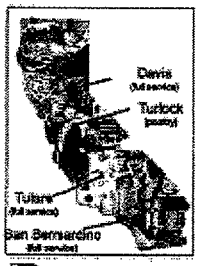
附件 4

California Animal Health & Food Safety Laboratory System (CAHFS)

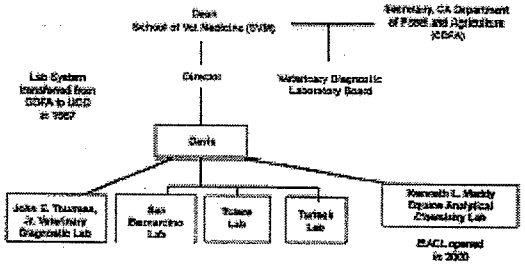


Overview of CAHFS

May 18, 2012

Dr. Richard Breitmeyer
Director

CAHFS' Organization



Total Faculty & Staff

Davis, Turlock, Tulare, San Bernardino

- 21 Faculty

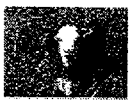
- 105 Technical Staff

- 7 Information Technology
- 6 Residents
- 27 Administration

CAHFS' Core Mission

Protect Animal Health, Public Health & Food Supply

- **Laboratory disciplines**
  - Pathology/Histology
  - Bacteriology
  - Virology/Molecular Diagnostics
  - Toxicology
  - Immunology/Epidemiology
  - Equine Chemistry/Pharmacology
- **Expertise – faculty and staff**
  - Advanced training and Board certification



CAHFS' Core Services

Protect Animal Health, Public Health & Food Supply

- Early warning system for foreign and emerging diseases in agriculture – Surveillance & Emergency Response
  - 30,000 accessions & ~1 million tests performed annually
  - Funding support from California Department of Food & Agriculture

Disease surveillance

Exotic Newcastle Disease 2002-2003

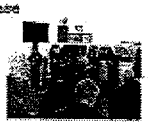
Veterinary 2007-08

Exotic Newcastle Disease Outbreak

Role of CAHFS – California 2002-2003

- Largest disease outbreak in 30 yrs in U.S.
- END first diagnosed at CAHFS
  - Backyard chickens in So. Calif. – Surveillance
- Impact – over $150 million eradication costs
- CAHFS response - >20,000 tests/month
  - Developed rapid PCR test
  - Prove freedom from disease

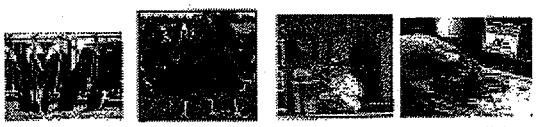
Chicken kill grows



CAHFS Core Services

Protect Animal Health, Public Health & Food Supply

- **Support all CDFA animal health and food safety regulatory programs**



Brucellosis Program | Avian Diseases – AI, END | Milk and Dairy Safety | Tuberculosis Program

CAHFS Core Services

Protect Animal Health, Public Health & Food Supply

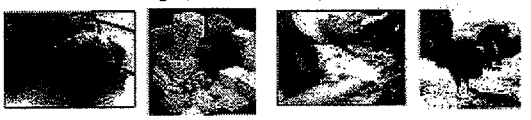
- Analytical Toxicology Services
  - Fully equipped analytical chemistry laboratory
- Food Emergency Response Network (FERN)
  - Partnership with FDA – milk, seafood, etc.

Instrumentation | Personnel | Collaboration

CAHFS Core Services

Protect Animal Health, Public Health & Food Supply

- Development and validation of laboratory tests to detect new diseases, toxic agents and drugs
  - New technologies, new demands, new discoveries



Melamine | Drug residues | Bluetongue virus | Exotic Newcastle disease

Accreditation and Quality Programs

- Accreditation by the American Assoc. of Veterinary Laboratory Diagnosticians – renewed 2010
- Quality standards
  - Document control
  - Corrective action
  - Preventive action
  - Internal audits
  - Client feedback
  - External assessments

NAHLN Overview

Beate Crossley, DVM, PhD, MPVM
NAHLN Coordinator at CAHFS

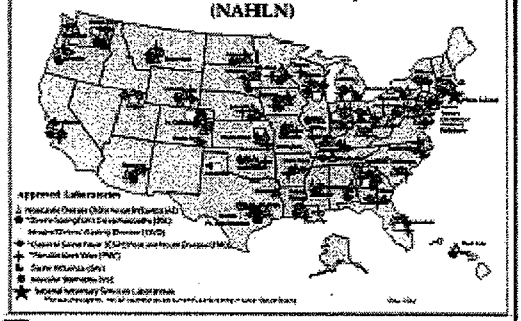
National Animal Health Laboratory Network

- Founded in 2002
- Member states:
  - 2002: 12 laboratories including California
    - Additional funding from USDA
  - 2012: 60 laboratories nationwide
- Partnership between
  - USDA
    - National Veterinary Services Laboratory
  - American Association of Veterinary Laboratory Diagnosticians (AAVLD)
  - NAHLN Laboratories

The purpose of NAHLN is....

- **Early detection:**
  - Targeted surveillance based on population density/risk
- **Rapid response:**
  - Capability and capacity to meet outbreak response needs
- **Rapid recovery:**
  - Surge capacity to enable post-outbreak recovery (e.g. demonstration of disease freedom for trade partners)

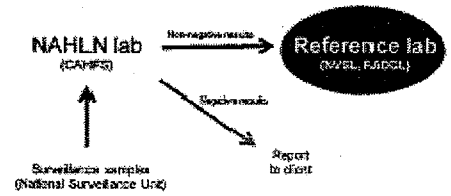
National Animal Health Laboratory Network (NAHLN)



Quality Requirements

- Quality standards
- Competency of laboratory personnel
- Standardized protocols and equipment
- Adequate biosafety/biosecurity
- Secure electronic communications and reporting
- Assessment of preparedness through scenario testing

Reporting of NAHLN results



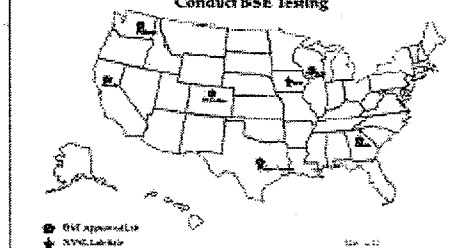
NAHLN testing at CAHFS:

Molecular assays:
- Avian influenza virus
  - Subtyping assays
- Avian Paramyxovirus-1 virus
  - Exotic Newcastle disease virus
- Classical Swine Fever virus
- Rinderpest virus
- African Swine Fever virus
- Foot-and-Mouth disease virus

Serological assays:
- Pseudorabies virus

TSE testing:
- Scrapie
- Bovine Spongiform Encephalopathy (BSE)

Laboratories Approved to Conduct BSE Testing



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Bovine Spongiform Encephalopathy Testing

Michelle Davidson
BSE Laboratory Manager

May 17, 2012

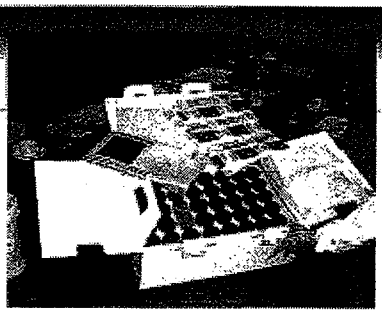
Tour of BSE Laboratories in Europe 2004

Visited Laboratories in England, Germany and Switzerland

May 17, 2012

European Laboratory Tour
Test methods and best practices

May 17, 2012



European Laboratory Tour
Specimen tracking methods

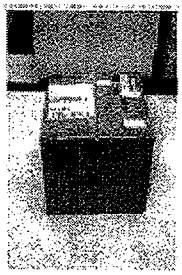
May 17, 2012

European Laboratory Tour
Safety and biosecurity measures

May 17, 2012

BSE Testing at CAHFS

Boxes of specimens delivered directly to the BSE Laboratory



May 17, 2012

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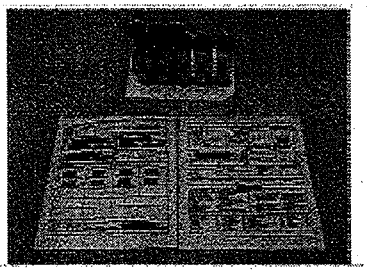
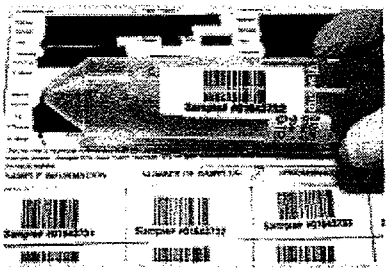
- Specimens arrive with USDA barcode labels
- USDA BSE Surveillance Submission Form
- USDA BSE Surveillance Data Collection Form

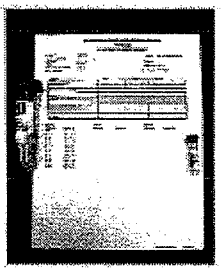


Barcode label on the specimen is matched to the submission form prior to scanning into the CAHFS Laboratory Information System

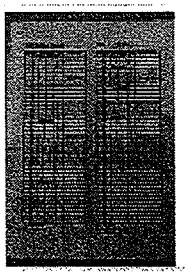


Specimen tube and grinding tube labeled

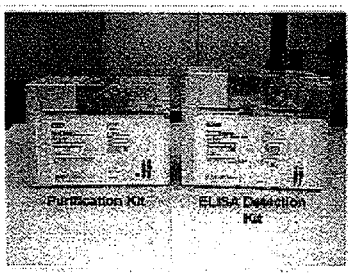
Chain of Custody Report



Specimen Preparation Record (BSE Cutting Record)

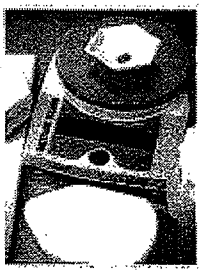


BIO-RAD Laboratories Test Method

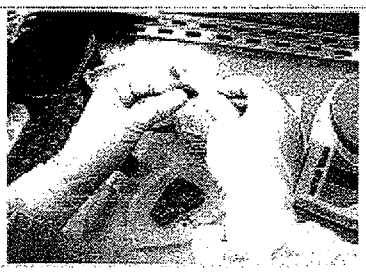


Select tissue from obex region of the brain stem

Weigh 0.35 g (+/- 0.040 g)

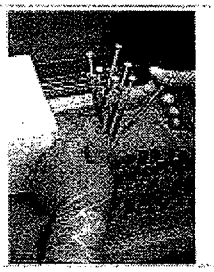


Put in grinding tube



Homogenizer

Transfer homogenized tissue to deep well plate



BIO-RAD
New Sample Processor
(NSP)

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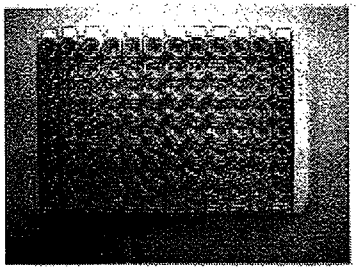
BIO-RAD
ELISA Detection



Summary

- April 18, 2012: Specimens collected at renderer
- April 19, 2012: Specimens delivered by rendering company employee to CAHFS for testing - 9 am
- April 19, 2012: CAHFS notified NVSL of initial reactor - 3:45 pm
- April 19, 2012: CAHFS notified NVSL of inconclusive result - 5:50 pm
- April 20, 2012: CAHFS shipped initial portion of specimen to NVSL
- April 23, 2012: CAHFS shipped remaining specimen to NVSL

附件 5

National Veterinary Services Laboratories	
Document Title: BSE ELISA	
Author/Position: Julie Lease, BSLT	Document Number: SOP-PL-0033.05
Page 1 of 9	Supersedes: SOP-PTS-0003.04

Table of Contents

1. **Purpose/Scope**

2. **Definitions**

3. **Safety Precautions**

4. **Equipment and Materials Required**

5. **Procedure**

   5.1. Receiving Samples
   5.2. Sample Log-in
   5.3. Sample Processing
   5.4. Running the Test
   5.5. Computer Data Entry and Reporting Sample Results
   5.6. Quality Control Parameters
   5.7. Sample Retention/Disposal

6. **Associated NVSL Quality Documents/References**

   - Biosafety in Microbiological and Biomedical Laboratories (BMBL), Current Edition
   - SOP-PL-0006, Procedures for Entering and Exiting BSL-3 Laboratory Pathobiology Laboratory
   - SOP-PL-0032, BSE Contract Laboratories to Receive and Test Bovine Brain Samples and Report Results for BSE Surveillance
   - SOP-PL-0036, Decontamination and Biological Accident Procedures Used in the Transmissible Spongiform Encephalopathy Laboratory (Building 21 – PL BSL3 Laboratory)
   - SOP-PL-0017, Disposal Procedure for BL3 Hazardous Waste and Reagent Waste
   - FM-PL-0027, BSE ELISA Checklist
   - EXT-WI-PL-5138, Bio-Rad Bovine Spongiform Encephalopathy Antigen Test Kit, ELISA
   - EXT-WI-PL- 5712, Bio-Rad NSP New Sample Pr(p)eparator Operator's Manual
   - WI-PL-5397, BSE ELISA Lab Ware Instructions

7. **Revision History**

8. **Appendices**

   Appendix 1: Photograph Identifying Brainstem Including Obex (Dorsal View)

Approved: /s/ Arthur J. Davis

Uncontrolled Copy

1. Purpose/Scope

The purpose of this Standard Operating Procedure (SOP) is to document the proper technical procedure for performing the enzyme-linked immunosorbent assay (ELISA) test for bovine spongiform encephalopathy (BSE) at the National Veterinary Services Laboratories (NVSL), Pathobiology Laboratory (PL).

2. Definitions

- **Detection plate identification (ID)** – Includes the date of testing and a number (beginning with 1 for the first plate of the day). Ex: 11-02-11-01
- **Obex** – A thin triangular lamina of gray matter in the medulla oblongata present just rostral to the point where the fourth ventricle narrows to form the central canal of the spinal cord. See Appendix for picture.
- **Lab Ware** – The current laboratory information management system designed to facilitate data collection and storage.

3. Safety Precautions

3.1. Personnel

- Precautions must be taken in the preparation of bovine central nervous system (CNS) tissues due to the potential exposure of humans to a distinct variant of Creutzfeldt-Jakob disease (vCJD), which seems to be associated with exposure to the agent that causes BSE. vCJD has been described in humans in the United Kingdom (UK) and in humans known to have been present in the UK during times of highest exposure to BSE infected cattle. In view of the BSE agent's resistance to conventional decontamination procedures and the fact that it may potentially be transmissible to humans, it is recommended that appropriate safety procedures are in place. The highest risks of infection appear to be from direct contact or splashing of highly infectious CNS material to eyes, mucous membranes, open wounds, abraded skin, or by swallowing. Aerosol transmission is considered the least likely route of exposure.
- Everyone working with potentially BSE-infected samples should understand and follow Biosafety Level-3 (BSL-3) guidelines to the extent the laboratory facilities permit. The Center for Disease Control (CDC) publication Biosafety in Microbiological and Biomedical Laboratories, current edition, should be consulted for recommendations concerning the handling of prion infected or potentially infected materials.
- Those working with prion infected or potentially infected materials need to have a general understanding of BSE and other transmissible spongiform encephalopathies (TSEs). Recommended reading is the CDC and World

Health Organization guides discussing biosafety for prions. Many good review articles are also available in the biomedical literature.

