TSE EURL
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TSE STRAIN CHARACTERISATION IN SMALL RUMINANTS

A TECHNICAL HANDBOOK FOR NATIONAL REFERENCE LABORATORIES IN THE EU

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Section 1

Introduction

INTRODUCTION

Purpose

- To support the statutory discriminatory testing of the different TSEs in small ruminants as laid down in Annex X to regulation (EC) No 999/20001.
- to document the properties which can be used to distinguish small ruminant TSE isolates requiring further investigation
- to provide sampling and testing strategies and protocols for discriminatory methods which have been approved by the EURL strain typing expert group
- to identify those isolates which have the characteristics of the BSE agent

Background

A TSE strain is currently defined as an agent that has distinct and reproducible biochemical and molecular characteristics and which, when serially passaged through congenic or transgenic mouse lines, produces consistent characteristics of relative incubation period, spongiform lesion profile, molecular profile and immunopathology.

BSE is defined as a disease of cattle. The apparent consistency of the disease leads to the suggestion that the BSE epidemic was caused by a single strain of agent, although the existence of two additional strain variants (defined as H-BSE and L-BSE) has been recognised, and the current classification requirements for bovine isolates are covered in a separate document.
http://www.izsto.it/index.php/centri-di-eccellenza/174-centri-di-eccellenza/2397-laboratorio-di-riferimento-europeo-per-le-encefalopatie-spongiformi-trasmissibili

Based upon primary passage in RIII mice, strain typing suggests that a single strain is responsible for the majority of suspect cases of BSE and vCJD tested so far. However, transgenic (Tg) mice are increasing being used to elucidate the biological properties of individual isolates. The most widely published and best-characterised models relevant to the characterisation of small ruminant isolates include Tg338 mice (which overexpress the ovine VRQ gene), Tg501 mice (which overexpress the sheep and goat ARQ gene) and Tg110 mice (which overexpress the bovine gene) amongst others.

Biochemical features cannot at present be equated with the definition of a strain,

but only as characteristics of a strain. They do, however, serve as a very useful initial screen to identify isolates which merit further investigation. Certain biochemical properties of PrP as seen in Western blots (in particular the glycoform ratio, the molecular weight of the unglycosylated band, and the

intensity of labelling with antibodies directed at the N terminal of the PrP) correlate well with BSE, vCJD, and with these agents as isolated in mice.

Whilst there may be a large number of TSE strains in small ruminants, current focus is on distinguishing the BSE agent from all others, on the basis that only the BSE agent is established as zoonotic.

It is generally assumed that zoonotic potential is an intrinsic property of the BSE agent. How this relates to any of the currently known and measurable properties of the agent is unknown. Therefore, in attempting to define the BSE agent, all that can be said at present is that it shows the following characteristics in all cases tested:

- Low molecular weight of the unglycosylated band of PrPres on Western blot (WB)
- PrP^{res} glycoprofile with a dominating diglycosyl moiety on WB.
- Poor or absent binding of antibodies directed at N-terminal epitopes (ovPrP93-97) in WB or ELISA formats under certain conditions
- Biological phenotype characteristics (e.g. IHC and vacuolation distribution, WB, relative incubation periods), which are indistinguishable from those of known BSE controls, in mouse bioassay.

These characteristics have been found to be 100% consistent for the BSE agent. However, on current evidence none of these characteristics, either alone or in combination, can be described as exclusive to the BSE agent.

Congruity with all of the above criteria will be interpreted as 'the agent in question cannot be distinguished from the BSE agent'.
Variation of one or more characteristics will be interpreted as 'the agent in question is not identical to the BSE agent'.

In 2002, the European Commission requested from the EURL comprehensive guidelines in support of the SSC Opinion "Strategy to investigate the possible presence of BSE in sheep" (adopted on 4-5 April 2002). To assist with this task, the EURL formed a strain typing 'Expert group' (STEG), with members drawn from those laboratories which (at that time) had experience with molecular methods which potentially offered some discriminatory potential between BSE and scrapie isolates. A requirement to subject all positive isolates to initial discriminatory testing is now written into the TSE surveillance regulations (999/2001 as

amended, 36/2005).

This manual contains the detailed protocols for these biochemical discriminatory methods for adoption by EU NRLs to enable discriminatory testing to be undertaken in each member state. These methods have been subjected to a blinded ring-trial to confirm their discriminatory potential and have been

approved by STEG**.**

Annual proficiency testing of the discriminatory test performance in each lab is co-ordinated by the EURL.

Section 2

Testing strategy

TESTING STRATEGY

(as required in the regulation 999/2001 AS AMENDED 36/2005 *(this strategy is summarised as a flow chart in Appendix1))*

Animal selection

Animals are currently selected for TSE testing through passive or active surveillance of the sheep and goat populations.

Sample requirements

The minimum sampling requirement for any animal from either source population is the **brainstem** (at the level of the obex) **and part of the cerebellum**, both of which can be accessed through the foramen magnum using a proprietary sampling spoon (See sampling guidance document http://www.izsto.it/index.php/centri-di-eccellenza/174-centri-di-eccellenza/2397-laboratorio-di-riferimento-europeo-per-le-encefalopatie-spongiformi-trasmissibili- for full details of sampling requirements and methods).

In summary, the quantity of tissue taken for testing should be sufficient to provide the following:

- A hemisection of fresh brainstem at the level of the obex, for the initial rapid test
- A fixedhemi-section of brainstem at the level of the obex for confirmatory immunohistochemistry and histopathology.
- A fixed section of cerebellum may be required for the confirmation of atypical scrapie using IHC..
- Sufficient remaining fresh-frozen medullary tissue (adjacent to the obex) and fresh-frozen cerebellum to provide fresh material for primary molecular testing (discriminatory WB) and possibly a range of secondary and tertiary testing (5-10 g whenever possible).
- Brain tissue from other areas in addition to the brainstem and cerebellum are always an advantage, as are lymphoreticular tissues when available. Although not essential for statutory purposes, they will be important in characterising the scrapie strain present if results differ from the expected confirmation at the level of the obex.

Initial TSE monitoring testing, as laid down in point 3.2(a) and (b) of Chapter C of Annex X to Regulation (EC) No 999/2001

Screening testing

- Active surveillance samples are subjected to one of the approved rapid tests (see point 4 of Chapter C of Annex X to Regulation (EC) No 999/2001 or the EURL website.
- Passive surveillance samples from clinical suspects may bypass rapid test and proceed directly to confirmatory testing.

Confirmatory testing

- Any animal found positive by any initial test, in either the active or passive surveillance programmes, shall be subject to confirmatory testing by one or more of the following methods – immunohistochemistry, Western blot, demonstration of characteristic fibrils by electron microscopy, or histopathological examination – as detailed in point 3.2(a) and (b) of Chapter C of Annex X to Regulation (EC) No 999/2001.
- Any TSE case which is confirmed, except atypical scrapie cases, should be subject to primary molecular testing to determine if BSE can be excluded or not.

Primary molecular testing with a discriminatory immuno-blotting method, as laid down in point 3.2(c)(i) of Chapter C of Annex X to Regulation (EC) No 999/2001

- Any positive, non-atypical scrapie, TSE isolate confirmed in sheep or goats pursuant to initial screening will be subjected to discriminatory WB using one of the protocols listed in part 4 of this Handbook. These tests have been assessed, and approved, using a ring trial coordinated by the EURL STEG which confirmed the ability of these tests to discriminate between BSE (either ovine or bovine) and classical scrapie (ovine).

- Possible outcomes of the primary molecular testing:
    - If the results of the primary molecular testing (discriminatory WB) are compatible with the defined characteristics of classical scrapie isolates (see below for interpretational criteria within the individual method protocols), the case is confirmed as classical scrapie positive and no further testing is required.
    - If the results of the primary molecular testing (discriminatory WB)

are inconclusive or different from above, and cannot exclude BSE, the case must be referred to the EURL STEG, who will advise on and coordinate secondary molecular testing with additional molecular testing methods. This will help to ensure that such unusual isolates are investigated in a consistent and comparable way, and with the relevant controls (which are a finite resource,

and can thus be conserved as much as possible).

- A poor quality WB (e.g. one that is faint, or overloaded) that does not enable clear identification of three bands (diglycosylated, monoglycosylated and unglycosylated) cannot be interpreted, and must be repeated, or referred to the EURL for a second opinion.

- Full details of test interpretation and specific criteria for referral can be found within the protocols for each method in the following section of this Handbook. Broadly, cases that require reference to the EURL include those with one or more of the following characteristics
  - Evidence of a low molecular weight for the unglycosylated band of PrPres, relative to scrapie.
  - Detectable reduction in immunoreactivity with the N-terminal specific antibody (binding to the epitope ovPrP 93-97)[1] when compared with the primary antibody (binding to the core region of PrP)[2].
  - Substantial reduction of signal with an N-terminal antibody relative to a core antibody following treatment of the sample with PK at a high concentration

Secondary molecular testing with additional molecular testing methods, as laid down in point 3.2(c)(ii) of Chapter C of Annex X to Regulation (EC) No 999/2001

- Any case referred to the EURL STEG will be further investigated using at least one alternative method, differing immunochemically from the original primary molecular method. The design of the secondary molecular testing, in accordance with the latest scientific knowledge and laboratory expertise, will be approved on a case-by-case basis by the EURL STEG, using some or all of the following methods:
  - a repeat WB using a different method/antibodies to a different epitopes
  - discriminatory IHC.
  - any available discriminatory method with proven ability to discriminate BSE from scrapie, even if based on technologies for the amplification of PrP^{Sc} (such as PMCA)

- The results will be interpreted by the EU reference laboratory assisted by the STEG, as well as a representative of the relevant national reference laboratory.
- If the isolate presents characteristics which are clearly identifiable as 'scrapie' or 'non-BSE', a report will be sent to the Commission at this stage, and no further testing will be undertaken.
- If the outcome of the testing is that the isolate is indistinguishable from BSE, or inconclusive with regard to BSE status it will go forward for mouse bioassay

[1] Table I in Langeveld et al., BMC Veterinary Research 2006, 2:19 doi:10.1186/1746-6148-2-19.
[2] Group B antibody as defined in Biacabe et al., 2007. Prion. 1:pp61-68

Tertiary testing strategy- Mouse bioassay.

As laid down in point 3.2(c)(iii) of Chapter C of Annex X to Regulation (EC) No 999/2001

- The optimal bioassay system for this purpose is the subject of active discussion by STEG, which will direct any incoming samples appropriately.

Much historical data on field isolates has been gathered in conventional congenic mouse lines, particularly C57Bl6, VM and RIII mice, which have been considered the 'gold standard' for strain characterisation. However, it is well known that a proportion of field isolates do not transmit to these mouse lines. Also in the past 10 years a profusion of transgenic mouse lines have become available, and data is accumulating with regard to the behaviour of BSE and scrapie isolates in these models. The STEG will direct the choice of bioassay for each individual isolate based on the data generated in the secondary testing phase, and the current state of scientific knowledge at the time of referral, but would propose a default panel of Tg 338 and Tg110 as a baseline group.

Section 3

Reporting and referral systems

REPORTING AND REFERRAL SYSTEMS

Data Collection and Provision

All cases reported should be accompanied by a breakdown of all the data requested, as prompted by the national competent authorities.

For cases being referred for secondary testing, the accompanying data should be broken down as indicated in the tabulation attached (appendix 2). This tabulation serves three purposes.

- Provision of a standardised data summary for each referred isolate, regardless of where the testing is performed.

- The collection and direct comparison of similar, objective data on each individual case from every MS, and the retrospective reinterpretation / regrouping of any individual parameter if required. It will assist with the classification, definition and preliminary epidemiological assessment of any previously unrecognised isolates that may arise in future.

How to refer an isolate

Each case referred to the EURL STEG should be notified in advance (by email, with the subject heading 'STEG referral {animal ID}') to the TSE reference laboratory mailbox (EURL.TSE@izsto.it) including a copy of the referral form (see appendix 2). It is particularly important that this notification includes an indication of the amount and quality of tissue available for subsequent testing.

Please supply all available tissue from each case (unless otherwise requested by the EURL STEG following notification).

- All movement of material must be covered by the relevant export and import permits. A copy of the import permissions can be obtained from the EURL on request.)

- Packages should be compliant with IATA regulations

- Packages should be addressed as follows:

  For the attention of EURL STEG
  Department of Nutrition, Food Safety and Veterinary Public Health, Istituto Superiore di Sanità, Viale Regina Elena 299, 00185, Rome, Italy

How results will be reported

Individual secondary molecular testing results will be reported to and collated by the EURL, who will compile a summary for the EURL STEG, and a decision will be reached on whether further testing by mouse bioassay is required. The referring NRL will be party to all discussion and interpretation of secondary testing results, as will the Commission. Results will be reported to the submitting NRL. The EURL will inform the Commission immediately about the outcome of the interpretation.

The formal reporting of the final results of secondary testing will be the responsibility of the competent authority in the referring MS.

Section 4

Discriminatory Western Blotting Methods

DISCRIMINATORY WESTERN BLOTTING METHODS

THE APHA PRIONICS-BASED HYBRID WESTERN BLOTTING METHOD

INTRODUCTION

The APHA Prionics based-Hybrid test is a protein extraction and Western immunoblotting technique based on the detection of abnormal prion protein (PrP^{Sc}) from the central nervous tissue of cattle and sheep, which can be distinguished from normal prion protein (PrP^{C}), both by its protease resistance and molecular size. This abnormal protein is a disease specific marker of Transmissible Spongiform Encephalopathy (TSE).

It is based on a modification of the commercially available Prionics® Check Western kit and utilises a double antibody detection system which enables discrimination to be made between cases of natural bovine BSE, natural ovine scrapie, and experimental BSE and natural scrapie in sheep.

SAFETY

Work with prions or potentially prion-containing materials has to be performed in strict accordance with National Safety Regulations.

Laboratories MUST adhere to National Safety Regulations, but the following information, published by The Advisory Committee for Dangerous Pathogens (ACDP) is available for guidance : "Transmissible Spongiform Encephalopathies (TSE) agents: safe working and the prevention of infection'. Copies can be obtained (ISBN 0113221665), from Department of Health (London) Stationery Office (Telephone +44 (20) 7873 9090). An update is available on the UK Department of Health web site (www.doh.gov.uk.cjd/tseguidance/).

MATERIALS

Chemicals and reagents

This Western immunoblotting protocol uses chemicals and reagents supplied with the Prionics® Check Western Kit (For local distributer please refer to www.prionics.com) and from other suppliers. The shelf life of all kit components is 1 year after production date if stored at +5±3°C. For actual expiry date see kit or components label.

Sigma Biotinylated Molecular Mass Standards	Sigma-Aldrich - Tel. No. +44 (0) 800 717181 Product No.B2787. To contain standards between approximately 10-200kDa. Diluted in SDS Sample buffer.
CDP-Star	PE Applied Biology (Tropix) -Tel No. +44 (0) 800 515840 Product No. T2147
Methanol	Analar
NuPAGE Antioxidant	Invitrogen - Tel. No. +44 (0) 800 269 210 Product No NP0005
NuPAGE Gels (12%Bis-Tris) 17well	Invitrogen - Tel. No. +44 (0) 800 269 210 Product No NPO349 Box
NuPAGE MOPS SDS Running Buffer (x20)	Invitrogen- Tel. No. +44 (0) 800 269 210 Product No NP0001 Dilute in distilled/deionised water 1:20 for use. Store at +5±3°C for up to 1 month.
Prionics Primary antibody mAb 6H4	Prionics® Check Western Kit reagent Store at +5±3°C
Primary Antibody RIDA® mAbP4	R-Biopharm Rhone LTD - Tel No. +44 (0) 141 9452924 Product No: R8007 Store at +5±3°C
Prionics Control Sample	Prionics® Check Western Kit reagent Store at +5±3°C
Prionics Digestion stop	Prionics® Check Western Kit reagent Store at +5±3°C
Prionics homogenisation buffer concentrate (x 5)	Prionics® Check Western Kit reagent Store at +5±3°C
Prionics Luminescence Buffer (x 10)	Prionics® Check Western Kit reagent Dilute in distilled/deionised water 1:10 for use. Store at +5±3°C
Prionics PAGE sample buffer	Prionics® Check Western Kit reagent Store at +5±3°C
Prionics PVDF Blocking buffer (x 5)	Prionics® Check Western Kit reagent Dilute in distilled/deionised water 1:5 for use. Store at +5±3°C
Prionics Proteinase K	Prionics® Check Western Kit reagent Store at +5±3°C
Tropix secondary antibody; goat anti-mouse-AP.	Applied Biosystems (Tropix) – Tel No. +44 (0) 800 515840 Product No. T2192 Use at 1:5000
Streptavidin - Alkaline phosphatase	Sigma-Aldrich - Tel. No. +44 (0) 800 717181 Product No S2890 . Stock solution 1mg in 8ml of TBS X1. For use; dilute stock at 1:2000 in TBST. Store at - 20°C or below.

Transfer Buffer (x1)	Add 1000ml of Transfer buffer (x10) to 8L of distilled/deionised water. Add 1000ml of methanol
Transfer Buffer (x10)	302.80g Tris(hydroxymethyl)methylamine 1441.3g Glycine Make up to 10L with distilled/deionised water.
Tris Buffered Saline (TBS) (x10)	80g NaCl 2g KCl 30g Tris(hydroxymethyl)methylamine Adjust to pH7.4 with HCl. Make up to 10L in distilled/deionised water. Store at +5±3°C
TBS (x1)	Add 1000ml of TBS x10 to 9L of distilled/deionised water. Store at +5±3°C
TBS with 0.05% Tween-20 (TBST)	Add 0.5ml of tween-20 to 1000ml of TBS x1. Store at +5±3°C
X-ray film developer	Kodak +44 (208) 3189441 Developer LX24 Product No 507 0933
X-ray film fixer	Kodak +44 (208) 3189441 Fixer AL4 Product No 507 1071

Equipment

0.5 ml x 96 well proteinase K digestion plate (v shaped wells)	Life System Design Tel no.+41 (0) 56 6645980 Product No 02-1402-0595
50 ml tubes (if using Omni homogeniser)	Falcon Fahrenheit Tel no. +44 (0) 1908 221212 Product No. 352070
Autoclave	Capable of maintaining 136°C for 20 minutes
Balance	With draft shield. Capable of weighing 0.4g ±0.5mg
Balance	Capable of weighing 310g ±0.01g
Biological Safety Cabinets	Class I (within a containment laboratory meeting national requirements for handling TSE's))
Chromatography Paper	Whatman Tel No +44 (0) 16622 674821 Product No. 3030 700
Circulating water bath	Grant Instruments Tel +44 (0) 1763 260811 Product no: LTD6G Capable of maintaining 4±3°C. With outlet and inlet pipes.
Centrifuge Rotor	Beckman Coulter Tel no +44 (0) 1494 441181 Product Name: TLA45 Rotor
Cooling coil	To fit transfer tank

Dispensette	To measure between 4-8ml (2% Accuracy, 2% Precision)
Disposable probe (if using Omni homogeniser)	Omni International Product no. 32750 (one per sample)
Beckman plastic 1.5ml centrifuge tubes	Cat No: 357448 Supplier: Beckman Coulter Tel no +44 (0) 1494 441181
Electrophoresis tanks	X cell Sure lock Mini-Cell - Invitrogen - Tel. No. +44 (0) 800 269 210 Product No E10001
Freezer	Capable of being controlled at –20°C or lower
Freezer	Capable of being controlled at –70°C or lower
Heating block	Life System Design +41 (0) 56 6645980 Product No. HBS-130 (To fit 96 well PCR plates. Able to maintain 50±1°C for 45 minutes and 105±3°C for 10 minutes)
Homogeniser	Omni International Tel no. 1-540-347-5331 Product no. Omni GLH220 (Camlab) or FASTH
Imaging machine and PC (if detecting results with imager)	BioRad -Tel No. +44 (0) 800 181134 Product Name: Fluor-S MultiImager or Versa Doc Model 1000
Magnetic Stirrer	Capable of maintaining 200rpm
Membrane incubation boxes	To fit the area of PVDF membrane used
Pasteur pipette	Plastic, disposable
pH meter	
Photographic trays (if detecting results by x-ray developing)	Three, to fit x-ray film
Pipette	5-40µl (Accuracy 4%, Precision 2.5%)
Pipette	40-200µl (2% Accuracy, 2% precision)
Pipette (multi-channel x 8)	5-40µl (Accuracy 4%, Precision 2.5%)
Pipette (multi-channel x 8)	40-200µl (2% Accuracy, 2% precision)
Pipette	200-1000µl (Accuracy 2%, Precision 2%)
Power supply	BioRad -Tel No. +44 (0) 800 181134 Product Name: PowerPac 200 Cat No: 165-5052 (To provide 200V constant for 40 minutes and 150V constant for 60 minutes) or equivalent
Rat-toothed forceps	Stainless steel
Refrigerator	Capable of maintaining +5±3°C

Rocking Platform	Bibby Sterilin Ltd Tel +44 (0) 1785 812121 Product No: Gyro-Rocker STR9
Scissors	Stainless steel
Small rolling pin	Such as plastic pipette
Stepper pipette	Capable of dispensing 0.01ml quantities
Transfer membrane (PVDF)	Millipore – Tel No: +44 (0) 870 900 46 45 Product No: IPVH 00010 (0.45um pore size. Immobilon -P from Millipore or equivalent)
Transfer sandwich cassette	BioRad -Tel No. +44 (0) 800 181134 Product No 170-3913 or Compatible with transfer tank
Transfer Sponges	BioRad -Tel No. +44 (0) 800 181134 Product No 170-3914 or Compatible with transfer tank.
Transfer Tank	BioRad -Tel No. +44 (0) 800 181134 Product No.170-3939 (Includes Sponges & Sandwich Cassette) or equivalent
Tray	To accommodate transfer sandwich assembly
Vortex Mixer	Fuson Whirlimixer Supplier: Thermo Instruments Tel No. +44 (0)1256 817282
Ultra Centrifuge	Beckman Coulter Tel no +44 (0) 1494 441181 Product Name: Beckman Optima TL Ultracentrifuge
X-ray cassette (if detecting results by x-ray developing)	To fit x-ray film
X-ray film (if detecting results by x-ray developing)	Amersham Biosciences Europe GmbH Tel No: +49 (0) 761 451 90 Tel No: Product No. Amersham ECL RPN2103K or equivalent

PROCEDURE / METHOD

Planning

A positive bovine classical BSE and positive classical ovine scrapie are always included on each run to validate the processing. Molecular mass markers are always included to allow for analysis of the banding patterns and molecular mass determination. The number of samples to be processed will vary on each run depending on the submissions and additional controls may be added if the samples are unusual and further examination required. The gel plan will be prepared to include all samples to be examined and relevant controls required.

Each gel is duplicated so that matching membranes will be produced to probe with different antibodies. This enables a comparison for each sample between its affinity for two antibodies targeted at different epitopes of the prion protein.
An example of a gel plan is shown in Fig 1.

Homogenisation
(Note: This stage is carried out in an appropriate safety cabinet according to National Safety Regulations)
Place a Falcon tube (if using Omni homogeniser) onto the balance with a draft shield and tare. Cut out the centre portion of spinal cord or medulla using scissors and forceps. Place between 0.45 – 0.75g of tissue into the tube and record the weigh to 2 decimal places.

Add Prionics homogenisation buffer (x1) to provide a 10% suspension, e.g. 5ml of buffer to 0.5g of tissue.
Homogenise tissue using Omni GLH220 for 1 minute ±10sec at 22±3°C.

Ensure that all the tissue has been homogenise completely. If necessary, repeat this homogenisation stage. (Use a new disposable Omni probe for each sample.)

Note: Omni probes can be re-used by soaking overnight in 20% hypochlorite solution and then rinsing 3x in tap water and 1x in Deionised

Water.

The homogenate may be frozen at -20°C or below at this stage and tested at a later date

Clarifying centrifugation
(Note: This stage is carried out in an appropriate safety cabinet according to National Safety Regulations)

Set a heating block to 50±1°C and another at 105±3°C. Allow them to reach a stable temperature before use. Label a 1.5ml Beckman centrifuge tube for each sample.

Vortex homogenates to ensure thorough homogenisation (Note: If homogenates have been frozen, ensure that they are completely thawed before vortexing). Using a plastic Pasteur pipette transfer 1.5ml of homogenate from the homogenisation vessel to a 1.5ml Beckman tube.

Centrifuge the samples for 5 mins at 1.127g (5,000rpm) at 10°C in Optima TL Ultracentrifuge using a TLA45 Rotor.

Protease Digest
(Note: This stage is carried out in an appropriate safety cabinet according to National Safety Regulations)
Label a digestion plate for processing the run. Add 10µl of Prionics Proteinase K solution to each well using a stepper pipette.

Remove 2 x 100µl of supernatant using a micropipette (40-200µl) and place in the duplicate pre-assigned wells on the digestion plate according to the plate plan and mix by gentle pipetting.

Place the digestion plate on the heating block set at 50±1°C. Incubate for 45±1 minutes.

Add 10µl of Prionics digestion stop to each well of the digestion plate using the stepper pipette and 100µl of Prionics sample buffer using a micropipette (40-200µl). Mix by gentle pipetting.

NOTE: The procedure at this point may be stopped and carried out the following day if the digestion plate is sealed (with a sealing film) and stored at -20°C or below.

Electrophoresis – Preparatory steps
Set up the electrophoresis tanks. Remove white tape from lower edge of gel. Remove plastic combs from gel and using a Pasteur pipette, gently flush wells with running buffer.

Place 2 gels in each tank, the shorter sides facing inward. Raise gels up and clamp in place. If a single gel is to be run then a blank plate is inserted in the tank. Place approx. 2 cm running buffer in the outer

chamber of each tank to prevent the gel base drying out whilst loading.

Sample Denaturation and Electrophoresis

Boil the samples in the digestion plate (105 ±3°C) on the heating block for 10 ±1 minutes. (Note: Stored digested samples previously boiled and tested should only be heated for 2-5 minutes at 65±3°C).

Boil the molecular weight standard solution (105 ±3°C) for 5±1 minutes.

Set the other heating block to 65±3°C. Place sufficient Prionics control, (10µl for each gel) into a 0.5ml Eppendorf. Heat for 2-5 mins.

Load 10µl of each sample into lanes according to the gel plan. The outside lanes of each edge are not used. 10µl of Prionics control is loaded into the far left-hand lane of each gel. 5µl of biotinylated molecular weight standard is loaded into the inner left lane and also the far right. Known bovine and ovine controls are loaded on the right hand side.

Repeat the loading on another gel, to give a duplicate gel which is identically loaded and can be probed with a different antibody.

Release the clamp, gently lower the gels and reclamp into position.

Slowly fill the inner buffer chamber with Running Buffer (x1). Fill the outer chamber with Running Buffer (x1) so that the level is approximately 3cm above the bottom of the gels. Add 500µl of Antioxidant to the inner chamber using a micropipette (200-1000µl). Place the lid onto the tank.

Run the gels at 200V until dye front is about 1-2cm from the bottom of the gel (approximately 45mins).

Protein Transfer – Preparatory steps

Fill the transfer tank halfway with transfer buffer (x1) and place the cooling coil next to the red side. Switch on the cooler tank. Ensure it is set at 4 ±3ºC. Add a magnet to the transfer tank and place the tank in a tray on a magnetic stirrer.

Cut the PVDF membrane to fit all the gels to be transferred (maximum 6 gels and 1 membrane per antibody). Handle the membrane with care, using forceps at the corner.

Pre-treat the PVDF membranes, by soaking them in methanol for a few seconds. Rinse in distilled/deionised water for 2 minutes. Equilibrate the membranes in transfer buffer (x1) for at least 10 minutes.

Cut 2 sheets of blotting paper to the size of each membrane.
After the gel electrophoresis has finished, remove the gels from the tanks and open the gel plates with a cleaver.

Trim the gels with the cleaver to remove the top 1/3 and the bottom just

below the dye-front.
Place the gels in transfer buffer (x1) and gently agitate for a few seconds before placing in sandwich.

Protein Transfer – Sandwich Assembly and Protein Transfer
In the safety cabinet, open the transfer cassette and place in a tray containing transfer buffer (x1) with the clear side at the bottom of the tray. (Ensure all following steps are immersed in transfer buffer).

On the clear side of the cassette place a sponge and a piece of wetted blotting paper, on top of this the membrane should be positioned. Write the reference number and antibody on the top right hand corner of the membrane with a china marker. The gels are placed on top of the membrane, ensuring they are in the correct orientation. The second piece of blotting paper is placed on top of the gels. The final sponge is placed on top of the blotting paper and the cassette is closed.

Note: After each layer is added to the sandwich, roll out air bubbles with a roller.

Place the cassettes in the transfer tank the clear side facing the red side of the tank and the black side facing the black side of the tank. Top up the tank with transfer buffer (x1) and position the lid on top (red to red, black to black).

Run the power supply at 150V for 60±2 minutes.

When the protein transfer is completed, disassemble the sandwich and place the membranes in incubation boxes containing 50ml Prionics PVDF blocking buffer (25ml for a 1 gel membrane, 50ml for a 6 gel membrane) for 30 ±5 minutes at 22 ±3°C.

Immunological detection
Dilute the primary antibodies in TBST, mAb6H4 (1:5000) and mAb P4 (1:5000). Prepare a volume sufficient to cover the membrane in the incubation boxes, one incubation box for each antibody. (25ml for a 1 gel membrane, 50ml for a 6 gel membrane).

Pour the blocking buffer into a discard container and add the primary antibody solutions to the appropriate membrane in the incubation box. Incubate on a rocking platform for 60 ±5 minutes (mAb 6H4) and 120 ±5 minutes (mAb P4) at 22 ±3°C, or 12-18h at 5±3°C.

Wash the membrane 3 times for approximately 7 minutes with TBST.

Dilute the appropriate secondary antibody solution (1:5000) and Streptavidin (1:2000 of stock solution). (25ml for a 1 gel membrane, 50ml for a 6 gel membrane). Add the secondary antibody solution and incubate on a rocking platform for a minimum 30 ±3 minutes at 22 ±3°C

Wash the membrane 3 times for approximately 7 minutes with TBST.

Signal detection using x-ray film

Equilibrate the membrane in Prionics luminescence buffer for a minimum of 5 minutes (25ml for a 1 gel membrane, 50ml for a 6 gel membrane).

Under appropriate safelight conditions in the dark room, blot off the excess luminescence buffer very gently, and transfer membrane to a clean glass/polythene surface.

Add up to 5ml of CDP-Star substrate to the surface of the membrane and distribute evenly, incubate for 5 ±1 minutes at 22 ±3°C. Blot off the excess substrate, and place the membrane in the development folder.

Place the development folder in the x-ray cassette. Place a sheet of x-ray film on top of the membrane and close the cassette.

Expose the membrane to the X-ray film. The actual exposure time will vary according to the samples processed and the suggested range is between 30 seconds and 20 minutes. The film should be exposed until a strong signal of the positive control and either the background or the proteinase K bands are visible. Expose for longer or shorter times for optimal signal visualisation.

Place the film in a tray of x-ray developer solution for 5 minutes with occasionally agitation.

Wash the film for 1 minute in a tray of tap water.

