

Fresh Tissue Diagnosis of Transmissible Spongiform Encephalopathies (TSE's) in Cattle and Sheep.

INTRODUCTION

This page includes the current methods used in the UK for routine statutory diagnostic, and confirmatory testing on fresh tissue for TSE disease in cattle and sheep.

Western immunoblotting is carried out on fresh or frozen brain tissue using the VLA Hybrid method as the routine statutory diagnostic test (Protocol 1). This method has also been used with double antibody detection to differentiate between natural scrapie and experimentally challenged BSE in sheep (Stack et al 2002) and is currently used as the discriminatory test within the UK on positive ovine cases.

An additional method used for confirmatory testing is a long method using PrP concentration steps (protocol 2), often referred to as the OIE-SAF Western blot method.

An alternative method incorporating a sodium phosphotungstic acid (NaPTA) precipitation step is sometimes used where insufficient tissue is available for OIE-SAF Western blot. This method is not included here.

Scrapie Associated Fibril (SAF) (Protocol 3) SAF extraction followed by examination using negative stain electron microscopy is carried out on fresh or frozen brain tissue. Historically SAF examination has been widely used for statutory diagnosis of BSE throughout the epidemic in the UK, as well as for scrapie in sheep. Whilst it is recognised that the sensitivity of this method is lower than for Western blotting or alternative immunohistochemical methods it is still a test option where material is badly autolysed, or where histological/immunohistochemical examination is precluded due to autolysis or tissue damage.

SUMMARY OF RESULT INTERPRETATION

Western Blot

POSITIVE

The PrP^{Sc} is reduced by proteinase K from its original size 32-35 kDa to 27-30 kDa (di-glycosylated band). There are usually 2 other bands found below the 27-30kDa band, which represent the mono- and un- glycosylated forms of the protein. The size can be estimated by comparing with the molecular weight standards, or accurately determined using Quantity One analysis software.

With double antibody discrimination; bovine samples have a lower molecular weight with mAb 6H4 compared to ovine samples and no signal detected with mAbP4.

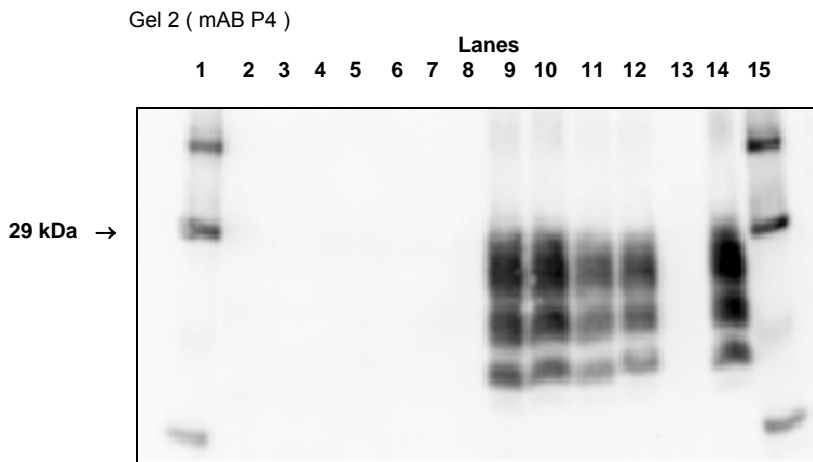
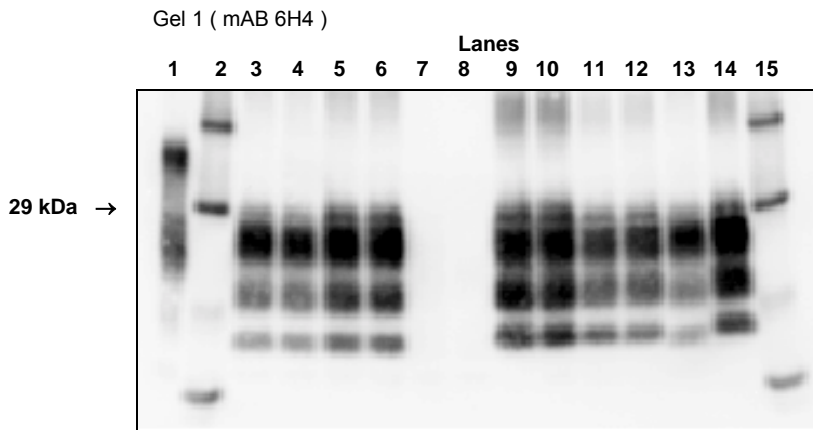
BSE in Sheep – experimental BSE in sheep gives a strong signal with mAb 6H4 and a low molecular weight compared to classical scrapie samples and a much reduced signal with mAb P4. Any ovine cases giving this type of profile should be referred to the Community Reference Laboratory (CRL) for further investigation.

