (for QA) Prionics - Check Priostrip (for test validation) IDEXX ELISA (for test validation) **T04 - 1266** Alberta original OD value: 3.215 repeat OD value: **** recut in duplicate: OD values 3.455 and 3.435 NCFAD: OD value from tissue trimmed at NCFAD: 2.938 AB homogenate 1 (OD value): 3.371 AB homogenate 2 (OD value): 3.405

Pathology No.	Results
T05 - 45	Positive **by confirmatory test (January 2005):** IHC: 6H4, F89, F99, BG4, DF7, FH11, KG9, MO2, L42 not by H & E: severe freezing artefact excluded histopathological diagnosis <u>by rapid molecular testing (prior to confirmation):</u> Prionics - Check Western WB <u>by molecular tests (after confirmation):</u> SAF Immunoblot (for technology transfer) BioRad Confirmatory WB (for technology transfer) BioRad Sheep & Goat WB (for technology transfer) BioRad TeSeE (for QA) Prionics Priostrip (for test validation) IDEXX ELISA (for test validation) **T05 - 54** <u>Alberta</u> original OD value: 3.445 *repeat* OD value: **** recut in duplicate: **** also manual purification step repeated as **** <u>NCFAD</u>: OD value from tissue trimmed at NCFAD A- medulla: 2.808 B- medulla (rostral): 2.622 C- cerebellum: 2.943 AB homogenate 1 (OD value): 3.320 AB homogenate 2 (OD value): 3.310

The following accompanying documents, related to laboratory test procedures, are provided for reference:

a) Detection of Prion Protein in Bovine Spongiform Encephalopathy using the BioRad TeSeE Purification and Detection Kit

b) Immunohistochemistry Detection of Prion Protein in Bovine Spongiform Encephalopathy using a Dako Autostainer

c) A comprehensive list of the antibodies available at the National BSE Reference Laboratory for use in the immunohistochemistry procedure.

d) Standard Operating Procedures - Hematoxylin and Eosin Staining for Bovine Spongiform Encephalopathy

e) Detection of Pathological Prion Protein in Bovine Spongiform Encephalopathy Using the Prionics-Check Western Blot

October 13, 2005.

DETECTION OF PATHOLOGICAL PRION PROTEIN IN BOVINE SPONGIFORM ENCEPHALOPATHY USING THE PRIONICS-CHECK WESTERN™ BLOT

Laboratories Directorate, CFIA
National Centre for Foreign Animal Disease
Centre of Expertise for Foreign Animal Disease Diagnosis

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Biosafety Level III

This document has been created as part of and/or to fulfil the requirements of the Laboratories Directorate, CFIA Quality Assurance Program, which was developed on the same basic principles as ISO/IEC 17025-1999, General requirements for the competence of testing and calibration laboratories.

Approved by: Head, Pathology, NCFAD	Date
Approved by: Director, NCFAD	Date
Reviewed by: Quality Assurance Manager, NCFAD	Date

This signature constitutes an endorsement of the method and procedure described herein as fit for a specific, defined and/or potential use. The method may or may not be currently for field use (for the production of official diagnostic results to be used for regulatory purposes) depending on client needs.

Revision Page

This document is version 1.1 and replaces "Detection of Pathological Prion Protein in Bovine Spongiform Encephalopathy using the Prionics-Check Western™ Blot", Version 1.0, May 2003.

Review due: February 2006

Trade names are used in this protocol solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the Canadian Food Inspection Agency or an endorsement over other products not mentioned.

SIGNATURE PAGE

(users of this document)

Signatures below indicate that those so identified have read, understood, and agree to abide by the contents of this document.

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1. INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative disorders which include genetic, infectious and sporadic forms. They are also classified as "prion diseases" because of the essential involvement of a host-encoded protein (prion protein) in the respective pathogenesis. Scrapie of sheep and goats is the oldest documented TSE, and thus considered to be the prototype disease. Other animal TSEs include bovine spongiform encephalopathy (BSE) of domestic and exotic bovidae, chronic wasting disease (CWD) of cervidae, transmissible mink encephalopathy (TME) and feline spongiform encephalopathy (FSE).

BSE was first diagnosed in 1986 in Great Britain. Since then, it has been confirmed in many European countries, in Japan and in Canada. In 1996, a distinct variant of Creutzfeldt-Jakob disease (vCJD) was described in ten humans in the UK (7.1). The cause of this new disease seems to be the exposure to the agent that causes BSE. In view of the BSE agent's resistance to conventional decontamination procedures and the fact that it seems potentially transmissible to humans, it is recommended that appropriate safety procedures are in place.