Place the film in x-ray fixer for 5 minutes. The light can be turned on once the film is fully submerged. Leave the film in the fixer for 5 minutes.

Thoroughly wash the film in running tap water for 1 minute and then hang the film up to dry.

Signal detection using a CCD imager

Equilibrate the membrane in Prionics luminescence buffer for 5-10 minutes (25ml for a 1 gel membrane, 50ml for a 6 gel membrane).

Blot off the excess luminescence buffer very gently and transfer membrane to a clean glass/polythene surface.

Add up to 5ml of CDP-Star substrate to the surface of the membrane and distribute evenly, incubate for 5 ±1 minutes at 22 ±3°C. Blot off the excess substrate and transfer to a clean polythene surface.

Place the membrane into the imager and detect the light signal using 1 & 10 minute exposures. Label the blot with sample and control details from gel loading plan and also include run number, method of processing,

antibody and detection time, and operator initials and date.

INTERPRETATION OF THE APHA PRIONICS BASED-HYBRID WESTERN IMMUNOBLOT RESULTS

This method is capable of identifying PrP^{Sc} profiles associated with classical bovine BSE, atypical H and L-type bovine BSE, classical ovine scrapie and experimental BSE in sheep.

The method is not suitable for detection of atypical scrapie

Using this method, clear but subtle differences in the molecular mass migration are observed (see Figure 2) between natural bovine BSE (lanes 7 & 13), natural ovine scrapie (lanes 2, 3, 10, 11 & 12), and experimental BSE in sheep (lanes 5, 6, 8 & 9) when detected with mAb 6H4.
Sheep passaged scrapie strain CH1641 (lane 4) mimics the same molecular weight and antibody affinity pattern as for experimental BSE in sheep.

A negative sample will have been totally digested by proteinase K and no signal will be visualised with either antibody, illustrated in figure 1b (lane 4).

With mAb P4, used at the dilution in this method, natural ovine scrapie is strongly detected (lanes 2, 3, 10, 11 & 12), there is a clear reduction in signal for experimental BSE in sheep and CH1641 (lanes 5, 6, 8, 9 & 4) and natural bovine BSE is not detected at all (lanes 7 & 13).

A negative sample will have been totally digested by proteinase K and so no signal will be visualised (lane 14).

Any samples that give a banding profile that is not consistent with classical scrapie should be referred to the EURL for a second opinion and further investigation where necessary.

LIMITATIONS OF THE TEST

- The method requires sufficient tissue from the optimal brain area, in order to maximize the amount of PrP^{Sc} loaded from each sample.
    - If the quantities of brain material are too low (a situation we have encountered with active surveillance), the number of Western blots that could be run from a single sample may not be sufficient to obtain an accurate interpretation and there may not be enough PrP^{Sc} in underweight samples to confirm a diagnosis even though the sample may be positive.

- It is important to compare the molecular weights of samples, relative to each other and the controls on the same gel.

- Care must be taken in interpretation, as samples that have not been digested correctly may initially appear positive. *These should be repeated.*

- Strong positive samples may give a saturated signal intensity which will obscure visual interpretation of the banding profile. *This type of sample requires repeat immunoblotting following dilution, so a clear banding pattern is observed.*

- The full banding profile may not be visualised in weaker samples.

We have shown that differences in the ovine genotype do not appear to give any variation in the banding obtained for scrapie samples but autolysed samples can run quicker through the gel and may resulting in a lower molecular weight than the equivalent fresh sample. At present we do not know whether mAb P4 results are affected by autolysis.

We have only assessed certain genotypes of experimental sheep-BSE and some molecular weight variability of natural sheep-BSE, if it is in the ovine population, cannot be ruled out at this stage.

RESULT SUMMARY:

Classical Bovine BSE:

- Signal and low molecular mass migration (compared to ovine) with mAb 6H4.
- No signal with mAb P4.
- Predominance of diglycosylated band.

(See Fig 2 below)

Classical Ovine scrapie:

- Visible signal and higher molecular mass migration (compared to bovine) with mAb 6H4.
- Visible signal with mAb P4.

(See Fig 2 below)

Experimental BSE in sheep:

- Strong signal and lower molecular mass migration (compared to ovine classical scrapie) with mAb 6H4.
- Much reduced signal with mAb P4.

(See Fig 2 below)

Negative
No signal with either mAb 6H4 or P4

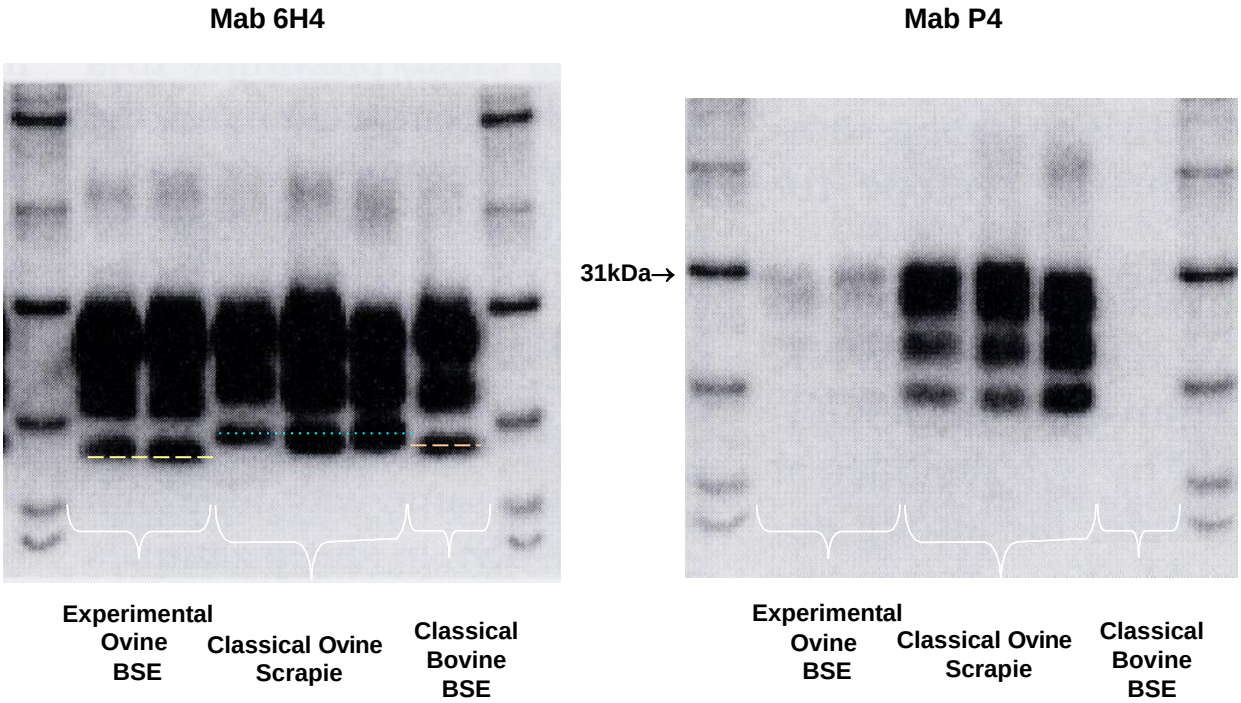
Figure 1

Gel plan – example of full layout

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Blank	Prionics Control	Biotin Marker	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5	Bov BSE +ve	Ov Scrapie +ve	Biotin Marker	Blank

Figure 2

Image showing clear differential molecular mass migration with core antibody (6H4) and differential N-terminal antibody affinity for classical scrapie, classical BSE and experimental ovine BSE.



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Cooley, W. A., Clark, J. K., Ryder, S. J., Davis, L. A., Farrelly, S. S. J., & Stack, M. J. (2001). Evaluation of a rapid western immunoblotting procedure for the diagnosis of bovine spongiform encephalopathy (BSE) in the UK. *Journal of comparative pathology*, *125*(1), 64-70.

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Stack, M., Chaplin, M., & Clark, J. (2002). Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies. *Acta neuropathologica*, *104*(3), 279-286.

ANSES DISCRIMINATORY WESTERN BLOT METHOD

Principle

A Western blotting technique using two different monoclonal antibodies (mAbs) BAR233 and P4 to identify BSE among scrapie cases in small ruminants, based on the differences in molecular masses and in reactivities with these two mAbs.
In this test, molecular differences between scrapie and BSE are the result of different resistances to proteinase K (pK) digestion. The abnormal prion protein is indeed more susceptible to pK cleavage than most scrapie cases. More precisely a larger part of the protein is digested by pK in BSE. This results in a lower molecular mass of the unglycosylated form of the pK digested PrP protein (PrP res). This molecular mass is slightly lower in BSE-in-sheep compared to cattle BSE, in contrast with most scrapie cases. Using mAb P4 recognising an epitope close to protease cleavage site, BSE also appears faintly, if at all, labelled, in contrast with most scrapie cases. Suspect molecular samples should be referred to the EURL for further analysis.

Reagent List

Solution 1 Loading buffer	Laemmli sample buffer (BIORAD # 161 0737) ➢ 28.5ml SDS (Euromedex, # 1012-B) ➢ 0.6 g ß-mercaptoethanol (Merck # 805740) ➢ 1.5ml
Solution 2 Gel buffer	36.2 g Tris Amino and 0.8 g SDS, add distilled water to 200 ml and adjust to pH 8.8 *by adding acidic HCl solution. Filter the solution with Whatman paper.* Store in a dark bottle at 4°C.
Solution 3 Stacking buffer	6.06 g Tris Amino and 0.4 g SDS, add distilled water to 100 ml and adjust to pH 6.8 *by adding acidic HCl solution. Filter the solution with Wattman paper. S*tore in a dark bottle at 4°C.
Solution 4 10 X Phosphate Buffered Saline (PBS) pH 7.4	NaCl (Euromedex # 1112) 80 g KH_2PO_4 (Merck # 805740) 2 g Na_2HPO_4(Prolabo # 28 026.292) 18.2 g KCl (Merck 4936) 2 g Adjust to 1000 ml with distilled water. Adjust to pH between 7.2 and 7.4. Store at room temperature.

Solution 5 Electrophoresis buffer	100 ml of TRIS GLYCINE SDS 10x buffer (Euromedex #EU0510) Adjust to 1000 ml with distilled water. Store at 4°C.
Solution 6 Blotting buffer	TRIS/CAPS 10x buffer (BIORAD #1610778) 100 ml Ethanol 125 ml Adjust to 1000 ml with distilled water. Store at 4°C
Solution 7 1 X Phosphate Buffered Saline (PBS) pH 7.4	100 ml Solution 6 and add 900 ml distilled water. Store at 4°C.
Solution 8 PBS-T	Solution 9 + 0.1 % Tween 20 (Euromedex # 2001B).
Solution 9 Blocking buffer for Bar 233	3% (P/V) Bovine Serum Albumin (Sigma #A7906) in Solution 10.
Solution 10 Blocking buffer for P4	5% (P/V) non fat dried milk (BIORAD) in Solution 10 (BIORAD # 1706404).
mAb Bar 233	SPI bio # A03223
mAb P4	R-Biopharm # R8007. E-mail: www.r-biopharm.com
Biotinylated marker	Sigma # B2787 or R&DSystem Ref MW001
Streptavidin-peroxidase	Sigma #55512
Secondary antibody conjugated to streptavidin-peroxidase	Clinisciences # 101005

15% Acrylamide gel preparation (for 2 gels with thickness of 1.5 mm)

	Gel 15 %	Stacking 4 %
Distilled water	5 ml	3,1 ml
Gel buffer (sol 2**)**	5 ml	
Stacking buffer (sol 3**)**		1,75 ml
A/B	10 ml	650 µl
APS (10% solution)	200 µl	30 µl
Temed	8 µl	7,5 µl

Materials required

* Tissue homogeniser such as RIBOLYSER (HYBAID) or TeSeE Precess® Biorad.
* Syringes of 1ml and 25G needles (BioRad).
* Heating block (used at 37°C and 100°C).
* Centrifuge for Eppendorf tubes.
* Electrophoretic and Blotting cells and apparatus(with cooling system).
* Versadoc 5000
* Micropipettes delivering volumes of 10, 50, 200 and 1000 µl.

Method

The starting amount of brainstem material (preferentially obex region) to be analysed may depend on the origin / quality of the sample and the amount of available material but in general 350 mg of tissue is used.

Purification and denaturation

As described in the BioRad TeSeE WB SOP (BIORAD #355 1169)

SDS-PAGE & Western Blot

1. Incubate each tube for 5 minutes at 100°C in a heating block just before loading samples on the acrylamide gel.

2. Run two identical gels with the same samples and respect gel plan described in annexe 1, that allows a comparison of each sample to be examined with a cattle BSE control loaded on the lane beside.

3. Electrophoresis is carried out at 200V for 1h20 with electrophoresis buffer (**solution 5**).

4. The two gels are transfered on to Hybond LFP 0.2µm PVDF membrane (Amersham # RPN303LFP) (previously re-hydrated with alcohol) at 115 V for 1h with Blotting buffer (**solution 6**).

5. The membranes are blocked in 10 ml of Blocking buffer (**solution 9 or 10**) for 1

hour.

6. Membranes are incubated 30 minutes at room temperature in 10 ml of a 1:10000 dilution of primary monoclonal antibody Bar 233 or 1:5000 dilution for primary monoclonal antibody P4 (1 mg/ml) in PBS-T buffer (**solution 8**) (NB MAb dilutions are not definitively defined. Working dilutions have to be evaluated for each batch.)

7. Membranes are washed in PBS-T buffer (**solution 8**) 20 minutes

8. Incubate the membranes in the secondary antibody (1:2500) (goat anti-mouse conjugated to streptavidine peroxidase) (Cliniscience # 101005) with Streptavidin-peroxidase (0,1µl/ml) for 20 minutes at room temperature.

9. They are then washed again in PBS-T (**solution 8**) 30 minutes, and a final wash with PBS (**solution 7**) during 5 minutes.

10. Then, membranes are incubated in chemiluminescence buffer E.C.L. (Amersham # RPN 2109) in order to take a picture on autoradiographics films (Amersham # RPN 1674K).

11. The labelling is visualised by means of the enhanced chemiluminescence system. Signals are quantified using Versadoc 5000 computer analysis (Quantity One, software Biorad UK Ltd) or equivalent, allowing the detection of chemiluminescent signals and quantitative analysis of the data.

12. If the band signal obtained with use of chemiluminescent buffer ECL is weak, after a wash (5 min) in PBS (**solution 7**), SuperSignal Dura chemiluminescence buffer (Interchim # 34075) could be used.

Data analysis

13. Using this system, apparent molecular weights of the unglycosylated band are measured by comparison to the biotinylated markers on the gel and the centre position for each sample band is determined to measure the apparent molecular weight. To obtain a comparison of molecular weights between the samples, results are expressed in differential molecular weights (dmw), corresponding to the differences between the apparent molecular weight of the sample and that of the cattle BSE control which is loaded on the lane beside (see annexe 2 of this section).

14. Before sample analysis some verifications must be made. The quality of the

electrophoretic mobility is checked with the use of two controls: Ovine scrapie control and cattle BSE control. The dmw of the ovine scrapie control loaded must be positive. If this is not the case the blot must be repeated. The homogeneity of the unglycosylated band molecular weight of the four cattle BSE control must be evaluated. All of them have to be similar, if one of them differs by more than 0.5 kDa, samples situated near cannot be interpreted. Mabs labelling is controlled with the visual analysis of the differential immune-reactivity for ovine scrapie control: for validation of analysis P4 labelling must be more intensive or equivalent of Bar233 labelling.

15. Check that the positive or negative dmw obtained following computer analysis are consistent with the apparent dmw on the film and that the technical quality of the blot allows a correct interpretation of the data.

16. Differential immuno-reactivity between WB Bar 233 and WB P4 for is observed for each sample, immuno-reactivities being assessed with both antibodies as ++, +, +/- or -, as illustrated in annexe 2 (of this method section).

Interpretation

See annexe 2 (of this method section) as an example

Differential Molecular Weights (dmw):

- A sample with a **Dmw > 0,200** is not suspect, and does not lead to further action.

- A sample with a **Dmw < 0,200** should be retested:
  - if the second analysis gives a result as: **Dmw < 0** then the sample is suspect and should be referred to the EURL for further analysis.
  - if the second analysis gives a result as: **Dmw > 0** then sample is not suspect and does not lead to further action.

Differential Immuno-Reactivity:
The immuno-reactivities are assessed with scores as ++, +, +/- or -. (see annexe 2)

- A sample with differential immuno-reactivity showing: **P4 signal ≥ Bar 233 signal** is not suspect, and does not lead to further action.

- A sample with differential immuno-reactivity showing: **P4 signal << Bar 233 signal** is suspect and should be referred to the EURL for further analysis.

Note about the influence of the PrP res quantity in the sample:

Negative or very weakly positive samples with 2 µl per lane should be retested with higher volumes of PrPres extracts. In this case, the same volumes of cattle BSE control should be loaded, but keeping the same quantity of PrPres loaded in these lanes; add Loading buffer to obtain the desired volume.
For repeatedly negative samples or very weak positive samples with BAR233, the interpretation of the apparent molecular weight of the unglycosylated band can be impossible. Nevertheless, if the signal with the mAb P4 is stronger than the signal with mAb BAR233, suspicion of BSE can be excluded.

For very strongly positive samples with mAb BAR233, the signal with mAbs P4 can be apparently strongly positive with a BSE sample and the interpretation of the molecular mass is sometimes difficult (large diffusion of the bands). In this situation, strongly positive samples should be retested following dilution and then re-run, beside the same volumes of cattle BSE control. The same quantities of PrPres extracts in the cattle BSE controls (as compared to the normally 2µl loaded, should be used; add Loading buffer to obtain the desired volume.

Transmission to EURL:

- ✔ Biological sample
- ✔ Results (figures of dmw and scores of immuno-reactivities)
- ✔ Film

ANNEX 1

Gel plan :

WB with mAb Bar 233

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Biotinylated Marker	Sample 1	Cattle BSE Control	Sample 2	Sample 3	Cattle BSE Control	Sample 4	Biotinylated Marker	Sample 5	Cattle BSE Control	Sample 6	Sample 7	Cattle BSE Control	Sample 8	Biotinylated Marker
2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl
	*			*				*			*			

WB with mAb P4

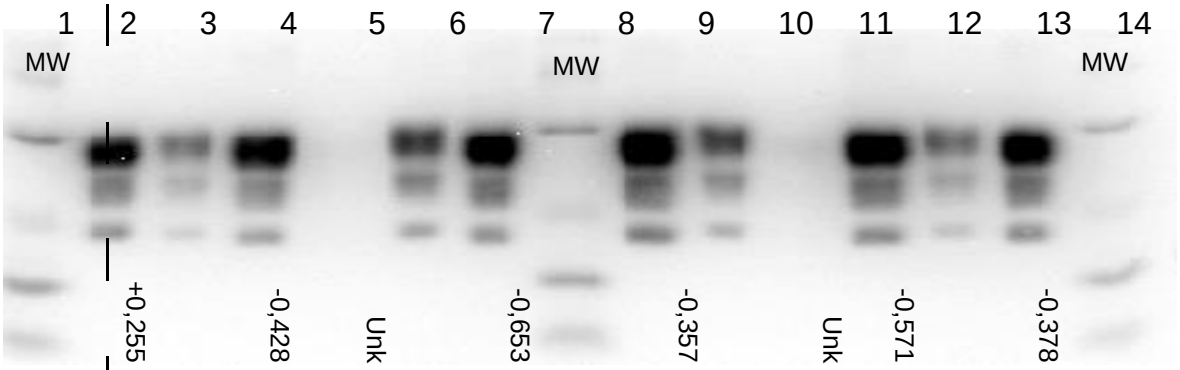
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Biotinylated Marker	Sample 1	Cattle BSE Control	Sample 2	Sample 3	Cattle BSE Control	Sample 4	Biotinylated Marker	Sample 5	Cattle BSE Control	Sample 6	Sample 7	Cattle BSE Control	Sample 7	Biotinylated Marker
2µl	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**	**2µl**

***** Comparison and calculation of the dmw of the unglycosylated band is performed only with the adjacent cattle BSE control.
For a better comparison of the pattern, equal volumes should be run on each well (if needed dilute the samples with Loading buffer).

Ovine scrapie control: ovine scrapie control is loaded in any sample position in gel plan for the two membranes.

ANNEX 2 (Example of interpretation)

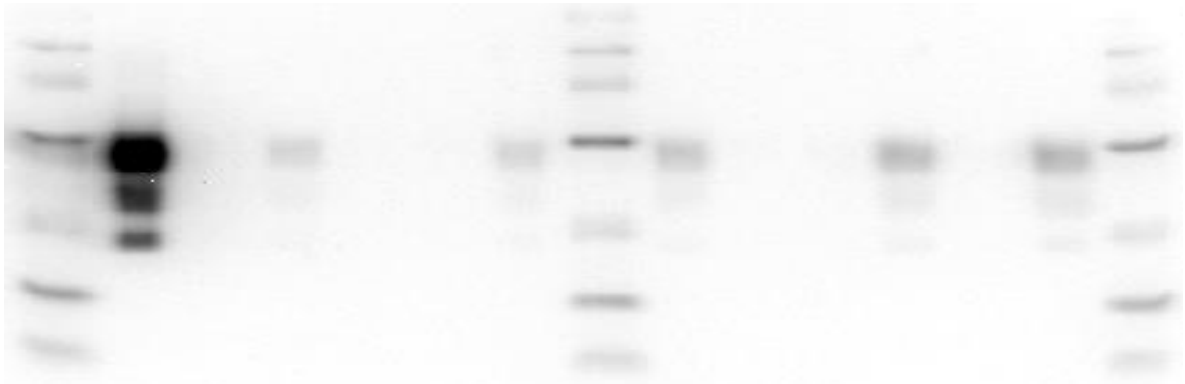
WB with mAb Bar 233



Dmw:

Signal: ++ + ++ - + ++ ++ + - ++ + ++

WB with mAb P4



Signal: ++ - +/- - - +/- +/- - - +/- - +/-

Interpretation: (see next page)

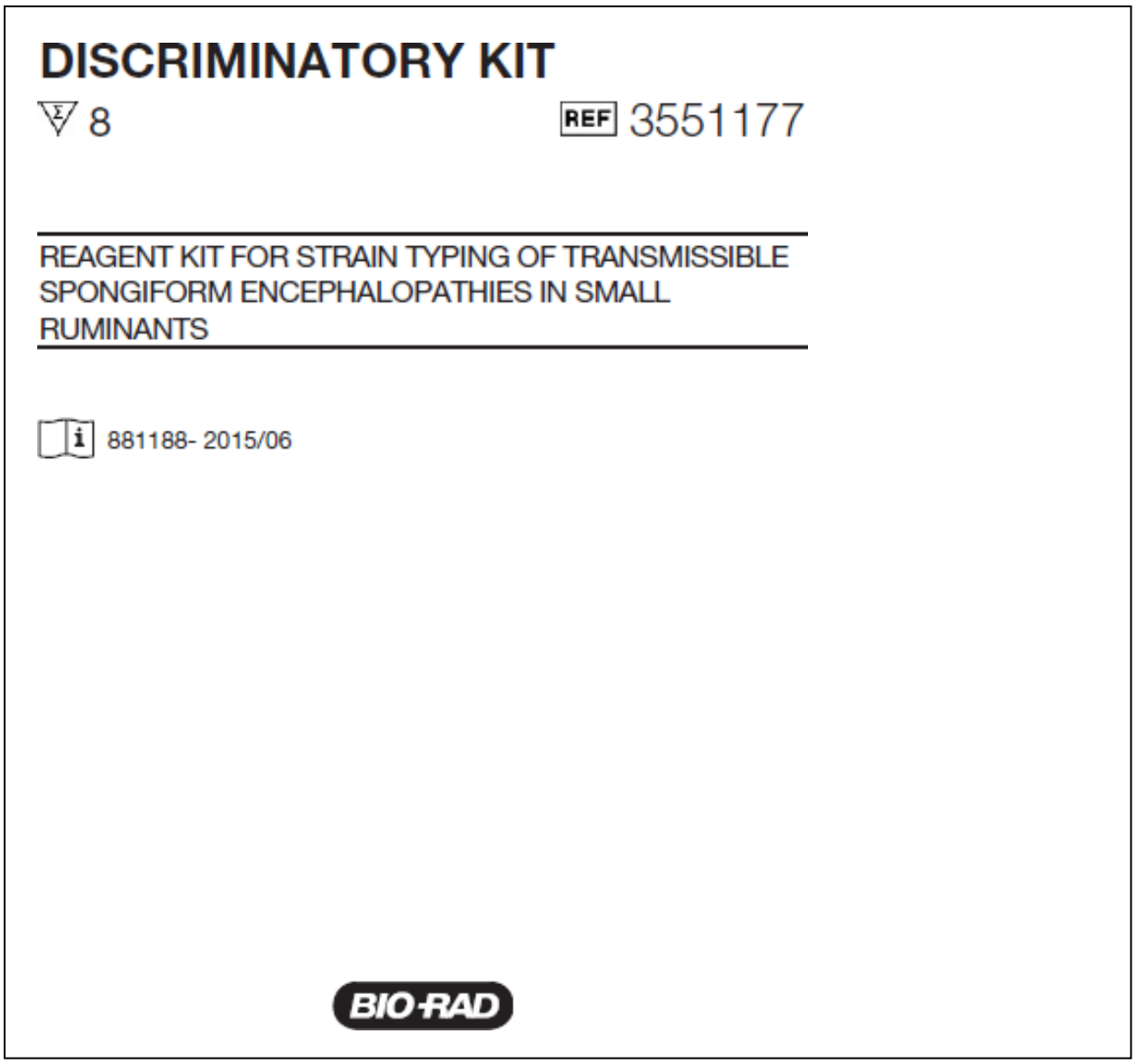
Interpretation

Sample 1 (lane 2)	Dmw	+0,255	Scrapie	Scrapie
	Immuno-reactivity	**P4 ≥ Bar 233**	Scrapie	
Sample 2 (lane 4)	Dmw	-0,428	Suspect	Suspect
	Immuno-reactivity	**P4 << Bar 233**	Suspect	
Sample 3 (lane 5)	Dmw	unknown	unknown	Should be repeated
	Immuno-reactivity	unknown	unknown	
Sample 4 (lane 7)	Dmw	-0,653	Suspect	Suspect
	Immuno-reactivity	**P4 << Bar 233**	Suspect	
Sample 5 (lane 9)	Dmw	-0,357	Suspect	Suspect
	Immuno-reactivity	**P4 << Bar 233**	Suspect	
Sample 6 (lane 11)	Dmw	unknown	unknown	Should be repeated
	Immuno-reactivity	unknown	unknown	
Sample 7 (lane 12)	Dmw	-0,571	Suspect	Suspect
	Immuno-reactivity	**P4 << Bar 233**	Suspect	
Sample 8 (lane 14)	Dmw	-0,378	Suspect	Suspect
	Immuno-reactivity	**P4 << Bar 233**	Suspect	

In lane 2, we can see a typical sample of a natural scrapie case, as those generally found in the field and in lanes 4,7,9,12 and 14, samples of sheep experimentally infected by BSE are shown in this example.

BIO-RAD DISCRIMINATORY TEST (BASED ON THE CEA DISCRIMINATORY WESTERN BLOT METHOD)

The Discriminatory Kit (BioRad reference 35 51177) is manufactured and distributed by Bio-Rad under **CEA** licence for Strain Typing (DTST) of Transmissible Spongiform Encephalopathies (TSEs) in small ruminants.


DISCRIMINATORY KIT

Σ 8

REF 3551177

REAGENT KIT FOR STRAIN TYPING OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES IN SMALL RUMINANTS

881188- 2015/06

BIO-RAD

CONTENTS

1 - AIM OF THE TEST

The DISCRIMINATORY KIT[1] for Strain Typing of Transmissible Spongiform Encephalopathies (TSEs) in small ruminants has been developed to discriminate BSE from scrapie and to confirm atypical scrapie strains.
Discriminating TSE strains in small ruminants encompasses 3 major steps:

- Detection of the TSE infected animals
By a rapid test with an ELISA detection in the screening laboratories.

- Confirmation with the TeSeE™ WESTERN BLOT assay (Product Ref.: 355169)
By the National Reference Laboratories on all the samples detected repeatedly positive in the screening laboratories. At this stage, unusual scrapie strains as Nor98 can already be identified by their migration pattern.

- Discrimination between BSE and scrapies
Confirmed samples are calibrated and evaluated with the **Discriminatory Kit (Product Ref.: 3551177)** for StrainTyping (DTST), developed by CEA. Discrimination between BSE and scrapie is based on the partial resistance of BSE infected samples to PK treatment. Comparison between confirmation assay result and discriminatory assay result is possible since the protocol of the discriminatory assay has been directly adapted from the **TeSeE™ WESTERN BLOT assay (Product Ref.: 3551169)**.
NB: TSE in small ruminants can also be confirmed by IHC.

The DTST does not only identify the strains exhibiting a BSE-like profile, but also confirms the PK sensitivity of unusual strains like Nor98 that would be previously identified by the **TeSeE™ WESTERN BLOT**.

[1] Discriminatory Kit is manufactured and distributed by Bio-Rad under "CEA" license.

2 - ASSAY PRINCIPLE

The test is based on the differential resistance to proteolysis of the N-terminal region of PrPres depending on the TSE strain. Indeed, depending on the conditions of proteolysis, the N-terminal region of PrPres in BSE can be digested, while it persists for classical strains of scrapie. Moreover, in these peculiar conditions of proteolysis, it is possible to completely digest PrPres in PK sensitive strains of scrapie, like Nor98.
The test is performed through two conditions of purification (LOW and HIGH conditions of proteolysis respectively), combined with two conditions of detection by western blotting, using two monoclonal antibodies recognizing either the N-terminal (TEST detection) or the core protein (CONTROL detection) regions respectively. The Discriminatory test results have to be interpreted taking into consideration the results of previous analysis, including:

i) Rapid test result for screening
ii) and confirmatory Western Blot profile for confirmation and scrapie strain classification.

3 - COMPOSITION OF THE KIT

Labelling	Type of reagents	Presentation	Storage
A1	Denaturing solution	1 vial 8 mL	+2°C to +8°C
A2	Denaturing solution additive	1 vial 0.4 mL	+2°C to +8°C
B	Clarifying solution Colouring: bromophenol blue Ready to use	1 vial 6 mL	+2°C to +25°C
PK	Proteinase K Colouring: phenol red	1 vial 0.2 mL	+2°C to +8°C
Ab Ctrl	Control antibody[1] Anti-PrP core monoclonal antibody (10x)	1 vial 8 mL	+2°C to +8°C
Ab Test	Test antibody[1] Anti-octapeptide monoclonal antibody (10x)	1 vial 4 mL	+2°C to +8°C
Ab II	Secondary antibody[1]: Sheep Anti-Mouse IgG(H+L)-HRP (10x)	1 vial 14 mL	+2°C to +8°C
Bl	Blocking solution[1] (10x)	1 vial 10 mL	+2°C to +8°C

[1] These reagents contain 0.1 % of ProClin™ 300 (preservative).