NEGATIVE

The sample will have been totally digested by proteinase K and so no signal will be visualised.

Care must be taken in interpretation, as samples that have not been digested correctly may initially appear positive. These samples should be repeated. In the event of unusual or inconclusive staining the sample should be referred to an experienced manager for advice on further testing.

**Interpretation of Western blot Results
(Hybrid method with double antibody discrimination)**



- | | |
|--------------------------|--|
| Lane 1 | = Prionics Control (*Not detected with mAB P4) |
| Lanes 2 & 15 | = Biotinylated Molecular Weight Markers |
| Lanes 3 & 4 | = Bovine Positive Sample (*Not detected with mAB P4) |
| Lanes 5 & 6 | = Bovine Positive Sample (*Not detected with mAB P4) |
| Lanes 7 & 8 | = Negative Sample |
| Lanes 9 & 10 | = Ovine Positive Sample |
| Lanes 11 & 12 | = Ovine Positive Sample |
| Lane 13 | = Positive Bovine Control (*Not detected with mAB P4) |
| Lane 14 | = Positive Ovine Control |

Scrapie Associated Fibrils.

POSITIVE - Identification of undisputed fibrils of any type within the definition of Merz et al (1981).

See Image below

NEGATIVE - No fibrils fitting the definitions found after completing a search of at least 20 grid squares in a 20 minute period.

INCONCLUSIVE – Where, on rare occasions, a sample cannot be classified, a further grid should be prepared either from the same extract or from another preparation from another part of the CNS. Then examine again and re-classify.

SAF



Magnification X 39,000

References

STACK MJ, CHAPLIN MJ, CLARK.J “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 Neuropathol (Berl)*. 2002 Sep; 104(3): 279-86

MERZ, P.A., SOMERVILLE, R.A., WISNIEWSKI, H.M. & IQBAL, K. (1981) Abnormal fibrils from scrapie-infected brain. *Acta Neuropathologica* 54, 63-74

GENERAL 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/).

PROTOCOLS

Protocol 1. THE VLA HYBRID WESTERN BLOT METHOD

The VLA 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).

The VLA Hybrid Western blotting test uses 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. The method is a modification of the Prionics[®] Check Western Kit which enables a cleaner, more defined signal of the PrP^{Sc} profile.

MATERIALS

Chemicals and reagents

The VLA Hybrid Western immunoblotting protocol uses chemicals and reagents supplied with the Prionics[®] Check Western Kit (For local distributor 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.

Biotinylated standards	molecular weight	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 Multimager 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 (0.45µm 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 known bovine and ovine positive are always included on each gel to validate the processing. Biotinylated markers are always included at both ends of the gel to allow for analysis of the banding patterns and molecular weight determination. Lanes 1 and 17 are not used in order to reduce any 'smiling' effects.

Figure 1 shows a suggested gel plan that allows for 5 samples to be tested in duplicate.

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

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 \pm 10sec at $22\pm 3^{\circ}\text{C}$. Ensure that all the tissue has been homogenised 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 $50\pm 1^{\circ}\text{C}$ and another at $105\pm 3^{\circ}\text{C}$. 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 and 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 10 μ l 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 duplicate 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 $4 \pm 3^{\circ}\text{C}$. 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 $22 \pm 3^{\circ}\text{C}$.