Various tissues of a prion-infected animal contain a pathologically altered, disease specific form of the prion protein. The altered prion protein is denominated PrP^{res}. The normal isoform of PrP is termed PrP^{c} (the cellular form of PrP). PrP^{res} differs from PrP^{c} in its protease resistance: Upon treatment with proteinase K, PrP^{c} is destroyed, while PrP^{res} is reduced from its original size of 32-35 kD to a smaller size of 27-30 kD. The remaining protease-resistant PrP^{res}-fragment is referred to as PrP^{Sc}. The Prionics®-Check WESTERN test achieves its high precision and reliability by monitoring three independent criteria: protease-resistance, glycosylation pattern and lower molecular weight of the protease-resistant PrP^{Sc}-fragment (27-30 kD) compared to normal, undigested PrP.

In a first step, protease treatment converts PrP^{res} to the PrPSc-fragment while destroying PrP^{c}. In a second step, PrP^{Sc} is identified by its immuno-reactivity with anti-PrP antibodies and by its size, in an optimized Western blotting procedure.

The unique properties of the buffer solutions used in Prionics®-Check and the high affinity of the antibody allow that the test can be performed directly with tissue homogenates combining the reliability of the Western blotting procedure with the speed needed for mass screening. The monoclonal antibody used in the detection procedure recognizes bovine, mouse, hamster and human prion protein allowing Prionics®-Check WESTERN to be used in each of these species. In 1999, the Prionics-Check Western was officially acknowledged by the EU as the only test to achieve 100% sensitivity and 100% specificity without retesting (7.2).

2. EQUIPMENT AND INSTRUMENTATION

Any compatible brand can be used unless otherwise specified.

2.1 Single channel pipettors: 10 uL, 100 uL,1000 uL (optional 20 uL, 200 uL), with suitable tips.

2.2 8-channel pipettors: 5-50 uL, 50-300 uL.

2.3 Balance, top loading, to measure 0.45-0.70g

2.4 Dispensette III Easy Cal 1-10 ml

2.5 Master Plate - 96 well plate that holds minimum 1ml in each well

2.6 Digestion Plate - 0.2 mL 96 well PCR plate

2.7 Plate sealers

2.8 Dry Block Heater: temperatures of 48°C and 96°C for a 96 well PCR plate
Modular Heating Block - for a 0.2 mL 96well PCR plate.

2.9 Xcell Surelock Mini-cell Gel chamber - Invitrogen (need min. of 6 chambers)

2.10 NuPage 12%Bis-Tris gels 1.0 mm, 17 wells

2.11 Power Supply: reach min. 200V, Capacity 2.0 Amps (need 2-3 power supplies)

2.12 Blotting Filter Paper: for transfer

2.13 Blotting Membrane: PVDF 0.45 um

2.14 Square plastic incubation trays with lids

2.15 Forceps - flat head, scissors, gel knife

2.16 Transfer Chamber - large chamber with cassettes, sponges and cooling coil

2.17 Cooling unit

2.18 Magnetic stirrer, stir bars

2.19 Plate Rocker - variable speed

2.20 Plastic or glass inserts to fit into film cassettes

2.21 Plastic wrap

2.22 Film, hyperfilm 8" x 10"

2.23 Film Cassette 8" x 10"

2.24 Film Developer

2.25 Film Fixer

2.26 Homogenizer - FASTH or Medi-FASTH

2.27 Prypcons

2.28 Refrigerator set at 4 ± 3°C

2.29 Freezer set at -20 $\pm$ 5°C and -70 $\pm$ 10°C

2.30 Bio safety cabinet

2.31 Autoclave with temperature capabilities of 135°C. A Pre-vac autoclave is preferred for high temperature TSE sterilization.

2.32 Hot air oven

2.33 pH meter, operational range from 0.00 to 14.00 $\pm$ 0.01 pH.

2.34 Scalpel handle and disposable blades.

2.35 Glassware: beakers, assorted sizes; bottles, autoclavable (100 mL to 500 mL); graduated cylinders, (50 mL to 1000 mL); specimen bottles; funnels.

2.36 Paper products (paper towels; facial tissues)

2.37 Safety equipment: nitrile gloves; gowns; face shields with elastic strap; plastic aprons; shoe covers.

2.38 Lab Timer.

2.39 Water purification system, Millipore Milli-Q UF Plus or similar system.

2.40 Cutting board.

2.41 Carboys, Nalgene or similar.

2.42 Reagent reservoir

2.43 Mini Centrifuge for antibody tubes.

3. REAGENTS

3.1 Materials Included in the Prionics-Check Western Kit

3.1.1 Chemicals

3.1.1.1 Homogenisation Buffer Concentrate (5x) - (5x concentrate, dilute before use).

3.1.1.2 PVDF Blocking Buffer Concentrate (5x) - (5x concentrate, dilute before use).

3.1.1.3 Digestion Buffer (1x) - (Ready-to-use). One vial of Digestion Buffer.

3.1.1.4 Digestion Stop (1x) - (Ready-to-use, Pefabloc® SC). One vial of Proteinase K Blocker to stop proteolytic activity of the Proteinase K.