4 - REAGENTS AND MATERIAL REQUIRED WITH CRITERION™ XT GEL

4.1- REAGENTS AND DISPOSABLES

Graduated pipettes (5, 10, 25 mL), conical tubes (50 mL), 2 mL polypropylene micro-test tubes with caps.
PARAFILM®M Sealing films.

Sample purification

Laemmli sample buffer	30 mL	Bio-Rad, cat. Nr. 1610737
2-Mercaptoethanol	25 mL	Bio-Rad, cat. Nr. 1610710
SDS	100 g	Bio-Rad, cat. Nr. 1610301
Calibration syringes	x 200	Bio-Rad, cat. Nr. 3551174

Gel electrophoresis

Kaleidoscope prestained standard	500 µL	Bio-Rad, cat. Nr. 1610324
MagicMark™ XP Western Standard (Molecular weight standard)	250 µL	Invitrogen, cat. Nr. LC5602
Criterion™ XT 12 % Bis-Tris	1 gel-18 wells	Bio-Rad, cat. Nr. 3450118
XT-MOPS (running buffer) (20x)	500 mL	Bio-Rad, cat. Nr. 1610788

Immunoblotting

Ethanol (Normapur)	1 L	VWR, cat. Nr. 20821-296
Tris/CAPS (transfer buffer) (10x)	1 L	Bio-Rad, cat. Nr. 1610778
Filter paper (transfer paper for Criterion™ XT precast gels)	50 sheets	Bio-Rad, cat. Nr. 1704085
PVDF membrane (0.2 µm)	10 sheets	Bio-Rad, cat. Nr. 1620175
Tween® 20	100 mL	Bio-Rad, cat. Nr. 1706531
PBS (washing buffer) (10x)	1 L	Bio-Rad, cat. Nr. 1610780
ECL (substrate for conjugate)	125 mL	Amersham, cat. Nr. RPN2109
ECL Hyperfilms (18 x 24 cm)	25 films	Amersham, cat. Nr. RPN2103K
Development folders	30 folders	Applied Biosystems, cat Nr. T2258
Developing solution	-	VWR or Kodak
Fixative solution	-	VWR or Kodak
Blotting Grade Blocker	300 g	Bio-Rad, cat. Nr. 1706404

4.2 - EQUIPMENT

Adjustable pipettes (10, 40, 200, 1000 µL).
Graduated cylinder (1 L and 2 L). plastic forceps, Trays, Vortex®.
Exposure cassette and red light for film development.

Sample purification	
TeSeE™ PRECESS 48™	Bio-Rad, cat. Nr. 3590200
TeSeE™ PRECESS 24™	Bio-Rad, cat. Nr. 3591070
Or Ribolyser®	Bio-Rad, cat. Nr. 3589158
Heating block	Bio-Rad, cat. Nr. 3589046
Heating block adaptor - 20 tubes	Bio-Rad, cat. Nr. 3589199
Centrifuge - 220/240V	Bio-Rad, cat. Nr. 3589190
Drum rotor	Bio-Rad, cat. Nr. 3589189
Rotor adaptors - (x6)	Bio-Rad, cat. Nr. 3589191
Gel electrophoresis	
Criterion™ XT Cell	Bio-Rad, cat. Nr. 1656001
PowerPac HC power supply 100/120V - 220/240V	Bio-Rad, cat. Nr. 1645052
Transfer	
Criterion™ XT blotter	Bio-Rad, cat. Nr. 1704070
Immunoblotting	
Western Processor base	Bio-Rad, cat. Nr. 3590098
Western Processor Criterion™ kit	Bio-Rad, cat. Nr. 1703985

4.3 - REAGENT PREPARATION

4.3.1 - Sample Purification

- **Proteinase K**

PK solutions must be prepared just before use and must not be stored.
HIGH PK solution:
Prepare first the mix A1+A2 by adding 50 µL of buffer A2 per mL of buffer A1 (volume for 4 samples as 250 µL of mix A1+A2 are strictly required per test sample).
Then to obtain the HIGH PK, add 40 µL of proteinase K per mL of mix A1+A2 according to the number of samples being tested - see table below.

Number of samples		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
HIGH PK	**Mix A1+A2**	1 mL	1 mL	1 mL	2 mL	2 mL	2 mL	2 mL	3 mL
	PK	40 µL	40 µL	40 µL	80 µL	80 µL	80 µL	80 µL	120 µL

Mix well by inverting until obtaining an homogenous solution.

LOW PK solution:
The LOW PK is made from a dilution of the HIGH PK.
Prepare first the mix A1+A2 by adding 50 µL of buffer A2 per mL of buffer A1 (volume for 4 samples as 250 µL of mix A1+A2 are strictly required per test sample).
Then to obtain the LOW PK, add 50 µL of HIGH PK per mL of mix A1+A2 according to the number of samples being tested - see table below.

Number of samples		1	2	3	4	5	6	7	8
LOW PK	Mix A1+A2	1 mL	1 mL	1 mL	2 mL	2 mL	2 mL	2 mL	3 mL
	HIGH PK	50 µL	50 µL	50 µL	100 µL	100 µL	100 µL	100 µL	150 µL

Mix well by inverting until obtaining an homogenous solution.

- **Laemmli Solution**

Solution of SDS + 2-Mercaptoethanol + Laemmli sample buffer:
- 0.6 g SDS
- 1.5 mL 2-Mercaptoethanol

Mix by inverting, and then add:
- 28.5 mL Laemmli sample buffer

And mix again.
Solution is aliquoted into 4 mL aliquots and stored at -20°C for 1 year maximum. Thawed aliquots can be re-frozen 3 times maximum. Mix thoroughly when thawed.

Note: It is recommended to prepare Laemmli solution one hour before use allowing SDS to be completely dissolved.

4.3.2 - Electrophoresis

2 Criterion gels will be required (one for the Control Ab condition + one for the Test Ab condition).

- **Kaleidoscope Prestained Standard**

The kaleidoscope prestained standard is prepared during the sample denaturation before loading on the acrylamide gel.
Prepare a 1/12 dilution in Laemmli solution (for example 10 µL of the kaleidoscope prestained standard + 110 µL of Laemmli solution).
Please refer to the kaleidoscope prestained standard insert for storage conditions.

- **MagicMark™ XP Western Standard**

The MagicMark™ XP molecular weight is prepared during the sample denaturation before loading on the acrylamide gel.
Prepare a 1/12 dilution in Laemmli solution (for example 10 µL of MagicMark™ XP + 110 µL of Laemmli solution).
Please refer to MagicMark™ XP insert for storage conditions.

• **Criterion™ XT migration buffer**
(1) Solution of MOPS (1x).
Prepare a 1/20 dilution. **1 L of diluted buffer is required for 1 tank:**
- 950 mL Distilled water
- 50 mL MOPS buffer (20x)

Mix thoroughly. Solution cannot be stored.

4.3.3 - Protein Transfer

• **Transfer buffer**
Solution of Tris/CAPS-Ethanol 15%. **Approximately 2 L is required for 1 transfer tank.**
- 750 mL Distilled water
- 150 mL Pure ethanol
- 100 mL Tris/CAPS (10x)

Mix thoroughly. Solution cannot be stored.

4.3.4 - Immunoblotting

• **Wash solution 1**
Solution of PBS (1x) + 0.1% Tween® 20. **Approximately 1 L is required for the complete process of 1 membrane.**
- 900 mL Distilled water
- 100 mL PBS (10x)
- 1 mL Tween® 20

Mix thoroughly. Solution can be stored at +2°C to +8°C, overnight.

• **Wash solution 2**
Solution of PBS (1x). **Approximately 200 mL is required for the complete process of 1 membrane.**
- 900 mL Distilled water
- 100 mL PBS (10x)

Mix thoroughly
Solution can be stored at room temperature (+18°C to +30°C) overnight.

• **Control blocking solution**
During the transfer step, dilute the blocking solution (BI) 1/10 in Wash solution 1.
40 mL of diluted blocking solution is required for 1 membrane.
- 36 mL Wash solution 1
- 4 mL Blocking solution (10x)

Mix by tube inverting.

- **Test blocking solution**

40 mL of Blotting Grade Blocker 5% is required for 1 membrane.

- 2.5 g Blotting Grade Blocker
- to 50 mL wash solution 1

Mix by tube inverting.
This solution must be prepared 30 minutes minimum before use

- **Diluted primary antibodies (Control and Test antibodies)**

Just prior to use, dilute each primary antibody 1/10 in Wash solution 1.
30 mL of diluted primary antibody is required for 1 membrane.

- 27 mL Wash solution 1
- 3 mL Primary antibody (10x)

Mix by tube inverting.

- **Diluted secondary antibody (conjugate)**

Just prior to use, dilute the secondary antibody 1/10 in Wash solution 1.
40 mL of diluted conjugate is required for 1 membrane.

- 36 mL Wash solution 1
- 4 mL Secondary antibody (10x)

Mix by tube inverting.

- **Substrate (ECL)**

Substrate (ECL) must be prepared just prior to use.
2 mL of substrate is required for 1 membrane.

- 1 mL Reagent 1
- 1 mL Reagent 2

Mix the solution.

- **Developing solution**
  - 800 mL Distilled water
  - 200 mL Development product

Mix the solution
Dilution ratios may vary from one manufacturer to another. Please follow manufacturer kit instructions when preparing the development solution.
Solution can be stored at room temperature (+18°C to +30°C), in a darkroom for 15 days maximum.

- **Fixative solution**
  - 800 mL Distilled water
  - 200 mL Fixative product

Mix the solution
Dilution ratios may vary from one manufacturer to another. Please follow manufacturer kit instructions when preparing the development solution.

Solution can be stored at room temperature (+18°C to +30°C), in a darkroom for 15 days maximum.

4.4 - ASSAY PROCEDURE WITH CRITERION™ XT GELS

4.4.1 - Sample Purification

• Sampling

Take a mass of 350 mg ± 40 mg of nervous tissue (preferably in the obex area). Deposit the sample in grinding tube, close firmly and proceed to the grinding step in the homogenizer (Ribolyser®, TeSeE™ PRECESS 24™ or TeSeE™ PRECESS 48™ - system).

• Sample grinding

Place the tubes in the crown of the homogenizer.
Perform one agitation cycle with the following instrument parameters.

	Ribolyser®	TeSeE™ PRECESS 48™ or TeSeE™ PRECESS 24™
	Nervous tissues	Nervous tissues
Time (sec.)	45	-
Speed	6.5	-
Program	-	Program 1

When grinding is insufficient, another 1 or 2 agitation cycles can be performed. Wait a 5 minutes pause between the 2 agitation cycles.

• Sample calibration

Remove the grinding tubes from the homogenizer, resuspend the homogenate by inverting before opening the tubes.

Note: For samples with a saturating OD signal with the Bio-Rad TeSeE™ SAP ELISA test (OD ≥ 2.500), dilute 1 volume of sample homogenate in 9 volumes of a 20% healthy brain homogenate confirmed negative by ELISA.

For samples presenting a non-saturating OD signal (OD < 2.500), test is performed on undiluted sample homogenate.

If OD ≤ 0.4, the results may not be accurate. Please refer to the EURL in case of doubt.

Transfer 250 µL of diluted or undiluted homogenate in two micro test-tubes (250 µL each) with the calibration syringe, taking care to immerse the needle below the level of ceramic beads to avoid poorly homogenized tissue fragments. Obtained homogenate is 20% weight/volume.

- **Proteinase K treatment**

Distribute 250 µL of LOW PK or HIGH PK (see paragraph 4.3.1) in the two micro test-tubes of each sample respectively.
Mix closed tubes by inversion (10 times).
Incubate at 37°C ± 2°C in a heating block incubator for 30 minutes.

- **Precipitation of PrPres with reagent B**

Remove the tubes from the incubator. Open them and distribute 250 µL of reagent B into each tube.
Mix by successive inversions until a transparent homogenous blue-green colour is obtained.

- **Concentration of the PrPres by centrifugation**

Centrifuge the tubes for 7 minutes at 15 000 g at 20°C.

- **Sample clarifying**

Discard the supernatant by inverting over a waste container. Then dry the tubes by inverting onto an absorbent paper for 5 minutes.
Distribute 50 µL of the Laemmli solution (see paragraph 4.3.1) into each micro test-tube.
Incubate 5 minutes at room temperature (+18°C to +30°C).
Completely resolubilize the pellet by aspiration/dispensing with a pipette.
Pierce the cap of each micro test-tube.
Incubate for 5 minutes at 100°C ± 5°C in a heating block incubator.
Remove the micro test-tubes from the incubator, homogenize with a Vortex®.
Centrifuge the micro test-tubes for 15 minutes at 15,000 g at 20°C.
Transfer the supernatant (=purified sample) to a new micro test-tube. Discard the tube containing the pellet.

Note: at this stage, the supernatant can be stored frozen at -20°C for up to 1 month if needed. The samples must be thawed at room temperature (+18°C to +30°C) prior to use.

4.4.2 - Calibration of the Sample Loading (Dot Blot)

The determination of the most appropriate sample dilution (=working dilution) is performed with the CONTROL antibody.

- **Sample loading**

Incubate each micro test-tube for 4 minutes at 100°C in a heating block incubator (if samples were frozen).
Prepare two-fold serial dilutions (Neat ; 1:2 ; 1:4 ; 1:8... to 1:128) of LOW and HIGH purified samples in Laemmli solution (see paragraph 4.3.1).

Prepare the 0.2 µm PVDF membrane. Soak it in ethanol for 10 seconds, and immerse it in distilled water for 5 minutes.
Eliminate distilled water, lay the membrane on a wet paper filter. Load onto the membrane 2 µL of each dilution starting from the neat sample. LOW and HIGH conditions are to be loaded nearby for each dilutions (see Fig. 1). Allow for drying for 5 minutes.

• **Immunoblotting**
Soak the PVDF membrane into diluted Control blocking solution (see paragraph 4.3.4) and incubate the membrane for 30 minutes under medium agitation.
40 mL of diluted Control blocking solution is required for 1 membrane.

Note: from this step, the Bio-Rad Western Processor can be used for agitation and washing steps (refer to instruction manual for settings).

Eliminate the Control blocking solution and incubate the membrane in diluted Control antibody (see paragraph 4.3.4) for 30 minutes at room temperature (+18°C to +30°C) under medium agitation.
30 mL of diluted Control antibody is required for 1 membrane.

Eliminate the Control antibody solution then briefly rinse the membrane with Wash solution 1, then wash twice for respectively 5 and 10 minutes, under fast agitation.
100 mL of Wash solution 1 is required for each cycle and for 1 membrane.

Eliminate Wash solution 1 and incubate the membrane for 20 minutes in diluted secondary antibody (see paragraph 4.3.4) at room temperature (+18°C to +30°C) under medium agitation.
40 mL of diluted secondary antibody is required for 1 membrane.

Eliminate the secondary antibody solution then briefly rinse the membrane with Wash solution 1, then wash for respectively 5, 10 and 10 minutes under fast agitation.
100 mL of Wash solution 1 is required for each cycle and for 1 membrane.

Eliminate Wash solution 1 and place the membrane in 100 mL of Wash solution 2 under slow agitation for 5 minutes minimum.
Drain the membrane on absorbent paper without blotting and place it in the plastic folder.

Add the substrate reagent (ECL - see paragraph 4.3.4). Eliminate the excess of reagent and air bubbles with absorbent paper. Place into the exposure cassette.
In a darkroom, cover the folder with a film and expose for 15 minutes. Film can be exposed longer or shorter time for optimal signal.
Immerse the film in developing solution for 45 seconds (see paragraph 4.3.4).
Rinse in distilled water. Immerse the film in fixative solution until the film becomes completely transparent.
Wash with distilled water and let the film dry.

- **Dot blot interpretation**

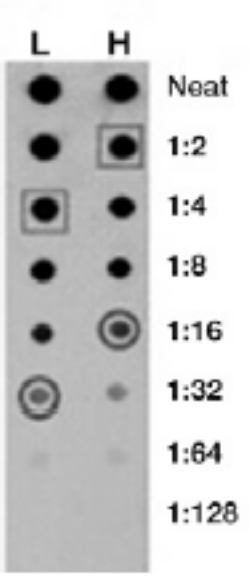


Fig. 1: Dot Blot

For each sample and for each PK condition (LOW and HIGH):
- determine the appropriate sample dilution corresponding to the first non-saturating signal intensity – see fig. 1: circles.
- count 3 dilutions above the selected dilution for each purification condition to select the most appropriate sample dilution ("Working dilution") – see Fig. 1: squares
- Check that the two spots (LOW ; HIGH) selected have comparable intensities.

For the Discriminatory Western Blot, samples must be tested according to the "Working dilution" determined above.

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Note:
- *Whatever the PK condition, when only the first sample dilution (neat) shows a signal, the working dilution to be used for the Discriminatory Western Blot is neat.*
- *If for a PK condition, all the spots produce a saturating signalwhatever the dilution, then do the dot blot again using a higher sample dilution (a 10-fold higher dilution is recommended in first intention)*
- *When a sample shows no signal for both LOW and HIGH PK conditions, sample purification must be repeated on neat samples.*

4.4.3 - Discriminatory Western Blot

Purified samples (LOW and HIGH PK conditions) have to be detected in parallel with two different monoclonal antibodies, *i.e.* TEST antibody directed against the octapeptide part of the PrP protein, and the CONTROL antibody directed against the core region of the PrP protein.

• Gel preparation

Place the Criterion™ XT gels (see paragraph 4.3.2) in the migration tank. Pour the migration buffer (see paragraph 4.3.2) into the tank on each side of the gels, up to the top of the wells. Carefully remove the combs and rinse each well with migration buffer, using a pipette.

• Sample loading

Incubate each tube (LOW and HIGH purified samples at the appropriate dilution previously determined) for 4 minutes at 100°C in a heating block incubator just before loading 15 µL per well on both CONTROL and TEST acrylamide gels. LOW and HIGH purifications for each sample have to be loaded nearby.
Load 15 µL of the diluted kaleidoscope prestained standard and 15 µL of the diluted MagicMark™ XP (see paragraph 4.3.2).

Example of loading for 8 samples:

First group of 2 gels (CONTROL + TEST)

LANE	1	2	3	4	5	6	7	8	9	10
Sample	K	Sample #1		Sample #2		Sample #3		Sample #4		MM
		LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	

Second group of 2 gels (CONTROL + TEST)

LANE	1	2	3	4	5	6	7	8	9	10
Sample	K	Sample #5		Sample #6		MM	Sample #7		Sample #8	
		LOW	HIGH	LOW	HIGH		LOW	HIGH	LOW	HIGH

K: Kaleidoscope ; MM: Magic Mark.

Note: In case several gels are run at the same time, make sure that you stagger the loading of markers (MM and K) into different lanes for easy identification.

- **Differential migration of the samples**

Run the gels at room temperature (+18°C to +30°C) for 50 minutes at 200 V.
The blue line must be out of the gel.

- **Protein Transfer Preparation**

Cut the membranes to the gel dimensions. Always use forceps when handling the membranes.
Immerse the membranes in pure ethanol for 15 seconds, rinse in distilled water for 5 minutes, then for 10 minutes in the transfer buffer.
Carefully remove the Criterion XT gel and let it equilibrate for 10 minutes in the transfer buffer.

- **Gel Sandwich Preparation**

Soak filter paper and fibre pads in the transfer buffer.
Open the transfer cassette, with red side on the left. Respectively place on the red side a fibre pad, a filter paper, the membrane* and the gel*.
Complete with a filter paper then a fibre pad and close the cassette.
Immerse it in the transfer tank previously filled to the indicated limit with transfer buffer.
*Remove any air bubbles which may have formed.

Note: In case several membranes are processed at the same time, label each membrane in the corner.

• **Transfer onto the PVDF membrane**

Agitate during the transfer by using a magnetic stirring bar and run for 60 minutes at 115 V.

• **Immunoblotting**

Western immunoblotting is performed according to the manufacturer's conditions, using the antibodies provided with the Discriminatory kit: Ab Ctrl for the CONTROL conditions, Ab Test for the TEST conditions, and Ab II for both CONTROL and TEST conditions.

a) Upon completion of the protein transfer, open the blotting assembly and remove the membrane for development. Quickly immerse the membrane in Wash solution 2 (see paragraph 4.3.4), then place it in ethanol for 10 seconds before rinsing for 5 minutes in distilled water.

Note: At this step, the membranes can be stored overnight in distilled water at +2°C to +8°C.
Let the membranes adjust to room temperature (+18°C to +30°C) before to start the immunoblotting.

b) Eliminate distilled water.

c) Incubate the membranes for 30 minutes in Control or Test blocking solution depending on the condition (see paragraph 4.3.4). Incubate under medium agitation.
40 mL diluted Control or Test blocking solution is required for 1 membrane.

Note: from this step, the Bio-Rad Western Processor can be used for agitation and washing steps (refer to instruction manual for settings).

d) Eliminate the blocking solutions. Incubate the first membrane in diluted Control antibody (see paragraph 4.3.4) and the second membrane in diluted Test antibody (see paragraph 4.3.4) for 30 minutes at room temperature (+18°C to +30°C) under medium agitation.
30 mL of diluted primary antibody is required for each membrane.

e) Eliminate the Control and Test antibody solutions. Briefly rinse the membranes with Wash solution 1, then wash twice with wash solution 1 for respectively 5 and 10 minutes, under fast agitation.
100 mL of Wash solution 1 is required for each cycle and for 1 membrane.

f) Eliminate Wash solution 1 and incubate the membranes for 20 minutes in diluted secondary antibody (see paragraph 4.3.4) at room temperature (+18°C to +30°C) under medium agitation.
40 mL of diluted secondary antibody is required for 1 membrane.

g) Eliminate the secondary antibody solution. Briefly rinse the membranes with Wash solution 1, then wash for respectively 5, 10 and 10 minutes under fast agitation.
100 mL of Wash solution 1 is required for each cycle and for 1 membrane.

h) Eliminate the Wash solution 1 and place the membranes in 100 mL of Wash solution 2 under slow agitation for 5 minutes minimum.

i) Drain the membranes on absorbent paper without blotting and place it in a plastic folder.

j) Add the substrate reagent (ECL - see paragraph 4.3.4). Eliminate the excess of reagent and air bubbles with absorbent paper. Place the folder into one exposure cassette.

k) In a darkroom, cover the folders with a film and expose for 15 minutes. Film can be exposed longer or shorter time for optimal signal.

Note: Time for exposure is depending on the system used in the lab. Please check that the Magic Mark marker are clearly seen before stopping exposure.

l) Immerse the film in developing solution for 45 seconds (see paragraph 4.3.4). Rinse in distilled water. Immerse the film in fixative solution until the film becomes completely transparent.

m) Wash with distilled water and let the film dry.

5 - REAGENTS AND MATERIAL REQUIRED FOR MINI BLOT™ GEL FORMAT

5.1 - REAGENTS AND DISPOSABLES

Graduated pipettes (5, 10, 25 mL), conical tubes (50 mL), 2 mL polypropylene micro-test tubes with caps.
PARAFILM®M Sealing films.

Sample purification

Laemmli sample buffer	30 mL	Bio-Rad, cat. Nr. 1610737
2-Mercaptoethanol	25 mL	Bio-Rad, cat. Nr. 1610710
SDS	100 g	Bio-Rad, cat. Nr. 1610301
Calibration syringes	x 200	Bio-Rad, cat. Nr. 3551174

Gel electrophoresis

Kaleidoscope prestained standard	500 µL	Bio-Rad, cat. Nr. 1610324
MagicMark™ XP Western Standard (Molecular weight standard)	250 µL	Invitrogen, cat. Nr. LC5602
Acrylamide 40% 29:1	500 mL	Bio-Rad, cat. Nr. 1610146
0.5 M Tris-HCl pH 6.8	1 L	Bio-Rad, cat. Nr. 1610799
1.5 M Tris-HCl pH 8.8	1 L	Bio-Rad, cat. Nr. 1610798
Bromophenol blue	10 g	Bio-Rad, cat. Nr. 1610404
Sucrose	1 kg	Bio-Rad, cat. Nr. 1610720
Ammonium persulfate	10 g	Bio-Rad, cat. Nr. 1610700
TEMED	5 mL	Bio-Rad, cat. Nr. 1610800
Tris/Glycine/SDS running buffer (10x)	1 L	Bio-Rad, cat. Nr. 1610732

Immunoblotting

Ethanol (Normapur)	1 L	VWR, cat. Nr. 20821-296
Tris/CAPS (transfer buffer) (10x)	1 L	Bio-Rad, cat. Nr. 1610778
Filter paper (transfer paper for Mini Blot™ handcast gels)	50 sheets	Bio-Rad, cat. Nr. 1703932
PVDF membrane (0.2 µm)	10 sheets	Bio-Rad, cat. Nr. 1620175
Tween® 20	100 mL	Bio-Rad, cat. Nr. 1706531
PBS (washing buffer) (10x)	1 L	Bio-Rad, cat. Nr. 1610780
ECL (substrate for conjugate)	125 mL	Amersham, cat. Nr. RPN2109
ECL Hyperfilms (18 x 24 cm)	25 films	Amersham, cat. Nr. RPN2103K
Development folders	30 folders	Applied Biosystems, cat Nr. T2258
Developing solution	-	VWR or Kodak
Fixative solution	-	VWR or Kodak
Blotting Grade Blocker	300 g	Bio-Rad, cat. Nr. 1706404

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5.2 - EQUIPMENT

Adjustable pipettes (10, 40, 200, 1000 µL).
Graduated cylinder (1L and 2L), plastic forceps, Trays, Vortex®.
Exposure cassette and red light for film development.

Sample purification	
TeSeE™ PRECESS 48™	Bio-Rad, cat. Nr. 3590200
TeSeE™ PRECESS 24™	Bio-Rad, cat. Nr. 3591070
Or Ribolyser®	Bio-Rad, cat. Nr. 3589158
Heating block	Bio-Rad, cat. Nr. 3589046
Heating block adaptor - 20 tubes	Bio-Rad, cat. Nr. 3589199
Centrifuge - 220/240V	Bio-Rad, cat. Nr. 3589190
Drum rotor	Bio-Rad, cat. Nr. 3589189
Rotor adaptors - (x6)	Bio-Rad, cat. Nr. 3589191
Gel electrophoresis	
Mini-PROTEAN® Tetra Cell, electrophoresis module	Bio-Rad, cat. Nr. 1658002
5 spacers plates	Bio-Rad, cat. Nr. 1653312
PowerPac HC power supply 100/120V - 220/240V	Bio-Rad, cat. Nr. 1645052
Transfer	
Trans-Blot® Cell	Bio-Rad, cat. Nr. 1703946
Immunoblotting	
Western Processor base	Bio-Rad, cat. Nr. 3590098
Western Processor Mini Blot™ kit	Bio-Rad, cat. Nr. 1703988

5.3 - REAGENT PREPARATION

5.3.1 - Sample Purification

- **Proteinase K**

PK solutions must be prepared just before use and must not be stored.
HIGH PK solution:
Prepare first the mix A1+A2 by adding 50 µL of buffer A2 per mL of buffer A1 (volume for 4 samples as 250 µL of mix A1+A2 are strictly required per test sample).
Then to obtain the HIGH PK, add 40 µL of proteinase K per mL of mix A1+A2 according to the number of samples being tested - see table below.

Number of samples		1	2	3	4	5	6	7	8
HIGH PK	Mix A1+A2	1 mL	1 mL	1 mL	2 mL	2 mL	2 mL	2 mL	3 mL
	PK	40 µL	40 µL	40 µL	80 µL	80 µL	80 µL	80 µL	120 µL

Mix well by inverting until obtaining an homogenous solution.

LOW PK solution:
The LOW PK is made from a dilution of the HIGH PK.
Prepare first the mix A1+A2 by adding 50 µL of buffer A2 per mL of buffer A1 (volume for 4 samples as 250 µL of mix A1+A2 are strictly required per test sample).
Then to obtain the LOW PK, add 50 µL of HIGH PK per mL of mix A1+A2 according to the number of samples being tested - see table below.

Number of samples		1	2	3	4	5	6	7	8
LOW PK	Mix A1+A2	1 mL	1 mL	1 mL	2 mL	2 mL	2 mL	2 mL	3 mL
	HIGH PK	50 µL	50 µL	50 µL	100 µL	100 µL	100 µL	100 µL	15 µL

Mix well by inverting until obtaining an homogenous solution.

- **Laemmli solution**

Solution of SDS + 2-Mercaptoethanol + Laemmli sample buffer:
- 0.6 g SDS
- 1.5 mL 2-Mercaptoethanol

Mix by inverting, and then add:
- 28.5 mL Laemmli sample buffer

And mix again.
Solution is aliquoted into 4 mL aliquots and stored at -20°C for 1 year maximum. Thawed aliquots can be re-frozen 3 times maximum. Mix thoroughly when thawed.

Note: It is recommended to prepare Laemmli solution one hour before use allowing SDS to be completely dissolved.

5.3.2 - Electrophoresis

- **Hand cast discontinuous acrylamide gel**

Just before use, prepare two gels (one for the Control Ab condition + one for the Test Ab condition).
The gels must be 1.5 mm thickness.
Using the Mini Blot™ casting module, the resolving gel (13.5% acrylamide, pH 8.8) is cast first, once the resolving gel is polymerized the stacking gel is added (3% acrylamide, pH 6.8).

Resolving gel (volumes are indicated for one gel)
- 2.8 mL Acrylamide 40%, 29:1
- 1.7 mL 1.5 M Tris-HCl buffer, pH 8.8 / SDS (1)
- 1.3 mL 50% sucrose solution (2)
- 2.5 mL distilled water

Mix by inverting, and at the last time add:
- 43 µL 10% Ammonium persulfate (3)
- 9 µL TEMED

Mix again.

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Pour 7 mL of the gel solution into the plates and retain the residual solution as a control of polymerization. Gently overlay to the top with 1 mL of 0.3 M Tris-HCl pH 8.8 / SDS buffer (4) so that the gel surface doesn't dry out.
Let the gel polymerize for 15-20 minutes at room temperature (+18°C to +30°C). Check the residual solution is polymerized.
Invert the plate assembly to eliminate excess of buffer.

Stacking gel (volumes are indicated for one gel)

- 4 mL 3% Acrylamide solution (7)
- 28 µL 10% Ammonium persulfate (3)
- 6 µL TEMED

Mix by inverting.
Gently pour the stacking gel onto the resolving gel and retain the residual solution as a control of polymerization. Position the comb, taking care not to trap any bubble in the well positions.
Let the gel polymerize for 5-10 minutes at room temperature (+18°C to +30°C).
Check the residual solution is polymerized.

(1) Solution of 1.5 M Tris-HCl buffer, pH 8.8/SDS

- 0.2 g SDS
- 50 mL 1.5 M Tris-HCl buffer pH 8.8

Mix by inverting.
Solution can be stored at +2°C to +8°C for 2 weeks.

(2) Solution of 50% Sucrose

- 25 g Sucrose
- to 50 mL Distilled water

Mix by inverting.
Sucrose solution can be stored at +2°C to +8°C for 2 weeks.

(3) Solution of 10% Ammonium persulfate

- 5 g Ammonium persulfate
- to 50 mL Distilled water

Mix by inverting.
Ammonium persulfate solution is aliquoted and stored at -20°C. Thawed solution can be stored at +2°C to +8°C for 2 weeks.