- Formalin, most routine disinfectant methods, and some incineration methods are not effective in inactivating prions, including the BSE agent. The NVSL employ one hour of wet contact time with half strength bleach (5.25% sodium hypochlorite with equal amount water, mixed fresh) for routine disinfection of equipment and workspaces when working with prion infected or potentially infected materials. For decontamination associated with biological accidents, please refer to SOP-PL-0036, Decontamination and Biological Accident Procedures.

3.2. Security of Sample Storage in the BSL-3 Laboratory

The NVSL PL is secured from the public. The exterior doors are kept locked 24 hours per day, 7 days per week, and employee entry is by a key card. All visitors must sign in and be issued a visitor's pass at either Guard Station. Visitors must sign in and out of the PL front office if the visit is during normal business hours. Visitors must be escorted while in the PL; visibly wear their visitor pass at all times, except in the BSL-3 Laboratory; and turn in the visitor's pass upon leaving. A security alarm is engaged at all times when employees are not in PL. A surveillance camera with a view of the main entry is monitored from the guard station at the NVSL Central Campus.

4. Equipment and Materials

- Scalpels or razor blades
- Weigh boats
- Forceps
- Weight Scale
- Biological Safety Cabinets
- Tissue Homogenizer: for example TeSeE® Precess 48
- Deep Well plates
- Calibration syringe and needles (supplied by Bio-Rad)
- NSP: New Sample Pr$_{(p)}$eparator (and NSP disposable tips)
- Bovine Spongiform Encephalopathy Antigen Test Kit, ELISA, TeSeE Short Assay Protocol, TeSeE Purification kit and TeSeE Detection Kit (Bio-Rad Laboratories, Inc., Hercules, California)
- 100°C ± 5°C Heating Block (for Deep well plate)
- Centrifuge (for Deep well plate)
- 37°C ± 2°C incubator
- 96 well micro titer plate
- DW40 Deep well plate washer
- 96 well micro titer plate washer
- 96 well micro titer plate reader

- Single channel pipettes and Multi channel pipettes and tips
- 5°C ± 3°C Refrigerator
- Freezer
- Autoclave
- Sharps containers
- Gloves, Lab Coat, Eye protection
- Reagent reservoir
- Sodium hypochlorite containing at least 2% available chlorine
- 15 and 50 ml tubes for mixing

5. Procedure

See SOP-NVSL-9076 Policy for Assigning Accessions and Reporting Results – Ames IA Location for addition information regarding assigning accessions and reporting results.

5.1. Receiving Samples

- Samples are received by the Laboratory Resources Unit (LRU) where accession numbers are assigned. LRU personnel deliver samples to the airlock of the laboratory at PL unless other arrangements have been made.
- Laboratory personnel retrieve samples from the airlock and place them on the downdraft table or a biological safety cabinet in the lab.

5.2. Sample Log-in

5.2.1. Open each submission individually and assign a sequentially unique Reference Assistance (RA) number. Use preprinted labels containing RA numbers for this purpose. This will be an E number. Ex: 11E021

5.2.2. Label each case folder and the submission forms with the RA number to create a case file.

5.2.3. Verify samples against what is listed on the submission forms.

5.2.4. Record "Samples verified", date received, number of samples received, and the initials of the technician on the submission form.

5.2.5. Circle or correct the total number of samples received on the form. Indicate if samples are missing and/or samples are not identified on the form.

5.2.6. Number the sample identification numbers (ID) on the submission form sequentially beginning with the number one. If the sample was received without a barcode, assign one to it at this time.

5.3. Sample Processing

5.3.1. Arrange samples in numerical order beginning with the lowest accession number.

5.3.2. Place samples in the same order as they were numbered on the submission forms.

5.3.3. Number the outside of the individual sample containers sequentially beginning with 1. For each sample, a Bio-Rad grinding tube is labeled with the RA number and sample number (either manually written on the tube or labels can be generated for this).

5.3.4. Place the tubes on the test rack in the same order they were logged in.

5.3.5. Number the tube caps sequentially beginning with 1.

5.3.6. Retain the paperwork in the case folder.

5.3.7. Take samples and Bio-Rad grinding tubes to the biological safety cabinet for cut-in.

5.3.8. Cut-in samples wearing disposable laboratory coat, gloves (double glove with non-white on the outside per SOP-PL-0006, Procedures for Entering and Exiting BSL-3 Laboratory Pathobiology Laboratory), and eye protection.

5.3.9. Determine the suitability of the sample for testing. Process all samples, including those that are received but are not clearly recognizable as brain stem. The obex area of the brain stem must be sampled for optimal detection of protease resistant prion protein (PrP^{res}). See Appendix 1 for correct sampling locations to extract the Dorsal Motor Nucleus of the Vagus (DMNV) Nerve from the obex.

5.3.10. Place a clean disposable weigh boat on the balance scale and zero. Use clean forceps and scapels for each sample.

5.3.11. Place the tissue to be cut in a separate weigh boat and cut the obex to extract a unilateral segment of the DMNV or, if obex is unavailable or unrecognizable, an alternate segment of brain stem (see Appendix 1).

5.3.12. Place the cut sample in the weigh boat on the scale. The sample mass should be 0.35 ± 0.04g. Add or remove tissue to meet this weight range.

5.3.13. Place the tissue into the grinding tube and then return it to the rack.

5.3.14. Discard scalpels following their use, into the appropriate biohazard container. Set forceps aside for rinsing if they are to be reused according to SOP-PL-0036, Decontamination and Biological Accident Procedures.

5.3.15. Cut subsequent samples as described above.

5.3.16. Homogenize the samples in the grinding tubes using the TeSeE® Process 48 or equivalent, load 350-500 µl of each sample into a deep well plate according to manufacturer's instructions.

5.3.17. Perform the purification process on the New Sample Pr(p)eparator (NSP) automated equipment following EXT-WI-PL- 5712.

5.4. Running the Test

5.4.1. Perform the detection process according to the current Bovine Spongiform Encephalopathy Antigen Test manufacturer's kit insert EXT-WI-PL-5138. Use FM-PL-0027 BSE ELISA Checklist to record your progress.

5.4.2. Once the plate is ready to be read, open the Microplate Manager run (open the appropriate BSE icon on the desk top).

5.4.3. Enter the technician name (same as your log on name, in capital letters) and Detection Plate ID.

- After the plate is read and results calculated by the software, a report is generated containing OD values as well as an interpretation including NVSL 1 (mean of negative controls + 0.21 then multiplied by 1), ND (Not Detected which is any value less than the mean of negative controls + 0.21 then multiplied by 0.9) or NVSL 1 (any value that lies between the NVSL 1 and ND values).

5.5. Computer Data Entry and Reporting of Sample Results Follow WI-PL-5397 BSE ELISA Lab Ware Instructions.

5.5.2 After the purification processes is complete on the NSP, back up the data and transfer it from the NSP computer to the computer that runs the plate reader.

5.5.3 Save the electronic file including the plate results using the plate ID as the file name in the folder of the current month. (i.e. Elisa Feb09- folder for February 2009).

5.5.4 Print a paper copy of the plate results and write the plate ID on this paper copy.

5.5.5 File the paper copy in the designated results binder.

5.5.6 Enter VSLS results for each sample tested by ELISA on the web at: https://cowebapps.aphis.usda.gov/vslabsub (once the submission information has been entered accurately).

5.6. Quality Control Parameters

- Samples that are received but are not clearly recognizable as brain stem will not be tested. Results will be reported as "NOT TESTED." For brain stem samples received that have no recognizable obex, samples will be processed and test results will be reported.
- In the event of a known splashing or pipetting error while using the Bovine Spongiform Encephalopathy Antigen Test Kit, document the error in the comments section of the BSE ELISA Checklist and report it to the supervisor or designee PRIOR to reading the plate.
- Sample controls run are analyzed according to manufacturer's recommendations.

- The test is repeated according to manufacturer's recommendations.
- Balances and weights will be calibrated through the NCAH Calibration Laboratory approximately yearly.
- The Precess, NSP, block heaters, plate washers and plate reader will be serviced by Bio-Rad Technical support approximately yearly for preventative maintenance.
- The centrifuge and block heaters will be checked through the NCAH Calibration Laboratory approximately yearly.
- Function-check the plate reader with the calibration plate at a minimum quarterly and print the results.

5.7. Sample Retention/Disposal

- Place remaining tissue back into the container and then place the sample containers into a clean bag with the date of cut-in on the outside of the bag.
- Retain all routine not detected samples for a minimum of five working days at 2-8° C before discarding.
- Hold positive or inconclusive slides, blocks, formalin-fixed and frozen tissues indefinitely in a secure safe or freezer at the BSL-3 laboratory.

6. Associated NVSL Quality Documents/References

- TeSeE® detection and purification kit used for the detection of BSE in rabies negative fresh samples of CNS tissue was designed by the Commissariat a l'Energie Atomique-CEA (French Atomic Energy Commission) and is produced and marketed by Bio-Rad.
- BioSafety in Microbiological and Biomedical Laboratories (BMBL), Current Edition
- SOP-PL-0006, Procedures for Entering and Exiting BSL-3 Laboratory Pathobiology Laboratory
- SOP-PL-0032, BSE Contract Laboratories to Receive and Test Bovine Brain Samples and Report Results for BSE Surveillance
- SOP-PL-0036, Decontamination and Biological Accident Procedures Used in the Transmissible Spongiform Encephalopathy Laboratory (Building 21 – PL BSL3 Laboratory)
- SOP-PL-0017, Disposal Procedure for BL3 Hazardous Waste and Reagent Waste
- FM-PL-0027, BSE ELISA Checklist
- EXT-WI-PL-5138, Bio-Rad Bovine Spongiform Encephalopathy Antigen Test Kit, ELISA
- EXT-WI-PL- 5712, Bio-Rad NSP New Sample Pr(p)eparator Operator's Manual
- WI-PL-5397, BSE ELISA Lab Ware Instructions

7. Revision History

Version .06 replaces version .05.

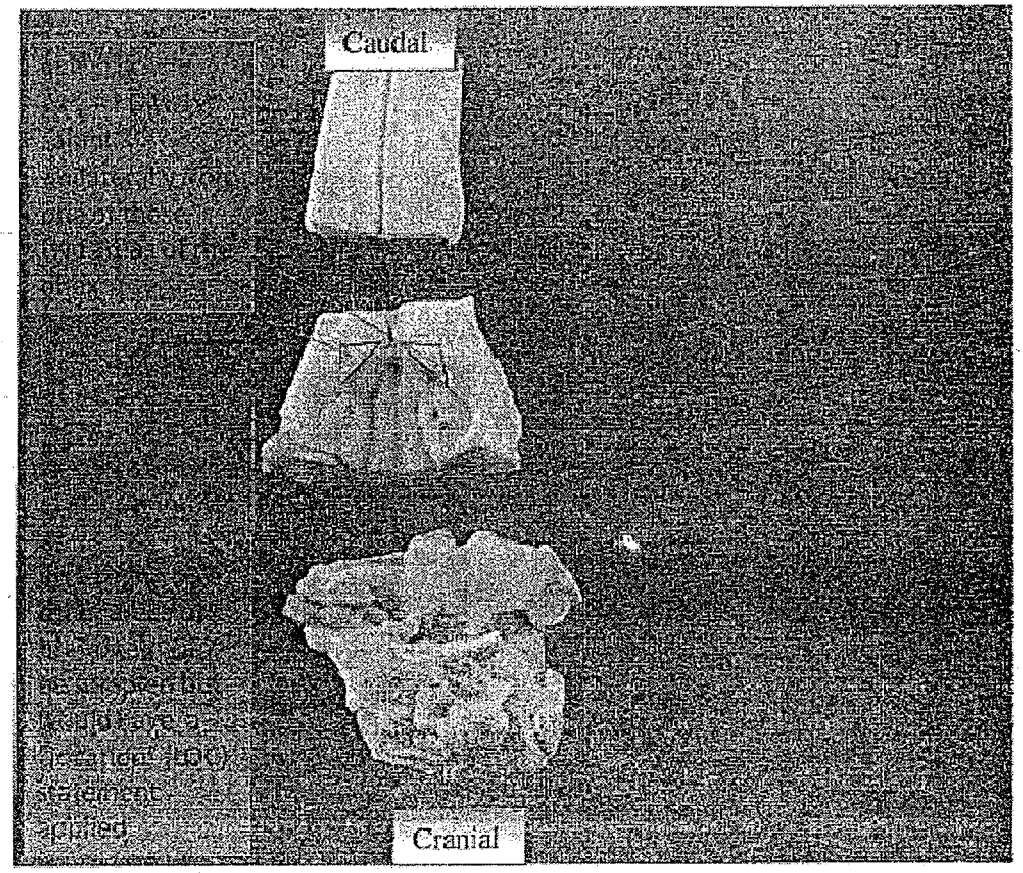
- Removed Lab Ware instructions and made into a separate document: WI-PL-5397 BSE ELISA Lab Ware Instructions
- Updated SOP to include critical equipment maintenance

- Version .05 replaces version .04.
  - Document number changed from SOP-PTS-0003 to SOP-PL-0033
  - Updated associated documents (section 6) throughout document

8. Appendices

Appendix 1: Photograph Identifying Brainstem Including Obex

Appendix 1: Photograph Identifying Brainstem Including Obex (Dorsal View)



附件 6

National Veterinary Services Laboratories	
Document Title: Bovine Spongiform Encephalopathy (BSE) Histology and Immunohistochemistry Procedures	
Author/Position: Sharon A. Lund, BSL Tech	Document Number: SOP-PL-0012.03
Page 1 of 13	Supersedes: SOP-PTS-0002.02

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- FM-PL-0007: BSE IHC Results Worksheet
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- FM-PL-0019: BSE Histology - IHC Laboratory Maintenance Log
- FM-PL-0020: Request for Histology Services
- SOP-PL-0005: NVSL Building 21 Pathobiology Laboratory Clothing Policy
- SOP-PL-0006: Procedures for Entering and Exiting BSL-3 Laboratory Pathobiology Laboratory
- SOP-PL-0007: Pathobiology Laboratory Security and Safety Handbook
- SOP-PL-0031: Histopathological Evaluation of Bovine Brain Specimens for Lesions of Bovine Spongiform Encephalopathy
- SOP-PL-0024: Tracking Procedure for Bovine Spongiform Encephalopathy Samples
- SOP-PL-0025: Immunohistochemical Interpretation and Terminology Used for Detection of Bovine Spongiform Encephalopathy (BSE) Diseases
- SOP-PL-0036: Decontamination and Biological Accident Procedures Used in the Transmissible Spongiform Encephalopathy Laboratory (Building 21 –PL BSL3 Laboratory)

Approved: /s/ Arthur Davis

- SOP-PL-0029: Confirming Inconclusive Results from Bovine Spongiform Encephalopathy Testing Laboratories at the NVSL
- SOP-PL-0040: Hematoxylin and Eosin Staining Procedure
- SOP-PL-0041: Coverslipping of Tissue Slides
- SOP-PL-0042: Microtomy of Paraffin Embedded Tissue Sections
- SOP-PL-0013: Paraffin Embedding of Tissue Samples
- SOP-PL-0044: Processing of Tissue Samples

7. Revision History

8. Appendices

Appendix 1: Protocol # 5 ultraView Red Paraffin

1. Purpose/Scope

This Standard Operating Procedure (SOP) establishes the histological and immunohistochemical methods for detection of proteinase resistant prion protein in paraffin embedded tissue sections of cattle tested at the National Veterinary Services Laboratories (NVSL), Ames, Iowa.

2. Definitions

Obex: A thin, triangular lamina of gray matter in the medulla oblongata present in the roof of the fourth ventricle just rostral to the point where the ventricle narrows to form the central canal of the spinal coral.