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 by 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 occasional 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 an 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 $22 \pm 3^\circ\text{C}$. 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.

Results

X-ray images are viewed under strong light conditions. The results from the imager are displayed on the computer screen and can be adjusted for optimal viewing.

Bovine BSE positive PrP^{Sc} and typical ovine scrapie PrP^{Sc} is reduced by proteinase K from its original size 32-35 kDa to 27-30 kDa (di-glycosylated band). The size can be estimated by comparing with the molecular weight standards. There are usually 2 other bands found below the 27-30kDa band, which represent the mono- and unglycosylated forms of the protein.

Interpretation

Using the Hybrid technique, clear but subtle differences in the molecular weight position are observed (see Figure 1) 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 antibody 6H4. Sheep passaged scrapie strain CH1641 (lane 4) mimics the same molecular weight and antibody affinity pattern as for experimental BSE in sheep.

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

With antibody 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).

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

Summary:

Bovine BSE:

Strong signal and low molecular weight (compared to ovines) with mAb 6H4.
No signal with mAb P4.

Typical ovine scrapie:

Strong signal and higher molecular weight (compared to bovines) with mAb 6H4.

Strong signal with mAb P4.

Experimental BSE in sheep or atypical scrapie:

Strong signal and lower molecular weight (compared to bovine BSE or typical ovine scrapie) with mAb 6H4.

Much reduced signal with mAb P4.

Ovine samples with this profile should be referred to the CRL.

Other samples that should be repeated and/or referred to the Community Reference Laboratory are:

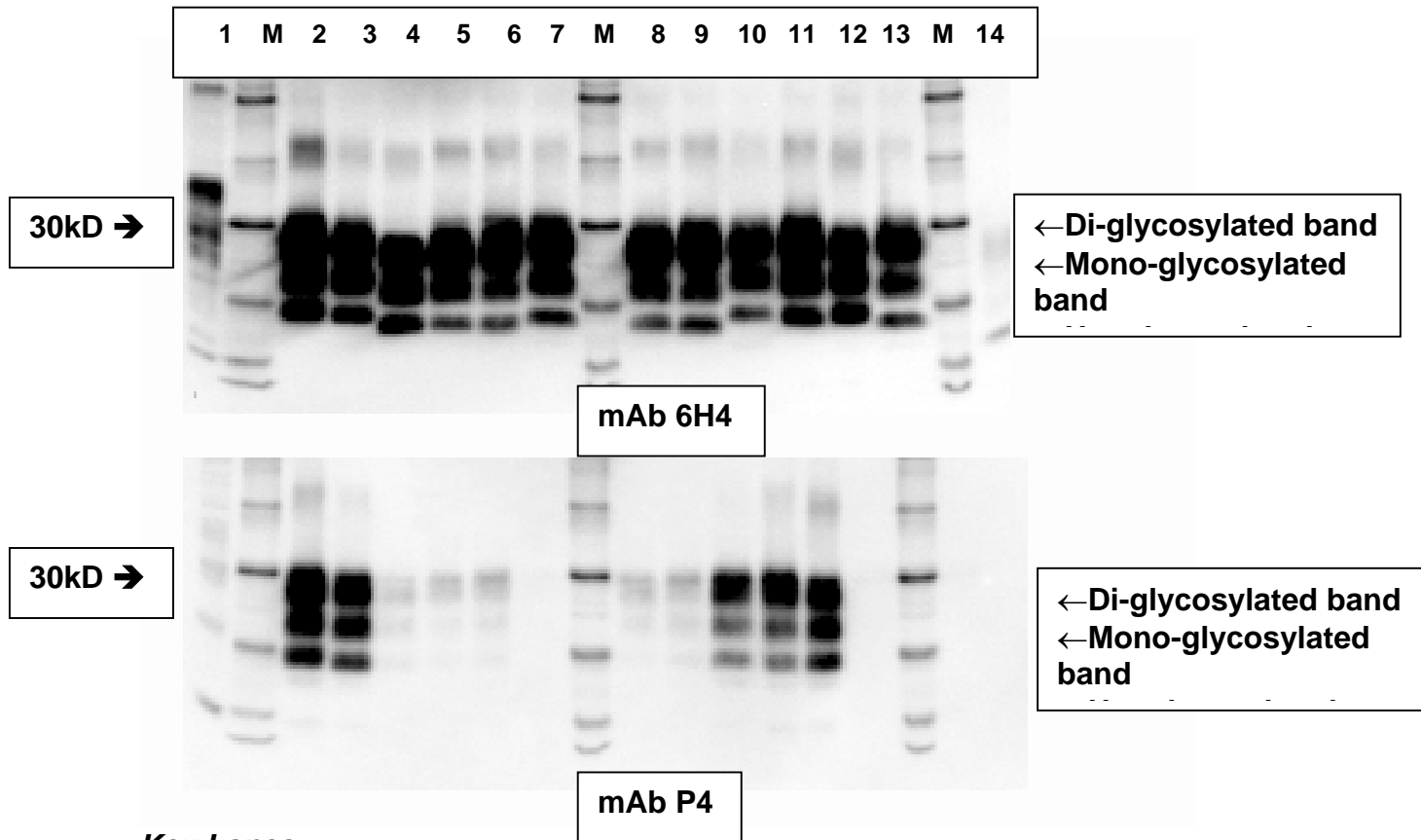
- samples that have not been digested correctly, that initially appear positive.
- Samples for which only the di-glycosylated band is detected.
- Samples in which only the di-glycosylated band and mono-glycosylated band are detected.