(4) Solution of 0.3 M Tris-HCl buffer, pH 8.8/SDS

- 40 mL Distilled water
- 10 mL 1.5 M Tris-HCl buffer pH 8.8 / SDS (1)

Mix by inverting.
Solution can be stored at +2°C to +8°C for 2 weeks.

(5) Solution of 0.5 M Tris-HCl buffer, pH 6.8/SDS

- 0.2 g SDS
- 50 mL 0.5 M Tris-HCl buffer pH 6.8

Mix by inverting.
Solution can be stored at +2°C to +8°C for 2 weeks.

(6) Solution of 1% Bromophenol Blue

- 0.5 g Bromophenol Blue
- 50 mL Distilled water

Mix by inverting.
Bromophenol Blue solution can be stored at room temperature (+18°C to +30°C) for 6 months.

(7) Solution of 3% acrylamide

- 3.8 mL Acrylamide 40%, 29:1
- 10 mL 0.5 M Tris-HCl buffer pH 6.8 / SDS (5)
- 6 mL Sucrose 50% (2)
- 500 µL Bromophenol Blue 1% (6)
- to 50 mL Distilled water

Mix by inverting.
Solution can be stored at +2°C to +8°C for 2 weeks.

- **Kaleidoscope prestained standard**

The kaleidoscope prestained standard is prepared during the sample denaturation before loading on the acrylamide gel.
Prepare a 1/12 dilution in Laemmli solution (for example 10 µL of the kaleidoscope prestained standard + 110 µL of Laemmli solution).
Please refer to the kaleidoscope prestained standard insert for storage conditions.

- **MagicMark™ XP Western Standard**

The MagicMark™ XP molecular weight is prepared during the sample denaturation before loading on the acrylamide gel.
Prepare a 1/12 dilution in Laemmli solution (for example 10 µL of MagicMark™XP + 110 µL of Laemmli solution).
Please refer to MagicMark™ XP insert for storage conditions.

- **Mini Blot™ migration buffer**

Solution of Tris-Glycine-SDS (1x).
Prepare a 1/10 dilution. **1 L of diluted buffer is required for 1 tank**:

- 900 mL Distilled water
- 100 mL Tris-Glycine-SDS buffer (10x)

Mix thoroughly. Solution cannot be stored.

5.3.3 - Protein Transfer

• Transfer buffer

Solution of Tris/CAPS-Ethanol 15%. **Approximately 2.5 L is required for 1 transfer tank.**

- 750 mL Distilled water
- 150 mL Pure ethanol
- 100 mL Tris/CAPS (10x)

Mix thoroughly. Solution cannot be stored.

5.3.4 - Immunoblotting

• Wash solution 1

Solution of PBS (1x) + 0.1% Tween® 20. **Approximately 500 mL is required for the complete process of 1 membrane.**

- 900 mL Distilled water
- 100 mL PBS (10x)
- 1 mL Tween® 20

Mix thoroughly.

Solution can be stored at +2°C to +8°C, overnight.

• Wash solution 2

Solution of PBS (1x). **Approximately 100 mL is required for the complete process of 1 membrane.**

- 900 mL Distilled water
- 100 mL PBS (10x)

Mix thoroughly

Solution can be stored at room temperature (+18°C to +30°C) overnight.

• Control blocking solution

During the transfer step, dilute the blocking solution (Bl) 1/10 in Wash solution 1.

20 mL of diluted blocking solution (1x) is required for 1 membrane.

- 18 mL Wash solution 1
- 2 mL Blocking solution (10x)

Mix by tube inverting.

• Test blocking solution

20 mL of Blotting Grade Blocker 5% is required for 1 membrane.

- 1.25 g Blotting Grade Blocker
- to 25 mL Wash solution 1

Mix by tube inverting.

This solution must be prepared 30 minutes minimum before use.

• **Diluted primary antibodies (Control and Test antibodies)**
Just prior to use, dilute each primary antibody 1/10 in Wash solution 1.
15 mL of diluted primary antibody is required for 1 membrane.
- 13.5 mL Wash solution 1
- 1.5 mL Primary antibody (10x)

Mix by tube inverting.

• **Diluted secondary antibody (conjugate)**
Just before use, dilute the secondary antibody 1/10 in Wash solution 1.
20 mL of diluted conjugate is required for 1 membrane.
- 18 mL Wash solution 1
- 2 mL Secondary antibody (10x)

Mix by tube inverting.

• **Substrate (ECL)**
Substrate (ECL) must be prepared just before use.
1 mL of substrate is required for 1 membrane.
- 0.5 mL Reagent 1
- 0.5 mL Reagent 2

Mix the solution.

• **Developing solution**
- 800 mL Distilled water
- 200 mL Development product

Mix the solution
Dilution ratios may vary from one manufacturer to another. Please follow manufacturer kit instructions when preparing the development solution.
Solution can be stored at room temperature (+18°C to +30°C), in a darkroom for 15 days maximum.

• **Fixative solution**
- 800 mL Distilled water
- 200 mL Fixative product

Mix the solution
Dilution ratios may vary from one manufacturer to another. Please follow manufacturer kit instructions when preparing the development solution.
Solution can be stored at room temperature (+18°C to +30°C), in a darkroom for 15 days maximum.

5.4 - ASSAY PROCEDURE WITH MINI BLOT GELS

5.4.1 - Sample Purification

- **Sampling**

Take a mass of 350 mg ± 40 mg of nervous tissue (preferably in the obex area). Deposit the sample in grinding tube, close firmly and proceed to the grinding step in the homogenizer (Ribolyser®, TeSeE™ PRECESS 24™ or TeSeE™ PRECESS 48™ - system).

- **Sample grinding**

Place the tubes in the crown of the homogenizer.
Perform one agitation cycle with the following instrument parameters.

	Ribolyser®	TeSeE™ PRECESS 48™or TeSeE™ PRECESS 24™
	Nervous tissues	Nervous tissues
Time (sec.)	45	-
Speed	6.5	-
Program	-	Program 1

When grinding is insufficient, another 1 or 2 agitation cycles can be performed. Wait a 5 minutes pause between the 2 agitation cycles.

- **Sample calibration**

Remove the grinding tubes from the homogenizer, resuspend the mix by inverting before opening the tubes.

Note: For samples with a saturating OD signal with the Bio-Rad TeSeE™ SAP ELISA test (OD ≥ 2.500), dilute 1 volume of sample homogenate in 9 volumes of a 20% healthy brain homogenate confirmed negative by ELISA.

For samples presenting a non-saturating OD signal (OD < 2.500), test is performed on undiluted sample homogenate.

If OD ≤ 0.4, the results may not be accurate. Please refer to the EURL in case of doubt.

Transfer 250 µL of diluted or undiluted homogenate in two micro test-tubes (250 µL each) with the calibration syringe, taking care to immerse the needle below the level of ceramic beads to avoid poorly homogenized tissue fragments. Obtained homogenate is 20% weight/volume.

- **Proteinase K treatment**

Distribute 250 µL of LOW PK or HIGH PK (see paragraph 5.3.1) in the two micro test-tubes of each sample respectively.
Mix closed tubes by inversion (10 times).
Incubate at 37°C ± 2°C in a heating block incubator for 30 minutes.

• **Precipitation of PrPres with reagent B**

Remove the tubes from the incubator. Open them and distribute 250 µL of reagent B into each tube.
Mix by successive inversions until a transparent homogenous blue-green colour is obtained.

• **Concentration of the PrPres by centrifugation**

Centrifuge the tubes for 7 minutes at 15 000 g at 20°C.

• **Sample clarifying**

Discard the supernatant by inverting over a waste container. Then dry the tubes by inverting onto an absorbent paper for 5 minutes.
Distribute 50 µL of the Laemmli solution (see paragraph 5.3.1) into each micro test-tube.
Incubate 5 minutes at room temperature (+18°C to +30°C).
Completely resolubilize the pellet by aspiration/dispensing with a pipette.
Pierce the cap of each micro test-tube.
Incubate for 5 minutes at 100°C ± 5°C in a heating block incubator.
Remove the micro test-tubes from the incubator, homogenize with a Vortex®.
Centrifuge the micro test-tubes for 15 minutes at 15,000 g at 20°C.
Transfer the supernatant (=purified sample) to a new micro test-tube. Discard the tube containing the pellet.

Note: at this stage, the supernatant can be stored frozen at -20°C for up to 1 month if needed. The samples must be thawed at room temperature (+18°C to +30°C) prior to use.

5.4.2 - Calibration of the Sample Loading (Dot Blot)

The determination of the most appropriate sample dilution (=working dilution) is performed with the CONTROL antibody.

• **Sample loading**

Incubate each micro test-tube for 4 minutes at 100°C in a heating block incubator (if samples were frozen).
Prepare two-fold serial dilutions (Neat ; 1:2 ; 1:4 ; 1:8... to 1:128) of LOW and HIGH purified samples in Laemmli solution (see paragraph 5.3.1).
Prepare the 0.2 µm PVDF membrane. Soak it in ethanol for 10 seconds, and immerse it in distilled water for 5 minutes.

Eliminate distilled water, lay the membrane on a wet paper filter. Load onto the membrane 2 µL of each dilution starting from the neat sample. LOW and HIGH conditions are to be loaded nearby for each dilutions (see Fig. 1). Allow for drying for 5 minutes.

- **Immunoblotting**

Soak the PVDF membrane into diluted control blocking solution (see paragraph 4.3.4) and incubate the membrane for 30 minutes under medium agitation.
40 mL of diluted control blocking solution is required for 1 membrane.

Note: from this step, the Bio-Rad Western Processor can be used for agitation and washing steps (refer to instruction manual for settings).

Eliminate the blocking solution and incubate the membrane in diluted control antibody (see paragraph 4.3.4) for 30 minutes at room temperature (+18°C to +30°C) under medium agitation.
30 mL of diluted control antibody is required for 1 membrane.

Eliminate the control antibody solution then briefly rinse the membrane with Wash solution 1. Wash again twice with Wash solution 1, for respectively 5 and 10 minutes, under fast agitation.
100 mL of Wash solution 1 are required for each cycle and for 1 membrane.

Eliminate Wash solution 1 and incubate the membrane for 20 minutes in diluted secondary antibody (see paragraph 4.3.4) at room temperature (+18°C to +30°C) under medium agitation.
40 mL of diluted secondary antibody are required for 1 membrane.

Eliminate the secondary antibody solution then briefly rinse the membrane with Wash solution 1, then wash for respectively 5, 10 and 10 minutes under fast agitation.
100 mL of Wash solution 1 are required for each cycle and for 1 membrane.

Eliminate Wash solution 1 and place the membrane in 100 mL of Wash solution 2 under slow agitation for 5 minutes minimum.
Drain the membrane on absorbent paper without blotting and place it in the plastic folder.

Add the substrate reagent (ECL - see paragraph 4.3.4). Eliminate the excess of reagent and air bubbles with absorbent paper. Place into the exposure cassette.
In a darkroom, cover the folder with a film and expose for 15 minutes. Film can be exposed longer or shorter time for optimal signal.
Immerse the film in developing solution for 45 seconds (see paragraph 4.3.4).
Rinse in distilled water. Immerse the film in fixative solution until the film becomes completely transparent.
Wash with distilled water and let the film dry.

- **Dot blot interpretation**

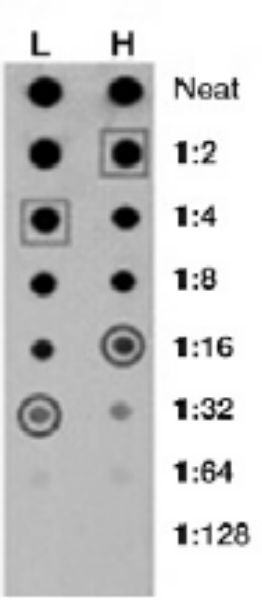


Fig. 1: Dot Blot

For each sample and for each PK condition (LOW and HIGH):
- determine the appropriate sample dilution corresponding to the first non-saturating signal intensity – see fig. 1: circles.
- count 3 dilutions above the selected dilution for each purification condition to select the most appropriate sample dilution ("Working dilution") – see Fig. 1: squares
- Check that the two spots (LOW ; HIGH) selected have comparable intensities.

For the Discriminatory Western Blot, samples must be tested according to the "Working dilution" determined above.

Note:
- *Whatever the PK condition, when only the first sample dilution (neat) shows a signal, the working dilution to be used for the Discriminatory Western Blot is neat.*
- *If for a PK condition, all the spots produce a saturating signal whatever the dilution, then do the dot blot again using a higher sample dilution (a 10-fold higher dilution is recommended in first intention)*
- *When a sample shows no signal for both LOW and HIGH PK conditions, sample purification must be repeated on neat samples.*

5.4.3 - Discriminatory Western Blot

Purified samples (LOW and HIGH PK conditions) have to be detected in parallel with two different monoclonal antibodies, *i.e.* TEST antibody directed against the octapeptide part of the PrP protein, and the CONTROL antibody directed against the core region of the PrP protein.

• Gel preparation

Place the acrylamide gels (see paragraph 5.3.2) in the migration tank. Pour the migration buffer (see paragraph 5.3.2) into the tank on each side of the gels, up to the top of the wells. Carefully remove the combs and rinse each well with migration buffer, using a pipette.

• Sample loading

Incubate each tube (LOW and HIGH purified samples at the appropriate dilution previously determined), for 4 minutes at 100°C in a heating block incubator just before loading 15 µL per well on both CONTROL and TEST acrylamide gels. LOW and HIGH purifications for each sample have to be loaded nearby.
Load 15 µL of the diluted kaleidoscope prestained standard and 15 µL of the diluted MagicMark™ XP (see paragraph 5.3.2).

Example of loading for 8 samples:

First group of 2 gels (CONTROL + TEST)

LANE	1	2	3	4	5	6	7	8	9	10
Sample	K	Sample #1		Sample #2		Sample #3		Sample #4		MM
		LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	

Second group of 2 gels (CONTROL + TEST)

LANE	1	2	3	4	5	6	7	8	9	10
Sample	K	Sample #5		Sample #6		MM	Sample #7		Sample #8	
		LOW	HIGH	LOW	HIGH		LOW	HIGH	LOW	HIGH

K: Kaleidoscope ; MM: Magic Mark.

Note: In case several gels are run at the same time, make sure that you stagger the loading of markers (MM and K) into different lanes for easy identification.

- **Differential migration of the samples**

Run the gels at room temperature (+18°C to +30°C) for 90 minutes at 150 V.
The blue line must be out of the gel.

- **Protein transfer preparation**

Cut the membranes to the gel dimensions. Always use forceps when handling the membranes.
Immerse the membranes in pure ethanol for 15 seconds, rinse in distilled water for 5 minutes, then for 10 minutes in the transfer buffer.
Carefully remove the gel from the glass plates and let it equilibrate for 10 minutes in the transfer buffer.

- **Gel sandwich preparation**

Soak filter paper and fibre pads in the transfer buffer.
Open the transfer cassette, with red side on the left. Respectively place on the red side a fibre pad, a filter paper, the membrane* and the gel*.
Complete with a filter paper then a fibre pad and close the cassette.
Immerse it in the transfer tank previously filled to the indicated limit with transfer buffer.
**Remove any air bubbles which may have formed.*

Note: In case several membranes are processed at the same time, label each membrane in the corner.

- **Transfer onto the PVDF membrane**

Agitate during the transfer by using a magnetic stirring bar and run for 60 minutes at 115 V.

- **Immunoblotting**

Western immunoblotting is performed according to the manufacturer's conditions, using the antibodies provided with the Discriminatory kit: Ab Ctrl for the CONTROL conditions, Ab Test for the TEST conditions, and Ab II for both CONTROL and TEST conditions.

a) Upon completion of the protein transfer, open the blotting assembly and remove the membrane for development. Quickly immerse the membrane in Wash solution 2 (see paragraph 5.3.4), then place it in ethanol for 10 seconds before rinsing for 5 minutes in distilled water.

Note: At this step, the membranes can be stored overnight in distilled water at +2°C to +8°C.
Let the membranes adjust to room temperature (+18°C to +30°C) before to start the immunoblotting.

b) Eliminate distilled water.

c) Incubate the membranes for 30 minutes in Control or Test blocking solution depending on the condition (see paragraph 5.3.4). Incubate under medium agitation.
20 mL of diluted Control or Test blocking solution is required for 1 membrane.

Note: from this step, the Bio-Rad Western Processor can be used for agitation and washing steps (refer to instruction manual for settings).

d) Eliminate the blocking solutions. Incubate the first membrane in diluted Control antibody (see paragraph 5.3.4) and the second membrane in diluted Test antibody (see paragraph 5.3.4) for 30 minutes at room temperature (+18°C to +30°C) under medium agitation.
15 mL of diluted primary antibody is required for each membrane.

e) Eliminate the Control and Test antibody solutions. Briefly rinse the membranes with Wash solution 1, then wash twice with wash solution 1 for respectively 5 and 10 minutes, under fast agitation.
50 mL of Wash solution 1 is required for each cycle and for 1 membrane.

f) Eliminate Wash solution 1 and incubate the membranes for 20 minutes in diluted secondary antibody (see paragraph 5.3.4) at room temperature (+18°C to +30°C) under medium agitation.
20 mL of diluted secondary antibody is required for 1 membrane.

g) Eliminate the secondary antibody solution. Briefly rinse the membranes with Wash solution 1, then wash for respectively 5, 10 and 10 minutes under fast agitation.
50 mL of Wash solution 1 is required for each cycle and for 1 membrane.

h) Eliminate the Wash solution 1 and place the membranes in 50 mL of Wash solution 2 under slow agitation for 5 minutes minimum.

i) Drain the membranes on absorbent paper without blotting and place it in a plastic folder.

j) Add the substrate reagent (ECL - see paragraph 5.3.4). Eliminate the excess of reagent and air bubbles with absorbent paper. Place the folder into one exposure cassette.

k) In a darkroom, cover the folders with a film and expose for 15 minutes. Film can be exposed longer or shorter time for optimal signal.

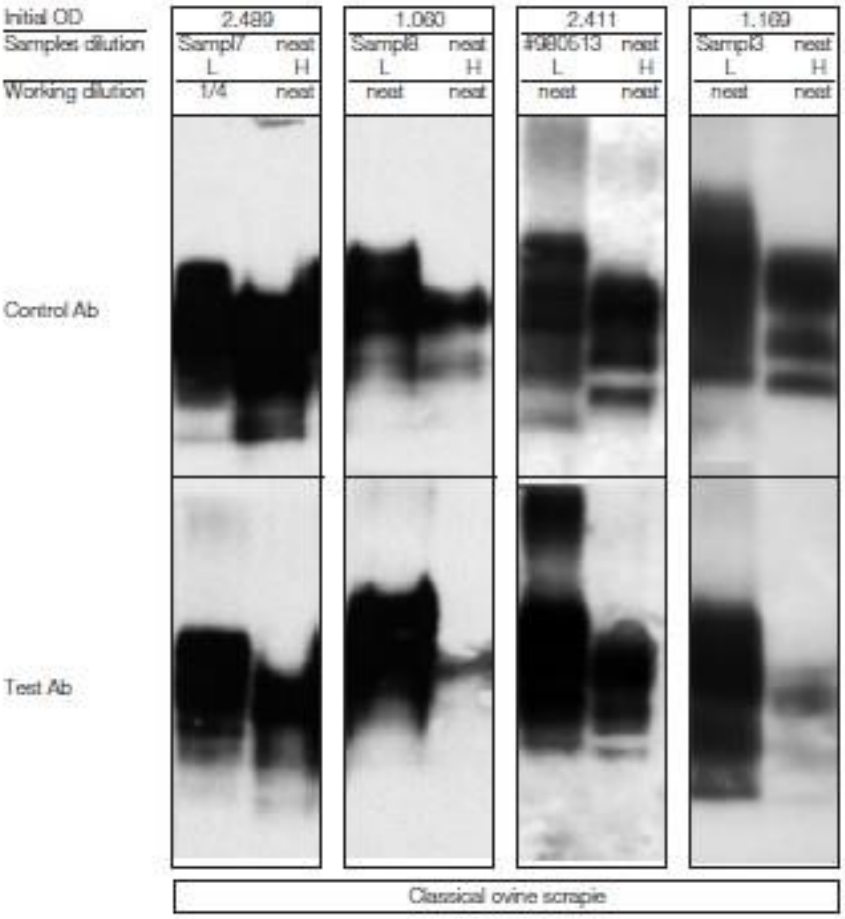
Note: Time for exposure is depending on the system used in the lab. Please check that the Magic Mark marker are clearly seen before stopping exposure.

l) Immerse the film in developing solution for 45 seconds (see paragraph 5.3.4). Rinse in distilled water. Immerse the film in fixative solution until the film becomes completely transparent.

m) Wash with distilled water and let the film dry.

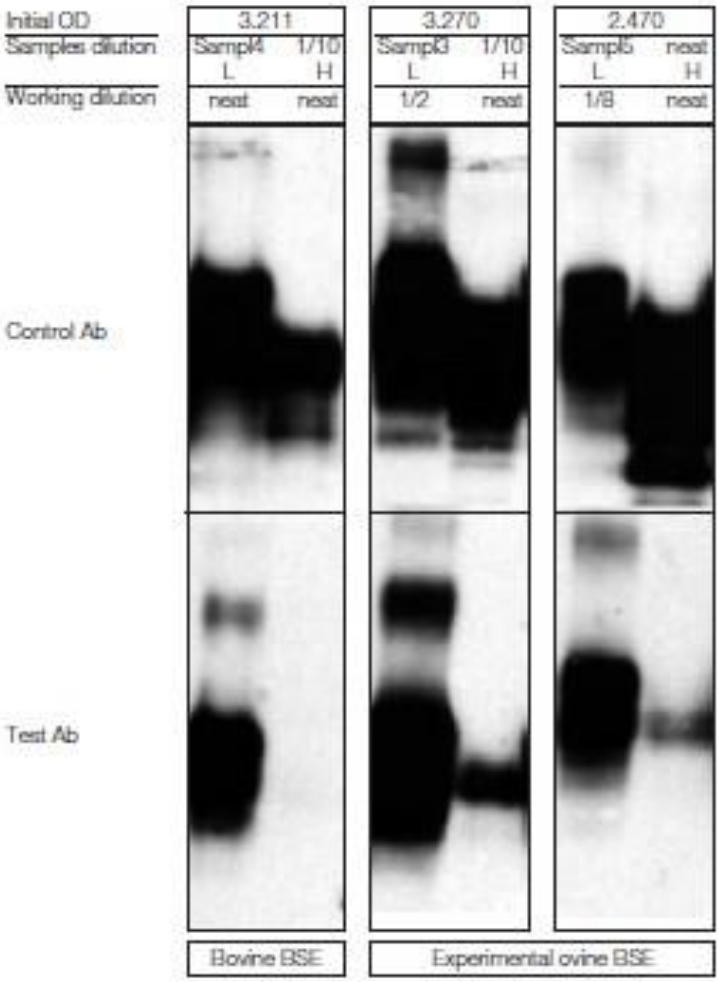
6 - INTERPRETATION OF DISCRIMINATORY KIT RESULTS

Classical strains of scrapie exhibit roughly similar signals for both LOW and HIGH conditions of purification in each condition of detection. Signals in TEST and CONTROL detections have to be present. They usually are on the same range of intensity.



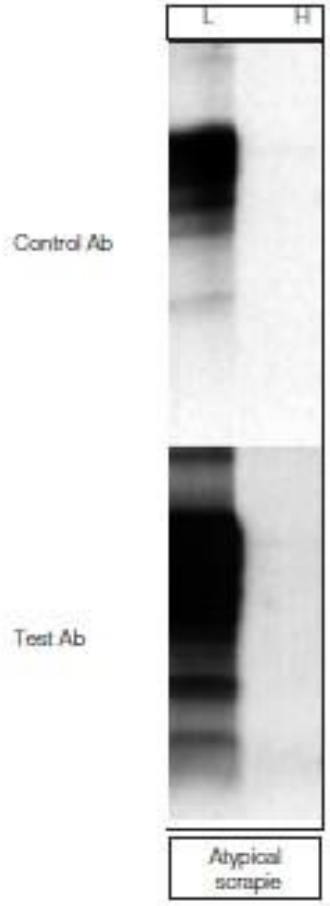
33 [EN]

detection should present no signal or a signal very much lower than the signal observed in CONTROL detection.



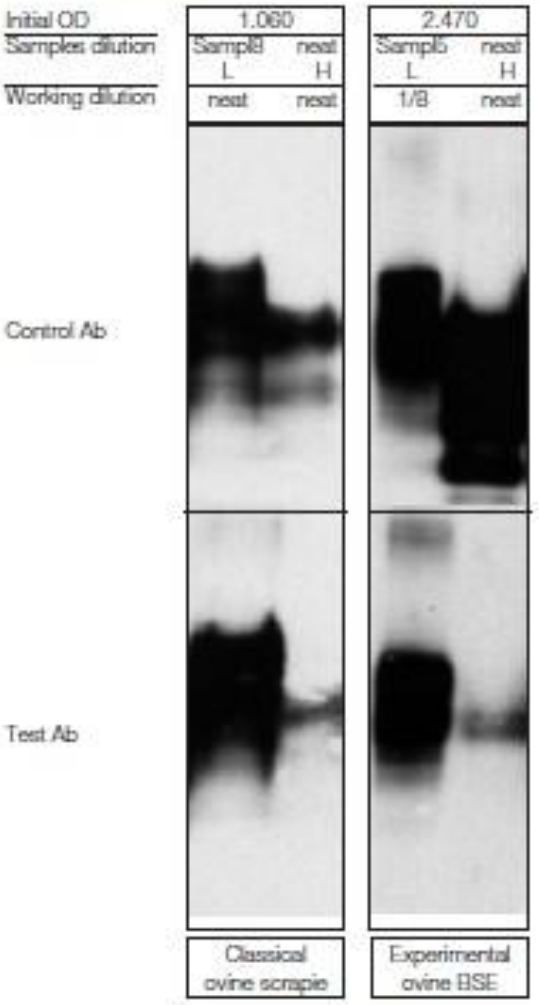
34 [EN]

Unusual PK-sensitive strains exhibit a normal signal for LOW condition of purification and no (or very weak) signal for HIGH condition of purification with both TEST and CONTROL detections.



35 [EN]

Taking into account the HIGH condition for both BSE and Scrapie samples, a BSE and a Scrapie can show quite similar signal for TEST detection. But for BSE a signal in TEST condition can only be observed if a really strong signal is observed on the CONTROL condition.



Conditions of Purification								
Conditions of Detection	Negative		Scrapie		BSE		PK sensitive	
	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH
Control	-	-	+	+	+	+	+	-
Test	-	-	+	+	+	- / (+)	+	-

Note: Discriminatory test results should not be interpreted without preliminary indications on prion strain provided by the TeSeE™ Western Blot assay (Product ref.: 3551169).

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7 - PRECAUTIONS

The quality of the data obtained depends on compliance with the following good laboratory practices:

- Reagents must be stored at the appropiate temperature (refer to supplier's indications).
- Do not use reagents whose shelf-life has expired.
- Do not use reconstituted proteinase K after 10 hours storage at room temperature (+18°C to +30°C).
- Do not mix or combine reagents derived from different batches of the DISCRIMINATORY KIT during the same manipulation, with the exception of grinding tubes, reagent A, reagent B and proteinase K.
- Allow the reagents and buffers to adjust to room temperature (+18°C to +30°C) for 30 minutes before use.
- Thoroughly reconstitute reagents, avoiding any contamination.
- Do not perform the test in the presence of reactive vapors (acids, alkalines, aldehydes) or dust, which could alter the enzymatic activity of the conjugate.
- The enzymatic reaction is very sensitive to all metals or metallic ions. Consequently, no metallic element must be in contact with the conjugate.
- Only use polypropylene tubes.
- Use clean glassware, rinsed in distilled water, or preferably disposable material.
- Use a new pipette tip for each sample.
- When starting electrophoresis and transfer, check that the 2 electrodes are in contact with buffer.
- All the rinsing times must be respected to avoid any excess background noise during final staining with ECL reagent.

8 - HYGIENE AND SAFETY INSTRUCTIONS

Generally, hygiene conditions, biosafety measures and good laboratory practices must be in agreement with the recommendations of national regulatory authorities.

- All reagents of the kit are intended for use in «in vitro» diagnosis.
- Wear disposable gloves when handling reagents and samples and wash your hands thoroughly after handling them.
- Do not pipette by mouth.
- Use polypropylene containers to avoid broken glass.
- All the materials directly in contact with the samples and the wash solutions must be considered as contaminated.

- Avoid splashing samples or solutions containing samples.
- Contaminated surfaces must be cleaned with 20 000 ppm sodium hypochlorite solution (bleach). When the contaminating liquid is an acid, contaminated surfaces must be first neutralized with sodium hydroxide before using bleach. Surfaces must be rinsed with distilled water, dried with ethanol and wiped with absorbent paper. The material used for cleaning must be discarded in a specific container for contaminated waste.
- Samples, material and contaminated products must be eliminated after decontamination:
  - either by soaking in 1 M sodium hydroxide (final concentration) for at least 1 hour at room temperature (+18°C to +30°C),
  - or by soaking in 20 000 ppm sodium hypochlorite solution for at least 1 hour at room temperature (+18°C to +30°C),
  - or by autoclaving at 134°C minimum for at least 18 minutes, under 3 bars of pressure.

Note: Never autoclave solutions containing bleach or reagent B.

- All operations involved in Transmissible Spongiform Encephalopathy (TSE) screening tests are subject to regulations and must be performed in an isolated, limited and controlled access laboratory devoted exclusively to this activity. A laboratory coat, overshoes, gloves, mask with visor or simple mask with safety glasses are required to ensure the operator's safety.
- Operators must receive specific training concerning the risks related to TSEs agents or prions and the validated modes of decontamination for unconventional agents. Biosafety measures must be in agreement with recommendations of regular authorities of the country.
- Neutralize and/or autoclave all wash solutions or wash wastes or any liquid containing biological samples prior to their elimination.
- For hazard and precaution recommendations relating to this test kit, please refer to the pictogram(s) displayed on reagent labels and the information supplied at the end of this instructions for use document. The Safety Data Sheet is available on www.bio-rad.com.