Clinical bovine spongiform encephalopathy (BSE) case: An animal with central nervous system (CNS) signs and for which the majority of the brain is submitted.

PrP Only: Cases in which only the obex is received and H & E slides are not made (excludes clinical cases and inconclusive ELISA cases).

3. Safety Precautions

Personnel working with prion infected or potentially infected materials need to have a general understanding of transmissible spongiform encephalopathies (TSEs). Additional information on prion bio-safety is located in the Center for Disease Control (CDC) and World Health organization (WHO) Guides and review articles in the biomedical literature.

Prior to entering the BSL3 area of the Pathobiology Laboratory (PL) individuals must be documented as having received yearly Agent Specific BSE training. Training will be provided from the Pathology Section of the PL. Material Safety Data Sheets for BSE should be reviewed.

Procedures in this SOP require the use of formalin and formic acid. These agents are hazardous and should be handled only in a fume hood. Precautions must be taken to avoid contact with eyes and skin, and to avoid breathing the fumes.

4. Equipment and Materials Required

- Chemical Fume Hood
- Refrigerator
- Flammable Liquids Storage Cabinet
- Acid Storage Cabinet
- Ventana NexES IHC Full System with Ebar Printer
- Biocare Medical Decloaking Chamber with digital controls
- Drying Oven – Set to 80 C

- Coverslipping supplies using permanent mounting medium
- Laboratory Tissue
- Biocare Medical Steam Monitor Strips
- Waste solvent storage
- Staining dishes
- Non metal slide holder
- Microscope slides – Colorfrost Plus, charged slides from Erie Scientific or others approved by the NVSL
- 95 – 98% formic acid
- Tris Buffer 0.1m Tris-HCl - pH 7.5, Media prep order #30146 or equivalent
- Timing devices
- Squirt wash bottle
- Biocare Medical RTU DIVA Retrieval Solution or other approved by the NVSL
- Xylene
- Absolute ethyl alcohol
- 95% ethyl alcohol
- Dawn dishwashing liquid, original formula
- Reverse Osmosis Water or other laboratory quality water
- Reagents from Ventana Medical Systems
  - 250-009 Liquid Coverslip
  - 250-042 APK Wash
  - 760-501 *ultraView* Red Detection Kit, 250 tests
  - 760-2021 Hematoxylin, 250 tests
  - 760-2037 Bluing Reagent, 250 tests
  - 790-2209 Antibody Anti-prion 99/97.6.1, 250 tests
  - 1418702 Ebar Bar Code Labels, 2500 labels
  - 1632900 Ebar Printer Ribbon
- Hematoxylin
- Eosin-Y (alcoholic)
- Potassium Acetate – 1% prepare daily
- Isopropyl Alcohol
- Glacial Acetic Acid

5. Procedure

5.1. Additional Considerations to be followed for BSE ELISA Inconclusive Samples.

All testing will be done in the BSL3 area of the Pathobiology Laboratory with two employees present to sign off on the sample verification, cut-in, slide-making and evaluation.

A Hematoxylin and Eosin (H & E) stained slide will be prepared from each block.

An immunohistochemistry stained slide will be prepared from the obex block.

Optional steps may be used to optimize IHC staining and evaluation with the concurrence of the Laboratory Director. This can include but is not limited to primary antibody, formic acid and/or Decloaker times, antigen retrieval solutions, and reagent kits. All variations will be documented and results reported to the Laboratory Director.

Times, temperatures and volumes are approximate measurements unless otherwise indicated.

NOTE: Follow BSE Tracking, SOP-PL-0024 for sample receiving, log-in, and cut-in procedures.

5.2. Formic Acid Treatment of Tissue in the Cassette

Formic acid treatment of tissues in the cassette is an optional treatment that may be used to classify the paraffin tissue block as containing noninfectious material. This procedure is not routinely performed at the NVSL.

5.2.1. Remove cassettes containing tissue sections from formalin and drain.

5.2.2. Immerse cassettes in 95% - 98% formic acid and incubate for approximately 60 minutes at room temperature.

5.2.3. Remove cassettes from formic acid and rinse overnight in running tap water.

5.2.4. Transfer the cassettes to fresh 10% neutral buffered formalin and hold for a minimum of twenty four hours to re-equilibrate.

5.2.5. Remove samples from formalin and move to the processor.

5.2.6. Formic acid can be used up to three times.

NOTE: Follow Processing of Tissue Samples, SOP-PL-0044; Paraffin Embedding of Tissue Samples, SOP-PL-0013; Microtomy of Paraffin Embedded Tissue Sections, SOP-PL-0042.

5.3. Slide Staining with Hematoxylin and Eosin (H&E)

- All blocks from clinical BSE cases will be stained by H & E.
- All blocks from ELISA Inconclusive Cases will be stained by H & E.

5.3.1. Hand stain or follow Hematoxylin and Eosin Staining Procedure, SOP-PL-0040 for automated staining.

• 1. Xylene	3 min
• 2. Xylene	3 min
• 3. Xylene	3 min
• 4. 100% Ethyl Alcohol	1 min
• 5. 100% Ethyl Alcohol	1 min
• 5. 95% Ethyl Alcohol	1 min
• 7. Tap Water	1 min
• 8. Hematoxylin	1.5 min
• 9. Running Tap Water	2 min
• 10. Clarifier	1 min
• 11. Running Tap Water	2 min
• 12. Potassium Acetate 1%	1 min
• 13. Tap Water	1 min
• 14. 95% Ethyl Alcohol	1 min
• 15. Eosin	1.5 min
• 16. 95% Ethyl Alcohol	1 min
• 17. 95% Ethyl Alcohol	1 min
• 18. 100% Ethyl Alcohol	1 min
• 19. 100% Ethyl Alcohol	1 min
• 20. Xylene	1 min
• 21. Xylene	1 min

5.3.2. When hand staining, change solutions after each batch of slides except for hematoxylin and eosin which are good for approximately 200 slides or one week if kept tightly covered.

NOTE: Follow Coverslipping of Tissue Slides, SOP-PL-0041.

5.4. Slide Staining for Immunohistochemistry (IHC)

- **Clinical Cases will have blocks A, B and D stained.**
- **PrP Only Cases will have the obex or best tissue available block stained.**
- **ELISA Inconclusive Cases will have the obex or best tissue available block stained.**

Staining dish containers should be dedicated to a particular reagent or process. Never use a dish that has had stain in it for this protocol.

5.4.1. One set of up to twenty slides for each NexES module available can be processed together. Run control tissues in slot #1 with each set of

nineteen or less samples. More control slides may be included in other slots if deemed necessary.

5.4.2. Complete slide, control and staining run information on the BSE Result Worksheet.

5.4.3. Load slides in non-metallic rack and dry a minimum of 15 minutes in an 80 C oven.

5.4.4. Remove a reagent carousel from the refrigerator for each module to be used. Check that all reagents required for the run are available on the carousel. Register reagents as needed with the reagent wand per applicable Ventana instructions.

5.4.5. Allow reagents to adjust to room temperature and then remove each cap from the tip of the dispenser and place it on the holding pin.

5.4.6. Print slide bar code labels per protocol #5 UltraView Red Paraffin (see Appendix 1). Follow Ventana instructions for setting up templates and protocols.

5.4.7. Remove slides from the oven and process to these minimum times.

- These solutions should be changed every 40 slides.
  - 3 – Xylenes 5 minutes each
  - 2 – 100% Ethyl Alcohols 1 minute each
  - 1 – 95% Ethyl Alcohol 1 minute
  - 1 – Reverse Osmosis Water 1 minute
  - 95 – 98 % Formic Acid for 15 minutes

- Formic acid can be used three times.
  - 3 – Tris Buffer pH7.5 Rinses 2 minutes each
  - pH the third rinse, if it is not at 7.5 do more rinses until the pH is 7.5

- Buffer rinses are used only once.
  - 1 – Reverse Osmosis Water 1 minute

5.4.8. Add 200 mls of target retrieval solution to a staining dish for each set of slides.

5.4.9. Put 500 mls of reverse osmosis water in the Decloaker and center the heat shield in the pan.

5.4.10. Move the rack of slides into the target retrieval.

5.4.11. Fill the Decloaker with four dishes of slides each run. Use a staining dish, gray rack, 20 blank slides and 200 mls of water to create filler sets as needed.

5.4.12. Place a Biocare Medical Steam Monitor strip across the top of a dish. Use one strip for each Decloaker run.

5.4.13. Close the lid and rotate clockwise to secure it.

5.4.14. Lower the weight over the vent nozzle.

5.4.15. Toggle the unit power switch ON. Use the display buttons to set these conditions:

- Temperature 121 C
- Time 30 minutes
- Second temp 85 C
- Second time 25 minutes
- Default temp 10 C
- Actual temp will display
- Return to the 121 C display.
- See manufacturer's instructions for further details.

5.4.16. Push the START button. The time will count down when proper pressure and temperature are achieved. An alarm will sound when the time is done. Push the STOP button to silence the alarm.

5.4.17. Start a timer for 25 minutes and let the Decloaker cool until this time is up.

5.4.18. Carefully rotate the handle and remove the lid. Contents will be HOT!

5.4.19. Confirm that the steam monitor strip has changed color to black and record the result.

5.4.20. Remove staining dishes containing case slides. Pour off most of the hot liquid and quickly refill with APK wash OR carefully move the slide rack to a dish of APK wash, and then move rack to a second dish of APK. DO NOT LET SLIDES DRY. Let set for a minimum of five minutes and not more than two hours.

5.4.21. The Decloaker must be cooled after each use to obtain proper operating conditions for the next run. The temperature of the pan should be 37 C or lower before using. Leave the Decloaker pan empty and dry with the lid ajar when not in use.

5.4.22. Remove a slide from the APK and dry the colored end with a laboratory tissue. Apply the proper bar code label with the top of the label lined up with the top of the slide. Press firmly and make particular note that the bottom of the label is secured to the slide. Do not let the slide dry. Load the labeled slide under the clip on the NexES wheel. Cover the slide with APK from a squirt bottle. Repeat until all the slides are labeled and loaded.

5.4.23. Assure appropriate reagent levels are in the APK wash bottle, liquid coverslip bottle and the waste container.

5.4.24. Place the prepared reagent carousel on the instrument and close the slide tray drawer.

5.4.25. Select the RUN button at the upper right of the screen, click on or push enter to the review statements, enter the number of slides for the run and enter.

5.4.26. Should the program not start and/or run to completion, take action to correct the problem as trained and/or per manufacturer's instructions (manuals, Customer Service Technical Support 800-227-2155), or other trained resource personnel consultation. Continue as appropriate per manufacturer's instructions and site specifications. If circumstances prevent the slides from being stained to completion record any error messages and actions taken. Notify the section supervisor and complete any further recommendations.

5.4.27. The staining protocol will take approximately 90 minutes on the NexES and an alarm will sound at completion unless it is disabled. Silence the alarm by clicking on the yellow note pad on the lower left of the screen. Open the slide tray door.

5.4.28. Remove slides from the wheel and place in a gray rack. Dip 30 times in 250 mls of tap water containing two drops of Dawn original formula dishwashing liquid.

5.4.29. Rinse the slides with running tap water for two minutes or at least three changes of 250 mls water.

5.4.30. Dip the slides 10 to 20 times each in:

- Reverse Osmosis Water
- 100% Ethyl Alcohol
- 50/50 Ethyl Alcohol/Xylene
- Xylene

- Change reagents after 40 slides.

NOTE: Follow Coverslipping of Tissue Slides, SOP-PL-0041.

5.4.31. Put reagents in the refrigerator, clean the staining module, equipment, and work area after use and record on BSE Laboratory Maintenance Log.

5.4.32. Print a copy of the Ventana Run Sheet per manufacturer's instructions, add the date and run letter to the top right corner of the page, and file in the designated binder.

5.5. Slide Evaluation

Slides are put in a flat with the BSE Results Worksheet, and are then ready for interpretation.

The pathologist will follow Histopathological Evaluation of Bovine Brain Specimens for Lesions of Bovine Spongiform Encephalopathy, SOP-PL-0031 to evaluate the H & E stained slides.

The pathologist will follow Immunohistochemical Interpretation and Terminology Used for Detection of Bovine Spongiform Encephalopathy (BSE) Diseases, SOP-PL-0025 to evaluate the IHC stained slides.

5.6. BSE Results Worksheet and Slide Return

5.6.1. Check that information is complete at the top of the form and that each slide has an appropriate result.

5.6.2. Positive (P) diagnosis must have a second pathologist's confirming initials.

5.6.3. For P, compare slide to block to assure they match.

5.6.4. For repeat (R), highlight the line.

5.6.5. Locate the block corresponding to the R diagnosis and start the slide making process per Microtomy of Paraffin Embedded Tissue Sections.

5.6.6. Initial and date the bottom of the form.

5.6.7. FAX or e-mail the completed BSE Results Worksheet along with all case documents to support staff for reporting.

5.6.8. Make a copy for the case file folder housed in the BSL3.

5.6.9. Place the completed original (yellow) BSE Results Worksheet in the designated binder.

5.7. Waste Disposal

5.7.1. All containers will be wiped down with bleach before removal from the PL BSL3 area.

5.7.2. Water and Ventana staining waste will be dumped into a drum located in the BSL3 airlock to be removed and disposed of by the NCAH Safety Unit.

5.7.3. Xylene and alcohol waste will be picked up from the PL BSL3 airlock by NCAH Safety Unit and delivered to an off site waste handler.

5.7.4. Water and Ventana waste that was used on a positive BSE sample will be treated with sodium hydroxide and then disposal will be the same as for other waste. Xylene and alcohol waste that was used on a positive BSE sample will require a chain of custody form and certification from the off site hauler that it was incinerated.