3.1.1.5 Luminescence Buffer Concentrate (10x) - (10x concentrate, dilute before use).

3.1.1.6 PAGE Sample Buffer (1x) - (Ready-to-use). One vial of Sample Buffer for SDS Polyacrylamide Gel Electrophoresis (PAGE).

3.1.2 Immunologicals

3.1.2.1 Antibody 6H4 - One vial of monoclonal antibody to PrP (mouse anti-PrP IgG1). Working dilution: 1:5000. (In case fluid sticks to wall or lid, the tube can be centrifuged).

3.1.2. 2 Antibody-AP - One vial of goat anti-mouse IgG-AP, an antibody to mouse IgG that is conjugated to alkaline phosphatase. Working dilution: 1:5000. (In case fluid sticks to wall or lid, the tube can be centrifuged).

3.1.2.3 Control Sample - (Ready-to-use). One vial of normal PrPC and molecular weight markers (97/66/45/30/20/14 kD) in PAGE Sample Buffer. Mix before use, e.g. by flicking the tube.

3.1.2.4 Proteinase K (20U/ml) - (Ready-to-use). One vial of Proteinase K.

3.2 Reagents Required

3.2.1 Chemicals

3.2.1.1 NuPAGE MOPS/SDS Running Buffer 10x

3.2.1.2 NuPAGE Antioxidant

3.2.1.3 Tris base, $C_4H_{11}NO_3$, F.W. 121.14g

3.2.1.4 Glycine, $C_2H_5NO_2$, F.W. 75.06g

3.2.1.5 Methanol 100% HPLC grade, CH_3OH, F.W. 32.04g

3.2.1.6 Sodium Chloride, NaCl, F.W. 58.44g

3.2.1.7 Potassium Chloride, KCl, F.W. 74.55g

3.2.1.8 Deionized water, 18.2Ωcm

3.2.1.9 Tween-20, $C_{58}H_{114}O_{26}$, F.W. 1227.54g

3.2.1.10 Ponceau S, $C_{22}H_{12}N_4Na_4O_{13}S_4$, F.W. 760.60g

3.2.2 Immunologicals

3.2.2.1 CDP-Star concentrate, Alkaline Phosphatase Substrate (APS) (12.5 mM or 25 mM), Roche.

4. PREPARATION FOR THE TEST

4.1 Preparation of Reagents

4.1.1 Transfer Buffer (10x)

4.1.1.1	Tris base	30.28 g
	Glycine	144.13 g
	Deionized Water	1000 ml

4.1.1.2 Mix well and refrigerate.

> **Note:** You can use purchased transfer buffer as well, as long as it has a final concentration of 25 mM Tris and 192 mM Glycine. No SDS.

4.1.2 Working Transfer Buffer (1x)

> **Note:** You can use purchased transfer buffer as long as it has a final concentration of *25 mM Tris and 192 mM Glycine.*

4.1.2.1	10x Transfer Buffer	200ml
	Deionized Water	1600ml
	Methanol (100%)	200ml

4.1.2.2 Chill to 4 ° C for use. Working solution is stable for 1 week at 4 °C.

> *Note:* (Methanol final concentration must be 10%)

4.1.2.3	20X Transfer Buffer	100 ml
	Deionized Water	1700 ml
	Methanol (100%)	200 ml

4.1.2.4 Chill to 4 ° C for use. Working solution is stable for 1 week at 4 °C. (Methanol final concentration must be 10%)

4.1.3 Tris Buffered Saline, TBS (1x) pH 7.4

4.1.3.1	NaCl	8 g
	KCl	0.2 g
	Tris base	3 g
	Deionized Water	*1000 ml*

4.1.3.2 Mix well on a magnetic stirring plate, adjust pH to 7.4 ± 0.05 with HCl.

4.1.3.3 Store at room temperature.

4.1.4 Tris Buffered Saline with Tween (TBST), pH 7.4

4.1.4.1 Use 1 L of TBS (4.1.2) with 0.5 mL of Tween-20 (final concentration is 0.05% (v/v))

4.1.5 Ponceau S (20x)

4.1.5.1	Ponceau S	2.5 g
	Glacial, Acetic acid	25 mL
	Deionized Water	475 mL

4.1.5.2 Mix well and store at room temperature.

4.1.6 Working Ponceau S (1x)

4.1.6.1	20x Ponceau S	10 mL
	TBST	190 mL

4.1.6.2 Mix well and store at room temperature. Staining volumes of Ponceau S can be reused 5-10 times before discarding.

Note:	Kit insert recommends 0.5% w/v Ponceau S with 5% v/v acetic acid for a 20x solution, then diluted with TBST to obtain a 1x solution.