(BG)	• Този продукт съдържа човешки или животински компоненти. Бъдете внимателни при работа с него.
(CN)	• 本产品包含人/动物成分，请小心处理。
(CN) Traditional	• 本產品包含人/動物成分，請小心處理。
(CZ)	• Tento výrobek obsahuje lidské nebo zvířecí komponenty. Zacházejte s nim opatrně.
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(DK)	• Dette produkt indeholder humane og animalske komponenter. Skal behandles med forsigtighed.
(EE)	• Käesolev toode sisaldab inim-või loomseid komponente. Käsitseda ettevaatlikult.
(EN)	• This product contains human or animal components. Handle with care.
(ES)	• Este producto contiene componentes humanos o animales. Manejar con cuidado.
(FI)	• Tässä tuotteessa on ihmisestä tai eläimistä peräisin olevia osia. Käsittele varovasti.
(FR)	• Ce produit contient des composants d'origine humaine ou animale. Manipuler avec précaution.
(GR)	• Αυτό το προϊόν περιέχει ανθρώπινα ή ζωικά στοιχεία. Χειριστείτε το με προσοχή.
(HR)	• Ovaj proizvod sadrži ljudske ili životinjske sastojke. Pažljivo rukovati.
(HU)	• A készítmény emberi vagy állati eredetű összetevőket tartalmaz. Óvatosan kezelendő.
(IT)	• Questo prodotto contiene componenti umane o animali. Maneggiare con cura.
(JP)	• 本製品にはヒトまたは動物由来の構成成分が含まれます。取り扱いにご注意下さい。
(KR)	• 본 제품은 사람 또는 동물유래의 성분이 포함되어 있습니다. 취급에 주의하시기 바랍니다.
(LT)	• Šiame produkte yra žmogiškosios arba gyvūninės kilmės sudėtinių dalių. Elgtis atsargiai.
(MT)	• Dan il-prodott fih komponenti umani jew tal-annimali. Uża b'attenzjoni.
(NL)	• Dit product bevat menselijke of dierlijke bestanddelen. Breekbaar.
(NO)	• Dette produktet inneholder humane eller animalske komponenter. Håndteres med forsiktighet.
(PL)	• Niniejszy produkt zawiera składniki pochodzenia ludzkiego lub zwierzęcego. Należy obchodzić się z nim ostrożnie.
(PT)	• Este medicamento contém componentes de origem humana ou animal. Manuseie com cuidado.
(RO)	• Acest produs conține materiale de origine umană sau animală. Manevrați-l cu grijă.
(SE)	• Denna produkt innehåller beståndsdelar från människa eller djur. Hantera produkten varsamt.
(SI)	• Izdelek vsebuje človeške ali živalske sestavine. Rokujte previdno.
(SK)	• Tento výrobok obsahuje ľudské alebo zvieracie zložky. Narábajte s ním opatrne.

H226 - H318 - H302 - H315 - H317
H334 - H335 - H336
P210 - P280 - P305+P351+P338
P302+P352 - P333+P313 - P501

(BG)
опасно
Запалими течност и пари. Предизвиква сериозно увреждане на очите. Вреден при поглъщане. Предизвиква дразнене на кожата. Може да причини алергична кожна реакция. Може да причини алергични или астматични симптоми или затруднения в дишането при вдишване Може да предизвика дразнене на дихателните пътища.
Да се пази от топлина. Тютюнопушенето забранено. Използвайте предпазни ръкавици/ предпазно облекло/предпазни очила/ предпазна маска за лице. ПРИ КОНТАКТ С ОЧИТЕ: Промивайте внимателно с вода в продължение на няколко минути. Свалете контактните лещи, ако има такива и доколкото това е възможно. Продължавайте да промивате. ПРИ КОНТАКТ С КОЖАТА: Измийте обилно със сапун и вода. При поява на кожно дразнене или обрив на кожата: Потърсете медицински съвет/помощ. Изхвърлете съдържанието/контейнера в съответствие с местните/регионалните/ националните/международните разпоредби.

(CN)
危险
易燃液体和蒸气. 引起严重的眼睛损伤. 吞咽有害. 引起皮肤刺激. 可能引起皮肤过敏性反应. 吸入可能引起过敏或哮喘症状或呼吸困难. 可刺激呼吸道. . 可引致睡意或晕眩。.
远离热源/火花/明火/热表面。 – 禁止吸烟。. 戴防护手套/穿防护服/戴防护眼罩/戴防护面具。. 如进入眼睛：用水小心冲洗几分钟。如戴隐型眼镜并可方便地取出，取出隐型眼镜。继续冲洗。如皮肤沾染：用大量肥皂和水清洗。. 如发生皮肤刺激或皮疹：求医/就诊。. 按照本地 / 地区 / 国家 / 国际规例处理内含物 / 容器。

(CN) Traditional
危險
易燃液體和蒸氣. 引起嚴重的眼睛損傷. 吞咽有害. 引起皮膚刺激. 可能引起皮膚過敏性反應. 吸入可能引起過敏或哮喘症狀或呼吸困難. 可刺激呼吸道. . 可引致睡意或暈眩。.
遠離熱源/火花/明火/熱表面。 – 禁止吸煙。. 戴防護手套/穿防護服/戴防護眼罩/戴防護面具。. 如進入眼睛：用水小心沖洗幾分鐘。如戴隱型眼鏡並可方便地取出，取出隱型眼鏡。繼續沖洗。如皮膚沾染：用大量肥皂和水清洗。. 如發生皮膚刺激或皮疹：求醫/就診。. 按照本地 / 地區 / 國家 / 國際規例處理內含物 / 容器。

(CZ)
Nebezpečí
Hořlavá kapalina a páry. Způsobuje vážné poškození očí. Zdraví škodlivý při požití. Dráždí kůži. Může vyvolat alergickou kožní reakci. Při vdechování může vyvolat příznaky alergie nebo astmatu nebo dýchací potíže. Může způsobit podráždění dýchacích cest.
Chraňte před teplem/jiskrami/otevřeným plamenem/ horkými povrchy. Zákaz kouření. Používejte ochranné rukavice/ochranný oděv/ochranné brýle/ obličejový štít. PŘI ZASAŽENÍ OČÍ: Několik minut opatrně vyplachujte vodou. Vyjměte kontaktní čočky, jsou-li nasazeny a pokud je lze vyjmout snadno. Pokračujte ve vyplachování. PŘI STYKU S KŮŽÍ: Omyjte velkým množstvím vody a mýdla. Při podráždění kůže nebo vyrážce: Vyhledejte lékařskou pomoc/ošetření. Obsah/nádobu likvidujte v souladu s místními/regionálními/národními/mezinárodními předpisy.

(DE)
Gefahr
Flüssigkeit und Dampf entzündbar. Verursacht schwere Augenschäden. Gesundheitsschädlich bei Verschlucken. Verursacht Hautreizungen. Kann allergische Hautreaktionen verursachen. Kann bei Einatmen Allergie, asthmaartige Symptome oder Atembeschwerden verursachen. Kann die Atemwege reizen.
Von Hitze/Funken/offener Flamme/heißen Oberflächen fernhalten. Nicht rauchen. Schutzhandschuhe/Schutzkleidung/Augenschutz/ Gesichtsschutz tragen. BEI KONTAKT MIT DEN AUGEN: Einige Minuten lang behutsam mit Wasser spülen. Vorhandene Kontaktlinsen nach Möglichkeit entfernen. Weiter spülen. BEI KONTAKT MIT DER HAUT: Mit viel Wasser und Seife waschen. Bei Hautreizung oder -ausschlag: Ärztlichen Rat einholen/ärztliche Hilfe hinzuziehen. Entsorgung des Inhalts / des Behälters gemäß den örtlichen / regionalen / nationalen/ internationalen Vorschriften.

(DK)
Fare
Brandfarlig væske og damp. Forårsager alvorlig øjenskade. Farlig ved indtagelse. Forårsager hudirritation. Kan forårsage allergisk hudreaktion. Kan forårsage allergi- eller astmasymptomer eller åndedrætsbesvær ved indånding. Kan forårsage irritation af luftvejene.
Holdes væk fra varme/gnister/åben ild/ varme overflader. Rygning forbudt. Bær beskyttelseshandsker/beskyttelsestøj/ øjenbeskyttelse/ansigtsbeskyttelse VED KONTAKT MED ØJNENE: Skyl forsigtigt med vand i flere minutter. Fjern eventuelle kontaktlinser, hvis dette kan gøres let. Fortsæt skylning. VED KONTAKT MED HUDEN: Vask med rigeligt sæbe og vand.

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Ved hudirritation eller udslet: Søg lægehjælp. Bortskaffelse af indholdet/beholderen i henhold til de lokale/regionale/nationale/internationale forskrifter.

(EE)
Ettevaatust
Tuleohtlik vedelik ja aur. Põhjustab raskeid silmakahjustusi. Allaneelamisel kahjulik. Põhjustab nahaärritust. Võib põhjustada allergilist nahareaktsiooni. Sissehingamisel võib põhjustada allergia- või astma sümptomeid või hingamisraskusi. Võib põhjustada hingamisteede ärritust.
Hoida eemal soojusallikast/sädemetest/leekidest/kuumadest pindadest. Mitte suitsetada. Kanda kaitsekindaid/kaitserõivastust/kaitseprille/kaitsemaski. SILMA SATTUMISE KORRAL: loputada mitme minuti jooksul ettevaatlikult veega. Eemaldada kontaktläätsed, kui neid kasutatakse ja kui neid on kerge eemaldada. Loputada veel kord. NAHALE SATTUMISE KORRAL: pesta rohke vee ja seebiga. Nahaärituse või _obe korral: pöörduda arsti poole. Sisu/konteineri käitlus vastavuses kohalike/regionaalsete/rahvuslike/rahvusvaheliste nõuetega.

(EN)
Danger
Flammable liquid and vapour. Causes serious eye damage. Harmful if swallowed. Causes skin irritation. May cause an allergic skin reaction. May cause allergy or asthma symptoms or breathing difficulties if inhaled. May cause respiratory irritation. May cause drowsiness or dizziness.
Keep away from heat/sparks/open flames/hot surfaces. No smoking. Wear protective gloves/protective clothing/eye protection/face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. IF ON SKIN: Wash with plenty of soap and water. If skin irritation or rash occurs: Get medical advice/attention. Dispose of contents/container in accordance with local/regional/national/international regulations.

(ES)
Peligro
Líquidos y vapores inflamables. Provoca lesiones oculares graves. Nocivo en caso de ingestión. Provoca irritación cutánea. Puede provocar una reacción alérgica en la piel. Puede provocar síntomas de alergia o asma o dificultades respiratorias en caso de inhalación. Puede irritar las vías respiratorias.
Mantener alejado de fuentes de calor/chispas/llama abierta/superficies calientes. No fumar. Llevar guantes que aíslen del frío/gafas/máscara. EN CASO DE CONTACTO CON LOS OJOS: Aclarar cuidadosamente con agua durante varios minutos. Quitar las lentes de contacto, si lleva y resulta fácil. Seguir aclarando. EN CASO DE CONTACTO CON LA PIEL: Lavar con agua y jabón abundantes. En caso de irritación o erupción cutánea: Consultar a un médico. Eliminar el contenido o el recipiente conforme a la reglamentación local/regional/nacional/internacional.

(FI)
Vaara
Syttyvä neste ja höyry. Vaurioittaa vakavasti silmiä. Haitallista nieltynä. Ärsyttää ihoa. Voi aiheuttaa allergisen ihoreaktion. Voi aiheuttaa hengitettynä allergia- tai astmaoireita tai hengitysvaikeuksia. Saattaa aiheuttaa hengitysteiden ärsytystä.
Suojaa lämmöltä/kipinöiltä/avotulelta/kuumilta pinnoilta. Tupakointi kielletty. Käytä suojakäsineitä/suojavaatetusta/silmiensuojainta/kasvonsuojainta. JOS KEMIKAALIA JOUTUU SILMIIN: Huuhdo huolellisesti vedellä usean minuutin ajan. Poista piilolinssit, _edical voi tehdä helposti. Jatka huuhtomista. JOS KEMIKAALIA JOUTUU IHOLLE: Pese runsaalla vedellä ja saippualla. Jos ilmenee ihoärsytystä tai ihottumaa: Hakeudu lääkäriin. Säilytä säiliö(t) noudattaen paikallisia/alueellisia/kansallisia/kansainvälisiä määräyksiä.

(FR)
Danger
Liquide et vapeurs inflammables. Provoque des lésions oculaires graves. Nocif en cas d'ingestion. Provoque une irritation cutanée. Peut provoquer une allergie cutanée. Peut provoquer des symptômes allergiques ou d'asthme ou des difficultés respiratoires par inhalation. Peut irriter les voies respiratoires.
Tenir à l'écart de la chaleur/des étincelles/des flammes nues/des surfaces chaudes. Ne pas fumer. Porter des gants de protection/des vêtements de protection/un équipement de protection des yeux/du visage. EN CAS DE CONTACT AVEC LES YEUX: rincer avec précaution à l'eau pendant plusieurs minutes. Enlever les lentilles de contact si la victime en porte et si elles peuvent être facilement enlevées. Continuer à rincer. EN CAS DE CONTACT AVEC LA PEAU: laver abondamment à l'eau et au savon. En cas d'irritation ou d'éruption cutanée: consulter un médecin. Éliminer le contenu/récipient conformément à la réglementation locale/régionale/nationale/internationale.

(GR)
Κίνδυνος
Υγρό και ατμοί εύφλεκτα. Προκαλεί σοβαρή οφθαλμική βλάβη. Επιβλαβές σε περίπτωση κατάποσης. Προκαλεί ερεθισμό του δέρματος. Μπορεί να προκαλέσει αλλεργική δερματική αντίδραση. Μπορεί να προκαλέσει αλλεργία ή συμπτώματα άσθματος ή δύσπνοια σε περίπτωση εισπνοής. Μπορεί να προκαλέσει ερεθισμό της αναπνευστικής οδού.
Μακριά από θερμότητα/σπινθήρες/γυμνές φλόγες/θερμές επιφάνειες. Μην καπνίζετε. Να φοράτε προστατευτικά γάντια/προστατευτικά ενδύματα/μέσα ατομικής προστασίας για τα μάτια/πρόσωπο. ΣΕ ΠΕΡΙΠΤΩΣΗ ΕΠΑΦΗΣ ΜΕ ΤΑ ΜΑΤΙΑ: Ξεπλύνετε προσεκτικά με νερό για αρκετά λεπτά. Εάν υπάρχουν φακοί επαφής, αφαιρέστε τους, εφόσον είναι εύκολο. Συνεχίστε να ξεπλένετε. ΣΕ ΠΕΡΙΠΤΩΣΗ ΕΠΑΦΗΣ ΜΕ ΤΟ ΔΕΡΜΑ: Πλύνετε με άφθονο νερό και σαπούνι.

Εάν παρατηρηθεί ερεθισμός του δέρματος ή εμφανιστεί εξάνθημα: Συμβουλευθείτε/ Επισκεφθείτε γιατρό. Απορρίψτε τα περιεχόμενα/δοχείο σύμφωνα με τους τοπικούς/εθνικούς/διεθνείς κανονισμούς.

(HR)
Opasnost
Zapaljiva tekućina i para. Uzrokuje teške ozljede oka. Štetno ako se proguta. Nadražuje kožu. Može izazvati alergijsku reakciju na koži. Ako se udiše može izazvati simptome alergije ili astme ili poteškoće s disanjem Može nadražiti dišni sustav.
Čuvati odvojeno od topline/iskre/otvorenog plamena/vrućih površina. – Ne pušiti. Nositi zaštitne rukavice/zaštitnu odjelo/zaštitu za oči/zaštitu za lice. U SLUČAJU DODIRA S OČIMA: oprezno ispirati vodom nekoliko minuta. Ukloniti kontaktne leće ukoliko ih nosite i ako se one lako uklanjaju. Nastaviti ispiranje. U SLUČAJU DODIRA S KOŽOM: oprati velikom količinom sapuna i vode. U slučaju nadražaja ili osipa na koži: zatražiti savjet/pomoć liječnika. Odložite sadržaje /spremnike u skladu s lokalnim/regionalnim/nacionalni/medunarodnim odredbama.

(HU)
Veszély
Tűzveszélyes folyadék és gőz. Súlyos szemkárosodást okoz. Lenyelve ártalmas. Bőrirritáló hatású. Allergiás bőrreakciót válthat ki. Belélegezve allergiás és asztmás tüneteket, és nehéz légzést okozhat. Légúti irritációt okozhat.
Hőtől/szikrától/nyílt lángtól/forró felületektől távol tartandó. Tilos a dohányzás. Védőkesztyű/védőruha/ szemvédő/arcvédő használata kötelező. SZEMBE KERÜLÉS esetén: Több percig tartó óvatos öblítés vízzel. Adott esetben a kontaktlencsék eltávolítása, ha könnyen megoldható. Az öblítés folytatása. HA BŐRRE KERÜL: Lemosás bő szappanos vízzel. Bőrirritáció vagy kiütések megjelenése esetén: orvosi ellátást kell kérni. Az edény tartalmát / a tartályt a helyi/regionális/nemzeti/nemzetközi szabályozásoknak megfelelően kell hulladékként elhelyezni.

(IT)
Pericolo
Liquido e vapori infiammabili. Provoca gravi lesioni oculari. Nocivo se ingerito. Provoca irritazione cutanea. Può provocare una reazione allergica cutanea. Può provocare sintomi allergici o asmatici o difficoltà respiratorie se inalato. Può irritare le vie respiratorie.
Tenere lontano da fonti di calore/scintille/ fiamme libere/superfici riscaldate. Non fumare. Indossare guanti/indumenti protettivi/Proteggere gli occhi/il viso. IN CASO DI CONTATTO CON GLI OCCHI: sciacquare accuratamente per parecchi minuti. Togliere le eventuali lenti a contatto se è agevole farlo. Continuare a sciacquare. IN CASO DI CONTATTO CON LA PELLE: lavare abbondantemente con acqua e sapone. In caso di irritazione o eruzione della pelle: consultare un medico. Smaltire il prodotto/recipiente in conformità con le disposizioni locali / regionali / nazionali / internazionali.

(JP)
危険
引火性液体及び蒸気. 重篤な眼の損傷. 飲み込むと有害. 皮膚刺激. アレルギー性皮膚反応を起こすおそれ. 吸入するとアレルギー，ぜん（喘）息又は呼吸困難を起こすおそれ. 呼吸器への刺激のおそれ. 眠気又はめまいのおそれ.
熱 / 火花 / 裸火 / 高温のもののような着火源から遠ざけること。−禁煙。.
保護手袋/保護衣/保護眼鏡/顔保護面の着用。. 眼に入った場合：水で数分間注意深く洗うこと。次にコンタクトレンズを着用していて容易に外せる場合は外すこと。その後も洗浄を続けること。皮膚に付着した場合：多量の水と石けん（鹸）で洗うこと。. 皮膚刺激又は発しん（疹）が生じた場合：医師の診断 / 手当てを受けること。. 現地/地域/国/国際規定に従い内容物・容器の露出.

(KR)
위험
인화성 액체 및 증기. 눈에 심한 손상을 일으킴. 삼키면 유해함. 피부에 자극을 일으킴. 알레르기성 피부 반응을 일으킬 수 있음. 흡입시 알레르기성 반응, 천식 또는 호흡 곤란을 일으킬 수 있음. 호흡기계 자극을 일으킬 수 있음. 졸음 또는 현기증을 일으킬 수 있음.
열·스파크·화염·고열로부터 멀리하시오 – 금연. (보호 장갑·보호 의·보안경·안면보호구)를 (을) 착용하시오. 눈에 묻으면 몇 분간 물로 조심해서 씻으시오.가능하면콘택트렌즈를 제거하시오. 계속 씻으시오. 피부에 묻으면 다량의 비누와 물로 씻으시오. 피부자극성 또는 홍반이 나타나면 의학적인 조치·조언을 구하시오. 현지/지역/국가/국제규정에 따라서 내용물/용기 노출.

(LT)
Pavojinga
Degūs skystis ir garai. Smarkiai pažeidžia akis. Kenksminga prarijus. Dirgina odą. Gali sukelti alerginę odos reakciją. Įkvėpus gali sukelti alerginę reakciją, astmos simptomus arba apsunkinti kvėpavimą. Gali dirginti kvėpavimo takus.
Laikyti atokiau nuo šilumos šaltinių/žiežirbų/ atviros liepsnos/karštų paviršių. Nerūkyti. Mūvėti apsaugines pirštines/dėvėti apsauginius drabužius/ naudoti akių (veido) apsaugos priemones. PATEKUS Į AKIS: Kelias minutes atsargiai plauti vandeniu. Išimti kontaktinius lęšius, jeigu jie yra ir jeigu lengvai galima tai padaryti. Toliau plauti akis. PATEKUS ANT ODOS: Nuplauti dideliu kiekiu muilo ir vandens. Jeigu sudirginama oda arba ją išberia: kreiptis į gydytoją. Turinį/talpą išpilti (išmesti) - šalinti pagal vietines / regionines / nacionalines / tarptautines taisykles.

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(NL)
Gevaar
Ontvlambare vloeistof en damp. Veroorzaakt ernstig oogletsel. Schadelijk bij inslikken. Veroorzaakt huidirritatie. Kan een allergische huidreactie veroorzaken. Kan bij inademing allergie- of astmasymptomen of ademhalingsmoeilijkheden veroorzaken. Kan irritatie van de luchtwegen veroorzaken.
Verwijderd houden van warmte/vonken/open vuur/ hete oppervlakken. Niet roken. Beschermende handschoenen/beschermende kleding/ oogbescherming/gelaatsbescherming dragen. BIJ CONTACT MET DE OGEN: voorzichtig afspoelen met water gedurende een aantal minuten; contactlenzen verwijderen, indien mogelijk; blijven spoelen. BIJ CONTACT MET DE HUID: met veel water en zeep wassen. Bij huidirritatie of uitslag: een arts raadplegen. De inhoud en de verpakking verwerken volgens de plaatselijke/regionale/ nationale/internationale voorschriften.

(NO)
Fare
Brennbar væske og damp. Forårsaker alvorlige øyeskader. Helseskader ved svelging. Irriterer huden. Kan forårsake allergiske hudreaksjoner. Kan forårsake allergi, astmalignende symptomer eller pusteproblemer ved innånding. Kan irritere luftveiene.
Holdes adskilt fra varme. Ikke røyk. Bruk vernehansker/vernekląer/vernebriller/ansiktsskjerm. VED KONTAKT MED ØYNENE: Skyll forsiktig med vann i opptil flere minutter. Fjern evt. kontaktlinser såfremt dette er lett mulig. Fortsett skyllingen. VED HUDKONTAKT: Vask med store mengder vann og såpe. Ved hudirritasjon eller -utslett: Kontakt / tilkall lege. Innholdet / emballasjen skal avhendes i henhold til de lokale / regionale / nasjonale / internasjonale forskrifter.

(PL)
Niebezpieczeństwo
Łatwopalna ciecz i pary. Powoduje poważne uszkodzenie oczu. Działa szkodliwie po połknięciu. Działa drażniąco na skórę. Może powodować reakcję alergiczną skóry. Może powodować objawy alergii lub astmy lub trudności w oddychaniu w następstwie wdychania. Może powodować podrażnienie dróg oddechowych.
Przechowywać z dala od źródeł ciepła/iskrzenia/ otwartego ognia/gorących powierzchni. Palenie wzbronione. Stosować rękawice ochronne/ odzież ochronną/ochronę oczu/ochronę twarzy. W PRZYPADKU DOSTANIA SIĘ DO OCZU: Ostrożnie płukać wodą przez kilka minut. Wyjąć soczewki kontaktowe, jeżeli są i można je łatwo usunąć. Nadal płukać. W PRZYPADKU KONTAKTU ZE SKÓRĄ: Umyć dużą ilością wody z mydłem. W przypadku wystąpienia podrażnienia skóry lub wysypki: Zasięgnąć porady/zgłosić się pod opiekę lekarza. Zawartość / pojemnik usuwać zgodnie z przepisami miejscowymi / regionalnymi / narodowymi / międzynarodowymi.

(PT)
Perigo
Líquido e vapor inflamáveis. Provoca lesões oculares graves. Nocivo por ingestão. Provoca irritação cutânea. Pode provocar uma reacção alérgica cutânea. Quando inalado, pode provocar sintomas de alergia ou de asma ou dificuldades respiratórias. Pode provocar irritação das vias respiratórias.
Manter afastado do calor/da faísca/da chama aberta/das superfícies quentes. Não fumar. Usar luvas de protecção/vestuário de protecção/ protecção ocular/protecção facial. SE ENTRAR EM CONTACTO COM OS OLHOS: enxaguar cuidadosamente com água durante vários minutos. Se usar lentes de contacto, retire-as, se tal lhe for possível. Continuar a enxaguar. SE ENTRAR EM CONTACTO COM A PELE: lavar com sabonete e água abundantes. Em caso de irritação ou erupção cutânea: consulte um médico. Eliminar o conteúdo/ recipiente de acordo com a legislação local/ regional/nacional/internacional.

(RO)
Pericol
Lichid şi vapori inflamabili. Provoacă leziuni oculare grave. Nociv în caz de înghiţire. Provoacă iritarea pielii. Poate provoca o reacţie alergică a pielii. Poate provoca simptome de alergie sau astm sau dificultăţi de respiraţie în caz de inhalare. Poate provoca iritarea căilor respiratorii.
A se păstra departe de surse de căldură/scânteI/ flăcări deschise/suprafeţe încinse. Fumatul interzis. Purtaţi mănuşi de protecţie/îmbrăcăminte de protecţie/echipament de protecţie a ochilor/ chipament de protecţie a feţei. ÎN CAZ DE CONTACT CU OCHII: clătiţi cu atenţie cu apă timp de mai multe minute. Scoateţi lentilele de contact, dacă este cazul şi dacă acest lucru se poate face cu uşurinţă. Continuaţi să clătiţi. ÎN CAZ DE CONTACT CU PIELEA: spălaţi cu multă apă şi săpun. În caz de iritare a pielii sau de erupţie cutanată: consultaţi medicul. Aruncaţi conţinutul/containerul în acord cu regulamentele locale/regionale/naţionale/ internaţionale.

(SE)
Fara
Brandfarlig vätska och ånga. Orsakar allvarliga ögonskador. Skadligt vid förtäring. Irriterar huden. Kan orsaka allergisk hudreaktion. Kan orsaka allergi- eller astmasymtom eller andningssvårigheter vid inandning. Kan orsaka irritation i luftvägarna.
Får inte utsättas för värme/gnistor/öppen låga/heta ytor. Rökning förbjuden. Använd skyddshandskar/ skyddskläder/ögonskydd/ansiktsskydd. VID KONTAKT MED ÖGONEN: Skölj försiktigt med vatten i flera minuter. Ta ur eventuella kontaktlinser om det går lätt. Fortsätt att skölja. VID HUDKONTAKT: Tvätta med mycket tvål och vatten. Vid hudirritation eller utslag: Sök läkarhjälp. Innehållet / behållaren avfallshanteras enligt lokala / regionala / nationella / internationella föreskrifter.

(SI)	(SK)
Nevarno Vnetljiva tekočina in hlapi. Povzroča hude poškodbe oči. Zdravju škodljivo pri zaužitju. Povzroča draženje kože. Lahko povzroči alergijski odziv kože. Lahko povzroči simptome alergije ali astme ali težave z dihanjem pri vdihavanju. Lahko povzroči draženje dihalnih poti. Hraniti ločeno od vročine/isker/odprtega ognja/ vročih površin. Kajenje prepovedano. Nositi zaščitne rokavice/zaščitno obleko/zaščito za oči/ zaščito za obraz. PRI STIKU Z OČMI: previdno izpirajte z vodo nekaj minut. Odstranite kontaktne leče, če jih imate in če to lahko storite brez težav. Nadaljujte z izpiranjem. PRI STIKU S KOŽO: umiti z veliko mila in vode. Če nastopi draženje kože ali se pojavi izpuščaj: poiščite zdravniško pomoč/oskrbo. Vsebino/vsebnik odstranite v skladu z lokalnimi/ regionalnimi/narodnimi/mednarodnimi predpisi.	**Nebezpečenstvo** Horľavá kvapalina a pary. Spôsobuje vážne poškodenie očí. Škodlivý po požití. Dráždi kožu. Môže vyvolať alergickú kožnú reakciu. Pri vdýchnutí môže vyvolať alergiu alebo príznaky astmy, alebo dýchacie ťažkosti. Môže spôsobiť podráždenie dýchacích ciest. Uchovávajte mimo dosahu tepla/iskier/otvoreného ohňa/horúcich povrchov. Nefajčite. Noste ochranné rukavice/ochranný odev/ochranné okuliare/ochranu tváre. PO ZASIAHNUTÍ OČÍ: Niekoľko minút ich opatrne vyplachujte vodou. Ak používate kontaktné šošovky a ak je to možné, odstráňte ich. Pokračujte vo vyplachovaní. PRI KONTAKTE S POKOŽKOU: Umyte veľkým množstvom vody a mydla. Ak sa prejaví podráždenie pokožky alebo sa vytvoria vyrážky: vyhľadajte lekársku pomoc/starostlivosť. Zneškodnenie obsahu/obalu v súlade s miestnymi/ oblastnými/národnými/medzinárodnými nariadeniami.

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THE FLI DISCRIMINATORY WESTERN BLOT METHOD

Introduction

The FLI-test includes the determination of molecular masses, antibody binding affinities and glycosylation pattern of the TSE induced abnormal prion protein. The method is based on a Western blotting technique which uses two monoclonal antibodies (L42, P4) to discriminate scrapie in small ruminants from BSE in small ruminants. (Gretzschel et al 2005).
The technique involves protein extraction from central nervous system followed by homogenization and digestion with proteinase K. Normal cellular prion protein (PrP^{C}) will be completely hydrolysed whereas the abnormal pathological form PrP^{Sc} harbours a partial resistance to proteinase K digestion and will therefore only be reduced by 80-100 amino acids from the amino terminus, while the core region and the carboxyterminus remain intact.

According to the FLI-Test, PrP^{Sc} in a sample will be judged BSE-like, if the sample conforms to all three biochemical attributes:

- the glycoform ratio shows a clear predominance of the diglycosylated form
- the signal obtained using mab L42 is clearly stronger that the signal obtained with mab P4
- the molecular mass of the unglycosylated PrP is lower than that of the scrapie control

In case these three points cannot be answered by visual interpretation, the blots needs to be analysed using the Quantity One software. If that does not lead to a clear result, the test needs to be repeated.