6. Associated NVSL Quality Documents / References

- Miller, J., A. L. Jenny, W .D. Taylor, et al. 1994. "Detection of Prion Protein in Formalin-fixed Brain by Hydrated Autoclaving Immunohistochemistry for the Diagnosis of Sheep," Journal of Veterinary Diagnostic Investigation 6:366-368.
- Biocare Medical Digital Decloaking Chamber Operating Handbook
- Drying Oven Operating Instructions
- Labconco Fume Hood User's Manual
- Ventana Medical Systems, Inc. Ebar Printer Manual
- Ventana Medical Systems, Inc. NexES Manual
- FM-PL-0007: BSE IHC Results Worksheet
- FM-PL-0017: BSE Cut-In Sheet
- FM-PL-0019: BSE Histology - IHC Laboratory Maintenance Log
- FM-PL-0020: Request for Histology Services
- SOP-PL-0005: NVSL Building 21 Pathobiology Laboratory Clothing Policy
- SOP-PL-0006: Procedures for Entering and Exiting BSL-3 Laboratory Pathobiology Laboratory
- SOP-PL-0007: Pathobiology Laboratory Security and Safety Handbook
- SOP-PL-0031: Histopathological Evaluation of Bovine Brain Specimens for Lesions of Bovine Spongiform Encephalopathy
- SOP-PL-0024: Tracking Procedure for Bovine Spongiform Encephalopathy Samples

- SOP-PL-0025: Immunohistochemical Interpretation and Terminology Used for Detection of Bovine Spongiform Encephalopathy (BSE) Diseases
- SOP-PL-0036: Decontamination and Biological Accident Procedures Used in the Transmissible Spongiform Encephalopathy Laboratory (Building 21 –PL BSL3 Laboratory)
- SOP-PL-0029: Confirming Inconclusive Results from Bovine Spongiform Encephalopathy Testing Laboratories at the NVSL
- SOP-PL-0040: Hematoxylin and Eosin Staining Procedure
- SOP-PL-0041: Coverslipping of Tissue Slides
- SOP-PL-0042: Microtomy of Paraffin Embedded Tissue Sections
- SOP-PL-0013: Paraffin Embedding of Tissue Samples
- SOP-PL-0044: Processing of Tissue Samples

7. Revision History

- Version .03
  - Document number changed from SOP-PTS-0002 to SOP-PL-0012
  - Minor format changes
  - Updated associated documents (section 6) throughout document
- Version .02
  - Document number changed from SPSOP0002 to SOP-PTS-0002
  - complete rewrite

8. Appendices

Appendix 1: Protocol #5: NexEs UV Red Procedure: ultraView Red Par NexES IHC Staining Module

Appendix 1: Protocol #5: NexEs UV Red
Procedure: ultraView Red Par
NexES IHC Staining Module

Step No.	Procedure Step
1.	*****Warmup rinse Buffer to 41.0 Deg C*****
2.	Rinse Slide
3.	Adjust Slide Volume, then Apply Coverslip
4.	*****Start Times Steps*****
5.	*****Warmup Slide Chamber to 37.0 Deg C*****
6.	*****Start Unlimited Steps*****
7.	Rinse Slide
8.	Adjust Slide Volume, then Apply Coverslip
9.	*****Start Times Steps*****
10.	Rinse Slide
11.	Adjust Slide Volume, then Apply Coverslip
12.	Apply One Drop of [Anti-Prion(99)] (Antibody), and Incubate for [32 Minutes]
13.	Rinse Slide
14.	Adjust Slide Volume, then Apply Coverslip
15.	Apply One Drop of UV Red UNIV MULT, and Incubate for 12 Minutes
16.	Rinse Slide
17.	Adjust Slide Volume, then Apply Coverslip
18.	Rinse Slide
19.	Adjust Slide Volume, then Apply Coverslip
20.	Skip Application & Incubate for 2 Minutes
21.	Apply One Drop of UV Red Enhancer, and Incubate for 4 Minutes
22.	Apply One Drop of UV Fast Red A and One drop of UV Red Naphthol, and Incubate for 8 minutes
23.	Apply One Drop of UV Fast Red B, and Incubate for 8 Minutes
24.	Rinse Slide
25.	Adjust Slide Volume, then Apply Coverslip
26.	Apply One Drop of [Hematoxylin] (Counterstain), and Incubate for [6 Minutes]
27.	Rinse Slide
28.	Adjust Slide Volume, then Apply Coverslip
29.	Apply One Drop of [Bluing Reagent] (Post Counterstain), and Incubate for [6 Minutes]
30.	Rinse Slide

Note: One Drop is one reagent dispense

附件 7

National Veterinary Services Laboratories	
Document Title: TSE Western Blot	
Author/Position: Nadine Beckwith, Biological Sciences Laboratory Technician	Document Number: SOP-PL-0021.02
Page 1 of 15	Supersedes: SOP-PS-0004.01

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   5.8. Quality Control Parameters
   5.9. Sample Retention/Disposal

6. **Associated NVSL Quality Documents/References**

- EXT-WI-PL-5140, TeSeE Western Blot
- SOP-PL-0006, Procedures for Entering and Exiting BSL-3 Laboratory Pathobiology Laboratory
- SOP-PL-0017, Disposal Procedure for BL3 Hazardous Waste and Reagent Waste
- SOP-PL-0022, Chain of Custody of ELISA BSE Samples Received from Contract Laboratories for Confirmation
- SOP-PL-0024, Tracking Procedure for Bovine Spongiform Encephalopathy Samples
- SOP-PL-0029, Confirming Inconclusive Results from Bovine Spongiform Encephalopathy Testing Laboratories at the NVSL.

Approved: /s/ Mark Hall

- SOP-PL-0032, Protocol for BSE Contract Laboratories to Receive and Test Bovine Brain Samples and Report Results for BSE Surveillance
- SOP-PL-0036, Decontamination and Biological Accident Procedures
- SOP-PL-0048, Log-In and Cut-In Procedures for Scrapie Sample
- WI-PL-0016, Work Instructions for TSE

7. **Revision History**

8. **Appendices**

Appendix 1: OIE Adapted Protocol - Amount of Reagent Added

1. Purpose/Scope

The purpose of this Standard Operating Procedure (SOP) is to document the proper technical procedure for performing Western Blots (WB) for transmissible spongiform encephalopathy (TSE) at the National Veterinary Services Laboratories (NVSL), Pathobiology Laboratory (PL). This testing includes samples for Bovine Spongiform Encephalopathy (BSE) and Scrapie.

2. Definitions

Obex - A thin triangular lamina of gray matter in the medulla oblongata present just rostral to the point where the fourth ventricle narrows to form the central canal of the spinal cord.

3. Safety Precautions

3.1. Personnel

- Precautions must be taken in the preparation of bovine CNS tissues due to the potential exposure of humans to a distinct variant of Creutzfeldt-Jakob disease (vCJD) which seems to be associated with exposure to the agent that causes BSE. vCJD has been described in humans in the UK and in humans known to have been present in the UK during times of highest exposure to BSE infected cattle. 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. The highest risks of infection appear to be from direct contact or splashing of highly infectious material (CNS) to eyes, mucous membranes, open wounds, abraded skin, or by swallowing. Aerosol transmission is considered the least likely route of exposure.

- Everyone working with potentially infected BSE samples should understand and follow BSL-3 guidelines to the extent the laboratory facilities permit. The CDC publication Biosafety in Microbiological and Biomedical Laboratories, current edition, should be consulted for current recommendations concerning the handling of prion infected or potentially infected materials.

- Those working with prion infected or potentially infected materials need to have a general understanding of BSE and other TSEs. Recommended reading is the CDC and World Health Organization guides discussing biosafety for prions. Many good review articles are also available in the biomedical literature.

- Formalin, most routine disinfectant methods, and some incineration methods are not effective in inactivating prions, including the BSE agent. The NVSL employ 1 hour of wet contact time with bleach for routine disinfection of equipment and work spaces when working with prion infected or potentially infected materials. For decontamination associated with biological accidents, please refer to Decontamination and Biological Accident Procedures, SOP-PL-0036.

3.2. Security of Sample Storage in the BSL-3 Laboratory

The NVSL, PL is secured from the public. The exterior doors are kept locked 24 hours per day, 7 days per week, and employee entry is by a key card. All visitor's must sign in and be issued a visitor's pass. Visitors must be escorted at all times while in the PL, visibly wear their visitor pass at all times, and turn in the visitor's pass upon leaving. A security alarm is engaged at all times when employees are not in PL. A surveillance camera with a view of the main entry is monitored from the guard station at the NVSL Central Campus.

4. Equipment and Reagents

4.1. Equipment

- Scalpel
- Forceps
- Weigh boat
- Weigh Scale
- Homogenizer- Precess instrument (Bio-Rad) for TeSeE kit assay
- Homogenizer- Power Gen 125, Fisher Scientific for OIE adapted assay
- Calibration syringe and needles (Bio-Rad)
- 1.5 ml snap cap tubes
- Pipettes
- Dry Block Heater
- Centrifuge
- Ultracentrifuge appropriate tubes
- Ultracentrifuge
- Falcon tubes
- Stirring water bath
- 12% Bis-Tris gel (Bio-Rad)
- Electrophoesis tank and power source (Bio-Rad)
- Sponges
- Filter paper
- PVDF membrane
- Transfer tank with cooling unit and power source (Bio-Rad)
- Trans-Blot Turbo Transfer System (Bio-Rad)

- Rocker table
- Versa Doc image analysis system (Bio-Rad)

4.2. Reagents

- **BioRad TeSeE Western Blot Confirmatory Assay** - TeSeE Western blot kit contains all reagents needed except:
  - Laemmli sample buffer
  - 2-Mercaptoethanol
  - SDS
  - 20X XT-MOPS (running buffer)
  - Molecular marker (MagicMark XP Western Standard, Kaleidoscope prestained standard)
  - 10X Tris/CAPS Buffer
  - Ethanol absolute
  - Tween 20
  - 10X Phosphate Buffered Saline (PBS)
  - ECL Western Blotting detection system (Enhanced Chemiluminescent, Amersham or Supersignal, Pierce)

4.3. OIE Adapted Western Blot

- Sodium Thiosulphate Pentahydrate
- Potassium Iodide
- 1M Tris/HCL Buffer pH7.4
- N-lauroylsarcosine –(SARC)
- Deionized Water
- 0.01M Sodium Phosphate Buffer pH7.4
- Phenylmethylsulfonyl Fluoride (PMSF)
- N-ethyl-maleimide (NEM)
- 1-Propanol
- Sodium Dodecylsulfate (SDS)
- 2-Mercaptoethanol
- Sucrose
- 1% Bromophenol Blue
- Tropix I-Block
- Tris Buffered Saline x1 (TBS)
- Tween 20
- Tris
- MgCl
- 6H4 antibody (Prionics)
- P4 antibody
- Secondary Ab Goat Anti-mouse

- ECL Western Blotting detection system (Enhanced Chemiluminescent, Amersham or Supersignal, Pierce)

5. Procedure

5.1. Sample Receipt

- BSE Samples: Process samples with inconclusive BSE ELISA test results submitted to the NVSL from contract laboratories for BSE Western Blot confirmation according to SOP-PL-0029 Confirming Inconclusive Results from Bovine Spongiform Encephalopathy Testing Laboratories at the NVSL.

***IMPORTANT: BSE samples must be submitted under seal and accompanied by the appropriate chain of custody form as directed in SOP-PL-0022 Chain of Custody of ELISA BSE Samples Received from Contract Laboratories for Confirmation and SOP-PL-0032 Protocol for BSE Contract Laboratories to Receive and Test Bovine Brain Samples and Report Results for BSE Surveillance.

- Scrapie Samples: Process samples according to SOP-PL-0048 Log-In and Cut-In Procedures for Scrapie Sample and WI-PL-0016 Work Instructions for TSE.

5.2. Sample Tracking

- Log and assign testing for BSE and Scrapie WB samples in LabWare.
- Log and track BSE samples accordance with SOP-PL-0024 Tracking Procedure for Bovine Spongiform Encephalopathy Samples and SOP-PL-0022 Chain of Custody of ELISA BSE Samples Received from Contract Laboratories for Confirmation. The steps of the tracking and testing procedures for BSE will require the presence of a second PL staff member for sample identification and verification.

5.3. Method Selection

The western blot method used for confirmation of TSE in fresh tissue samples will be determined by the appropriate section head and/or by the Director of the Pathobiology Laboratory. The following are two acceptable procedures for WB:

- The TeSeE Western Blot Confirmatory Assay from Bio-Rad Laboratories
- The protocol of SAF Western Blot based on the procedure adapted at the OIE Reference Laboratory in VLA/Weybridge.

5.4. Protocol for TeSeE Western Blot Confirmatory Assay

Test should be run according to manufacturer's instructions: See EXT-WI-PL-5140 TeSeE Western Blot.

Times, temperatures, voltages, and gravitational force are approximate unless otherwise noted.

5.4.1 Obtain sample from the obex region in the following manner:

5.4.1.1 Remove 0.31g to 0.39g of sample obex.

5.4.1.2 Place in a Bio-Rad grinding tube.

5.4.2 Homogenize using the Precess instrument mode **1** to create a 20% brain homogenate.

5.4.3 Transfer 500µl of the homogenate into a 1.5ml snap cap tube and add 500µl of the Proteinase K and detergent solution (20µl Proteinase K added to 1ml Reagent A).

5.4.4 Incubate for 10 min at 37 °C.

5.4.5 Add 500µl of reagent B.

5.4.6 Centrifuge at 15,000*g* for 7 minutes.

5.4.7 Pour off the supernatant and dry the pellet for 5 minutes.

5.4.8 Add 100 ml of Laemmli solution to solubilize the pellet.

5.4.9 Incubate at 100 °C for 5 minutes.

5.4.10 Centrifuge at 15,000g for 15 minutes and transfer supernatant to a new snap cap tube.

5.4.11 Heat supernatants at 100 °C for 5 minutes before electrophoresis.

5.4.12 Load samples on the same blot as the controls and molecular marker to be able to judge the shift in molecular weight associated with various strains of the disease.

5.4.13 Run samples by electrophoresis at 200V for 50 minutes.

5.4.14 Wet the membrane in methanol for 5 minutes and then equilibrate in transfer buffer for at least 10 minutes.

5.4.15 Prepare transfer sandwich in the following manner:

5.4.15.1 Sponge
5.4.15.2 Filter paper
5.4.15.3 PVDF membrane wet in transfer buffer
5.4.15.4 Gel wet with transfer buffer
5.4.15.5 Filter paper
5.4.15.6 Sponge

5.4.16 Transfer the proteins onto a PVDF membrane at 115V for 60 minutes.

5.4.17 Block the membrane using Bio-Rad blocking solution for 30 minutes at room temperature.

5.4.18 Incubate the membrane with Sha 31 (Bio-Rad Abl) against YEDRYYRE (156–163) bovine PrP sequence for 30 minutes at room temperature.

5.4.19 Wash in PBST (two times) - 5 minutes and then 10 minutes.

5.4.20 Incubated the membrane with goat antimouse IgG antibody conjugated to horseradish peroxidase (Bio-Rad AbII) for 20 minutes.

5.4.21 Wash in PBST (three times) - 5 minutes, 10 minutes and then 10 minutes.

5.4.22 Incubate the membrane in ECL Western Blotting detection system (Enhanced Chemiluminescent, Amersham or Pierce) for 5 minutes.

5.4.23 Image the membrane using either the Versa Doc image analysis system (Bio-Rad) or an imager as determined by the head of the Pathology Section and/or by the Director of the Pathobiology Laboratory.

5.5. Protocol for OIE Adapted Western Blot

5.5.1. Obtain sample from the obex region in the following manner:

Type of Sample	Location and Amount of Sample
Negative control	4g of brainstem or not less than 0.31g-0.39g
Positive control	4g of brainstem or not less than 0.31g-0.39g
Test sample	1-4g or available amount not less than 0.31g-0.39g OD >2.5 Bio-Rad ELISA 1g of brainstem (all treated with PK) OD 1-2.5 Bio-Rad ELISA 2g of brainstem (may all be treated with PK)

5.5.2. Add NEM and PMSF to the BLB inside a BSC.

5.5.3. Homogenize each of the controls and test samples in BLB containing NEM and PMSF to create a 20% homogenate.

5.5.4. Transfer the homogenates to new tubes and equilibrate the volumes using BLB.

5.5.5. Centrifuge the samples at 20000g for 30 minutes at 10 °C using one of the following rotors:

- 21000 rpm using a TLA 55 rotor
- 17000 rpm using a 70TI rotor

5.5.6. Transfer the supernatant to a clean tube.

5.5.7. Equilibrate the tube volumes with BLB.

5.5.8. Centrifuge the samples at 177000g for 2 hours 15 minutes at 10 °C using one of the following rotors:

- 49000 rpm using a TLA 55 rotor
- 46000 rpm using a 70TI rotor

5.5.9. Discard the supernatant and suspend the pellet in deionized water and Tris/HCL according to Appendix 1.

5.5.10. Transfer the samples to clean tubes.

5.5.11. Incubate the samples in a stirring water bath at 37 °C for 15 minutes.

5.5.12. Add 15% KI-HSB according to Appendix 1 and incubate in a stirring water bath at 37 °C for 30 minutes.

5.5.13. Divide the negative and positive control samples into two aliquots for a PK and a non-PK treatment in clean tubes.