4.1.7 Homogenisation Buffer Concentrate (1x)

4.1.7.1	5x concentrated Homogenisation Buffer	200 mL
	Deionized Water	800 mL

4.1.7.2 Mix and store at 4 ± 3°C.

4.1.8 NuPage MOPS/SDS Running Buffer (1x)

4.1.8.1	20x MOPS/SDS Running Buffer	50 mL
	Deionized Water	950 mL

4.1.8.2 Mix and store at room temperature.

4.1.9 PVDF Blocking Buffer Concentrate (1x)

4.1.9.1	5x concentrated PVDF Blocking Buffer	10 mL
	Deionized Water	40 mL

4.1.9.2 Make fresh just before use.

4.1.10 Luminescence Buffer Concentrate (1x)

4.1.10.1	10x concentrated Luminescence Buffer	5 mL
	Deionized Water	45 mL

4.1.10.2 Make fresh just before use.

Note:	All reagents (except TBS) are stable for one week unless specified.

4.2 Preparation of Equipment/Instrumentation

4.2.1 Refer to the operating manuals, SOPs provided with the equipment/instruments, routine maintenance and trouble-shooting procedures.

4.3 Certification of Laboratory Personnel

4.3.1 Technologists have completed training on sample receiving, case assignment, tissue processing, and reporting as per SOP # FA-QA-014

4.3.2 Technologists have completed Workplace Hazard Materials Information System (WHMIS) training, have been instructed on all hazards associated with the test (biological and chemical) and are familiar with the Material Safety Data Sheets (MSDS) located in the laboratory.

4.3.3 Laboratory scientists and/or technologists have certified training with Prionics-Check WESTERN provided by Prionics/Roche or by scientists/technologists certified by Prionics/Roche.

4.3.4 Scientists and/or technologists are experienced in the reading and trouble shooting of western blots.

4.3.5 All staff should have been vaccinated against rabies.

4.4 Preparation of Samples

Note: Prior to trimming brain samples in the 2BII biosafety cabinet, the following items should be available: 2% bleach, paper towels, scale, scalpel plus blades, forceps, cutting board, cut-resistant gloves and nitrile gloves.

4.4.1 Assign a pathology number to the sample

4.4.2 All samples are to be tracked by completing worksheets in SOP #HI-QA-001

4.4.3 Tissues are trimmed in a 2BII biosafety cabinet.

Note: 1. Gloves and protective clothing are worn when handling fresh or fixed brains. Personnel are appropriately trained to work in the biosafety cabinet.

2. All specimens received must be examined and trimmed by a pathologist (or personnel authorized by a pathologist). Lab number and eartag information are written on worksheet - Worksheet for Western Blot for BSE (Appendix 8.1).

4.4.4 Cut a piece of the obex region of the medulla oblongata in the brain stem. Place obex section into a pre-tared Prypcon on a balance in the 2BII hood. The tissue piece should be in the range of 0.45-0.70 g. 0.5 - 0.55 g is the most ideal.

4.4.5 Use cutting board to trim tissues and paper towels to catch excess blood. Disinfect cutting board and paper towels with 2% bleach for one hour. Rinse well. Soak instruments in 2N NaOH for two hours. Rinse well. Place paper towels into open biohazard bags in biosafety cabinet, autoclave at 135°C for 30 minutes.

4.4.6 Add ten times the volume of 1x Homogenisation Buffer (w/v) to the Prypcon with the tissue piece in it. Close the prypcon.

4.4.7 *The tissue is then homogenized for 45 sec automatically using the* FASTH or MediFASTH homogenizer.

4.4.8 For each sample, pipette two 900 uL to1000 uL of homogenated sample in a 96-well 1.2 ml sample master plate.

From now on, each step will be done with duplicates = two samples/ original homogenate. The duplicate samples are done one above the other in the master plate and digestion plate

(eg. Sample #1 goes into H1 and H2, Sample #2 goes into G1 and G2 etc) see appendix 8.1 for plate layout. Master plate can be stored at 4°C or long term at -20°C or -70°C.

5. PERFORMANCE OF THE TEST

Note:	1.	All steps are performed at room temperature except where indicated.
	2.	Master plate and digestion plate have 48 samples per plate.
	3.	Turn on all heating blocks to allow them to reach desired temperature.
	4.	Label all plates with plate name from Appendix 8.1.

5.1 *Digestion of samples is done in a 0.2 mL 96 well PCR plate.*

5.2 Add 10 uL of Digestion Buffer to each 0.2 ml well of the digestion plate.

5.3 Add 10 uL of Proteinase K to each 0.2 mL well of the digestion plate. This allows you to ensure that each well receives Digestion Buffer and Proteinase K before the sample is added.

5.4 Transfer 100 uL of each homogenate with a multichannel pipette from the master plate into the digestion plate and mix well by up and down pipetting. Be sure to change pipet tips after each set of samples.

CRITICAL CONTROL POINT #1

CC:	Improper mixing of samples will result in under-digestion and the sample may need to \ be repeated. Mix well (min 8-10 times).