2. Materials

2.1. Chemicals and reagents

*(Those marked with * MUST be from the named sources)*

Acrylamide	Carl Roth GmbH Tel: 0800-5699000 Rotiphorese Gel 30 Product-No: 3029.1

Alkaline-phosphatase-conjugated anti-mouse immunoglobulin*	Dianova, Tel.: +49 040 450670 Product No : 115-055-003 Dilute in 1.0 ml deionized water. Add 1.0 ml glycerine. Store at -20 C°
Benzonase*	Novagen, Tel 0800 6931 000 Product-No: 70664-3
Benzonase stem solution	10,000 U Bensonase/ml deionised water (=10U/µl)
Blocking solution	5 g not-fat milk powder in 100 ml PBS/Tween Store at 4°C
Blotting buffer (1x)	3.03g Tris, 14.4g Glycine, 200 ml Methanol, 2g SDS Make up to 1.0 l in deionized water.
CDP-Star*	Appelera GmbH Product-No: T2147
Electrophoresis buffer (10x)	30g Tris, 144g Glycine, 10g SDS Make up to 1.0 l in deionized water.
Homogenization buffer	14.364g Sucrose, 0.5g deoxycholic acid sodium salt (DOC) 0.5g Nonidet P40 (NP 40) Make up to 100 ml in deionised water.Store at 4°C.
Phosphate buffered saline (PBS) (x10)	80g NaCl, 2g KCl, 11.5g $Na_2HPO_4x2H_2O$-11.5g KH_2PO_4 Make up to 1.0 l in deionised water
Magnesium chloride hexahydrate	Carl Roth GmbH. Tel 0800 5699000. Product no: 2189.2. Prepare a 0.1M stem solution
PBS/Tween (PBS/T)	Add 1.0 ml Tween-20 to 1000 ml PBS (1x)

Pefabloc* (0.1M)	100 mg Pefabloc. Make up to 4.17 ml in deionised water. Store at –20°C. Roche Product-Nr. 11429868001
Phosphotungstic acid*	Sigma Product-Nr: P4406 PTA solution: 4 g PTA 691 mg MgCl2 Fill to 100 ml with deionized water Adjust pH to 7.4 with NaOH
Primary antibody RIDA mAb P4*	R-Biopharm Tel.: +49 6151 8102 - 0
	R-Biopharm Tel.: +49 6151 8102 - 0 Product-No: R8007 Store at 4°C
Primary antibody RIDA mAb L42*	R-Biopharm Tel.: +49 6151 8102 - 0 Product-No: R8005 Store at 4°C
Proteinase K*	Sigma Tel.: 0800-5155000 Product-No: P5568 Store at 4°C
RGS-His antibody*	Qiagen, Tel: +49 2103 2912000 Product-No: 34610
N-laurylsarkosine sodium salt*	Sigma tel: 0800 5155000. Product No: L5125
Tris polyacrylamide gel (16%) (separating gel)	3.0 ml deionised water 3.75 ml 1.5 M Tris/HCl (pH 8.8) 8.0 ml Acrylamide 750 µl Sodium dodecyl sulphate (10%) 100 µl Ammoniumperoxidisulphate (10%) 10 µl Tetramethylethylenediamine (TEMED)

Stacking gel (4%)	6.0 ml deionised water 2.5 ml 0.5 M Tris/HCl (pH 6.8) 1.3 ml Acrylamide 500 µl Sodium dodecyl sulphate (10%) 200 µl Ammoniumperoxidisulphate (10%) 20 µl Tetramethylethylenediamine (TEMED)
Molecular weight marker*	Precision Plus Dual Color Marker (BioRad) Product-No. 161-0374
Tween-20	Merck Product-No: 8.170-722.500 Tel.: +49 6151 39720
Sample Buffer (10x)	2 g sodiumdodecylsulphate, 5 ml Tris/HCl (1M, pH 7,4), 5 ml 2-Mercaptoethanol, 3 g sucrose, ca. 20 drops 1% brom-phenolblue, fill up to 20 ml with deionised water, adjust pH to 6.8 with HCl.
Sample Buffer (2x) (working solution)	Dilute sample buffer (10x) 1:5 in deionised water

2.2. Equipment

Balance (0.002g-32g)	
Biological safety cabinet	
Chromatography paper	Whatman Tel. +49-551-50686-0 Product-Nr. 3030 917
Centrifuge (maximum 14,000g) No temperature control necessary	Eppendorf, Tel: +49 4053801-0 Product-No: 5417R

Electrophoresis cell	Biorad, Tel: +49 89 318 840 Product-No: 165-2944
Power supply	Immobilon PVDF membrane Millipore Product-No:JPVH 00010
Freezer (4°C, -20°C)	
Heating block (37°C, 55°C, 95°C)	
Pipette(0.5 -2.5 µl) Pipette(2.0 -20 µl) Pipette (10-100µl) Pipette (0.1-1.0 ml)	<6.0% precision <2.0% <0.7% <0.3%
Power supply	
Immobilon PVDF membrane	Millipore Tel: +49 01805 045 645 Product-No:JPVH 00010
Ribolyser	Thermo Electron Hybaid Product-No: FP120 HY-230
Transblot Semidry Cell	Biorad, Tel: +49 89 318 840 Product-No: 170-3949
Versadoc Imaging system	Biorad, Tel: +49 89 318 840 Product-No: 170-8050

3. Procedure/Method

All steps with infectious material are carried out in an appropriate safety cabinet.

3.1. Purification and proteinase K digestion

- Prepare 10% (% weight in volume [w/v]) homogenate from brainstem samples, in homogenization buffer by using a Ribolyser (Hybaid, Heidelberg, Germany) set for 2 x 45 seconds; velocity unit 6.5.
- Remove gross cellular debris by centrifugation at 6.000 g for 1 min at room temperature.
- Add 2.5µl Benzonase (10.000 U/ml) and 5 µl MgCl2 (100 mM) to 500 µl homogenate and incubate samples for 30 min at 37°C with constant agitation (550 rpm).
- Add 25 µl PK solution (1 mg/ml) and incubate at 55°C for 1 h.

- Stop digestion by addition of 10 µl Pefabloc (0.1 M) and heating for 5 min at 95°C.
- Mix samples with 500 µl PBS containing 4% (w/v) sarkosyl and incubate for 30 min at 37°C with constant agitation (550 rpm).
- samples are adjusted with 78.2 µl of a stock solution containing 4% (w/v) sodium phosphotungstic acid and 34 mmol/l magnesium chloride to give a final concentration in the sample of 0.3 % (w/v) sodium phosphotungstic acid.
- Incubate samples at 37°C for 60 min with constant agitation (550 rpm) before centrifugation at 14.000g for 30 min at room temperature

3.2. SDS-PAGE and Western blot

- After careful isolation of the supernatant, resuspend pellets in 40 µl sample buffer, heat for 5 min at 95°C and centrifuge shortly.
- Load samples on 16% Tris-polyacrylamide gels. Each sample is loaded on at least two gels. The sample volume to be loaded on the gel depends on the signal of the diagnostic sample in comparison with the positive controls (see section 3.3). A sheep scrapie, a bovine BSE and a negative control must be included in each assay.
- Electrophoresis is carried out at 100V (stacking gel) followed by 200V (separating gel) until the dye front reaches the bottom of the gel (approximately 90 mins).
- Transfer proteins onto polyvinylidene fluoride membrane (Millipore, Billerica, USA) using a semi-dry blotter set at 15 V and 0.3 A/Gel for 45 minutes.
- Incubate membranes for 1 h in 5 % (w/v) non-fat milk powder in PBS containing 0.1 % (v/v) Tween-20 (PBST) to block unspecific binding
- One gel is incubated with the monoclonal antibody L42 (binds to amino acids 145 to 163 of the ovine PrP) at a concentration of 0.4 µg/ml in 5% (w/v) non-fat dry milk powder in PBST
- The second gel is incubated with the monoclonal antibody P4 (binds to amino acid 89-104 of the ovine PrP) at a concentration of 0.4 µg/ml in 5% (w/v) non-fat dry milk powder in PBST

- Incubate membranes for 1.5 h at room temperature
- Wash three times for ten minutes in PBST
- Incubate in alkaline phosphatase-conjugated anti-mouse immunoglobulin (0.15 µg/ml) for 1 h at room temperature
- Wash three times for ten minutes in PBST
- Equilibrate membranes two times for two minutes in Assay buffer
- Add 1.5 ml CDP Star substrate on the membrane and incubate for five minutes
- Visualize signal using either a digital imaging system (e.g. VersaDoc) or an X-ray developing system

3.3. Data analysis

The blots are analysed visually, evaluating the three points mentioned in the introduction:
According to the FLI-Test, PrP^{Sc} in a sample will be judged BSE-like, if the sample conforms to all three biochemical attributes:

- the glycoform ratio shows a clear predominance of the diglycosylated form
- the signal obtained using mab L42 is clearly stronger that the signal obtained with mab P4
- the molecular mass of the unglycosylated PrP is lower than that of the scrapie control

In case these three points cannot be answered by visual interpretation, the blots needs to be analysed using the Quantity One software. If that does not lead to a clear result, the test needs to be repeated.

Testing must also be repeated if the results of the positive or negative controls are untypical or if the result of the diagnostic sample cannot be interpreted following the above described method. This might be:
- very faint signals (repeat FLI-test with a higher amount of brain homogenate)
- very strong signals (dilute resuspended sample in an appropriate volume)

Annex-1 - Interpretation of the results

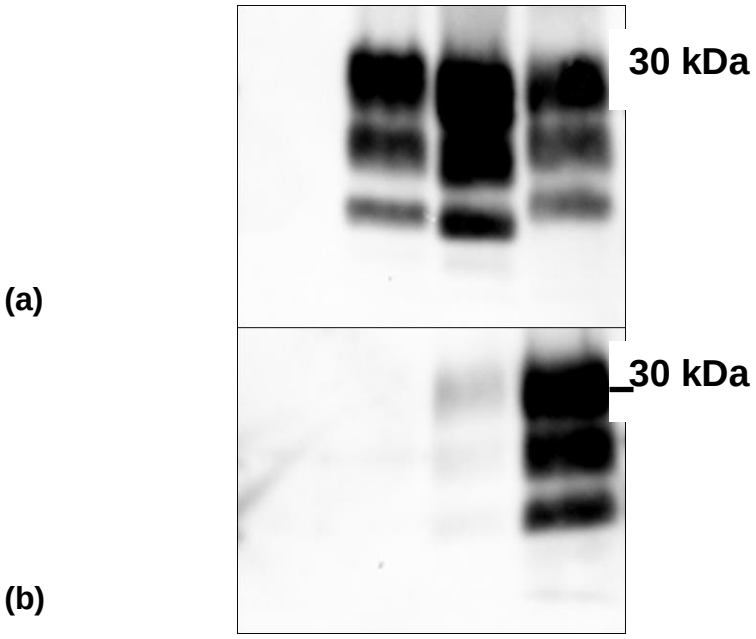


Figure 1 Comparison of electrophoretic profiles and antibody labeling of PrP^{Sc} after proteinase K digestion, PTA-precipitation and immunoblotting using mAb L42 (a) or mAb P4 (b). Both blots are loaded with the same quantities of precipitated PrP^{sc} of each sample. Negative sheep (lane 1), BSE in cattle (lane 2), BSE in sheep (lane 3) and scrapie in sheep S 33/02 (lane 4).

Annex-2 Gel loading plan (example)

Lane	Sample
1	Negative control without PK
2	Negative sheep (negative control) + PK
3	Protein marker
4	Sample 1
5	Sample 2
6	Sample 3
7	BSE in cattle control
8	Scrapie in sheep control

Reference

Gretzschel, A., Buschmann, A., Eiden, M., Ziegler, U., Lühken, G.,Erhard, G. and Groschup, M.H. (2005) Strain typing of German transmissible spongiform encephalopathies field cases in small ruminants by biochemical methods. *J Vet Med B Infect Dis Vet Public Health*. **52**(2):55-63

THE CIDC-LELYSTAD DISCRIMINATORY METHOD

1. Introduction

This procedure describes a method to discriminate between BSE and scrapie-like status in brain isolates of confirmed TSE-positive sheep and goats. The intention is to find BSE-like infections in small ruminants. The method described is based on the findings of Thuring *et al*. (2004). It consists of two Western blots run in parallel with the same set of samples, using mostly materials and procedures of the Prionics Check Western test for BSE testing. Essential difference is the use of the two blots and the their development with different PrP-specific monoclonal antibodies, P4 and 94B4. The use of two blots obviates the need of molecular weight estimation of the PrP^{res} protein bands, and thus is not subject to variations in such measurements. Visual inspection will already in most cases yield a diagnosis, but with difficult cases a density scan of photographic film can be recorded and from this a BSE-like diagnosis can be based on the density ratio 94B4/P4 using appropriate software. Nevertheless, other characteristics can still be estimated like molecular weight - provided molecular weight markers are run alongside - and glycoprofile. However, these measurements are not the purpose of the method.

TSE-positive isolates are usually initially detected by routine testing for TSE in small ruminants and confirmed positive by histopathology and immunohistochemistry. The sample must be retested from homogenate together with known bovine BSE, ovine scrapie and, if available but not necessary, experimental BSE sheep isolate as reference samples to confirm the validity of the test.

The behaviour of atypical scrapie material in this method is discussed in the interpretation of the results (paragraph 5.5.6).

Definitions

PrP	Prion protein
PrP^{c}	Normal isoform of prion protein
PrP^{Sc}	Disease associated isoform of PrP
PrP^{res}	Prion protein resistant to proteinase K, a diagnostic marker for disease.
BSE	Bovine Spongiform Encephalopathy
TBST	Tris Buffered Salt Solution with 0,05% Tween 20
MAb	monoclonal antibody

3. Materials

3.1 Chemicals and reagents

Pipette tips 0-10 µl	
Pipette tips 10-250 µl	
Pipette tips 100-1000 µl	
H_2O	demineralized or distilled water
96-well digestion plate 0,2 ml	Life Systems Design, cat.no. 02-1402-0595
Lid for microplates	Greiner, cat.no. 656101
Proteinase-K	Prionics Check Western
Digestion stop buffer	Prionics Check Western
PAGE sample buffer	Prionics Check Western
1/1 Diluted PAGE sample buffer	PAGE sample buffer/H_2O 1/1 (v/v)
Homogenisation buffer 5x concentrated	Prionics Check Western
12% NuPAGE gels, 17-lanes	Invitrogen, cat.no. NP 0349
NuPAGE MOPS/SDS running buffer 20x	Invitrogen, cat.no. NP 0001
running buffer 1x	50 ml running buffer 20x, 950 ml H_2O
NuPAGE anti oxidant	Invitrogen, cat.no. NP 0005
Distilled water	
Control sample	Prionics Check Western

Magic Mark XP	InVitrogen, cat. no. LC5602; dilute 2 µl in 10 µl 1/1 PAGE sample buffer; do not heat.
PVDF membrane, 0.45 µm	Millipore, cat.no. IPVH00010
Chromatography Paper, 3MM Chr,	Whatman, cat.no. 3030917
Methanol 100%	Merck, cat.no. 1.06009
PVDF blocking buffer 5x concentrated	Prionics Check Western
MAb 94B4	CIDC-Lelystad, Lelystad, The Netherlands
MAb P4	R-Biopharm, Almere, Netherlands
Conjugate	Prionics Check Western or rabbit anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Dako, Glostrup, Denmark).
Luminescence buffer 10x	Prionics Check Western
CDP-star, ready to use	Roche, cat.no. 2 041677
15 & 50 ml conical BD Falcon tubes	BD Biosciences, #352196&352070
Tissues	
Saran foil	Dow
ECL- hyperfilm	Amersham, cat.no. RPN3103K
X-ray film developer	Konica, cat.no. 102665
X-ray film fixer	Konica, cat.no. 102663
Transfer buffer 10x	30,28 g Tris base, 144,13 g Glycine, add distilled water to 1000 ml

Transfer buffer 1x	Add sequentially in a container: 300 ml transfer buffer 10x, 2.6 L H_2O, 300 ml methanol 100%. Use as cold solution.
TBST	8 g NaCl, 0,2 g KCl, 3 g Tris base, add distilled water to 1000 ml, adjust to pH 7,4. Add 0,5 ml Tween 20
Ponceau S 40x	5 g Ponceau S, 1000 ml distilled water , 50 ml 100% acetic acid
Ponceau S 1x	25 ml Ponceau S 40x, 975 ml TBST

3.2 Equipment

- Biological Safety Cabinets, Class IIb

- Heating block 50°C
- Heating block 96°C
- Power Supply
- Electrophoresis unit
- Transfer tank
- Transfer cassette
- Transfer sponges
- Perspex plates (size 23 x 17 cm)
- Cooling unit transfer tanks
- Multichannel pipettes : 5-50 µl, 20-200 µl
- Single channel pipettes: 2-20 µl, 20-200 µl, 100-1000 µl
- Rocking platform
- Membrane incubation boxes
- Vortex
- Film developing machine
- X-ray cassette (24x18 cm)
- Glassware (flasks): 100 ml, 500 ml and 1 liter
- 20°C freezer

3.3 Reference materials

Confirmed positive brain isolates each of bovine BSE and ovine scrapie (and if available, but not necessary, an ovine BSE isolate from an experimentally infected sheep) are used as reference material.

The Prionics control sample in this test is used as control sample for Western blotting and immunoassay.

A molecular weight marker kit is used for molecular weight estimations like Magic Mark XP from InVitrogen. The protein reference standards in this kit react with the anti-mouse Ig or anti-rabbit Ig alkaline phosphatase conjugates.

5. Method

5.1 Safety

Work with TSE agents or prions or potentially prion containing materials has to be performed in strict accordance with National Safety Regulations.

Laboratories must adhere to National Safety Regulations, but the following information, published by The Advisory Committee for Dangerous Pathogens (ACDP) is available for guidance: "Transmissible Spongiform Encephalopathy agents: safe working and the prevention of infection", Department of Health, London, UK (can be ordered at the Stationery Office, ISBN 0113221665, phone number +44 (20) 7873 9090). An update is available on the UK department of Health CJD website (http://www.doh.gov.uk.cjd/tseguidance/)

5.2 Equipment

- Start the Biological safety cabinet 15 minutes before use.
- Preheat the heating block for incubation at 50°C 1 hour before use.
- Preheat the heating block for incubation at 96°C 1 hour before use.
- Precool the cooling unit used for blotting at 8°C 1 hour before use.
- Switch on the X-ray film developing machine 20 minutes before use.
- The development time of the X-ray film varies between 0.5 -12 minutes.

5.3 Flow diagram: Scrapie

positive isolate
↓
PK digestion homogenate
↓
Electrophoresis of digested samples (PAGE)
↓
dual Western blotting (two membranes)
↓
Protein staining with Ponceau S
↓
Immuno assays (2 separate antibodies)
↓
Antigen detection with X-ray film

5.4 Procedure

5.4.1 Proteinase K digestion

- Add 10 µl proteinase K to the wells of the digestion plate.
- Add 100 µl homogenate to the wells of the digestion plate.
- Mix 5 times by up and down pipetting.
- Cover the digestion plate with a lid for microplates.
- Incubate 1 hour at 50°C in the heating block.
- Add 10 µl digestion stop solution to the wells of the digestion plate.
- Add 100 µl sample buffer to the wells of the digestion plate
- Mix 5 times by up and down pipetting.
- Boil the samples 5 minutes at 96°C in the heating block.
- Cool the digestion plate for 5 minutes at room temperature
- Heat the control sample 2 minutes at 65°C.
- Place an aliquot of Magic Mark XP from freezer to room temperature.

5.4.2 Electrophoresis: each sample is loaded onto 2 gels

- Fill the electrophoresis unit with 3 cm running buffer (1x times concentrated).
- Remove the tape from the gel cassette.
- Remove the comb from the gel cassette.
- Place the gel cassettes in the electrophoresis unit (the side of the sample application is directed to the inner chamber).
- Raise the gel and clamp into the electrophoresis unit
- Fill the lanes with running buffer (1 times concentrated).
- Add 10 µl control sample
- Add 5 µl of Magic Mark XP in lane 2.
- Add 10 µl reference and test sample into the lanes according to the test protocol and the next table:

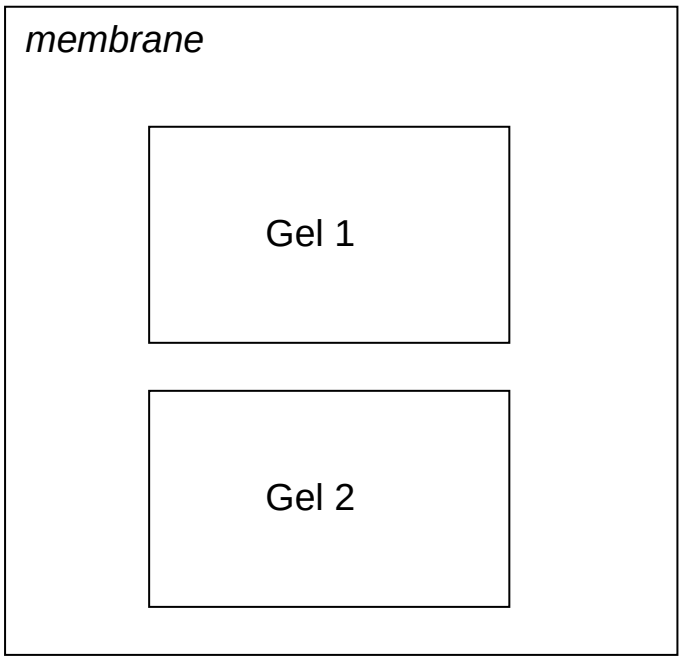
Lane 1	Control sample
Lane 2	Magic Mark XP
Lane 3	ovine scrapie reference sample
Lane 4	bovine BSE reference sample
Lane 5	Test sample 2
Lane 6	Test sample 3
Lane 7	Test sample 4
Lane 8	Test sample 5
Lane 9	Test sample 6
Lane 10	Test sample 7
Lane 11	Test sample 8
Lane 12	Test sample 9
Lane 13	Test sample 10
Lane 14	Test sample 11
Lane 15	Test sample 12
Lane 16	Test sample 13
Lane 17	ovine BSE reference sample (or bovine BSE reference sample)

- Release the clamp, lower the gel cassette to the bottom of the electrophoresis unit and clamp the gel cassette again.

- Fill the inner chamber with running buffer 1x until the running buffer is above all lanes.
- Add 500 µl NU PAGE anti oxidant to the inner chamber.
- Fill the outer chamber till equal level of running buffer in the inner chamber.
- Electrophorese the samples 45 minutes at 200V.

5.5.3 Western blotting

- Soak the PVDF membrane for 10 seconds in methanol.
- Incubate the membrane for 15 minutes in transfer buffer on a shaker.
- Place the clear side of the transfer cassette in a tray filled with transfer buffer 1x.
- Put a sponge on the clear side of the transfer cassette.
- Place a chromatography paper on the sponge.
- Place the equilibrated membrane on the chromatography paper.
- Open the gel cassette with the gel knife.
- Place the gels in the correct position on the membrane (lane one from the gel at the left side of the membrane).



- Mark with a pencil the position of the gels on the membrane.
- Put a chromatography paper on the gels.
- Place a sponge on the chromatography paper.
- Close the transfer cassette.
- Fill the transfer tank with transfer buffer 1x.
- Place the transfer cassette in the transfer tank with the dark side of the transfer cassette to the dark side (cathode or negative pole) of the transfer tank.
- Close the transfer tank, connect the electrode and blot 1 hour at 150V at 8°C or overnight at 30V at 8°C.

5.5.4 Ponceau S staining

- Stain the blotted membrane 1 minute with Ponceau S 1x .
- Mark with a pencil the position of the molecule weight markers of the lane with Prionics control sample.
- Mark the position of the proteinase-K band where visible.

- Wash the membrane 2-3 times with TBST.

5.5.5 Immuno assay

- Incubate the membrane in blocking buffer 1x for 30 minutes at room temperature on a rocking platform.
- Dilute the primary monoclonal antibodies P4 and 94B4 in TBST to 0.2 µg IgG/ml. *(Substitutes for PrP-core antibody 94B4 are either MAb 6H4 [1:5000, source Prionics Check Western test,] or 9A2 [0.2 µg/ml, source CIDC-Lelystad]).*
- Cut the membrane in two parts, corresponding to gel 1 and 2.
- Mark the two membrane parts with a pencil for gel number (1 and 2) and monoclonal antibody to be used in the assay (resp. P4 and 94B4).
- Incubate the membranes one hour with monoclonal antibodies P4 and 94B4 at room temperature on a rocking platform.
- Wash the membranes 3x5 minutes with TBST on a rocking platform.
- Dilute the conjugate 1:5000 in TBST.
- Incubate the membranes 30 minutes with conjugate at room temperature on a rocking platform.
- Wash the membranes 5x5 minutes with TBST at room temperature on a rocking platform
- Equilibrate the membranes 5 minutes with luminescence buffer 1x at room temperature on a rocking platform.
- Add CDP-star (ready to use) to the membranes.
- Distribute the substrate equally over the membranes, and incubate for 5 minutes at room temperature
- Discard the CDP-star.
- Place the 2 membranes besides each other on a perspex plate.
- Dry the membranes with tissue.
- Place the perspex plate with the membrane in Saran foil.

- Place the perspex plate in the X-ray cassette.

- Place in the dark an X-ray film on top of the plate and close the cassette.

- Expose the film for 0.5, 1, 5, and 15 minutes in the dark.

- Develop the X-ray film in the film developing machine. The film should have the lanes not overexposed which means that PrP^{res} bands per lane should be visible as separate bands. Sometimes films with different exposure times are required to have all samples on an acceptable density.

5.5.6 Visual Interpretation

- BSE and scrapie appear with a characteristic PrP^{res} 3 banding pattern, the lowest visible band producing the weakest signal (upper band = diglycosylated, middle = monoglycosylated and lower = unglycosylated PrP^{res}). In a weak sample only the stronger upper 1 or 2 bands are visible).

- Discriminating BSE-like isolates from scrapie-like isolates in TSE infected sheep: For **BSE-like** isolates a relative difference is noted in staining intensity with P4 (weak or negative) clearly weaker than 94B4. MAb 94B4 (and Mab's 6H4 and 9A2) detects all BSE- and scrapie-isolates well. MAb P4 detects only scrapie isolates well. From this follows, that **Scrapie-like** isolates hardly exhibit a difference in staining intensity between both antibodies per sample. If there are **doubts** about the interpretation, a density scan can be made. Again: the diagnosis is only valid if the blots were run together, and if they were exposing the same film. See paragraph below: *digitalisation and interpretation of results*.

- Molecular weights of PrP^{res} bands: MAb 94B4 (and MAb 6H4 and 9A2) detects also slight modifications in molecular weight of PrP^{res} bands between scrapie and BSE isolates related to the respective control scrapie samples on the same gel.

- The Prionics control sample (see Fig. 1, lanes 1) reacts strongly with MAb 94B4 (and MAb's 6H4 and 9A2) and -relative to this - MAb P4 yields only weak staining. This is due to the relatively low affinity of P4 for bovine PrP. P4 has a much better affinity for ovine PrP than for bovine PrP.

- The recently detected Nor98-like cases and potentially other atypical forms of TSEs in sheep and goat (Benestad *et al*., 2003; Orge *et al*., 2004; Buschmann *et a*l., 2004; Gavier-Widen *et al*., 2004; De Bosschere *et al*.; 2004; Onnasch *et al*., 2004; LeDur *et al*., 2005; Everest *et al*., 2006) differ from BSE and classical scrapie in sheep, because of a very different PrP^{res} banding pattern and a reduced resistance to PK treatment. Atypical cases will, therefore, not show up in this procedure and, if they do, these will have a prominent reactivity with antibody P4 which is stronger than with core specific antibodies 6H4 or 94B4.

Digitalisation and interpretation of dubious results from visual inspection:

Usually, the diagnosis negative (NEG) and BSE-like (BSE) can be concluded by visual inspection of the film. Lanes which contain bovine or ovine BSE samples will show a weak to negative staining with P4, while 94B4 (or 6H4 or 9A2) will exhibit a clearly stronger than P4 staining.

However, when the difference between the P4 and 94B4 blot is less clear for a certain sample, the data can be subjected to a more quantifiable approach. In that case the film exposed to the two blots with monoclonals 94B4 (or 6H4, 9A2) and P4 have to be scanned using a scanner for density recording. For those samples which have been designated TSE-positive *i.e.* reactive with 94B4 (or 6H4, or 9A2), densities of protein bands can be recorded from the film with e.g. an Agfa Duoscan T200XL scanner, further processed with GelPro software (MediaCybernetics, Silver Spring, Md.), or other software dedicated to process electronic data from blots. By using similar measuring windows for each set of lanes (94B4 and P4) the densities of the two blots per sample can be derived by the Gelpro software. The GelPro software can express the data as integrated optical densities (IOD). These IOD values are further used for density ratio calculations by hand, or by digital processing in a spread sheet program like Microsoft Excel. The ratio to be calculated is: 94B4/P4 (or 6H4/P4, or 9A2/P4). Any ratio value >1.5 is indicative for a BSE-like diagnosis (see Thuring *et al*., 2004; see example of ring trial samples in Figures 1 and 2).

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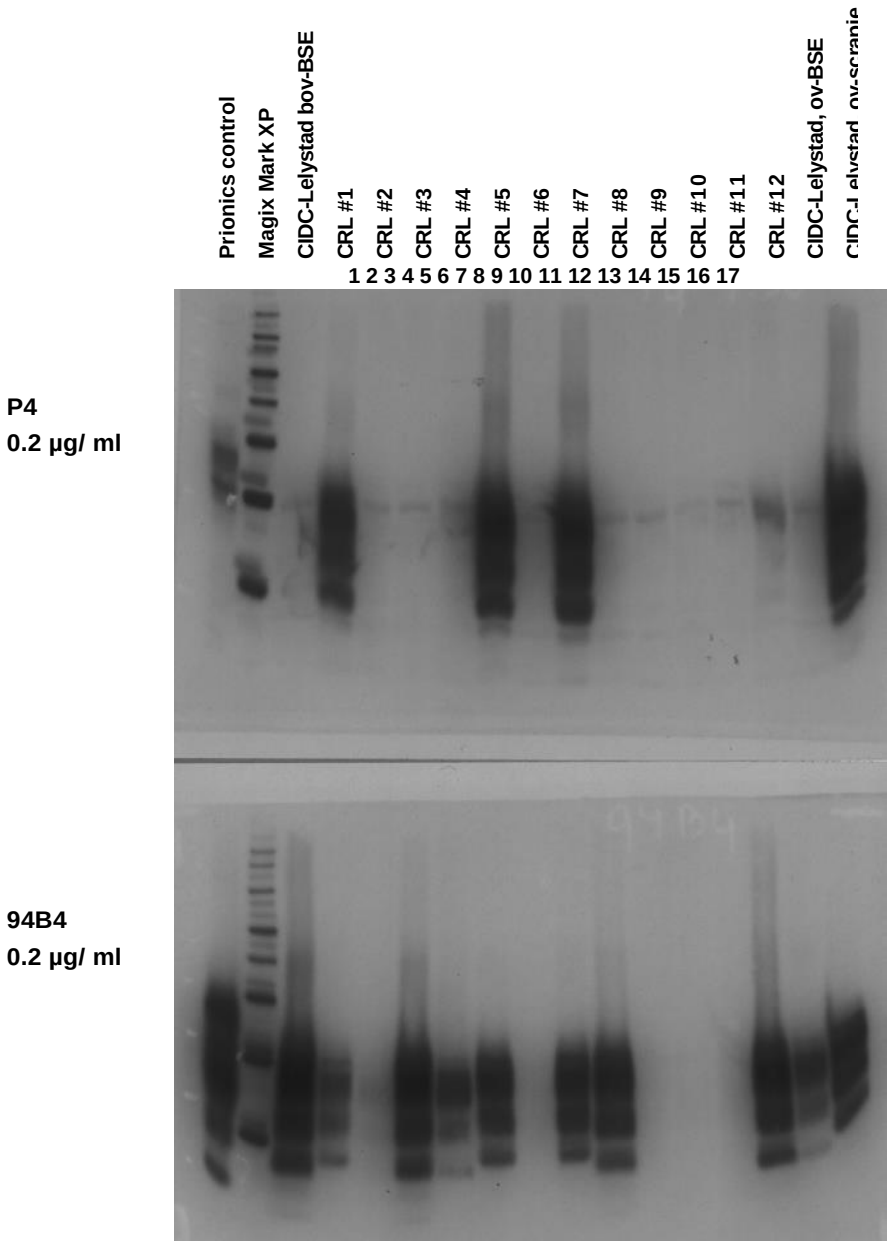

Figure 1: Example of BSE and scrapie samples obtained for a ring trial. Two blots are from gels run in parallel, one developed with MAb 6H4, the other with MAb P4 as indicated. Films were exposed for 30 seconds to obtain densities with limited fusion between the PrP^{res} triplet bands. This is a very short exposure time, but longer exposure of films will be required for less intense staining samples (like in lane 5), or even rerun when positive neighbouring samples are overshining. Samples: in lanes 4-15 EURL# samples have been applied, in lane 1 the Prionics control sample (bovine brain homogenate plus markers), lane 2 molecular weight marker lane with Magic Mark XP (M_r from

bottom to top of respectively 20, 30, 40, 50, 60, 80, 100, 120, and 200 kDa; the latter is not visible in the example), lane 3 bovine BSE brain, lane 16 experimental ovine BSE brain, and lane 17 ovine scrapie brain. Lanes 3-17 were all subjected to digestion with proteinaseK.