Figure 1 Suggested Gel plan

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

INTERPRETATION OF HYBRID WESTERN BLOT RESULTS –
DISCRIMINATION OF NATURAL BOVINE BSE, NATURAL OVINE
SCRAPIE, AND BSE AND SCRAPIE IN SHEEP



Key Lanes:

1. Prionics control – normal bovine brain
2. Scrapie positive (Romney VRQ/VRQ)
3. Scrapie positive (Cheviot ARQ/ARQ)
4. CH1641 (sheep passaged scrapie strain)
5. BSE in sheep (Cheviot VRQ/VRQ)
6. BSE in sheep (Romney VRQ/VRQ)
7. BSE positive
8. BSE in sheep (Cheviot VRQ/VRQ)
9. BSE in sheep (Romney VRQ/VRQ)
10. Scrapie positive (Swaledale ARQ/VRQ)
11. Scrapie positive (Swaledale ARQ/VRQ)
12. SSBP1 (sheep scrapie brain pool)
13. BSE positive
14. Bovine negative
- M. Molecular Weight Standard

Limitations

Tissue quantities and quality of sample

The method requires that enough brain tissue of optimal area is available as frozen material and is dependent on the quantity of PrP^{Sc} in the sample. 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 run quicker through the gel and may result in a lower molecular weight than the equivalent fresh sample.^[3] At present we do not know whether mAb P4 results are affected by autolysis. The test works best with fresh/frozen caudal medulla/brain stem/spinal cord. 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.

Routine use of the tests

The VLA hybrid WB technique, being based on a validated test, commercially available in kit form, can and has been used on large series of samples in several countries. The method does require a very high quality of blots for any quantitative measurements, so weak positives or samples which do not give a measurable signal for the un-glycosylated protein band cannot be fully assessed (eg by glycoform analysis). We have only assessed certain genotypes of experimental sheep-BSE, and only secondary and primary passaged material has been compared in such genotypes; some molecular weight variability of natural sheep-BSE, if it is in the ovine population, cannot be ruled out at this stage.

Standardisation of mAb P4

The following procedure is used to standardise new batches of RIDA[®] mAbP4.

A known ovine scrapie positive and a known bovine BSE positive control samples are homogenised and serially diluted in negative ovine and negative bovine brain homogenates respectively, down to 1:2048 dilution.