5.5 Cover digestion plate with a plate sealer and place in a block heater for 40 min at 48°C.

Note:	During this incubation prepare the 17 slot 12% NuPAGE gels by removing the comb carefully and peel away the white plastic foil at the bottom of the gel. You will need 6 gels for every 48 samples(one master plate). The gels are numbered (1-6) on the left hand side under the wells and placed into the gel chambers. Prepare them for loading by gentle flushing out the wells with SDS-MOPS. Add 3-5 cm of MOPS to the bottom of the gel chambers. You can also fill transfer unit with 1x Transfer Buffer and turn on cooling unit to pre-chill the Transfer Buffer.

5.6 After incubation, remove plate sealer and add 10 uL of Digestion Stop to each 0.2 ml well of the digestion plate to stop the proteolytic reaction. The same pipette tips can be used but avoid to touch the wells with the pipet tips to prevent cross contamination.

5.7 Add 100 uL of PAGE Sample Buffer to each 0.2 ml well of the digestion plate, mix well by pipetting up and down. Be sure to change pipet tips after each set of samples.

5.8 Incubate for 5 min at 96°C, in a block heater, **do not use a plate sealer.** (Old samples that were previously incubated at 96°C should be heated to 65°C for 2 min).

5.9 Heat the Control Sample to 65°C for 2min (this can be done in the hot air oven).

5.10 Load 8 uL of the Control Sample in the first lane above the gel number on the left hand side(we only use 8 uL due to the controls strong signal).

5.11 Load 10 uL of the heated samples per lane.

5.12 Fill up inner chamber with 1x Electrophoresis SDS-MOPS Running Buffer, ensuring that the buffer covers the top of the gels.

5.13 Add 500 uL of Antioxidant to INNER chamber only.

5.14 Run gels at 200V for 30-35 mins until the dye front is about 1-2 cm from the bottom of the gel.

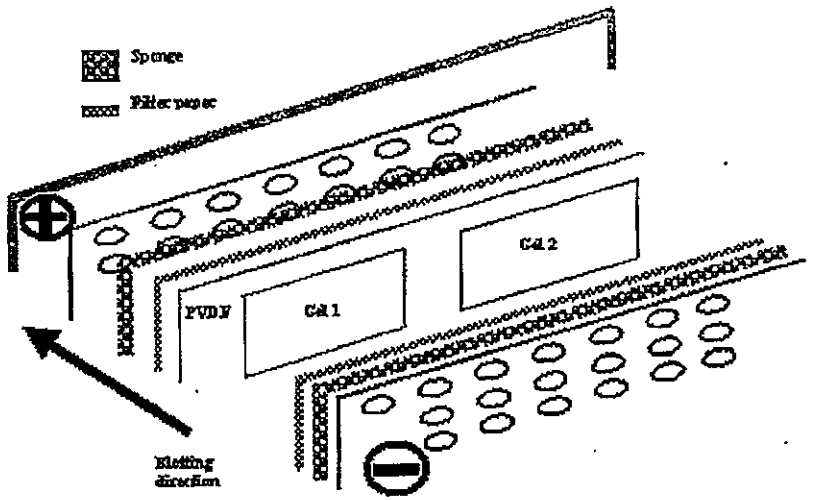
5.15 While the gels are running, the PVDF membrane is prepared. Cut the PVDF membrane to the desired size (see Appendix 8.2). Wet the membrane in methanol (100%) for a few seconds in a plastic incubation tray, pour off methanol. Equilibrate the membrane for at least 10 min in 1x Transfer Buffer in a plastic incubation tray on the plate rocker.

5.16 In a container with 1x Transfer buffer, moisten one sponge, place a piece of filter paper on top of the sponge so that it is moistened. Place PVDF membrane on the filter paper and write the plate number on the top left corner of membrane. Make sure that there is enough buffer in the container so that the membrane will not dry out while placing gels on it.

5.17 Crack open plastic frame of NuPAGE gel with a gel knife. Cut and remove the top part of the gel containing the slots and cut the bottom part below the dye front. Make a small notch in the corner where the gel number is to help orientate the gel on the membrane.

5.18 Place the gel on the membrane, as in Figure 5.1 be sure to avoid air-bubbles between the gel and the membrane, as they will interfere with the transfer.