MAb 6H4																	
Lanes:		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rows		(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)
window density		1257.6	1218.7	711.17	308.58	951.41	835.97	888.59	NEG	953.69	751.25	NEG	NEG	NEG	1129.8	657.35	790.87
MAb P4																	
Lanes:		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rows		(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)
density /lane window		344.05	230.22	831.4	110.23	87.323	238.06	1033.6	NEG	1028.2	153.38	NEG	NEG	NEG	160.61	223.37	980.16
MAb 94B4																	
Lanes:		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rows		(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)
density /lane window		1203.9	1205.8	547.57	289.61	1049.9	593.85	728.34	NEG	726.01	791.44	NEG	NEG	NEG	905.41	565.64	750.77
MAb 9A2																	
Lanes:		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rows		(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)	(IOD)
density /lane window		1028.5	1275.6	1087.6	561.61	1129.4	1044.2	1353.9	NEG	1236.7	827.56	NEG	NEG	NEG	1165.4	803.55	1223.7
ratio criterium		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
6H4/P4	>1.5	3.66 B	5.29 B	0.86 S	2.80 B	10.90 B	3.51 B	0.86 S	N	0.94 S	4.90 B	N	N	N	7.03 B	2.94 B	0.81 S
94B4/P4	>1.5	3.49 B	5.24 B	0.66 S	2.63 B	12.02 B	2.48 B	0.70 S	N	0.71 S	5.16 B	N	N	N	5.64 B	2.54 B	0.77 S
9A2/P4	>1.5	2.98 B	5.55 B	1.31 S	5.09 B	12.93 B	4.39 B	1.31 S	N	1.20 S	5.40 B	N	N	N	7.25 B	3.60 B	1.25 S

Figure 2: In this table the IOD density values recorded with a scanner from film, and further processed in GelPro are presented. Blots were run parallel and exposed together in a single casette to photographic film. While in figure 1 only two blots are shown, in fact four blots were developed with respectively MAb's 6H4, P4, 94B4, or 9A2 at 1/5000, 0.2, 0.2 and 0.2 µg IgG/ml. Those samples which by density scanning received a 6H4/P4 density ratio of more than 1.5 are designated BSE-like, if 1.5 or smaller scrapie-like. The same cut-off level holds for the ratio's: 94B4/P4 and 9A2/P4. B, BSE-like diagnosis; S, scrapie-like diagnosis, N, TSE-negative.

THE ISS DISCRIMINATORY WESTERN BLOT METHOD

Introduction

The ISS discriminatory Western Blot is an immunoblotting technique based on the detection of abnormal prion protein (PrPSc) from the central nervous tissue of sheep and goat. The test allows the determination of the molecular weight and uses a double antibody detection method which enables discrimination to be made between cases of scrapie and experimental BSE.

Safety

Work with prions or potentially prion-containing materials has to be performed in strict accordance with National Safety Regulations.
Laboratories MUST adhere to National Safety Regulations, but the following information, published by The Advisory Committee for Dangerous Pathogens (ACDP) is available for guidance : "Transmissible Spongiform Encephalopathies (TSE) agents: safe working and the prevention of infection'. Copies can be obtained (ISBN 0113221665), from Department of Health (London) Stationery Office (Telephone +44 (20) 7873 9090). An update is available on the UK Department of Health web site (http://www.doh.gov.uk.cjd/tseguidance/).

Materials

Critical reagents, equipment and materials that should be necessarily obtained from the mentioned manufacturers are indicated with *****

Commercial reagents

Methanol

NuPage Antioxidant *****	INVITROGEN Tel. 0039029822201 Prod. n°.NP0005 Store at +4°C
NuPage LDS Sample Buffer (4x) *****	INVITROGEN Tel. 039029822201 Prod. n°.NP0007 Store at +4°C

NuPage MOPS/SDS *****Running Buffer (20x)	INVITROGEN Tel. 0039029822201 Prod. n°.NP0001 Store at RT
NuPage MOPS/SDS Transfer Buffer (20x) *****	INVITROGEN Tel. 039029822201 Prod. n°. NP0006-1 Store at +4°C
NuPage Reducing Agent (10x)	INVITROGEN Tel. 039029822201 Prod. n°.NP0004. Use 1:10 in distilled water Store at +4°C
NuPage 12% Bis-Tris Gel 1.0mm 12well *****	INVITROGEN Tel. 039029822201 Prod. n°.NP0342 box Store at +4°C
Precision StrepTactin-HRP Conjugate	BIORAD Tel. 003902216091 Prod. n°. 161-0380 Use 1:1000000 in PBS-T Store at +4°C
Precision Plus Protein Standards	BIORAD Tel. 003902216091 Prod. n°. 161-0363 Store at -20°C
Primary antibody : mAb P4*	RIDASCREEN-Biopharm Tel. 00 39 059652504 Prod. n°. R8007 Dilute at 1mg/ml in distilled water and store at +4°C
Primary antibody : mAb SAF84 *****	SPI-BIO Tel. 00330139306260 Prod. n°. A03208 Dilute at 0.1mg/ml in distilled water and store at+4°C
Proteinase K*	SIGMA Tel. 00390233417321 Prod. n°. P6556
PVDF Membrane	MILLIPORE Tel. 0039848845645 Prod. n°. IPVH00010

Super Signal West Femto includes: Lumino/Enhancer solution Stable Peroxide solution HRP-conjugate Goat anti-mouse	PIERCE Tel. 0039023819560 Prod. n°. 34095 Store at +4°C

Reagents

Blocking Buffer
Phosphate Buffered Saline (PBS) 1x pH7.4 + 1% (w/v) Skim milk powder Oxoid #L31). Store at +4°C up to 10h .

Digestion buffer
1.2g TrisHCl (M-Mediacl #IB70162) in 100ml distilled water, adjusted to pH 7.4 by adding HCl (Carlo Erba reagents #7647-01-0) and NaOH (J. T. Baker #0402). Add 4g Sarcosyl (Sigma #L5125). Store at +4°C up to 1 month.

Digestion stop solution (phenylmethylsulfonyl fluoride - PMSF)
0.087g PMSF (Sigma #P-7626) (w/v) in 5ml 100% ethanol. Store at -20°C up to 1 year.

Homogenisation buffer
1.2g TrisHCl (M-Mediacl #IB70162) in 100ml distilled water, adjusted to pH 7.4 by adding HCl (Carlo Erba reagents #7647-01-0) and NaOH (J. T. Baker #0402). Store at +4°C up to 1 month.

Loading buffer 1x
NuPage LDS Sample Buffer (4x): NuPage Reducing Agent: distilled water 1: 0.4: 2.6. Use freshly prepared solution.

Loading buffer 2x
NuPage LDS Sample Buffer (4x): NuPage Reducing Agent: distilled water 1: 0.4: 0.1. Use freshly prepared solution.

Phosphate Buffered Saline (PBS) 20x pH7.4
80g NaCl (J. T. Baker #0278), 2g KCl (ICN Biomedicals #151944), 14.42g Na2HPO4 (ICN Biomedicals #191440) and 2g KH2PO4 (ICN Biomedicals #194727) in 1000ml distilled water, adjusted to pH 7.4 by adding HCl (Carlo Erba reagents #7647-01-0) and NaOH (J. T. Baker #0402). Store at +4°C up to 2 months.

Phosphate Buffered Saline (PBS) 1x pH7.4

50ml Phosphate Buffered Saline (PBS) 20x pH7.4 in 950ml distilled water. Use freshly prepared solution or store at +4°C up to 10h.

Phosphate Buffered Saline Tween 20 (PBST) pH7.4
Phosphate Buffered Saline (PBS) 1x pH7.4 + 0.05% Tween 20 (Sigma #P5927). Use freshly prepared solution or store at +4°C up to 10h.

Primary Antibody Buffer
Phosphate Buffered Saline (PBS) 1x pH7.4 + 0.05% Tween 20 (Sigma #P5927) + 1% (w/v) Skim milk powder(Oxoid#L31). Use freshly prepared solution or store at +4°C up to 10h.

Proteinase K solution
Add 1ml distilled water to PK batch, mix and make up to 5ml with distilled water in 15ml eppendorf. Store at –20°C in 320µl aliquots for up to 1 year. Thawed aliquots should be used within the day and cannot be re-frozen.

Running buffer
40ml NuPage MOPS/SDS Running buffer (20x) in 760ml distilled water. Use freshly prepared solution or store at +4°C up to 10h.

Secondary Antibody buffer
Add 2µl Precision StrepTactin-HRP Conjugate to 10ml Primary Antibody buffer (solution A) and then dilute solution A 1:200 in Primary Antibody buffer. Use freshly prepared solution or store at +4°C for up to 4h.

Isopropanol/Butanol solution
Mix equal volumes of Isopropyl alcohol (Carlo Erba Reagents #415154) and Butyl alcohol (J. T. Baker #8017). Use freshly prepared solution.

Transfer buffer
40ml NuPage MOPS/SDS Transfer Buffer (20x), 80ml Methyl alcohol (Carlo Erba reagents #414814), 0.8ml NuPage Antioxidant in 680ml distilled water. Use freshly prepared solution or store at +4°C up to 10h.

Equipment

- BalanceCapable of weighing 0,3g ± 0,5mg
- Balance — Capable of weighing 70g ± 0,01g
- Biological safety cabinets — Class II
- Centrifuge (capable of centrifuging — EPPENDORF

• 1.5ml Eppendorf tubes at • RCF at 20°C	Tel. 00390255404120,000 Prod. name 5810R Prod. No. 5811 000.010
• Centrifuge rotor	EPPENDORF Tel. 003902554041 Prod. No. 5804 726.006
• Electrophoresis tanks *****	INVITROGEN Tel. 0039029822201 Prod. No. EI0001
• Eppendorf tubes 1,5 ml	EPPENDORF Tel. 003902554041 Prod. n°. 0030120086
• Freezer	Capable of maintaining -20°C
• Homogeniser	OMNI INTERNATIONAL Tel. 18007764431 Prod. name OMNI GLH220
• Imaging machine	BIORAD Tel. 003902216091 Prod. name VersaDoc 1000 or equivalent
• Magnetic stirrer (heated)	VELP SCIENTIFICA s.r.l. Tel. 0039039628811 Prod. No. 100162 Able to maintain 300rpm and 70°C
• Omni Tip Disposable Generator Probe (one per sample)	OMNI INTERNATIONAL Tel. 18007764431 Prod. No. 30750
• Pipette	1-10µl
• Pipette	10-100µl
• Pipette	40-200µl
• Pipette	200-1000µl

•	
• PH meter	HANNA INSTRUMENTS Italy s.r.l. Tel. 00390773562014
• HI223Refrigerator	Capable of maintaining +4°C
• Polystyrene tubes 5ml	SARSTEDT Tel. 00390458510114 Prod. n°. 55526006
• Power supply	BIORAD Tel. 003902216091 Prod. name Power Pac 200 Prod. No. 165-5053 Prod. name Power Pac 300 Prod. No. 165-5051 Ables to provide 200V constant for one hour and 30V constant for one hour
• Rocking platform	
• Software *	BIORAD Tel. 003902216091 Quantity One
• Stainless steel forceps	Disposable
• Stainless steel scalpels	Disposable
• Thermomixer	EPPENDORF Tel. 003902554041 Prod. No 5350000013 Able to maintain heat at 38°C for one hour, at 25°C for 30 minutes and at 90°C for 10 minutes
• Transfer unit *	INVITROGEN Tel. 0039029822201 Prod. No. EI9051
• Whatman paper	WHATMAN

Procedure

Planning

A known ovine positive sample (scrapie control) is always included in each run.
Molecular weight markers are always included to allow the analysis of banding patterns and molecular weight determination.

Each gel is duplicated so that matching membranes will be produced to probe with different antibodies. This enables a comparison of each sample for its affinity for the two antibodies targeted at different epitopes of the prion protein.

Homogenisation

Work in an appropriate safety cabinet

Take 250-350mg of brainstem material, put into a 5 ml polystyrene tube and record the weight.

Add homogenisation buffer to provide 20% w/v suspension (e.g. 1.5ml of buffer to 300mg of tissue).

Place a clean homogenisation probe into the Omni homogeniser and homogenise for 1 min, with the homogeniser speed set at "3".

The homogenate may be kept frozen at -30°C and tested at a later date. The homogenate is stable for 3 weeks at -30°C.

Clarifying centrifugation and protease digestion

Work in appropriate safety cabinet

Set a thermomixer to 25°C.

Label a Eppendorf tube for each sample and distribute 150µl of digestion buffer in each tube with a micropipette (40-200µl).

Transfer 150µl of homogenate from the homogenisation tube to a 1.5ml Eppendorf tube with a micropipette (40-200µl) and mix thoroughly.

Place the Eppendorf tubes in the thermomixer (1000 rpm) and incubate for 20 mins.

Set a thermomixer to 38°C.

Place the Eppendorf tubes in a centrifuge, ensuring rotor is balanced. Centrifuge for 10 mins at 1,000g at room temperature.

Label a Eppendorf tube for each sample and add 50µl of proteinase K solution to each tube using a micropipette (10-100µl).

Remove 250µl of supernatant using a micropipette (200-1000µl), place in the labelled Eppendorf tube and mix thoroughly by pipetting.

Place the tubes in the thermomixer set at 38°C and at 1000 rpm. Incubate for 60 mins.

Remove the tubes from the thermomixer and add 10µl of digestion stop solution to each tube using a micropipette (10-100µl).

Label a new Eppendorf tube for each sample and put 30µl of loading buffer 2x into each tube using a micropipette (10-100µl).

Add 50µl of digested homogenate into each Eppendorf tube using a micropipette (10-100µl) and mix thoroughly.

Keep the remaining digested homogenate frozen at -30°C for later use (see Sample concentration). The digested homogenate is stable for 1 week at -30°C.

Sample concentration (to be used only for samples negative after the first analysis – see Data analysis)

Weakly positive or negative samples should be retested after PrPSc concentration. In these cases the scrapie control should also be concentrated.

Defrost the digested homogenate and mix thoroughly using a micropipette (200-1000 µl)

Label a new Eppendorf tube for each sample and put 200µl of isopropanol/butanol solution in each tube using a micropipette (40-200µl).

Add 200 µl of digested homogenate in the Eppendorf tubes using a

micropipette µl (40-200µl) and mix thoroughly

Centrifuge at 20000g for 30 mins at 20°C.

Discard the supernatant and dry tubes by inverting onto absorbent paper for 5 mins.

Add 50µl of loading buffer 1x to each Eppendorf tube using a micropipette (10-100µl) and mix thoroughly.

Electrophoresis

Set a thermomixer to 90°C.

Prepare gels according to manufacturer's conditions. Set up the electrophoresis tanks. Label the gels. Place two gels in each tank, the shorter sides facing inward. Clamp the gels in place. Fill the upper and lower buffer chambers with running buffer.

Heat the samples in the Eppendorf tubes in a thermomixer set at 90°C for 10 mins.

Centrifuge the tubes at 10000g for 5 mins at room temperature.

Heat the molecular weight standard at 90°C for 2 mins. Add 5µl of molecular weight standard to 65µl of loading buffer 1x (70µl of diluted molecular weight standard are needed for two gels).

Load 10µl of either sample, molecular weight standard or loading buffer 1x in the lanes of two identical gels, according to the gel plan described in annex 1.

Add 500µl of Antioxidant into the upper chamber. Place the lid onto the tank.

Connect the red electrode on the electrophoresis tank lid into the red socket on the power supply and the black electrode into the black socket. Set the power supply to 200V constant and run for 55-60 mins.

Protein transfer

While the gels are running, cut the PVDF membrane with scissors to fit all the gels to be transferred. One membrane per antibody. Handle the membrane with care, using forceps at the corner.

Pre-treat the PVDF membranes, by soaking them in methanol for 15

seconds. Transfer to distilled/deionised water for 2 minutes. Equilibrate the membranes in transfer buffer for at least 15 minutes. Cut two sheets of Whatman paper to the size of each membrane. Wet the sponges and the sheets of Whatman paper in transfer buffer.

When electrophoresis has finished, remove the gels from the tanks and open the gel plates with a cleaver.

Trim the gels with the cleaver to remove the top 1/3 and the bottom just below the dye-front. Cut a corner of the gel corresponding to the upper part of the control lane. Place the gels in transfer buffer and gently agitate for a few seconds before placing in sandwich.

Open the transfer unit and place in a tray containing transfer buffer with the deeper part of the transfer cassette facing upwards. (Ensure all following steps are immersed in transfer buffer). After each layer is added to the sandwich roll out air bubbles with a roller. On the bottom of the sandwich place two sponges, a piece of wetted Whatman paper and the gel. Label the PVDF membrane and put it on the gel. The second piece of Whatman paper is placed on top of the membrane. The final two sponges are placed on top of the blotting paper and the cassette is closed.

Place the cassettes in the electrophoresis tank. Top up the inner chamber with cold transfer buffer and the outer chamber with cold distilled water. Position the lid on top and connect to the power supply (red to red, black to black). Run the power supply at 30V constant for 60 minutes.

When the run has finished, disassemble the sandwich and cut a corner of the membrane corresponding to the corner of the gel previously identified, before placing the membranes in incubation boxes in PBST buffer for 10 minutes at room temperature on a rocking platform.

Immunological detection

Place the membrane in 25ml of blocking buffer on a rocking platform for 50 mins at room temperature.

After a wash with 50ml of PBST, the membranes are incubated for 50 mins on a rocking platform at room temperature in 15ml of a 1:250 dilution of primary antibody SAF84 or 1:5000 of primary antibody P4 in primary antibody solution.

Wash the membranes five times for 5 mins with 50ml PBST.

Incubate the membranes for 50 mins on a rocking platform at room temperature in 15ml of a 1:1000 dilution of HRP-conjugated goat anti- mouse antibody in freshly prepared secondary antibody solution.

Wash the membranes five times for 5 mins with 50ml PBST.

Prepare chemiluminescence Super Signal West Femto (800µl Lumino/Enhancer solution + 800µl Stable Peroxide solution for each membrane).

Blot off the excess PBST very gently and transfer membrane to a clean glass surface.

Add chemiluminescence Super Signal West Femto to the surface of the membrane and distribute evenly, incubate for 5 minutes at room temperature.

Blot off the excess substrate and transfer to a clean polythene surface. Place the membrane into the Versa Doc 1000 imager or equivalent, equipped with the Quantity One software, and detect the light signal.

Take a first image after 1 min of exposure. Check that samples do not saturate the maximum light signal detectable by the imager (highlight saturated pixels in the "transform" dialog of the Quantity One software). Take a shorter exposure if any sample contain saturated pixels and then proceed with longer exposures, up to 20 mins.

Data Analysis

The method described allows the quantification of the molecular weight of the non-glycosylated band and the SAF84/P4 ratio relative to the scrapie control.

Ensure that signals to be analysed do not contain saturated pixels.

Samples negative or weakly positive, so that the non-glycosylated band is not clearly identified in the blot, should be retested after PrPsc concentration (see Sample concentration).

The molecular weight is measured on the SAF84 blot, by comparison with molecular weight standard run in three lanes of each gel.

Define the 10 lanes by means of the "frame lanes" dialog of Quantity One software and then edit the frame to follow the lanes.

Automatically detect bands ("detect bands" command) and then remove all bands with the exception of the non-glycosylated bands. Do not remove

bands from the molecular weight standard lanes and ensure that all protein standards are detected in these lanes.

Select the standards (match>standards), previously inserted into the Quantity One software, and apply to the three standard lanes. See the molecular weight of the test lanes (band attributes>molecular weight).

Save the image and record the molecular weight of each sample, or print the report "all lanes report".

The SAF84/P4 ratio is the ratio of the chemiluminescence signal produced by a given sample when revealed separately with SAF84 and P4 monoclonal antibodies relative to the SAF84/P4 ratio of the control scrapie, and is measured by parallel measurements of replica blots developed with SAF84 and P4. These analyses are made by means of the "volume tools". A volume is the total signal intensity inside a defined rectangle drawn on the image.

Select the "volume rectangle tool" in the "volume menu". Draw a rectangle surrounding the three PrPSc bands for each sample and the scrapie control. Draw three rectangles in representative background regions of the blot. Double click on these three rectangles and select the "background" option.

Quantitate volumes using the "volume analysis report". Select the options "name" and "adjusted volume", and "global" as background subtraction. When done, the software will display the analysis report. Save the image and record the volumes of each sample with measured in the two blots (or print the "volume analysis report" for both, the SAF84 and the P4 blots).

In order to obtain the SAF84/P4 ratio, calculate the absolute ratio of SAF84/P4 volumes for each sample and the scrapie control and then divide the absolute ratio of each sample by the absolute ratio of the scrapie control.

Check that the data obtained following computer analysis are consistent with those observed by careful visual inspection of the blot.

Interpretation
(see annex 2 of this section)

Samples with a molecular weight lower than the scrapie control of more than 0.5 kDa and with a SAF84/P4 ratio higher than 2 are suspect and should be referred to the EURL.

Samples with a molecular weight higher than the scrapie control or lower by less than 0.5

kDa and with a SAF84/P4 ratio lower than 2 do not lead to further action.

Negative or very weakly positive samples with should be retested after PrPSc concentration (see Sample concentration)

Atypical Nor98 samples give very weak signals with SAF84 and are characterised by the presence of a band of 11-12 kDa, detected only by P4. For these samples the analyses of the molecular weight and the SAF84/P4 ratio cannot be done. Nevertheless, samples with these characteristics are not suspect and do not lead to further action.

For repeatedly negative samples or very weak positive samples with SAF84, the interpretation of the apparent molecular weight of the non- glycosylated band can be impossible. Nevertheless, if the signal with P4 is stronger than the signal with SAF84, suspicion of BSE can be excluded.

Samples very strongly positive (compared to the scrapie control) may occasionally give a SAF84/P4 ratio slightly higher than 2, while their molecular weight is similar to the scrapie control. These samples should be retested diluted.

Samples of bovine BSE (see annex 2 as an example) give no signal with P4 and thus the SAF84/P4 ratio give very high values. The molecular weight of bovine BSE is lower than scrapie control and higher then experimental BSE in sheep.

References

Lezmi, S., Martin, S., Simon, S., Comoy, E., Bencsik, A., Deslys, J. P., ... & Baron, T. (2004). Comparative molecular analysis of the abnormal prion protein in field scrapie cases and experimental bovine spongiform encephalopathy in sheep by use of Western blotting and immunohistochemical methods. *Journal of virology*, *78*(7),

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Stack, M., Chaplin, M., & Clark, J. (2002). Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies. *Acta neuropathologica*, *104*(3), 279-286.

Thuring, C. M. A., Erkens, J. H. F., Jacobs, J. G., Bossers, A., Van Keulen, L. J. M., Garssen, G. J., ... & Langeveld, J. P. M. (2004). Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein. *Journal of Clinical Microbiology*, *42*(3), 972-980.

Annex 1

Gel plan:

For each group of 6 samples run two identical gels according to the plan depicted below

WB with mAb SAF84

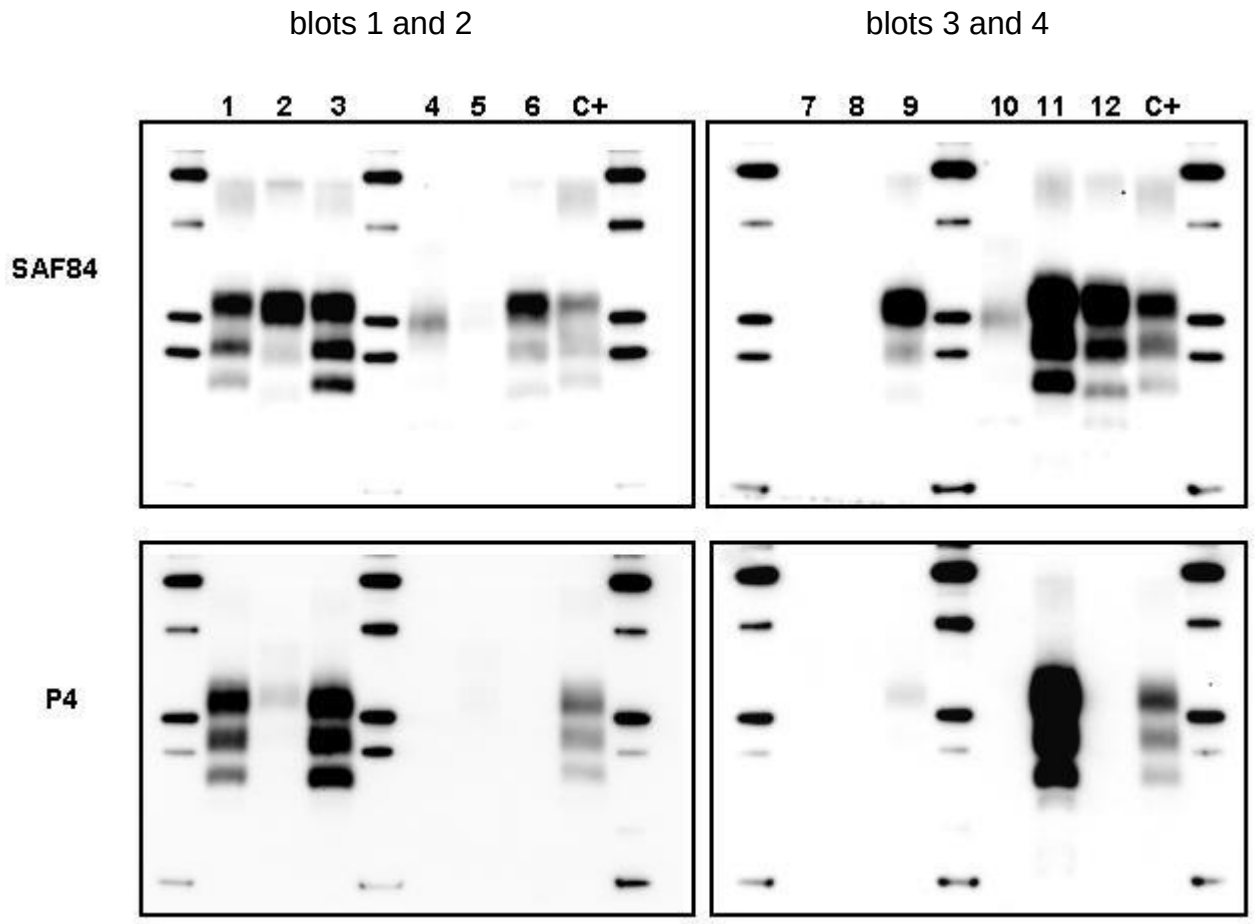
10µl	Loading buffer 1x	1
10µl	Molecular weight standard	2
10µl	Sample 1	3
10µl	Samp le 2	4
10µl	Sample 3	5
10µl	Molecular weight standard	6
10µl	Sample 4	7
10µl	Sample 5	8
10µl	Sample 6	9
10µl	Scrapie control	10
10µl	Molecular weight standard	11
10µl	Loading buffer 1x	12

WB with mAb P4

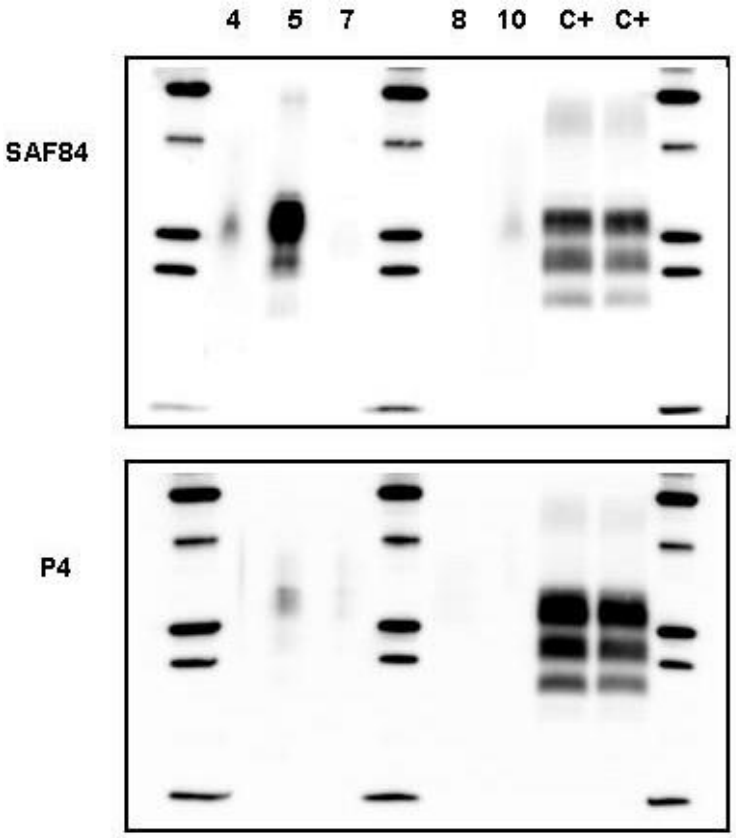
10µl	Loading buffer 1x	1
10µl	Molecular weight standard	2
10µl	Sample 1	3
10µl	Sample 2	4
10µl	Sample 3	5
10µl	Molecular weight standard	6
10µl	Sample 4	7
10µl	Sample 5	8
10µl	Sample 6	9
10µl	Scrapie control	10
10µl	Molecular weight standard	11
10µl	Loading buffer 1x	12

Annex 2

As an example of results interpretation, in the following blots the analysis of a group of 12 coded samples is reported. In blots 1-4 were loaded samples not concentrated, while in the blots 5 and 6 were loaded samples retested after concentration.



blots 5 and 6



		Molecular weight (kDa)	SAF84/P4 ratio	Interpretation	Decoded samples
Blots 1 and 2	Sample 1	18,11	0,96	scrapie	scrapie
	Sample 2	**17,42**	**10**	**suspect**	**sheep BSE**
	Sample 3	18,20	0,87	scrapie	scrapie
	Sample 4			repeat	
	Sample 5			repeat	
	Sample 6	17,70	**68**	**suspect**	**bovine BSE**
	Scrapie control	18,19	1		
Blots 3 and 4	Sample 7			repeat	
	Sample 8			repeat	
	Sample 9	**17,49**	**11**	**suspect**	**sheep BSE**
	Sample 10			repeat	
	Sample 11	18,22	0,62	scrapie	scrapie
	Sample 12	17,76	**56**	**suspect**	**bovine BSE**
	Scrapie control	18,06	1		

Blots 5 and 6 (samples retested after concentration)	Sample 4			negative	negative
	Sample 5	**17,40**	**15**	**suspect**	**sheep BSE**
	Sample 7			negative	negative
	Sample 8			negative	negative
	Sample 10			negative	negative
	Scrapie control	18,17	1		
	Scrapie control	18,19	0,96		

THE APHA BIO-RAD TeSeE-BASED HYBRID WESTERN BLOTTING METHOD

INTRODUCTION

The Bio-Rad TeSeE™ Western blot assay is a commercially available test kit that allows for the detection of PrP^{Sc} in nervous or peripheral tissues collected from infected animals (bovine, ovine, caprine and cervids), which can be distinguished from normal PrP, both by its protease resistance and molecular size. This abnormal protein is a unique identifier of Transmissible Spongiform Encephalopathy (TSE). This test has been developed as a confirmatory test of initial reactive samples identified by rapid screening tests and uses single antibody detection.