Two gels (one for each serially diluted sample) are then run and tested against:

- the newly reconstituted RIDA[®] mAbP4 (1ml/1mg: r-Biopharm Rhone Ltd)
- and the currently used mAb P4 for comparison. (Current Dilution is 1:5000).

The ovine scrapie signal detection limit from both batches needs to be matched for the serially diluted scrapie positive control sample. No signal should be detected with the bovine BSE positive control.

If the new mAbP4 appears to give a weaker signal than the previous batch at an equivalent dilution, a stronger mAb P4 dilution should be used. Alternatively if the signal appears to be too strong, then a weaker dilution of mAb P4 should be used and run against the above panel of known samples again.

Once the signal is matched the new batch at its standardised dilution is then used in parallel with the old batch of mAbP4. If the mAbP4 signals are equivalent after use on 30 field case submissions then the new batch can be used.

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Protocol 2. THE OIE-SAF WESTERN BLOT METHOD

Detection of PrP^{res} by a scrapie associated fibril (SAF) purification method, followed by Western immunoblotting techniques (35, 50, 79), is carried out on fresh (unfixed) or frozen brain or spinal cord material and is particularly useful as a confirmatory test when histological examination and immunohistochemistry results are equivocal due to autolysis. Improvements in purification methods for extracting PrP^{res} (7, 22) have contributed to increased sensitivity of this method. Where sufficient sample size remains, this methodology frequently provides a sensitive method of confirming diagnosis following initial suspicion of disease using more recent rapid tests (see below). For that reason, and in the absence of a full and accessible published method elsewhere, the method for SAF-Western immunoblotting used at the OIE Reference Laboratory is reproduced below.

- **Protocol for the SAF-Western Blot for TSE diagnosis**

The following is an example of a protocol for the purification and detection of the disease specific isoform of the prion protein (PrP^{res}) from unfixed brainstem material. Purification of the SAFs is first achieved by ultracentrifugation, followed by a proteinase K treatment to digest residual cellular PrP^C. A formic acid clearance step is used to reduce background signals prior to immunoblot detection of the PrP^{res}.

- **Selection of the starting weight**

Material must be taken from the optimum brain region. This will generally be brainstem (Obex region) but in the case of confirming atypical TSE cases cerebellum or cortex regions would be preferable. These areas tend to accumulate more PrP^{Sc} in such cases.

The amount taken depends on the prior results; with the following guidelines: -

- Negative Control: 1g PK treated
- Positive Control: 0.5g PK treated (This applies to VERY Strong Positive)
- Sample:
 - o Strong Rapid Test Positive: 1g (all PK treated)
(Biorad ELISA reading >2.5)
 - o Moderate Rapid Test Positive: 2g (all PK treated)
(Biorad ELISA reading 1 – 2.5)
 - o Weak Rapid Test Positive: 4g (2g PK treated + 2g Untreated PK)
(Biorad ELISA reading <1)

If a negative result occurs in a sample that has less than 2g tested, this sample should be repeated with at least 2g of PK Treated tissue if available.

NB: This method is typically for 4g of original tissue - Untreated & PK Treated.

Reagent List:

Solution 1 - Brain Lysis Buffer (BLB):

10 g N-Lauroylsarcosine, sodium salt (SIGMA #L5125) in 100 ml of 0.01 M sodium phosphate buffer pH 7.4 prepared fresh for each sample preparation procedure. (Store at 4°C for maximum 3 days)

Preparation of 0.01M sodium phosphate buffer:

- a) dissolve 0.345g NaH_2PO_4 in 250ml distilled water
- b) dissolve 0.9 g Na_2HPO_4 in 500 ml distilled water
- c) adjust to pH 7.4 by adding the acidic NaH_2PO_4 solution to the basic Na_2HPO_4 solution-store at 4°C

Solution 2 - 100 mM PMSF: 0.435 g Phenylmethylsulfonylfluoride in 25 ml propan-1-ol stored in a dark bottle for 4 weeks at 4°C