5.5.14. Add PK to the appropriate control samples and to the test samples according to Appendix 1.

5.5.15. Incubate for 1 hour in a stirring water bath at 37 °C.

5.5.16. Transfer all samples to new tubes and add 10% KI-HSB to both PK and non-PK treated samples. Equilibrate the volumes with BLB.

5.5.17. Centrifuge the samples at 189000g for 1 hour at 10 °C

- 55000 rpm using a TLA 55 rotor
- 51000 rpm using a 70TI rotor

5.5.18. Carefully discard the supernatant and suspend the pellet in 40µl Sample Buffer.

5.5.19. Heat the samples at 100 °C for 5 minutes and give a quick centrifuge spin to concentrate the sample to the bottom.

OIE Process Stopping Point: May store the samples at -20 °C overnight.

5.5.20. Heat the samples at 95 °C for 5 minutes.

5.5.21. Load 15µl of the sample onto a 12% Bis-Tris gel.

5.5.22. Load 8µl of the control and 10µl of Magic Mark XP onto the same gel.

5.5.23. Run the gel at 200V for 50 minutes.

5.5.24. Equilibrate gel in transfer buffer for at least 10 minutes.
5.5.25. Wet the membrane in ethanol for 15 seconds, rinse in deionized water for 5 minutes and then equilibrate in transfer buffer for at least 10 minutes.

5.5.26. Prepare transfer sandwich in the following manner:

5.5.26.1. Sponge
5.5.26.2. Filter paper
5.5.26.3. PVDF membrane wet in transfer buffer
5.5.26.4. Gel wet with transfer buffer
5.5.26.5. Filter paper
5.5.26.6. Sponge

5.5.27. Run the transfer at 115V for 60 minutes.

5.5.28. Block the membranes in Tropix blocking buffer.

5.5.29. Incubate the membrane in primary Ab 6H4 1:5000 (diluted in TBS-T) for 1 hour.

5.5.30. Wash in TBST (three times) 5 minutes each.

5.5.31. Incubate the membrane in secondary Ab Goat Anti-mouse 1:5000 (diluted in TBS-T) for 30 minutes.

5.5.32. Wash in TBST (three times) 7 minutes each.

5.5.33. Equilibrate the membrane in 1X Luminescence buffer for 5 minutes.

5.5.34. Incubate the membrane with ECL Western Blotting detection system (Enhanced Chemiluminescent, Amersham or Supersignal, Pierce) for 5 minutes.

5.5.35. Image the membrane using either the Versa Doc image analysis system (Bio-Rad) or an imager as determined by the head of the Pathology Section and/or by the Director of the Pathobiology Laboratory.

5.6. Control Parameters

5.6.1. Negative control - BSE and/or Scrapie negative bovine and/or ovine brain sample

5.6.2. Positive Control - For routine unknown sample:

5.6.2.1. Classical BSE positive bovine brain sample

5.6.2.2. Scrapie positive ovine brain sample

5.6.3. Positive Controls - For further characterization of samples found positive by western blot, IHC, or inconclusive by ELISA:

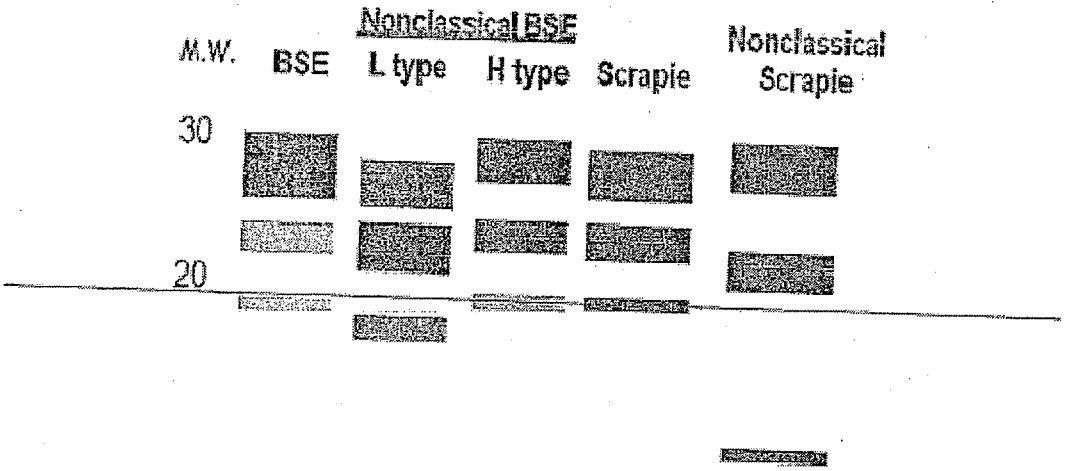
5.6.3.1. Classical BSE positive bovine brain sample
5.6.3.2. H-type BSE positive bovine brain sample
5.6.3.3. L-type BSE positive bovine brain sample when available
5.6.3.4. Scrapie positive ovine brain sample
5.6.3.5. Nor 98-like Scrapie positive ovine brain sample

5.7. Interpretation of Results

Negative control	Not treated with PK	Weak signal of undigested PrPC (33-35 kDa) or no signal
	Treated with PK	No signal
Positive control*	Not treated with PK	Very strong signal, often too strong to differentiate single PrP bands, highest signal at 33-35 kDa
	Treated with PK	Clear three-band signal ranging from 17-30 kDa

*A clear shift in molecular weight must be visible between the undigested and the digested fraction of the positive control and/or between another undigested PrPC control sample and the digested fraction of the positive control.

IF...	THEN diagnosis is...
there is a three-banded signal ranging from 19-30 kDa	positive for classical BSE
a low band measuring less than the low band of classical BSE	L-type
a low band measuring more than the low band of classical BSE	H-type
no detectable signal	not detected



5.8. Quality Control Parameters

5.8.1. Testing must be repeated if the results of the positive or negative control are incorrect or if the result of the diagnostic sample cannot be interpreted following the described methods.

5.8.2. Problem results may include:

5.8.2.1. Very faint signals- (repeat preparation with a higher amount of brain material).

5.8.2.2. Banding pattern does not match the positive control- (repeat procedure; use other diagnostic methods in addition).

5.9. Sample Retention/Disposal

5.9.1. Hold positive samples including the remaining homogenate and associated slides, blocks, formalin fixed and frozen tissues indefinitely in a secure safe or freezer with the BSL-3 laboratory of PL at NVSL.

5.9.2. Retain all routine not detected samples for five (5) working days at 2-8 °C before discarding unless otherwise directed by the section head.

6. Associated NVSL Quality Documents/References

- EXT-WI-PL-5140, TeSeE Western Blot
- SOP-PL-0006, Procedures for Entering and Exiting BSL-3 Laboratory Pathobiology Laboratory
- SOP-PL-0017, Disposal Procedure for BL3 Hazardous Waste and Reagent Waste
- SOP-PL-0022, Chain of Custody of ELISA BSE Samples Received from Contract Laboratories for Confirmation
- SOP-PL-0024, Tracking Procedure for Bovine Spongiform Encephalopathy Samples
- SOP-PL-0029, Confirming Inconclusive Results from Bovine Spongiform Encephalopathy Testing Laboratories at the NVSL.
- SOP-PL-0032, Protocol for BSE Contract Laboratories to Receive and Test Bovine Brain Samples and Report Results for BSE Surveillance
- SOP-PL-0036, Decontamination and Biological Accident Procedures
- SOP-PL-0048, Log-In and Cut-In Procedures for Scrapie Sample
- WI-PL-0016, Work Instructions for TSE
- The CDC publication Biosafety in Microbiological and Biomedical Laboratories, current edition, should be consulted for current recommendations concerning the handling of prion infected or potentially infected materials.

7. Revision History

Version .02

- Document number changed from SOP-PS-0004 to SOP-PL-0021
- Minor format changes
- Updated associated documents (section 6) throughout document
- Incorporated Scrapie Western Blot into this process
- Added tables

8. Appendices

Appendix 1: OIE Adapted Protocol - Amount of Reagent Added

Appendix 1: OIE Adapted Protocol - Amount of Reagent Added

Tissue	Original tissue 4 g		Original tissue 2 g		Original tissue 1 g	Original tissue 0.35 g
	No PK 2 g	PK treated 2 g	No PK 1 g	PK treated 1 g	All PK treated 1 g	All PK treated 0.35 g
Deionized water	3 ml	NA	1.5 ml	NA	1.5 ml	0.5 ml
Tris/HCL	50 µl	NA	25 µl	NA	25 µl	8 µl
15% KI-HSB	6 ml	NA	3 ml	NA	3 ml	1 ml
PK (1mg/ml)	NA	45 µl	NA	22.5 µl	22.5 µl	8 µl

附件 8

National Veterinary Services Laboratories	
Document Title: Quality Assurance: Proficiency Testing and Quality Monitoring of BSE ELISA Contract Laboratories by NVSL	
Author/Position: Dr. Mark Hall, VMO	Document Number: SOP–PS–0033.05
Page 1 of 3	Supersedes: GPPISOP0033.04

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1. **Purpose/Scope**
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   - 5.3 **Real Time Performance (Weekly) Monitoring**
6. **Associated NVSL Quality Documents / References**
7. **Revision History**
8. Appendices

Approved: /s/ Arthur Davis

1. Purpose/Scope

As a part of the United States Department of Agriculture's (USDA) ongoing bovine spongiform encephalopathy (BSE) surveillance, the Department's Animal and Plant Health Inspection Service (APHIS) is working to ensure both quality and accuracy of BSE test results reported by BSE contract laboratories. Therefore, APHIS has determined that a standard operating procedure (SOP) is needed to help insure quality of test results from these contract laboratories. This SOP is specific to the Bio-Rad ELISA test system, and additional or amended SOPs will be required for other test methods.

2. Definitions

- N/A

3. Safety Precautions

- N/A

4. Equipment and Materials Required

- N/A

5. Procedure

5.1. Evaluation Methods

APHIS will use two major methods to evaluate the quality of BSE ELISA testing at BSE contract laboratories.

1. Proficiency test (PT) - an initial proficiency test to evaluate contract laboratories administered by the Pathobiology Laboratory (PL) prior to their beginning real time BSE testing. Upon successful completion of the initial PT, subsequent PTs will be administered by the PL annually in a time not to exceed 24 months between tests. More frequent tests may be administered if deemed necessary by the Director of the PL.
2. Weekly trend monitoring of test performance and comparison of this performance to other labs in the network and to previous performance of the State lab will be conducted.

Each BSE contract laboratory will receive the results of the proficiency panel and updates from the ongoing evaluation of all laboratories testing history. Each laboratory will provide an electronic copy of test results to National Veterinary Services Laboratories (NVSL) - PL to assist the NVSL and the network laboratory in detecting performance change.

Weekly trend monitoring will be performed by the NVSL/PL until no longer deemed necessary by the Director of the NVSL PL, or designee. At such time the weekly trend

monitoring of the optical density (OD) data shall become the responsibility of each individual laboratory.

5.2. Proficiency Tests

Each laboratory will run a set of 5 unknown samples prepared and distributed by the NVSL PL at startup and thereafter as stated in #1 in 5.1 above. Positive tissues will be either Scrapie, chronic wasting disease, or other positive testing material that may be available in the future. The samples will be distributed as homogenates or as spiked tissues samples, when appropriate. Each laboratory will run the panel once on 2 different days, for a total of 2 runs from the same homogenate tubes. If laboratories have more than one person performing the BSE ELISA testing, the two runs should be performed by different personal. In the event that more than two people are performing the BSE ELISA testing, the PT testing personnel should be rotated with the next administered PT. Laboratories will be required to derive 5/5 correct answers on each run. Failure to do so will result in consultation, possible re-training, and, in rare cases, site visits and recorded audits. Laboratories that are initially starting up will not be allowed to perform real time testing until the proficiency panel is successfully completed.

5.3. Real Time Performance (Weekly) Monitoring

Each laboratory will save, and until advised otherwise, will provide the NVSL with an Excel spreadsheet output from all runs covering ELISA testing for the previous 7 days. The NVSL will collate the data. This data will allow both the NVSL and the partner labs to observe the ODs on control and surveillance samples, which can indicate technical proficiency and allow problems to be identified before they affect program integrity. It will also allow validation of numbers of samples run in the network. Once it is determined that the NVSL no longer needs to monitor this data, each individual laboratory will be required to monitor their own OD data and compare it to historical data. Any abnormalities should be promptly reported to the NVSL. In all cases, the testing laboratories shall maintain electronic copies of all OD data, and make that data available to the NVSL upon request.

6. Associated NVSL Quality Documents / References

- N/A

7. Revision History

- Version .05: Changed the number of samples to be used and described the testing intervals for administering PTs. Also added comment regarding rotation of personnel performing PTs for BSE ELISA.

8. Appendices

- N/A

附件 9 National Veterinary Service Laboratories	
Document Title: Confirming Inconclusive Results from Bovine Spongiform Encephalopathy Testing Laboratories at the NVSL	
Author/Position: Christie M. Loiacono, VMO	Document Number: SOP-PS-0034.06
Page 1 of 1	Supersedes: GPPISOP0034.05

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1. **Purpose/Scope**

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   - **5.1 Sample Receipt**
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   - **5.3 Testing Procedures Used for Confirmation**
   - **5.4 BSE ELISA Testing**
   - **5.5 BSE Immunohistochemistry Testing**
   - **5.6 IHC Interpretation by Pathologists and Reporting Results**
   - **5.7 BSE Western Blot**
   - **5.8 Interpretation and Summary of Confirmatory Testing Results**

6. **Associated NVSL Quality Documents/References**

   - SOP-PL-0032, Protocol for BSE Contract Laboratories to Receive and Test Bovine Brain Samples and Report Results for BSE Surveillance
   - SOP-PS-0002, BSE Histoprocedures
   - SOP-PS-0003, BSE ELISA
   - SOP-PS-0004, BSE Western Blot
   - SOP-PS-0005, Chain of Custody of ELISA BSE Samples Received from Contract Laboratories for Confirmation
   - SOP-PS-0007, Tracking Procedure for Bovine Spongiform Encephalopathy Samples
   - SOP-PS-0008, Immunohistochemical Interpretation and Terminology Used for Detection of Bovine Spongiform Encephalopathy (BSE) Diseases

7. **Revision History**

8. **Appendix**

Appendix 1: Flow Chart of Sample Testing Protocol

Approved: /s/ Mark Hall

1. Purpose/Scope:

The purpose of this Standard Operating Procedure (SOP) is to document the proper procedure for confirming inconclusive results from bovine spongiform encephalopathy (BSE) ELISA tests submitted to the National Veterinary Services Laboratories (NVSL) by the NVSL contract laboratories.

2. Definitions: NA

3. Safety Precautions: NA

4. Equipment and Materials Required: NA

5. Procedure:

5.1 Sample Receipt

All samples with inconclusive test results submitted to the NVSL from contract laboratories for BSE confirmation must be submitted under seal and accompanied by the appropriate chain of custody form as directed in the current version of NVSL SOP "Chain of Custody of ELISA BSE Samples Received from Contract Laboratories for Confirmation" (SOP-PS-0005) and in accordance with the current version of "Protocol for BSE Contract Laboratories to Receive and Test Bovine Brain Samples and Report Results for BSE Surveillance" (SOP-PL-0032). As outlined in the current version of SOP-PL-0032, the remaining homogenate including the homogenate from ALL three wells of the New Sample Pr(p)eparator (NSP) plate from the contract lab must be submitted to NVSL along with all other tissues. **Homogenates and test well sample remains must be submitted frozen. The obex must be submitted refrigerated.**

5.2 Sample Tracking

All submitted inconclusive samples shall be logged in and tracked through the Pathology Section (PS) in the Pathobiology Laboratory (PL) of NVSL in accordance with the current versions of NVSL SOP "Tracking Procedure for Bovine Spongiform Encephalopathy Samples" (SOP-PS-0007) and "Chain of Custody of ELISA BSE Samples Received from Contract Laboratories for Confirmation" (SOP-PS-0005). The steps of the tracking and testing procedures will require the presence of a second PL staff member for sample identification and verification. These steps are outlined in the following sections.