The assay procedure includes the following steps: Sample homogenisation, digestion of PrP^{c} with Proteinase K, purification and concentration of PrP^{Sc}, electrophoretic migration of the protein and transfer onto a membrane, immunoblotting and detection of signal.

Here, this confirmatory test is used with a double antibody detection method which enables discrimination to be made between cases of natural bovine BSE, natural ovine scrapie, and experimental BSE and natural scrapie in sheep.

SAFETY

Work with prions or potentially prion - containing materials has to be performed in strict accordance with National Safety Regulations.

Laboratories MUST adhere to National Safety Regulations, but the following information, published by The Advisory Committee for Dangerous Pathogens (ACDP) is available for guidance: "Transmissible Spongiform Encephalopathies (TSE) agents: safe working and the prevention of infection'. Copies can be obtained (ISBN 0113221665), from Department of Health (London) Stationery Office (Telephone +44 (20) 7873 9090). An update is available on the UK Department of Health web site (www.doh.gov.uk.cjd/tseguidance/).

MATERIALS

Chemicals and reagents

This Western immunoblotting protocol uses chemicals and reagents supplied with the TeSeE™ Universal Western Blot Kit (refer to www.bio-rad.com for distributors) and from other suppliers. The shelf life of all kit components is 1 year after production date if stored at + 5±3°C. For actual expiry date see kit or components label.

TeSeE™ Universal Western Blot kit Bio-Rad Tel No: +44 (0) 17585 812121
Cat No: 355 1169. Store at +5±3°C

Kit includes:
Grinding tubes (containing ceramic beads in a buffer solution)
A Denaturing Solution
B Clarifying Solution
PK Proteinase K
Ab I Primary antibody: anti PrP monoclonal antibody (10x)
Ab II Secondary antibody: Goat anti-mouse IgG (H+L)-HRP (10x)
Bl Blocking solution (10x)

Additional Reagents

Laemmlli Sample buffer	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0737 Store at 18°C to 30°C
2-Mercaptoethanol	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0710 Store at 18°C to 30°C
SDS	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0301 Store at 18°C to 30°C
Criterion XT 12% Bis-Tris	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 345 0118 Store at 18°C to 30°C
XT-MOPS x20	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0788 Store at 18°C to 30°C
Ethanol (Normapur)	VWR – Tel No: +44 (0) 1455 558600 Cat No: 20821-296 Store at 18°C to 30°C
Tris/CAPS x10	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0778 Store at 18°C to 30°C
Tween 20	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 170 6531 Store at 18°C to 30°C
PBS x10	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 355 1169 Store 18°C to 30°C
ECL Substrate	Amersham - Tel No: +49 (0) 761 451 90 Cat No: RPN2109 Store at +5±3°C
Goat Anti-Mouse HRP	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 172-1011 Store at –20°C or lower
Non-fat milk blocking reagent	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No 170-6404 Store at 18°C to 30°C

Primary Antibody RIDA® mAbP4	R-Biopharm Rhone LTD -Tel No: +44 (0) 141 9452924 Product No: R8007 Store at +5±3°C
Sigma Biotinylated Molecular Mass Standards	Sigma-Aldrich -Tel No: +44 (0) 800 717181 Product No.B2787. To contain standards between approximately 10-200kDa. Diluted in SDS Sample buffer. Store at -20°C or lower
Streptavidin Peroxidase - HRP	Sigma-Aldrich - Tel No: +44 (0) 800 717181 Cat No. S2438 Store at -20°C or lower
***For X-ray development only:*	
**X-ray film developer	Kodak - Tel No: +44 (208) 3189441 Developer LX24 Product No 507 0933 Store at 18°C to 30°C
**X-ray film fixer	Kodak - Tel No: +44 (208) 3189441 - Fixer AL4 Product No 507 1071 Store at 18°C to 30°C
**X-ray cassette	To fit x-ray film
**X-ray film	Amersham Tel No: +49 (0) 761 451 90 ECL RPN2103K or equivalent Store at 18°C to 30°C
**Development folders	Applied Biosystems Tel No: +44 (0) 800 515840 Cat no: T2258

Equipment

1ml Calibration syringes	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 355 1174
2ml Micro test tubes with safe lock caps	Eppendorf - Tel No: +44 (0) 7884263628 Cat No: 0030 120.086
Graduated pipettes	5, 10, 25ml
PVDF membrane (0.2 μm pore size)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 162 0175
Filter paper	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 170 4085
Cotton buds	

TeSeE PRECESS 48	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 359 0200
Heating Block (to fit 2ml Eppendorf tubes)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 358 9046
Centrifuge (capable of 15 000g)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 358 9190
Criterion XT Cell (Includes Roller)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 165 6001
PowerPac HC power supply	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 165 5052
Criterion XT Blotter (Includes 2 sandwich cassettes and 4 sponges)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 170 4070
Balance	With draft shield. Capable of weighing 0.4g ±0.5mg
Balance	Capable of weighing 310g ±0.01g
Biological Safety Cabinets	Class I (within a containment laboratory meeting national requirements for handling TSE's)
Freezer	Capable of being controlled at –20°C or lower
Freezer	Capable of being controlled at –70°C or lower
Imaging machine and PC (if detecting results with imager)	Bio-Rad - Tel No: +44 (0) 17585 812121 Fluor-S MultiImager or Versa Doc Model 1000
Magnetic Stirrer	Capable of maintaining 200rpm
Membrane incubation boxes	To fit the area of PVDF membrane used
Pasteur pipette	Plastic, disposable
Photographic trays (if detecting results by x-ray developing)	Three, to fit x-ray film
Pipette	5-40µl (Accuracy 4%, Precision 2.5%)

Pipette	40-200µl (2% Accuracy, 2% precision)
Pipette	200-1000µl (Accuracy 2%, Precision 2%)
Refrigerator	Capable of maintaining +5±3°C
Rocking Platform	Bibby Sterilin Ltd - Tel No: +44 (0) 1443 830830 Product No: Gyro-Rocker STR9
Tray	To accommodate transfer sandwich assembly
Vortex Mixer	Thermo Instruments - Tel No: +44 (0) 870 609 9203 Product Code: Fuson Whirlimixer

PROCEDURE/METHOD

Planning

A positive bovine classical BSE and positive classical ovine scrapie are always included on each run to validate the processing. Molecular mass markers are always included to allow for analysis of the banding patterns and molecular mass determination. The number of samples to be processed will vary on each run depending on the submissions and additional controls may be added if the samples are unusual and further examination required. The gel plan will be prepared to include all samples to be examined and relevant controls required.

Each gel is duplicated so that matching membranes will be produced to probe with different antibodies. This enables a comparison for each sample between its affinities for two antibodies targeted at different epitopes of the prion protein.
An example of a gel plan is shown in Fig 2.

Sample Purification

(Note: This stage is carried out in an appropriate safety cabinet according to National Safety Regulations)

Sampling and homogenisation:
~~Dissect and deposit 350mg ± 40mg of b~~rain tissue in a grinding tube.
Grind the sample in the Ribolyser for 45 sec at a speed of 6.5 for neural tissue.
Mix by inversion. Aspirate 500µl with a calibration syringe.
Transfer each 500µl into a 2ml micro-test tube.

Sample Digestion

Distribute 500µl of reconstituted proteinase K solution *(see kit insert for method)* into each micro-test tube and mix thoroughly by inversion (10 times).
Incubate at 37°C ± 2°C for 10 mins.

Precipitation of PrP^{Sc} and concentration

Distribute 500µl of reagent B into each tube and mix thoroughly by inversion (10 times)
Centrifuge at 15 000g at 20°C for 7 minutes.

Sample Clarifying

Discard the supernatant and dry the tube for 5 mins by inversion.
(Note: Use a disposable cotton bud to remove any excess liquid from the tube and lid, taking care not to disturb the pellet).

Distribute 100µl of Laemmlli solution *(see kit insert for method)* and incubate for 5 mins at room temperature (18°C to 30°C).

Completely resolubilise the pellet by aspiration/dispensing with a pipette.
Incubate at 100°C ± 5°C for 5 minutes, then homogenise by vortexing.

Centrifuge the tubes for 15 mins at 15,000g at 20°C.

Transfer the supernatant into a new micro-test tube. The procedure can be split over 2 processing days at this point and the denatured sample frozen at -20°C for 24 hours. The samples should be thawed prior to use.

Protein Separation

(Note: This stage is carried out in a safety cabinet)

Gel preparation

Remove the plastic strip on the bottom of the plate and place the gels in the migration tank.

Pour the MOPS x 1 migration buffer *(see kit insert for method)* on each side of the gel up to the top of the wells and into the Criterion XT Cell electrophoresis migration tank.
Remove the combs and rinse each well with migration buffer.

Heat the samples and known controls at 100°C ± 5°C for 4 minutes.

Immediately load 15µl of each sample per well according to the gel plan.

Load 5µl of Sigma Biotinylated Marker diluted 1:4 in Laemmlli Buffer and heated at 37'C for 2 hours as per reagent instructions (or other markers according to their specific instructions) in the outer most wells each side.

Repeat the loading on another gel, to give a duplicate gel that is identically loaded and can be probed with a different antibody.

Run the gels at room temperature (18°C to 30°C) for 50 minutes at 200V.

Protein Transfer

Protein Transfer preparation
Cut the membrane to the gel dimensions. Label each membrane in the corner so they can be correctly identified at a later stage.
Immerse the membranes in pure ethanol for 15 seconds.
Rinse in distilled water for 5mins
Equilibrate in transfer buffer *(see kit insert for method)* for 10 minutes.

Gel sandwich preparation
Equilibrate the gel in transfer buffer for 10mins.
Pre-soak the fibre pads and filter paper in transfer buffer.
Respectively place on the red side of the cassette: fibre pad, filter paper, membrane, gel, filter paper, and fiber pad.

Transfer onto PVDF membrane
Fill the Criterion XT blotter to the indicated limit with approximately 1.5Lof transfer buffer. Place an ice pad in the holder of the transfer tank and run with agitation using a magnetic stirrer for 60mins at 115V.

Immunoblotting

Upon completion of transfer, open the blotting assembly and remove the membranes for development. Soak membranes in Wash solution 2 *(see kit insert for method)* then in pure Ethanol for 10 seconds. Rinse in distilled water for 5mins.

Incubate the membrane to be detected with the kit antibody (SHA31) in 40ml of blocking solution *(see kit insert for method)* for 30mins under medium agitation.

Incubate the membrane to be detected with P4 in 50ml of Bio-rad non-fat milk blocking reagent blocking solution for 30mins under medium agitation (Make up a 5% solution with Wash solution 1 and dissolve for at least 1 hour before use).

Note: The non-kit Bio-rad Non-fat milk blocking reagent is used for probing with any other primary antibodies. Make up a 5% solution with Wash solution 1 and dissolve for at least 1 hour before use.

Dilute the kit primary antibody *(see kit insert for method)*

Dilute the P4 primary antibody 1:5000 (1mg/ml stock solution).
(5µl mAb P4 in 25mls Wash solution1).

Eliminate the blocking solution from both membranes.

Incubate the first membrane in 30ml of diluted kit primary antibody SHA31 at 18°C to 30°C for 30 mins under medium agitation.

Incubate the second membrane in 25ml of diluted mAb P4 primary antibody at 18°C to 30°C for 60 mins under medium agitation.

To allow all membranes to be taken through the washing and detection stages together, the SHA31 membrane should be placed at +4°C for a further 30mins whilst the P4 membrane continues to the end of the 60 mins incubation period.

Using Wash solution 1, briefly rinse then wash twice for respectively 5 and 10 mins under fast agitation (100ml per membrane and per cycle).

Dilute the kit secondary antibody for the SHA 31 membrane *(see kit insert for method).*

Dilute the secondary antibody for the P4 membrane, Bio-rad Goat Anti-Mouse HRP 1:5000. (10µl in 50mls Wash solution1).

Note: If using Sigma Biotinylated Markers add Sigma HRP (dilution 1:30 000).

Incubate the membranes in the appropriate diluted secondary antibody at 18°C to 30°C for 20mins under medium agitation.

Using wash solution 1, briefly rinse then wash three times respectively for 5, 10 and 10mins under fast agitation (100ml per membrane and per cycle). Place the membrane in Wash solution 2 under medium agitation.

Prepare ECL Substrate reagent *(see kit insert for method).*
Eliminate the wash solution 2 and drain the membrane on absorbent paper without blotting. Place the dried membrane onto a plastic transparency and add the substrate solution. Cover with another transparency and distribute the solution evenly over the membrane eliminating excess and air bubbles. Incubate for 1min.

Signal detection using a CCD imager

Blot away any excess liquid on the transparency before placing it into the Imager.
Place the membrane into the Bio-Rad Multi–Imager and detect the light signal using 1 & 10 minute exposures.
The membrane should be exposed until a strong signal of the positive control is visible.
Expose longer or shorter times for optimal signal visualisation.
Label the blot with sample and control details from gel loading plan and also include run number, method of processing, antibody and detection time, and operator initials and date.

Signal detection using x-ray film

Place the development folder in the x-ray cassette and turn off the safelight.

Place a sheet of x-ray film on top of the membrane and close the cassette. Expose the membrane to the X-ray film. The actual exposure time will vary according to the samples processed and the suggested time is 15mins. Expose the film longer or shorter times to achieve optimal signal visualisation.

Place the film in a tray of x-ray developer solution for 45 seconds with occasional agitation.
Wash the film for 1 minute in a tray of tap water
Place the film in x-ray fixer until the film becomes transparent.

Thoroughly wash the film in distilled water and then hang the film up to dry.

INTERPRETATION OF THE APHA BIO-RAD BASED-HYBRID WESTERN IMMUNOBLOT RESULTS

This method is capable of identifying PrP^{Sc} profiles associated with classical bovine BSE, atypical H and L-type bovine BSE, classical ovine scrapie, atypical ovine scrapie and experimental BSE in sheep.

Using this Western blot method with monoclonal antibodies SHA31 and P4, clear but subtle differences in the molecular weight position and antibody affinity are observed between the different PrP^{Sc} profiles described in the summary. A representative image of each type is shown below in figures 1a-d.

A negative sample will have been totally digested by proteinase K and no signal will be visualised with either antibody, illustrated in figure 1b (lane 4).

Any samples that give a banding profile that is not consistent with either classical or atypical scrapie should be referred to the EURL for a second opinion and further investigation where necessary.

LIMITATIONS OF THE TEST

- The method requires sufficient tissue from the optimal brain area, in order to maximize the amount of PrP^{Sc} loaded from each sample.
    - If the quantities of brain material are too low (a situation we have encountered with active surveillance), the number of Western blots that could be run from a single sample may not be sufficient to obtain an accurate interpretation and there may not be enough PrP^{Sc} in underweight samples to confirm a diagnosis even though the sample may be positive.

- It is important to compare the molecular weights of samples, relative to each other and the controls on the same gel.

- Care must be taken in interpretation, as samples that have not been digested correctly may initially appear positive. *These should be repeated.*

- Strong positive samples may give a saturated signal intensity which will obscure visual interpretation of the banding profile. *This type of sample requires repeat immunoblotting following dilution, so a clear banding pattern is observed.*

- The full banding profile may not be visualised in weaker samples or where the optimal tissue for atypical scrapie has not been submitted.

- Atypical TSE's differ in their PrP^{Sc} distribution and so the correct brain area is critical for clear, accurate results e.g. medulla for classical scrapie and cerebellum for atypical scrapie.

We have shown that differences in the ovine genotype do not appear to give any variation in the banding obtained for scrapie samples but autolysed samples can run quicker through the gel and may resulting in a lower molecular weight than the equivalent fresh sample. At present

we do not know whether mAb P4 results are affected by autolysis.

We have only assessed certain genotypes of experimental sheep-BSE and some molecular weight variability of natural sheep-BSE, if it is in the ovine population, cannot be ruled out at this stage.

Although this test was capable of accurately discriminating the panel of blind samples supplied by the CRL, it is possible for a false positive BSE in sheep to be detected using this method, since some unusual scrapie samples can exhibit a BSE-like profile (e.g. CH1641). The purpose of the Bio-Rad TeSeE Western blot is for confirmation and, as such, purification buffers and antibodies were developed and selected for their sensitivity. When strain typing was developed by CEA, the purpose was to cleave the octapeptide domain in BSE but maintain it for the other ovine prion strains. Unfortunately, the purification buffer from this confirmatory test was not protective enough for scrapie octapeptides: some unusual scrapie samples could be considered as BSE-like. A new purification buffer was developed for the Bio-Rad discriminatory kit, allowing a better protection of octapeptides in non-BSE samples. Moreover, the test used an anti-octapeptide antibody rather than the P4 antibody, because the former epitope is clearly removed in ovine BSE while the P4 epitope can persist. Therefore, the perceived major risk of using Bio-rad TeSeE western blot coupled to SHA31 and P4 for strain typing may increase numbers of false BSE-like samples, However, any potential BSE type cases are referred to the Strain Typing Expert Group (STEG) for extensive investigation using alternative differential methods and this process will aid resolution of such cases if they occur.

RESULT SUMMARY:

Classical Bovine BSE:

- **Signal and low molecular mass migration (compared to ovine) with mAb SHA31.**
- **No signal with mAb P4.**
- **Predominance of diglycosylated band.**

(See Fig 1a-d)

Classical Ovine scrapie:

- **Visible signal and higher molecular mass migration (compared to bovines) with mAb SHA31.**
- **Visible signal with mAb P4.**

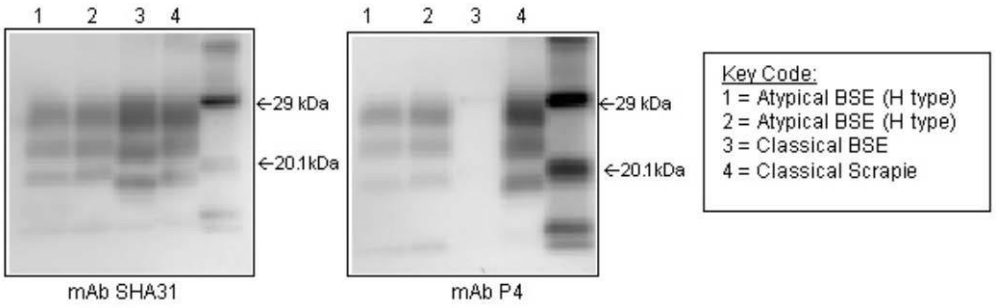
(See Fig 1a-d)

Unusual Bovine BSE:
Potential 'H' Type

- **Visible signal and high molecular mass migration (compared to bovine control) with mAb SHA31.**
- **Equivalent signal with mAb P4.**
- **Predominance of diglycosylated band.**

(See Fig 1a below)

Figure 1a



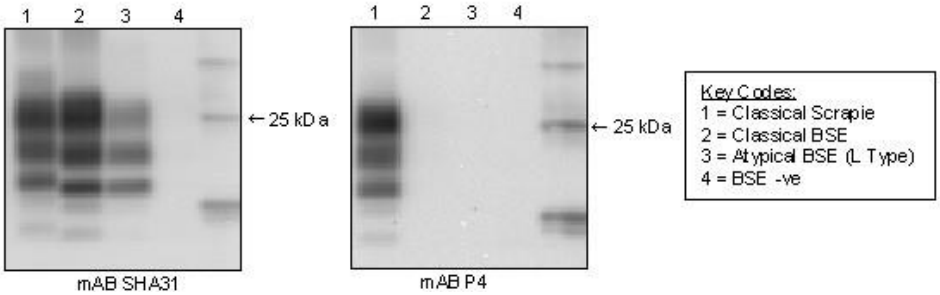
Potential 'L' Type

- **Visible signal with mAb SHA31.**
- **Molecular mass migration can be equivalent or lower than bovine control with mAb SHA31**
- **No signal with mAb P4.**
- **Reduction in the intensity of the di-glycosylated band compared to the monoglycosylated band. This may give the appearance of equal intensity of both bands or a predominance of the mono-glycosylated band.**

NB The glycoprofile can be difficult to determine if the signal is saturated, therefore dilution of the sample may be necessary.

(See Fig 1b below)

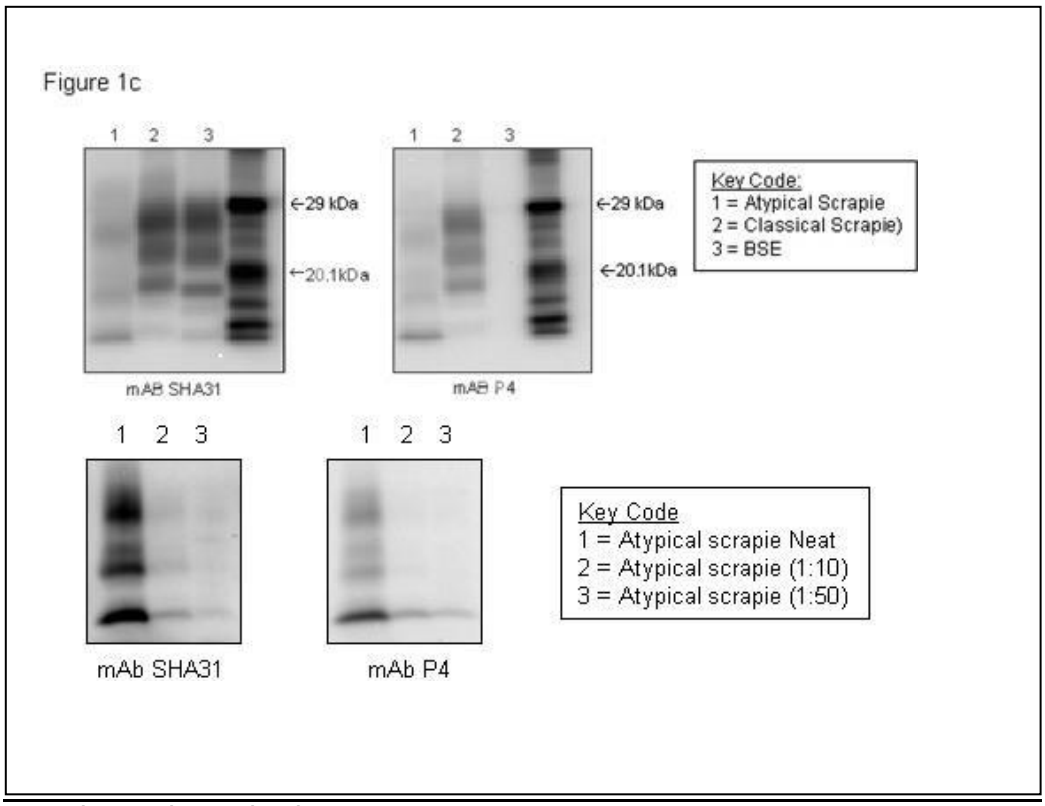
Figure 1b



Atypical Ovine Scrapie:

- **A multiple band profile is seen with mAb SHA31 and mAb P4.**
- **The bands show a significant downwards shift compared to the classical scrapie control with the fourth band being lower than 15kD (typically around 12kD).**

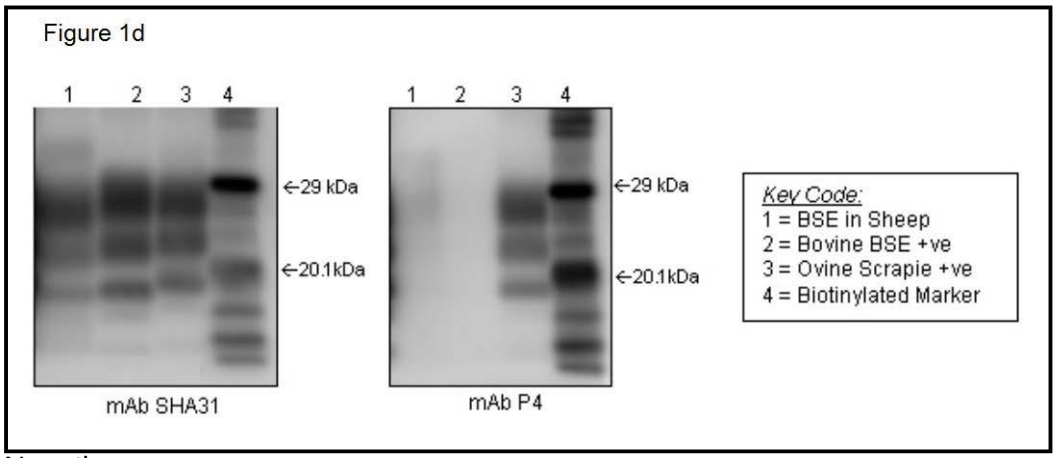
(See Fig 1c below)



Experimental BSE in sheep:

- **Strong signal and lower molecular mass migration (compared to ovine scrapie) with mAb SHA31.**
- **Much reduced signal, or no signal with mAb P4.**

(See Fig 1d below)



Negative
No signal with either mAb SHA31 or P4

Figure 2 – Suggested gel plan

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Biotin Marker	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Bov BSE +ve	Ov Scrapie +ve	Biotin Marker

REFERENCES

Manufacturers instructions – included in Bio-Rad TeSeE Western blot kit.

TSE EURL CONTACT DETAILS

For any issues related to discriminatory tests please contact Romolo Nonno or Laura Pirisinu at:

romolo.nonno@iss.it

laura.pirisinu@iss.it

For other issues please contact the TSE EURL general mailbox:

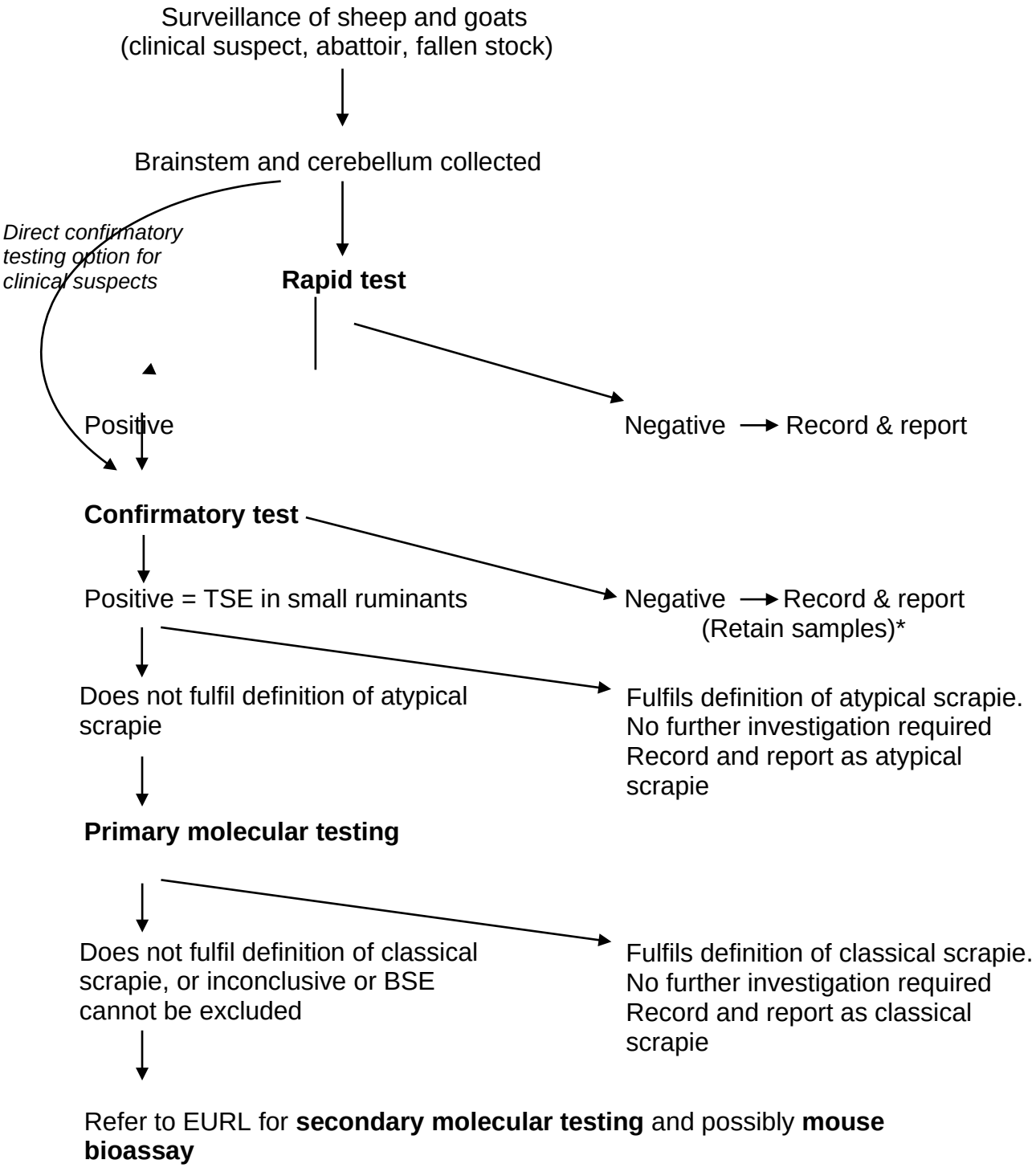
EURL.TSE@izsto.it

This document is largely based on a previous one that originally was made available by APHA (UK) as EURL for TSEs. After the transition of the EURL to our consortium, in the documentation that we are making available, some minor changes were needed to update information, contacts and references

Section 5

Appendices

APPENDIX 1: TESTING STRATEGY FLOWCHART



[9] Current criteria for definition can be found in http://www.efsa.europa.eu/en/efsajournal/pub/276.htm

APPENDIX 2: REFERRAL FORMAT FOR SR TSE POSTIVE ISOLATES to the EURL STEG for discriminatory testing

The following information should be recorded

Animal ID	
Age	
Genotype	
Breed	
Rapid test used **- result**	
Confirmatory test used	
- overall result	
(Ab used for IHC)	
If IHC unusual (i.e. different neuroanatomical distribution, or morphological type *see website/ guidelines)*	
Ab used	
Distribution of immunoreactivity (i.e. present/absent)	
DNV?	
V?	
Sol tract?	
Other?	
Discriminatory WB:	
Method used	
MW of unglycosylated band	
Relative staining intensity: PrP N-terminus specific < = or > PrP-core specific antibody).	
Amount and type of material remaining from this case?	
Referred for ring trial (date)	
SRT number (issued by EURL STEG)	

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