Solution 3 - 100 mM NEM: 0.313 g N-ethyl-maleimide in 25 ml propan-1-ol store in a dark bottle for 3 months at -20°C (or at 4°C for 2 weeks)

Solution 4 - 1 M Tris/HCl pH 7.4 : 121.14g Tris in 1L distilled water adjust to pH 7.4 using HCl

Solution 5 10% or 15% - KI-HSB: 1.5 g Sodium thiosulphate, 1.0 g N-lauroyl-sarcosine in 1 ml 1M Tris/HCl add 10 g (for 10%) or 15 g (for 15% solution) potassium iodide make up to 100 ml with distilled water - store at 4°C

Solution 6 - Proteinase K (PK): 1 mg / ml (in distilled water)

Solution 7 - 1M NaCl: 5.844g NaCl in 100ml distilled water

Solution 8 - Formic Acid: Formic Acid (98% Analar)

Solution 9 - 1 x sample buffer:

- 2 ml SDS (20%)
- 1 ml Tris/HCl (1M, pH 7,4)
- 1 ml Mercaptoethanol
- 0,6g sucrose
- 1-3 drops Bromphenol blue
- 15 ml distilled water

Solution 10 – Running Buffer: NuPAGE MOPS SDS Running Buffer

Solution 11 – Antioxidant: NuPAGE Antioxidant

Solution 12 – Transfer Buffer: Tris (25 mM) 3,03 g/l
Glycine (192 mM) 14,4 g/l
Methanol (10%) 100 ml/l

Solution 13 - Blocking buffer: 0.2% I-Block in Tris Buffered Saline (TBS – solution 15) +
0.1% Tween 20

Solution 14 – TBS: 8g NaCl, 0.2g KCl, 3g TRIS in 1L distilled water. Adjust to
pH 7.4 using HCl

Solution 15 – TBS Tween (TBST) : TBS + 0.5 % Tween 20

Solution 16 – Luminescence buffer: 2.42g TRIS in 1L distilled water. Adjust pH to 9.8
using HCl. Add 0.2g MgCl₂

• Detergent extraction and proteinase K treatment

1. Take appropriate amount of brainstem material.
2. Cut into small pieces (carefully remove the dura mater) and add 5 ml of BLB (**Solution 1**) with 10µl of 100 mM PMSF (**Solution 2**) and 10 µl of 100 mM NEM (**Solution 3**).
3. Homogenise thoroughly in a falcon tube using an Omni homogeniser.
4. Add another 2-5 ml of BLB (**Solution 1**) into the falcon tube and re-homogenise if necessary. Transfer homogenate to a Beckman 39.2ml plastic centrifuge tube. Top up with BLB until at least $\frac{2}{3}$ full to prevent the tube collapsing during centrifugation.
5. Centrifuge at 20,000 g (13400 rpm) for 30 minutes at 10°C in a Beckman Ultra-Centrifuge Rotor 55.2Ti at rMax.
6. Carefully remove the supernatant into clean centrifuge tubes Fill $\frac{2}{3}$ of the tube up with BLB to prevent it collapsing during centrifugation. Centrifuge at 177,000 g (39700rpm) for 2 h 15 minutes at 10°C in a Beckman Ultra-Centrifuge Rotor 55.2Ti at rMax.
7. Discard the supernatant and suspend the pellet in 3 ml distilled water with 50 µl 1 M Tris/HCl pH 7.4 (**Solution 4**) by gentle aspiration with a pipette. For less than 2g of medulla, 1.5ml distilled water with 25 µl of Solution 4 is used. Transfer to a bijoux.
8. Incubate in a water bath at 37°C for 15 minutes with constant stirring.
9. Add 6 ml of 15% KI-HSB (**Solution 5-15%**) and incubate for a further 30 minutes as in step 8. **For less than 2g of medulla, 3ml of Solution 5 – 15% is added.**
10. Divide the solution into two aliquots of 4.5 ml (only for samples that are divided into aliquots for plus and minus PK treatment).
11. To one aliquot add 1mg/ml PK (**Solution 6**) and incubate for one hour as in step 8, amount of PK solution to be added depending on weight of starting material:
1g +PK: 22,5 µl
2g +PK: 45 µl
4g +PK: 90 µl
12. To both aliquots add 4.5 ml of 10% KI-HSB (**Solution 5-10%**) and fill the tubes to around $\frac{2}{3}$ with more 10% KI-HSB to prevent them collapsing during centrifugation. Centrifuge at 189,000 g (41100rpm) for 1 hour at 10°C in a Beckman Ultra-Centrifuge Rotor 55.2Ti at rMax.