5.3 Testing Procedures Used for Confirmation

Official BSE confirmatory testing for samples submitted to NVSL will be done using IHC and Western blot procedures. BSE ELISA testing is repeated at the NVSL for purposes of quality evaluation.

5.4 BSE ELISA Testing

The submitted homogenates will be tested as documented in the current version of NVSL SOP "BSE ELISA" (SOP-PS-0003).

Two further samples will be taken from the submitted fresh tissue as close to the obex as possible and homogenized for further testing. At the discretion of the head of PS, the head of PL, or those acting for them, the homogenate may be used for BSE ELISA testing and/or Western Blot testing. The remaining fresh obex tissue will be preserved in formalin for immunohistochemistry (IHC) procedures. If these fresh tissue homogenates are tested by BSE ELISA, they should be placed in adjacent wells using the procedure outlined in the current version of NVSL SOP "BSE ELISA" (SOP-PS-0003).

5.5 BSE Immunohistochemistry Testing

Bovine spongiform encephalopathy IHC testing on submitted ELISA inconclusive samples will be performed as outlined in the current versions of NVSL SOPs "Tracking Procedure for Bovine Spongiform Encephalopathy Samples" (SOP-PS-0007) and "BSE Histoprocedures" (SOP-PS-0002). As stated in SOP-PS-0002, "There will be a minimum of two individuals present during all procedures associated with "Suspect" or "Inconclusive" BSE cases.

5.6 IHC Interpretation by Pathologists and Reporting Results

A PS pathologist will read and interpret stained IHC slides utilizing the criteria outlined in "Immunohistochemical Interpretation and Terminology Used for Detection of Bovine Spongiform Encephalopathy (BSE) Diseases" (SOP-PS-0008). All IHC slides will be independently reviewed by at least one other pathologist. Following review, the pathologist's results will be verbally reported to the Head of the PS Section of PL and/or the Director of PL (or their acting), who will in turn verbally report results to the Director of NVSL (or acting Director). Results will be entered in the BSE Reporting Tool by a PL employee. A final written report will be issued as appropriate to the BSE Network Laboratory initiating the inconclusives as well as other appropriate recipients.

5.7 BSE Western Blot

The official Western Blot procedure for USDA confirmation of BSE will be completed in the Pathobiology Laboratory of the NVSL in Ames, Iowa according to the NVSL SOP "BSE Western Blot" (SOP-PS-0004). At the discretion of the head of PS, the PL Director, or those acting for them, the homogenate produced from fresh brain tissue submitted by the NVSL contract laboratory and/or the homogenates submitted by the NVSL contract laboratory may be used for confirmatory western blot testing. Also at the discretion of the head of PS, the PL Director, or those acting for them, Western blots will be completed according to the methodology described in the current edition of the World Organization for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for

Terrestrial Animals or according to manufacturer's instruction included with the Bio-Rad TeSeE Western Blot Confirmatory Assay as described in the NVSL SOP "BSE Western Blot" (SOP-PS-0004).

5.8 Interpretation and Summary of Confirmatory Testing Results

Interpretation of confirmatory test results is summarized in **Appendix 1.**

If either or both the IHC and Western blot are positive the case will be considered officially as positive. If both IHC and Western blot are negative (PrP not detected), the case will be considered officially as negative.

6. References/Associated Quality Documents

- SOP-PL-0032, Protocol for BSE Contract Laboratories to Receive and Test Bovine Brain Samples and Report Results for BSE Surveillance
- SOP-PS-0002, BSE Histoprocedures
- SOP-PS-0003, BSE ELISA
- SOP-PS-0004, BSE Western Blot
- SOP-PS-0005, Chain of Custody of ELISA BSE Samples Received from Contract Laboratories for Confirmation
- SOP-PS-0007, Tracking Procedure for Bovine Spongiform Encephalopathy Samples
- SOP-PS-0008, Immunohistochemical Interpretation and Terminology Used for Detection of Bovine Spongiform Encephalopathy (BSE) Diseases

7. Revision History:

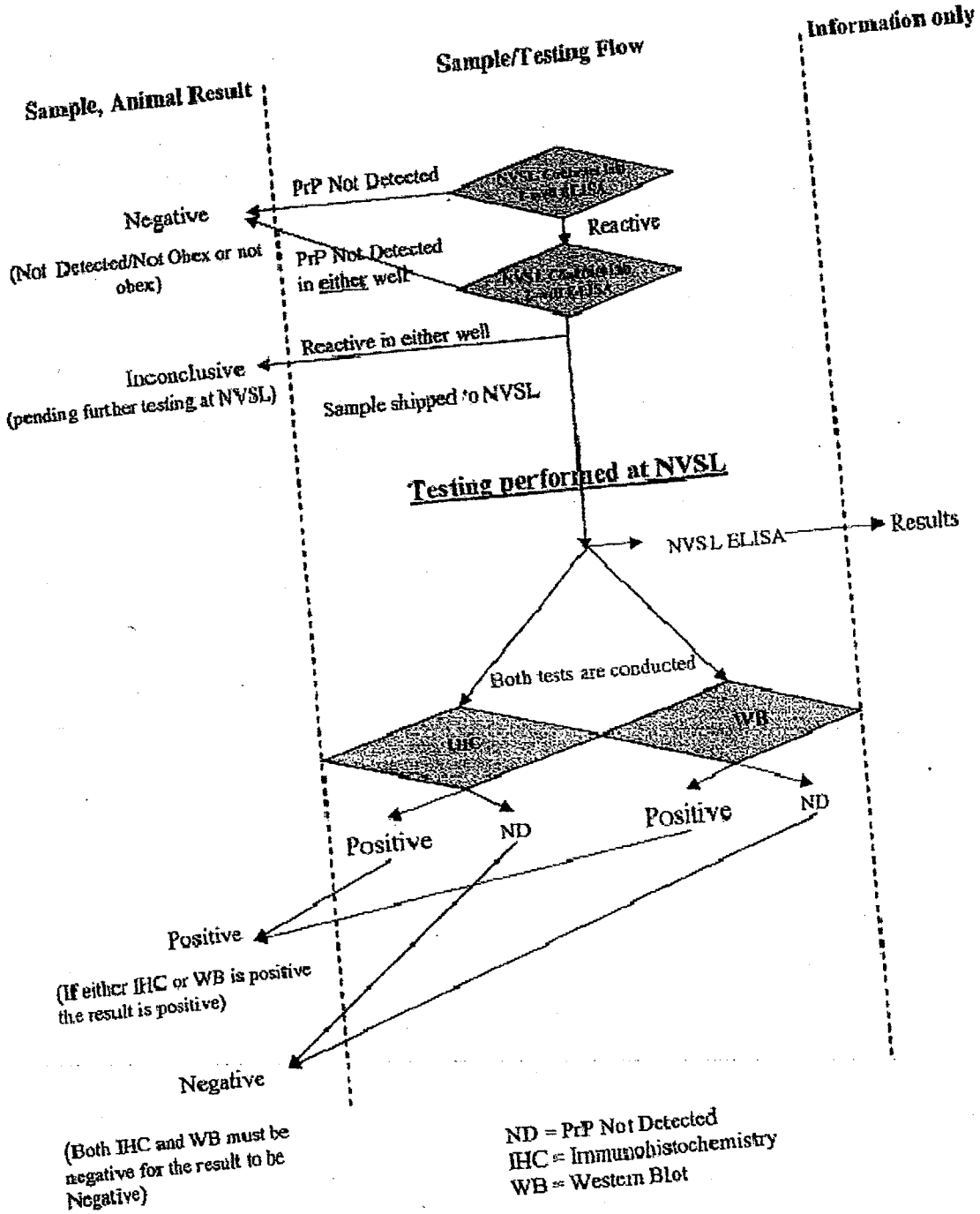
- Version .06
  - Page 2-
    - Updated SOP titles and document numbers for Chain of Custody BSE Tracking, and BSE ELISA
    - Removed reference to ELISA tracking SOP
  - Page 3
    - Updated SOP titles and document numbers for BSE ELISA, BSE Tracking, and BSE histoprocedures
    - Removed the four procedural modifications
  - Page 4-
    - Removed the final two procedural modifications continuing from page 3
    - Updated the Western Blot information and added SOP title and document number
    - Updated reference SOP titles and document numbers

Appendix 1- Updated the flow chart of sample testing protocol

8. Appendix:

Appendix 1: Flow Chart of Sample Testing Protocol

Appendix 1: Flow Chart of Sample Testing Protocol



附件 10

National Veterinary Services Laboratories	
Document Title: Bovine Microsatellite / Identity Testing	
Author/Position: James Higgins, Team Leader, Molecular Biology Team	Document Number: SOP-MB-9314.02
Page 1 of 9	Supersedes: SOP-MB-9314.01

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   5.1 Test Standards and Controls
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   5.3 Preparation of the Stockmarks PCR Reaction Mix
   5.4 Thermal Cycling Conditions
   5.5 Preparation of PCR Products for Electrophoresis on the ABI 3500xL Instrument
   5.6 Electrophoresis on the ABI 3500xL Instrument
   5.7 Interpreting the Results
   5.8 Data Recording and Management

6. **Associated NVSL Quality Documents/References**

   - FM-MB-6664, Record of Transfer of Samples from PL and DBL-MB for Testing
   - WIA-MB-5173, ABI 3500xl Genetic Analyzer General Operation and Plate Setup
   - WI-PL-5249, DNA Samples Transfer Between PL-TB PCR and DBL-MB
   - SOP-MB-2219, Bead-Beater™ DNA Extraction Method for Mycobacterial DNA
   - SOP-MB-9317, DNA Extractions Using the Qiagen DNeasy Kit
   - WI-MB-2219, Bead-Beater™ DNA Extraction Method for Mycobacterial DNA
   - SOP-PL-0047, Detection of Mycobacterial Nucleic Acid in Formalin-Fixed Paraffin-Embedded Tissues (FFPE) by Polymerase Chain Reaction

7. **Revision History**
8. **Appendices**

Approved: /s/ Suelee Robbe-Austerman

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1. Purpose/Scope

The purpose of this Standard Operating Procedure (SOP) is to provide a procedure for bovine microsatellite / identity testing using the Stockmarks® reagent kit marketed by Applied Biosystems (ABI). The Stockmarks® assay can provide quality control / quality assurance for processing and analysis of bovine tissues received at the NVSL.

For example, when suspected bovine tuberculosis samples are received at the NVSL, they are split into two lots, one of which is forwarded to the MB Section, and the other to the Pathobiology Laboratory, for bacterial culture and histology / PCR, respectively. The Stockmarks® assay provides confirmation that samples processed by both departments originated from the same animal, and that mixture of the bovine tissues with those of unrelated animal(s), leading to false-negative or false-positive results, has not occurred.

The Stockmarks® assay provides this confirmation because it generates a DNA 'fingerprint' unique to an animal. Multiple tissue samples from the same animal will generate the same fingerprint. Should a particular tissue sample generate a fingerprint different from those present in other tissues from the same animal, it would constitute evidence that mislabeling or inadvertent switching of that tissue has taken place.

The Stockmarks® kit analyzes bovine DNA for the presence of microsatellites existing in each of 11 loci. For some loci, depending on the genetic makeup of the animal being tested, two PCR products, rather than one, may be generated for a given locus.

The Stockmarks® kit includes the PCR primers necessary to amplify all 11 loci, as well as a positive control.

2. Definitions

- Accession Number – a specific number assigned by Labware of the NVSL for tracking purposes of submitted cases.
- Microsatellite / identity testing – detection of selected repetitive DNA sequences, referred to as short tandem repeats (STR), or 'microsatellites', within the genome of a animal. The resultant STR profile can be used to establish a genetic 'fingerprint' unique to an individual animal.
- Locus / loci – region of the genome that contains STRs or microsatellites. The Stockmarks® kit assays 11 loci within the bovine genome.

3. Safety Precautions

- The bovine identity testing assay is a BSL-2 level procedure and does not involve handling any pathogenic microorganisms. Standard safety procedures for conducting work in a molecular biology laboratory are to be observed.

4. Equipment and Materials Required

- Equipment
  - Applied Biosystems 3500xL Genetic Analyzer
  - Thermocycler (Applied Biosystems, GeneAmp PCR System 9700 or equivalent)

- Allegra 25R Centrifuge with S5700 rotor and plate buckets (Beckman Coulter or equivalent)
- Pipettes x 3 for each work area
  - Rainin LTS 2 or equivalent
  - Rainin LTS 10 or equivalent
  - Rainin LTS 100 or equivalent
  - Rainin LTS 1000 or equivalent
- Multichannel pipettes
  - Pipet-lite LTS 8-channel pipet 0.5-10 µl, or equivalent
  - Pipet-lite LTS12-channel pipet 20-300 µl, or equivalent
- EDP3-Basic 10-200 µl multi-dispensing pipettes, or equivalent
- PCR hood for preparing PCRs (Misonix PCR prep system or equivalent)
- Titer plate shaker (Lab Line or equivalent)
- Microcentrifuge (Sorvall Biofuge, Fresco max speed 16,000 × g or equivalent)
- Vortex
- ABI septa for 3500xl Genetic Analyzer
- Cooling blocks for 96 well plates
- ABI GeneMapper™ fragment size analysis software (GeneMapper v4.1 or equivalent)

- Materials/Reagents/Cultures
  - CAPMat 96 round well PCR plate (Dot Scientific, Inc. #CM-651-5 or equivalent)
  - Pipette tips
    - Rainin RT-L10F or equivalent
    - Rainin RT-L200F or equivalent
    - Rainin RT-L1000F or equivalent
    - Rainin RT-L300F or equivalent
  - 1.5 ml sterile microcentrifuge tubes (Fisher brand premium, 05-408-129 or equivalent)
  - 1.5 ml microcentrifuge tube racks
  - 96-well Half Skirted 0.2 ml PCR plate (DOT scientific #951-PCR or equivalent)
  - Stockmarks®® for Cattle Bovine Genotyping Kit (ABI part number 4307480)
  - Aluminum film for plate covering (AlumaSeal II film DOT scientific #T488 or equivalent)
  - GeneScan 600 LIZ Size Standard v2.0 (ABI part number 4408399)
  - POP-7™ Polymer for 3500/3500xL Genetic Analyzers, 384 samples,
  - (ABI Cat. No. 4393708 or equivalent)
  - Anode Buffer Container (ABC) 3500 Series, (ABI Cat. No. 4393927 or
  - equivalent)
  - Cathode Buffer Container (CBC) 3500 Series, (ABI Cat. No. 4408256 or
  - equivalent)
  - Hi-Di Formamide (ABI 4311320 or equivalent)
  - Water, molecular biology grade (Sigma W4502, or equivalent) or MilliQ water

5. Procedure

5.1 Test Standards and Controls

- Run the positive control with every assay that is provided in the Stockmarks bovine microsatellite kit.
- Run a negative control consisting of a reagent tube containing water in lieu of bovine DNA with every assay.
- Store the Stockmarks kit / positive control at -15 °C to -25 °C.