Figure 5.1



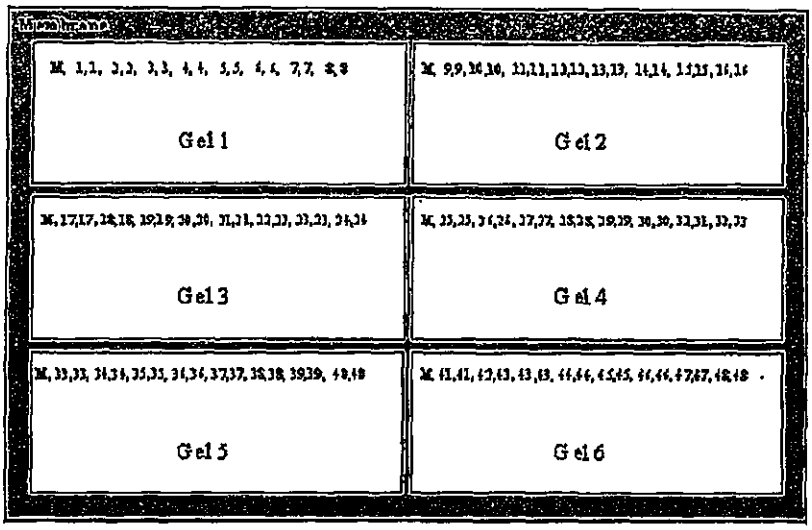
96 well plate filled with 48 duplicate samples, transferred to six 17-well gels.
Numbers indicate the probes 1-48 (M=molecular size marker with undigested PrP^C).

5.19 Overlay gels with moistened filter Paper, place second sponge on top.

5.20 Transfer sandwich into transfer cassette carefully as not to create bubbles in sandwich.

5.21 Close transfer cassette and place in transfer unit. Proteins are negatively charged and move towards the positive (red) pole of the transfer unit. Make sure that the cassette is inserted with the PVDF membrane towards the positive pole and the gels towards the negative pole. Black to black, clear to red. (See Figure 5.2)

Figure 5.2



5.22 Transfer at 140-150V for 1h at +4°C with continuous cooling and mixing.

5.23 Once transfer is completed, remove the membrane from the sandwich and place into a plastic incubation tray to complete the rest of the testing. At this point the PK bands may be visible without staining. Add 1x Ponceau S to the membrane and place on plate rocker/shaker until the stain shows the bound protein of the control.

5.24 Rinse off the Ponceau S with TBST until the red color has disappeared (approx. 2x 1 min).

NOTE: This staining step is not necessary and can be skipped.

5.25 Pour off the TBST and add the appropriate amount of PVDF Blocking Buffer according to the size of the membrane (see Appendix 8.2). Incubate at room temperature on a plate rocker with gentle agitation for 30 mins.

5.26 Pour off the PVDF Blocking Buffer. Prepare the appropriate amount of the Primary antibody (6H4) in TBST according to the size of the membrane(see Appendix 8.2). Add the dilution to the membrane and incubate at room temperature on a plate rocker with gentle agitation for 1 hour or overnight at 4°C.

Note: The membrane can be left in a wash stage in TBST for a longer period of time if necessary.

5.27 Wash membranes 3x for 5 min each with approximately 50 mL of TBST.

5.28 Pour off the last TBST wash. Prepare the appropriate amount of the secondary antibody (AP) in TBST according to the size of the membrane (see Appendix 8.2). Add the dilution to the membrane and incubate at room temperature on a plate rocker with gentle agitation for 30 mins .

5.29 Wash membranes 5x for 5 min each with approximately 50 mL of TBST.

5.30 Pour off the last TBST wash. Prepare 50 mL of 1x Luminescence Buffer in Deionized water. Remove required amount of Luminescence buffer, into a separate tube, (see Appendix 8.2). Pour remainder of the Luminescence Buffer onto the membrane to equilibrate the membrane and gentle agitate for 5 mins.

Note: For steps 5.30 to 5.32 all quantities are dependent on the size of the membrane used see Appendix 8.2 for volumes.

5.31 Add the appropriate amount of CDP-Star (12.5mM; 50x) or (25mM; 100x)(see Appendix 8.2), to the poured off portion of the Luminescence buffer. Keep in the dark until needed.

5.32 Pour off the Luminescence buffer, remove the membrane from the plastic incubation tray and place the membrane on the plastic or glass plate insert. Distribute the appropriate amount of diluted CDP-Star solution (from 5.31), evenly on the membrane and incubate for 5 min at room temperature. Make sure that the entire membrane is kept moist during this stage.

5.33 Remove any remaining Luminescence buffer from the membrane with a soft Kleenex tissue (do not totally dry out membrane). Immediately cover the membrane with plastic wrap and smooth out any folds and bubbles from under the plastic wrap. Place plastic or glass insert into the film cassette.

5.34 Proceed to the dark room. Expose the membrane to an X-Ray film (HyperFilm) until a strong signal of the positive control and either the background or the Proteinase K bands are visible (appr. 5 to 20 min). Expose longer or shorter times for optimal signal visualization. Alternatively use a CCD-Camera Detection System.