13. Carefully tip off supernatants and drain the tubes well.
14. Re-suspend pellet in 1ml 1M NaCl (**Solution 7**). Carefully transfer re-suspended pellet to 1.5ml Eppendorf tubes. Centrifuge at 11600g for 15mins at room temperature. (13000rpm MSE Micro Centuar Rotor at rMax).
15. Remove supernatant carefully and discard. Added 50µl Formic Acid (98% Analar) (**Solution 8**) to the pellet. (Note: It will become gluey and hard).
16. Dry the pellet in a SpeedVac set for 1 hour at 45°C. Allow an extra 15mins for cooling.
17. Resuspend samples in 40µl 1 x sample buffer (**Solution 9**). Stored at -20°C overnight.
20. Samples are now ready for sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) and Western immunoblotting.

- **SDS-PAGE and Western immunoblotting (Some steps specific to Invitrogen Novex minigel electrophoresis system and Bio-Rad transfer system).**

- i) Set a heating block to 95±1°C.
- ii) Thaw, mix and transfer the samples into 0.5ml Eppendorf tubes if necessary or leave in the 1.5ml Eppendorfs if using a larger welled heating block. Centrifuge briefly (Quick pulse spin in the MSE Micro Centaur Rotor) to concentrate the sample in the bottom of the tube.
- iii) Set up the electrophoresis tanks using pre-cast Invitrogen NuPage 12% Bis-Tris gels. Remove white tape from lower edge of gels. Remove plastic combs from gel and using a Pasteur pipette, gently flush wells with running buffer (**Solution 10**). 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 glass plate is inserted in the tank. Place approx. 2 cm running buffer (**Solution 10**) in the outer chamber of each tank to prevent the gel base drying out whilst loading.
- iv) If sufficient sample is available, serially dilute it in OIE sample buffer (up to 10 wells) to run on an extra gel. The number of gels to be run is dependant on the number of monoclonal antibodies to be used for detection.

Boil the samples at 95±1°C on the heating block for 5 minutes. Boil the molecular weight standard solution at 105±1°C for 5 minutes.

- v) 10µl of sample, controls (If the Positive controls are strong, 3-5µl loaded onto the gels will give a clearer cleaner signal) and 5µl Biotinylated Markers are loaded onto the first gel. Release the clamp, gently lower the gels and re-clamp into position.
Please Note: PK treated and untreated PK samples along with PK treated and untreated PK positive and negative controls should always be run on the same gel.
- vi) Slowly fill the inner buffer chamber with Running Buffer (**Solution 10**). Fill the outer chamber with Running Buffer (**Solution 10**) so that the level is approximately 3cm above the bottom of the gels. Add 500µl of Antioxidant (**Solution 11**) to the inner chamber using a micropipette (200-1000µl). Place the lid onto the tank.
- vii) Run the gels at 200V until the dye front is approximately 1-2cm from the bottom of the gels (approximately 45mins)