5.2 Samples for Testing

- Perform the Stockmarks bovine microsatellite kit on DNA extracted from bovine tissues, hair, blood, or exudate associated with the internal (in contact with the animal) portion of the ear tag. Any approved DNA extraction method can be used, including but not limited to:
  - SOP-MB-2219, Bead-Beater™ DNA Extraction Method for Mycobacterial DNA
  - SOP-MB-9317, DNA Extractions Using the Qiagen DNeasy Kit
  - WI-MB-2219, Bead-Beater™ DNA Extraction Method for Mycobacterial DNA
  - SOP-PL-0047, Detection of Mycobacterial Nucleic Acid in Formalin-Fixed Paraffin-Embedded Tissues (FFPE) by Polymerase Chain Reaction

5.2.1. Samples retrieved from the Pathobiology Laboratory are accompanied by FM-PL-6425, Record of Transfer of Samples from PL and DBL-MB for Molecular Testing. This form is filled out by the individual retrieving the samples, and is kept in the file cabinet in the office of the MB Section Molecular Team Leader. Retrieved samples are stored in a -65 ± 2 ° C freezer at the MB Section.

5.2.2. Use 10 – 25 ng in a 2 µl volume for DNA extracted from sodium borate – stored tissue as the quantity of DNA used as template in the Stockmarks assay. For FFPE extract, use 2 µl as template for PCR (note that the FFPE-derived DNA is a comparatively crude extract, and is not as readily amenable to quantitative or qualitative analysis as DNA extracted using the Bead-Beater technology).

5.2.3. Use 10 – 25 ng as template for ear tag – associated tissues and hair. For best results, DNA with an A_{260}/A_{280} ratio of 1.8 – 2.0 should be used; however, DNA with absorbance values outside these limits is also capable of generating successful Stockmarks data. Quantitative or qualitative analysis of DNA can be performed using the NanoDrop™ and QuBit™ instruments.

5.2.4 FFPE (or 'block') – derived tissue should originate from the same sample subjected to PCR at the Pathobiology Laboratory. Because of the comparatively crude nature of the FFPE-derived DNA, it may not provide satisfactory Stockmarks results; if this is observed, re-extract the FFPE using the method described in SOP-MB-9317, DNA Extractions Using the Qiagen DNeasy Kit.

5.2.4 Store extracted DNA at 4 ± 2 ° C for 7 - 10 days if it is anticipated that it will be used in PCR during that interval; if a longer interval prior to use in PCR is anticipated, then store the DNA at – 20 ± 2 ° C. For long term storage (years), storage at – 65 ± 2 ° C is recommended.

5.3 Preparation of the Stockmarks PCR Reaction Mix

5.3.1 Follow the instructions below excerpted from the ABI Stockmarks® manual for the appropriate amount of samples.

To prepare the eleven-plex reaction:

1. Combine the following reagents in a 0.2- or 0.5-mL tube to create a master mix for the eleven-plex reactions:

Component	Volume (µL) for One Sample	Volume (µL) for Ten Samples[a]
StockMarks PCR Buffer	3.0	33.0
dNTP mix	4.0	44.0
AmpliTaq Gold DNA Polymerase	0.5	5.5
Amplification primer mix	5.5	60.5
Deionized water	1.0	11.0
Total volume	14.0	154.0

a. The total volume per reagent equals the amount for one sample multiplied by the number of samples, plus one. The additional volume accounts for the loss that occurs during reagent transfers.

2. Vortex the master mix.
3. For each animal to be tested, transfer 14 µL of master mix to a separate thin-walled PCR tube.
4. Add 1.0 µL of template DNA at 10 ng/µL to 100 ng/µL to each tube. Cap the tubes.

   Note: DNA concentration may vary. Adjust volumes of template DNA and deionized water accordingly. The final volume of the PCR reaction is 15 µL.
5. Vortex the tubes briefly.
6. Spin the tubes briefly in a microcentrifuge to ensure that all of their contents are collected in the bottoms of the tubes.

5.4 Thermal Cycling Conditions

5.4.1 Follow the instructions below excerpted from the ABI Stockmarks® manual.

Thermal Cycler	Times and Temperatures						
	Initial Step[a]	Each of 31 Cycles			Final Extension	Final Step	Hold
		Melt	Anneal	Extend			
GeneAmp® PCR System 9700[b]	1 cycle 95 °C 10 min	94 °C 45 sec	50% ramp 61 °C 45 sec	80% ramp 72 °C 60 sec	1 cycle 72 °C 60 min	1 cycle 25 °C 2 hr	4 °C HOLD
GeneAmp® PCR System 9600	1 cycle 95 °C 10 min	94 °C 45 sec	30 sec ramp 61 °C 45 sec	30 sec ramp 72 °C 60 sec	1 cycle 72 °C 60 min	1 cycle 25 °C 2 hr	4 °C HOLD

a. The initial 10-min, 95 °C heating step is required to activate the AmpliTaq Gold DNA Polymerase.
b. Program the 9700 System in 9600 Emulation Mode.

5.5 Preparation of PCR Products for Electrophoresis on the ABI 3500xL Instrument

5.5.1 Dilute each PCR product with 150 µl deionized, molecular biology grade water.

5.5.2 Combine 1.0 µl diluted PCR product, 8.5 µl Hi-Di™ formamide, and 0.5 µl LIZ® 600 Size Standard.

5.5.3 Briefly spin the mixtures to collect all the liquid at the bottom of the tube. Heat at 95 °C for approximately 2 minutes and then immediately place on ice for a minimum of 3-5 minutes.

5.6 Electrophoresis on the ABI 3500xL Instrument

- Refer to 'Applied Biosystems 3500/3500xL Genetic Analyzer User Guide' for an overview, program description, operating the system, and routine maintenance.
- Refer to work instruction 'WI-MB-5173, ABI 3500xL Genetic Analyzer General Operation and Plate Setup' for operation and setup.
- Refer to the manuals 'Applied Biosystems GeneMapper® 4.1 Microsatellite analysis: getting started guide' and the 'Applied Biosystems GeneMapper® 4.1: quick reference guide' for information on the use of the GeneMapper software to identify and size Stockmarks® PCR products / fragments electrophoresed on the ABI 3500xL instrument.

5.7 Interpreting the Results

5.7.1 The expected size ranges for the fragments (i.e., PCR products) generated for the 11 loci are provided in the table below (excerpted from the ABI Stockmarks® manual). Fragments (i.e., PCR products) lying outside these values, although rare, may be considered legitimate if the (y-axis) Relative Fluorescence Units for that peak / fragment are equivalent to those of the kit positive control peaks.

Locus	Dye	Color	Expected Size Range (bp)[a]
TGLA227	FAM™	Blue	64–115
BM2113	FAM	Blue	116–146
TGLA53	FAM	Blue	147–197
ETH10	FAM	Blue	198–234
SPS115	FAM	Blue	235–265
TGLA126	JOE™	Green	104–131
TGLA122	JOE	Green	134–193
INRA23	JOE	Green	193–235
ETH3	NED	Yellow	90–135
ETH225	NED	Yellow	136–165
BM1824	NED	Yellow	170–218

5.7.2 The Stockmarks® kit positive control should generate the following results (with an anticipated variation of ± 1 bp for each fragment), while no fragments should be observed in the negative control sample.

Marker	Peak 1	Peak 2
BM1824	176.5	180.73
BM2113	122.3	129.93
ETH10	211.13	217.0
ETH225	134.9	
ETH3	116.09	134.9
INRA23	210.89	
SPS115	242.29	259.77
TGLA122	138.42	140.62
TGLA126	118.05	120.12
TGLA227	83.1	94.02
TGLA53	155.78	159.88

- If the kit positive control does not generate an amplicon for each of the 11 markers (loci), then the results are invalid and the assay needs to be repeated. If there are PCR products

present in the negative ('no template' control), then the results are invalid and the assay needs to be repeated.

- Each sample should register at least one fragment / PCR product for each of its 11 markers (loci). However, depending on the quantity and quality of the DNA being analyzed, complete Stockmarks® profiles for all 11 loci may not be generated. In these cases, the graph below (excerpted from Rodriguez-Ramirez et al., Genetics and Molecular Research, 2011, 10: 2358 - 2365) is helpful in delineating the probability of two samples possessing matching Stockmarks results as a function of the number of loci analyzed.

- As is indicated in the graph, the chance of two animals sharing the same microsatellite markers for 7 loci is approximately 1×10^{-7}, or 1 in 10,000,000, while the chance of two animals sharing the same microsatellite markers for 3 loci is approximately 1×10^{-2}, or 1 in 100. Rodriguez et al. (2011) recommend that acceptable Stockmarks data be generated for a minimum of 7 or more loci for a given animal, in order to make valid conclusions about identity testing. Failure to obtain successful Stockmarks calls for at least 7 loci requires the assay to be repeated.

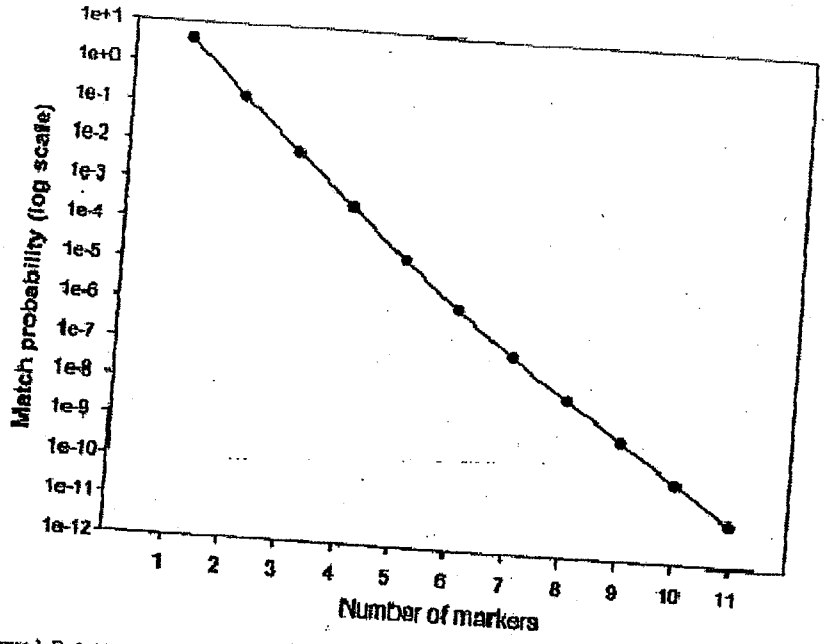


Figure 1. Probability of two samples matching by chance as a function of the number of microsatellite markers evaluated.

5.8 Data Recording and Management

5.8.1 GeneMapper data from each Stockmarks assay are retained on the ABI-associated server maintained by the NVSL IT staff (\\Iaamvsabiserver\mycobacteria\StockMarks-Bovine).

5.8.2 Data are reported in the format of a 'Stockmarks Genotyping Report' Word / pdf file, for distribution to VS staff and affiliates. The report is compiled by the

MB Molecular Team Leader; hard copies of each report are printed and placed in the binder labeled 'Bovine Identity Testing' in the Team Leader's office.

5.8.3 Hard copies of completed FM-PL-6425, Record of Transfer of DNA Samples from PL-TB PCR and DBL-MB for Microsatellite Testing, are maintained in the Team Leader's office.

6. Associated NVSL Quality Documents/References

- Applied Biosystems, 'Stockmarks® Horse, Cattle, and Dog Genotyping Kits: Protocol' http://www3.appliedbiosystems.com/cms/groups/applied_markets_support/documents/generaldocuments/cms_041413.pdf
- Applied Biosystems 3500/3500xL Genetic Analyzer User Guide
- Applied Biosystems GeneMapper® 4.1 Microsatellite analysis: getting started guide
- Applied Biosystems GeneMapper® 4.1 Microsatellite analysis: quick reference guide
- Rodriguez-Ramirez, R., et al. 2011. Molecular traceability of beef from synthetic Mexican bovine breeds. Genetics and Molecular Research 10: 2358 - 2365
- Vasquez, J. F., et al. 2004. Practical application of DNA fingerprinting to trace beef. J. Food Protection 67: 972 - 979
- FM-MB-6664, Record of Transfer of Samples from PL and DBL-MB for Testing
- WIA-MB-5173, ABI 3500xl Genetic Analyzer General Operation and Plate Setup
- WI-PL-5249, DNA Samples Transfer Between PL-TB PCR and DBL-MB
- SOP-MB-2219, Bead-Beater™ DNA Extraction Method for Mycobacterial DNA
- SOP-MB-9317, DNA Extractions Using the Qiagen DNeasy Kit
- WI-MB-2219, Bead-Beater™ DNA Extraction Method for Mycobacterial DNA
- SOP-PL-0047, Detection of Mycobacterial Nucleic Acid in Formalin-Fixed Paraffin-Embedded Tissues (FFPE) by Polymerase Chain Reaction

7. Revision History

- Version .02, clerical changes throughout.

8. Appendices

- Not Applicable (N/A)

附件 11

METHOD FOR THE PROVISIONAL CLASSIFICATION OF BOVINE TSE ISOLATES

The examination, by histopathology, immunohistochemistry, Western blotting and bioassay, of bovine isolates from individuals with clinical signs throughout the BSE epidemic has supported the hypothesis that the epidemic has been sustained by a single type, or strain, of BSE. However the development of sensitive PrP^{res} immuno-detection diagnostic techniques, and their application through active surveillance in non-suspect populations, has lead to the detection of a small number of geographically widespread sporadic cases of deviant types, predominantly in older animals. These isolates have now been confirmed in mice as distinct strains, and have been operationally defined as H- (high) or L- (low) type based on the molecular mass of the unglycosylated fragment of PK resistant PrP in Western blot.

L-type behaves in Western blots like the cases initially identified in Italy (initially described as BASE; bovine amyloidotic spongiform encephalopathy). For the time being L-type and BASE are considered to be the same. H-type and L-type cases have up till now not been detected in clinically suspect animals, but all these cases occurred in animals aging 8 years and older.

The following blotting protocol has been prepared on behalf of the EU Community Reference Laboratory for TSE Strain Typing Expert Group by Prof. Jan Langeveld, Lelystad, based on the 2007 publication by Jacobs et al.

Please note: This method cannot be included into the range of tests for which External Quality Assurance is provided by the CRL due to lack of adequate representative H- or L-type positive control material. The method must therefore be considered to be 'out of the scope' for accreditation purposes.

In order to have confidence in the results of such a test, it is vital that the appropriate controls should be run on the same gel as the suspect sample. Appropriate controls would include samples previously confirmed as C, H and L type BSE, either in the laboratory of origin, or through referral of the sample to a laboratory with the correct control materials available.

If you suspect that you have an unusual sample that requires further characterization, it is recommended that you contact the CRL (tseeucrl@vla.defra.gsi.gov.uk) to discuss the options available to you.

A 2-blot protocol for PrP^{res} typing in BSE from cattle.

Introduction

The initial classification of C-type, H-type and L-type BSE using different parameters (as summarized in table I) has been undertaken by the European funded NeuroPrion network of excellence. The following parameters are used:

1. molecular migration of PrP^{res} bands
2. differential binding to PrP-specific antibodies (to this purpose antibody groups A, B and C have been defined, see Table II)
3. PrP^{res} glycoprofiles
4. susceptibility to proteinase K (PK)
5. number of non-glycosylated PrP^{res} bands

Table I: Discrimination between BSE-types based on molecular properties of PrP^{res} (from: Jacobs et al., 2007)

BSE type	size difference[a] in kDa	binding to 12B2	glycoprofile[b] di-glyc (%)	deglycosylation with PNGase F[c] (163-242 epitopes)	proteolytic susceptibility pH 8/pH 6.5[d]
C	ref	no	>50	1 band	> 0.7
H	+1.4	yes	dual character[e]	2 bands	< 0.6
L	-0.3	no	<50	1 band	< 0.6

[a] Approximate difference value with C-type for the nonglycosylated band of the PrP^{res} population in 17-19 kDa region; tested with group B antibodies 9A2, L42 or 6H4.
[b] Percentages of diglycosylated fraction should be compared together with C-type.
[c] Two bands can only be observed with group C antibodies like 94B4 and SAF84 that bind to the C-terminal domain 163-242 of bovine PrP.
[d] Ratio calculation, see Jacobs et al., 2007.
[e] Depending on the use of antibodies of groups A & B or of group C (see Table II).