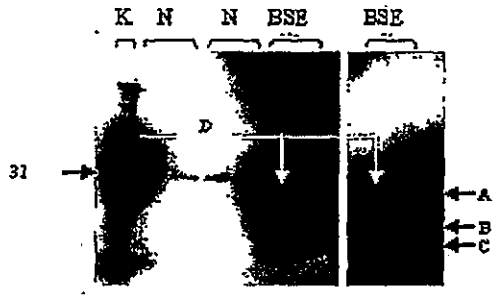
6. INTERPRETATION OF THE TEST

6.1 Reading the Films

6.1.1 The following figure shows the expected band patterns of BSE-negative, BSE-positive and control samples, respectively. The control sample (K) contains the normal isoform of the prion protein (PrP^C) which is visualized via immunological detection. The corresponding diffuse band is spread from 25-35 kD due to glycosylation of PrP^C which causes a heterogeneous distribution.

6.1.2 Negative samples (N) do not show a specific signal. The 31 kD band (not always visible) results from unspecific binding of the secondary antibody to proteinase K and can be used as an orientation aid.

6.1.3 Positive samples (BSE strong; BSE weak) exhibit a signal consisting of three bands, the top one (A) corresponding to a protein with an approximate molecular weight of 30 kD. The signal intensity of all bands (in particular that of the lower bands B and C) can be weaker than depicted here, but the top band (A) should be clearly visible. The arrow (D) illustrates the difference in molecular weight between digested, pathological prion protein and the undigested, normal protein.



6.2 Interpretation of results

6.2.1 Samples negative with Prionics-Check Western are considered negative, although they may contain infectious agent with a titer below the detection limit of the assay.

6.2.2 Samples considered inconclusive after testing, will be repeated by Prionics-Check Western. If the result remains inconclusive the sample (frozen and wet tissue) is to be forwarded to the National BSE Reference Laboratory at NCFAD/CFIA, Winnipeg for confirmation.

6.2.3 Samples considered positive by Prionics-Check Western will be called "suspicious/suspect" and the sample (frozen and wet tissue) are to be forwarded to the National BSE Reference Laboratory at NCFAD/CFIA, Winnipeg for confirmation.

7. REFERENCES and LITERATURE

7.1 Almond, J. and Pattison, J. (1997) Human BSE. Nature 389: 437-438.

7.2 The evaluation of tests for the diagnosis of transmissible spongiform encephalopathy in bovines. (8. July 1999). European Commission, DG24, Directorate B, Unit B3. (http://europa.eu.int/comm/food/fs/bse/bse12_en.html)

7.4 Doherr M.G. et al. (1999) Targeted surveillance for bovine spongiform encephalopathy. Veterinary Record 145: 672.

7.5 Schaller, O. et al. (1999) Validation of a Western immunoblotting procedure for bovine PrP^{Sc} detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). Acta Neuropathologica, 98:437-443.

7.6 Oesch, B. et al. (2000). Application of Prionics Western blotting procedure to screen for BSE in cattle regularly slaughtered at Swiss abattoirs. Archives of Virology, Supp. 16: 189-195

SOP # FA-EQ–016 - Accumet Model 115 pH Meter

SOP # FA-QA-014 - Technical Training and Certification of Diagnostic Test Analysts.

SOP # HI-HS-002 - Waste Disposal in the TSE Unit.

SOP # HI-QA- 001 - Specimen Receiving, Storage and Discard Procedures for the TSE Unit.

Appendix 8.1

Worksheet for Western Blot for BSE

DATE Rec'd _____ Trimmed by _____

Rec'd By _____

PlateName _____ Technologists _____

H1 Plate Layout

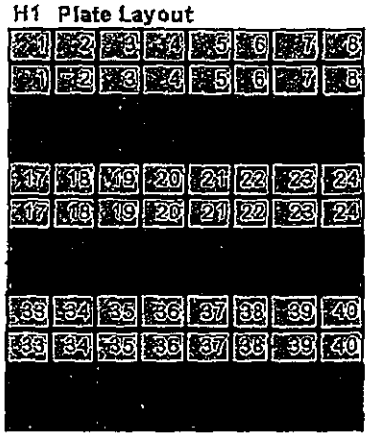


Plate Placement, Sample #, Ear Tag # and Result

	T03 #	Ear Tag #	R		T03 #	Ear Tag #	R
1				33			
2				34			
3				35			
4				36			
5				37			
6				38			
7				39			
8				40			
17							
18							
19							
20							
21							
22							
23							
24							

Homogenization solution _____

Kit lot # _____

MOPS _____

Transfer Buffer _____

Ponseau S _____

CDP Star _____

TBS'T _____

Comments:

Pathologist _____ Date _____

Appendix 8.2

Volumes and sizes for different number of samples

Number of gels	Tray size	Membrane size	TBST	1. Antibody	TBST	2. Antibody	Luminescence Buffer	CDP-Star	
								12.5 mM	25 mM
6	Large (22.4 x 15.6 cm)	13 x 17 cm	50 ml	10 µl	50 ml	10 µl	5 ml	100 µl	50 µl
4	Large (22.4 x 15.6 cm)	9 x 17 cm	50 ml	10 µl	50 ml	10 µl	4 ml	80 µl	40 µl
3	Medium (15 x 11.4 cm)	13 x 8.5 cm	25 ml	5 µl	25 ml	5 µl	3 ml	60 µl	30 µl
2	Medium (15 x 11.4 cm)	9 x 8.5 cm	25 ml	5 µl	25 ml	5 µl	3 ml	60 µl	30 µl
1	Small (5.5 x 9.5 cm)	4.5 x 8.5 cm	5 ml	1 µl	5 ml	1 µl	2 ml	40 µl	20 µl

Suggested Dilutions for larger volumes of buffers

Homogenisation Buffer HOB 1x	200 ml 5 x HOB 800 ml Deionized water
	1000 ml

Blocking Buffer 1 x	100 ml 5 x 400 ml Deionized water
	500 ml

Running Buffer (Gels) 1 x MOPS	50 ml MOPS Buffer (20x) 950 ml Deionized water
	1000 ml

Luminescence Buffer 1 x	27 ml 10 x 243 ml Deionized water
	270 ml

1 x Transfer Buffer	200 ml Transfer Buffer (10x) 1600 ml Deionized water 200 ml Methanol 100 %
	2000 ml

1 x TBST	1000 ml of TBS 0.5 mL Tween 20
	1000 ml

1 x Ponceau S	50 ml 20 x 950 ml 1 x TBST
	1000 ml

Appendix 8.3 Prionics Check Western Blot for Bovine Spongiform Encephalopathy

Date _____ Tech._____

Turn on heating plates!! Thaw samples.

1. Trim the obex region of brain tissue samples (0.45g-0.70g)
2. **Homogenization:** Add 10X the volume of 1X homgenisation buffer to the tissue sample
   Place the prypcon tubes in the automatic homogeniser
3. Pipette 900ul of homogenate into a master plate (in duplicate, one under the other)
4. **Digestion:** Add 10uL of Digestion buffer

   10uL of PK to sample wells of the digestion plate
   Transfer 100uL of homogenate sample to digestion plate and mix well
5. Incubate in heating block at 48°C (***Seal plate with plate sealer***) 40 minutes ☐
1. **Stop:** Add 10uL of Digestion-Stop buffer after incubation
7. Add 100uL of PAGE sample buffer to each sample and mix well
8. Boil samples in plate at 96°C inside the hood (***Cover plate with a tip box lid***) 5 minutes ☐
9. Prepare 12% NuPAGE gels (carefully remove comb and white foil) and place in gel boxes with a small volume of MOPS
   Also add mops solution into the wells to avoid any bubbles.
10. Heat control sample at 65°C in the incubator 2 minutes ☐
11. Load 8uL of control sample (ladder) in the first lane of the gel.
12. Load 10uL of the remaining samples in duplicate on the gel and lock gel in place.
13. Fill inner chamber of the gel box with 1X SDS-MOPS buffer enough to cover wells
14. Add 500uL of Antioxidant to ***inner chamber only.***
15. Run gel at 200V 35 minutes ☐
16. Fill the transfer unit with 1X Transfer buffer. ***Turn on cooling unit*** *(fill just before when using smaller unit)*
17. Wet the PVDF membrane in 100% methanol for a few seconds.
18. Place the membrane in 1X Transfer buffer for at least 10 minutes on the shaker or until ready to use.
19. Prepare sandwich: check sponge, filter paper, membrane, gel, filter paper, sponge
    (The PVDF membrane should be towards the positive pole and the gel should be towards the negative pole). Date the membrane on the top left corner with a black pen.
20. **Transfer** at 150V, 4°C (PK bands should be visible) 60 minutes ☐
21. **Block** the membrane in 50mL of PVDF blocking buffer with agitation at RT. 30 minutes ☐
    (Pour off blocking buffer before adding 1° Ab)
22. Dilute 10uL of 1° Ab in 50mL of TBST (Large blot)
    Add antibody to membrane and incubate at RT 60 minutes ☐
    (Membrane can be incubated with 1° Ab on rocker overnight at 4°C)
23. Wash membrane 3 times with TBST. 5 min/ time ☐
24. Spin 2° Ab in small centrifuge for 10 seconds.
    Dilute 10uL of 2° Ab in 50mL of TBST (Large blot)
    Add Ab to membrane and incubate at RT. 30 minutes ☐
25. Wash membrane 5 times with TBST. 5 min/ time ☐
26. Take 50ml of 1X Luminescence Buffer and add 45ml to membrane. 5 minutes ☐
27. Add 100uL of CDP-Star (12.5mM) or 50uL of CDP-Star (25mM) to the remaining 5ml of Luminescence Buffer. (Keep in dark until ready to use)
28. Place the membrane on glass plate and distribute the diluted CDP-Star on to the membrane.
    Incubate at RT. 5 minutes ☐
29. Remove excess liquid with kleenex and cover the membrane with saran wrap. The membrane is ready for X-ray exposure.