Table II: grouping of antibodies for recognition of bovine BSE-types updated from Biacabe et al., 2007

antibody group	antibody	region of binding in boPrP
group A	SAF32; 4F2; 12B2**; P4	62-107
group B	9A2; RB1; 12F10[a]; 6C2; F89/160.1.5; Bar233; L42**; Sha31**; 6H4	108-157
group C	SAF70; SAF60; SAF84**; 94B4**, F99/97.6; R524	157-242

** Antibodies marked with asterisks are the preferred antibodies for protocols A-D because of high affinity.

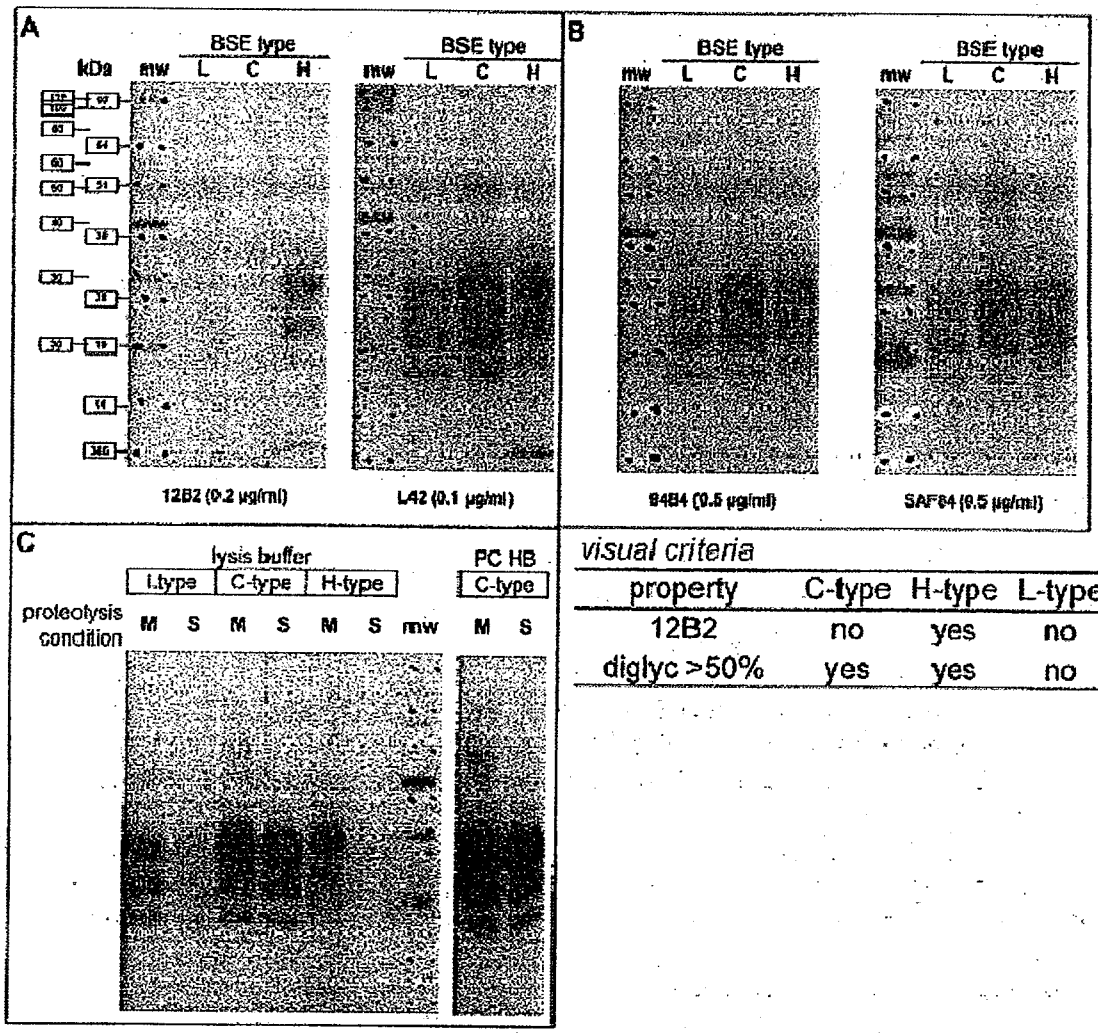
For standard discrimination of these three types only 2 parameters are required, and can be performed by visual inspection using antibodies of sufficiently high affinity to detect PrP^{res} (Fig. 1A):

1. Binding to PrP^{res} *N*-terminus specific antibody 12B2 (a group A antibody) compared to L42 (a group B antibody). Migration position of PrP^{res} bands of H-type is higher up than that of C- and L-type, due to the *N*-terminal epitope of 12B2 which is retained during digestion with PK in substantial amounts only in H-type (Fig. 1A).
2. Glycoprofile differences between L-type on the one hand, and C- and H-type on the other hand using L42 (group B antibody) (Fig. 1A).
3. This double blot test of Fig. 1A yields sufficient criteria for discrimination of three types (see table "visual criteria").

Other differences can be detected:

4. Using a group C antibody like 94B4 or SAF84, the PrP^{res} glycoprofile of H-type cases is basically different from the profile obtained with a group A or B antibody (compare H-type lanes between Fig. 1B and 1A).
5. The susceptibility of C-type for proteinase K is nearly the same between mild or stringent digestion condition, while on the contrary PrP^{res} of L-type and H-type hardly survives the stringent condition (Fig. 1C). This is the case for homogenates prepared in different homogenisation buffers like lysis buffer and a homogenisation buffer from a commercial test kit for bovine BSE detection (Prionics®-Check Western) (see lanes "PC HB", Fig. 1C).

Figure 1



visual criteria

property	C-type	H-type	L-type
12B2	no	yes	no
diglyc >50%	yes	yes	no

Fig. 1: Features of BSE types. A, H-type reacts in nearly equal intensity to antibody 12B2 and L42, while C-type largely and L-type completely are devoid of the 12B2 epitope due to *N*-terminal cleavage of amino acids 101-104 by PK under standard digestiong conditions (pH7.2, PK 50 µg/ml, 37°C, 60 min, in lysis buffer). Also, a 7 kDa band is present (L42 panel) which is most prominent in H-type. The glycoprofile of the L-type is clearly different from C- and H-type, because in L-type diglycosyl fraction is about 50% or less of the total PrP^{res} signal, while in C-type and H-type it is the diglycosylated band represents the predominant fraction. B, The glycoprofile of H-type is dependent of the antibody used. With group C antibodies it exhibits a similar intensity of bands running at a position of di- and mono-glycosylated PrP^{res} while on the contrary with group A or B antibodies the diglycosyl band represents the most intense band (cfr. Fig. 1B with Fig. 1A). C, Further evidence for difference of H-type and L-type from C-type comes from using two digestion conditions. Antibody used: group B antibody L42 at 0.1 µg/ml. Only C-type resists in large a stringent PK digestion (S) compared to mild digestion (M). Applied tissue equivalents, 0.5 mg per lane. After digestion samples were first precipitated with 1-propanol. Lane mw, molecular weight markers Magic Mark™ XP and SeeBlue® Plus2 (Invitrogen). Geltype: 12% Bis/Tris NuPAGE using MOPS running buffer (Invitrogen). Developed with CDP*, film exposure 1 min. S = stringent digestion at pH8, ~1 µg PK/100µg wet tissue; M = mild digestion at pH6.5, ~0.1 µg PK/100 µg wet tissue.

Protocols:
These protocols are based on the report by Jacobs et al., 2007.

Solutions/materials:

water	distilled or reversed osmosis grade water
PBS	8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.38 g KH_2PO_4, water to 1 L. pH=7.2 (10.8 mM Pi, 138 mM NaCl, 2.7 mM KCl)
lysis buffer	0.5% (w/v) TritonX-100, 0.5% (w/v) sodium-deoxycholate, in PBS
1M $CaCl_2$	1.47 g $CaCl_2.2H_2O$, dissolve in water; final volume 10 ml
4M HCl	add 5 ml $HCl_{concentrated}$ to 10 ml of water; final volume 15 ml
Tris/Ca	0.61g Tris, dissolve in 100ml water, bring at pH8.0 with ~1ml 4M HCl, add 100µl 1M $CaCl_2$ → 1mM Ca in 50mM Tris pH8.0
Adjustment buffers:	
pH6.5 buffer	200mM PO_4 pH6.5 prepared as follows A, dissolve 13.8g $NaH_2PO_4.1H_2O$ in 500ml water; B, dissolve 14.2g Na_2HPO_4 in 500ml water; titrate 3 volumes of A with ~1-2 volumes of B till pH6.5.
pH8 buffer	200mM Tris+HCl pH8.0 prepared as follows. Dissolve 12.1g Tris in ~450ml water. Titrate to pH8.0 with ~11.5ml of 4M HCl. Make up final volume to 500ml with water.
PK	proteinase K (30 mAnson-U/mg lyophilisate, Merck 1.24568)
500 µl vial	500 µl Eppendorf safelock vial
thermostat	incubator with heating blocks with tight fitting for 500 µl vials
centrifuge	table top microtube centrifuge such as Heraeus Biofuge Pico
PK-stock	11mg PK in 1ml Tris/Ca (aliquot 50µl portions and store at -20°C; retains activity for at least 12 mo)
PK-fresh	on day of use prepare by diluting 50 µl PK-stock with 950 µl PBS → 0.55 mg PK/ml
PK-fresh conc	dilute PK-stock with equal volume of PBS → 5.5 mg PK/ml
Pefa-stock	Pefabloc SC (AEBSF) hydrochloride [4-(2-aminoethyl)-benzene sulfonylfluoride hydrochloride] (Roche - 11585916), 30 mg in 1 ml water. Immediately after dissolving aliquot 100 µl portions and store at -20°C (retains activity for 2 mo)
Pefa-fresh	Pefa-stock diluted 10x with water, prepared directly before application
1-propanol	HPLC quality, from Sigma Chromasolv 34871
LB	loading buffer for SDS-PAGE, containing Na- or Li- dodecylsulfate, glycerol, BPB, reducing agent, and buffer. All at a 2x concentration; to be used to mix with equal volume of test sample
LB1/1	mix LB with water 1/1; to be used for empty lanes or for dissolving precipitated PrP^{res} pellets.
TBS	8g NaCl, 0.2g KCl, 3g Tris, dissolve in ~900ml water, bring to pH7.4 with ~2ml $HCl_{concentrated}$; final volume 1000ml with water
TBST	0.5ml Tween-20 per 1000ml TBS

Blocking buffer	3% skimmed milk powder in TBST (like Blotto, Santa Cruz, cat.# sc-2324).
1st Antibodies	Diluted in TBST. group B: L42, use at 0.1 µg/ml (RIDA R8005 from R-Biopharm, Darmstadt, Germany). group A: 12B2 at 0.2 µg/ml (from Central Veterinary Institute of Wageningen UR at Lelystad, The Netherlands). group C antibodies: SAF84 at 0.5 µg/ml (A03208 from SpiBio, France). 94B4 at 0.5 µg/ml (from Central Veterinary Institute of Wageningen UR at Lelystad, The Netherlands).
2nd antibody	Rabbit anti-mouse Ig conjugated to alkaline phosphatase (DAKO: D0314) Use at 1/5000 when developing with CDP* as chemoluminescent substrate.

Protocols A-D.

A: Protocol for preparing homogenates from brain tissue.

1. Weigh ~0.5 g brain tissue.
2. Add 5 ml of lysis buffer (or homogenisation buffer from Prionics®-Check Western BSE kit).
3. Homogenise with homogeniser of your choice (as an example of the procedure at Lelystad - the Consul FASTH instrument and disposable Prypcon homogenisation vials; 45 sec at 20000 rpm at RT). Let settle down for 15 min (if there are still visibly particles present a 2nd homogenisation could be carried). Before storage in freezer (!): transfer homogenate into a 15 ml Falcon tube and remove coarse debris by spinning at 500xg for 5min@RT. Collect supernate and store at -20°C.

If desired, store also the pellet at -20°C e.g. for DNA preparation and genotyping.

B: Protocol for preparation of PrP^{res} and discrimination of bovine BSE-types by using PrP-site specific antibodies from group A and B.

This protocol consists of preparation of PrP^{res}, precipitation, and Western blotting using two PrP-site specific antibodies. The step removes cross-reactive components like PK and tissue matrix components. The procedure yields a near 100% recovery of PrP^{res}, and removes components that might alter migration. General: work in 500 µl vials; mixing is performed with microtip pipets, tilting or tipping the vials.

Preparation of PrP^{res}:

1. Digest 100 µl homogenate: add 10 µl of PK-fresh, mix, incubate for 60min@37°C. Stop: by adding 10 µl of Pefa-fresh.
2. Add an equal volume of 1-propanol to digest: thus e.g. 100-120 µl 1-propanol to 120 µl of digest. Vortex briefly.
3. Spin at 16000xg for 5min@RT.
4. Carefully remove (use pipet with 200 µl microtip) and discard the clear supernatant (~1µl pellet and ~4µl remaining supernatant left).

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5. Add 100 µl LB1/1. Carefully redissolve the pellet by scratching with the pipet-tip and pipetting up-and-down several times. After there are no solid pellet parts visible in the solution anymore, still continue pipetting up-and-down for at least 10 times.
6. Incubate sample for 5-10min@96□C.
7. Take the sample out of the thermostat incubator. Let vials cool down. Mix.
8. Centrifuge 5min@16000xg@RT to pellet undissolved debris
9. Without mixing take a 10µl of supernatant for a slot of a 15-wells or 17-wells SDS-PAGE gel (compares with 500 µg TE).

Discrimination of bovine BSE-types by using PrP-site specific antibodies from group A and B in a parallel blot:

10. Have PrP^{res} of C-type BSE sample as reference.
11. Perform parallel SDS-PAGE for two blots with the same sample organisation and TE loading. As standards use a mixture of Invitrogen markers: 0.5 µl Magic Mark XP, and 5 µl SeeBlue plus 2 per lane (see example Fig 1A).
12. After electrotransfer: mark on both blots with a pencil the positions of the visible SeeBlue markers.
13. Stain one blot with 12B2 (0.2 µg/ml) and the other with L42 (0.1 µg/ml). Use for both blots exact the same conditions of incubation times, secondary antibody-alkaline phosphatase conjugate and film exposure times.
14. Discriminative features (see table "visual criteria" in Fig. 1):
    a. L-type is characterized by: no binding by 12B2; and < 50% di-glycosylated fraction of PrP^{res} using L42.
    b. H-type is characterized by: a nearly as high staining intensity of PrP^{res} with L42 and 12B2; and >50% di-glycosylated fraction of PrP^{res} using L42.
    c. C-type is characterized by: a poor binding to 12B2 compared to L42; and >50% diglycosylated of PrP^{res} fraction using L42.
    d. Migration position of non-glycosylated PrP^{res} is also indicative for H-type which is higher in the blot than that in C-type and L-type; for L-type and C-type the migration difference is less obvious and therefore difficult. Thus, differentiation above in a-c using the two antibodies is decisive for bovine BSE typing.

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