- viii) 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. On the bench, fill the transfer tank halfway with transfer buffer (**Solution 12**) and place the cooling coil next to the red side. Switch on the water bath and ensure it is set at $4\pm 3^{\circ}\text{C}$. Add a magnet to the transfer tank and place the tank in a tray on a magnetic stirrer.
- ix) Pre-treat the PVDF membrane, by soaking it in methanol for 15 seconds. Rinse with De-ionised Water. Equilibrate the membrane in transfer buffer (**Solution 12**) for at least 10 minutes. Cut 2 sheets of blotting paper to the size of the membrane.
- x) When electrophoresis has finished, remove the gels from the tanks and open the gel plates with a cleaver.
- xi) 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 (**Solution 12**) and gently agitate for a few seconds before placing in sandwich.
- xii) In the safety cabinet, open the transfer cassette and place in a tray containing transfer buffer (**Solution 12**) with the clear side at the bottom of the tray. (Ensure all following steps are immersed in transfer buffer).
- xiii) After each layer is added to the sandwich, roll out air bubbles with a roller. On the clear side of the cassette place a sponge and a piece of 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.
- xiv) Place the cassette 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 (**Solution 12**) and position the lid on top (red to red, black to black).
- xv) Run the power supply at 150V for 60 ± 2 minutes.
- xviii) Take membrane out of the transfer cell and block in 25 ml I-Block (**Solution 13**) for a 1 gel membrane (50ml for a 6 gel membrane) for 30 ± 5 minutes at $22\pm 3^{\circ}\text{C}$ on a rocker.
- xix) Dilute the primary antibody of choice in TBS + 0.05 % Tween 20 (**Solution 15**). Prepare a volume sufficient to cover the membrane in the incubation box, one incubation box for each antibody. (25ml for a 1 gel membrane, 50ml for a 6 gel membrane). Add the antibody solution and incubate on a rocking platform for minimum 1hr at $22\pm 3^{\circ}\text{C}$ or overnight at $5\pm 3^{\circ}\text{C}$.
- xx) Wash three times for 7 minutes each in TBS + 0.5 % Tween 20 (**Solution 15**) on a rocker.
- xxi) Dilute the secondary antibody (1:5000) and Streptavidin (1:2000) in TBS + 0.05 % Tween 20 (**Solution 15**). (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 room temperature.
- xxii) Wash three times for 7 minutes each in **Solution 15** at room temperature on a rocker.

- xxiii) Incubate in luminescence buffer (**Solution 16**) for 5-10 minutes at 22±3°C on a rocker (25ml for a 1 gel membrane, 50ml for a 6 gel membrane).
- xxiv) If using an enhanced chemiluminescence system, add up to 5ml of CDP-Star Tropix (New England BioLabs) 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 on a flat sheet of polythene.
- xxv) Visualise bound antibodies using an enhanced chemiluminescence system and quantify signals by a photoimager system such as a Fluor S Multimager, (BioRad) or by X-ray film for 1-10 minutes on average. To verify a negative result in a suspect sample, exposure times should be as long as necessary to produce substantial background staining.

- **Interpretation of results**

As this protocol aims at the concentration of the insoluble PrP^{res} by ultracentrifugation, no signal would be expected to be seen from the digested negative control sample and the undigested aliquot, containing only soluble PrP^C, is likely to give only a weak signal. Prion protein has two glycosylation sites and as a consequence, after PK digestion, PrP^{res} yields bands at m.m. 30–27 kDa, 26–24 kDa and 21–19 kDa in the immunoblot. For BSE PrP^{res} the upper band is most prominent. This is also true for most scrapie PrP^{res} signals, but banding intensities can also be different.

Negative control:

- Not treated with PK: no or only weak PrP^C (33–35 kDa) signal;
- Treated with PK: no PrP specific signal.

Positive control:

- Not treated with PK: very strong signal, often too strong to differentiate single PrP-bands, highest signal intensity at 33–35 kDa;
- Treated with PK: three PrP^{res} bands visible.

A shift in molecular weight should be visible between the undigested and the digested fraction of the positive control to verify the PK activity.

Diagnostic sample:

- The PK treated diagnostic sample is diagnosed as positive if PrP^{res} signals are clearly visible. Diagnostic samples should preferentially be loaded on the same blot as the PK treated positive control. They must always be loaded on the same gel as an undigested PrP^C control sample to be able to judge the shift in molecular weight.
- The PK treated diagnostic sample is diagnosed as negative if there is no detectable PrP^{res} specific signal.
- Testing must be repeated if the results of the positive or negative control are untypical or if the result of the diagnostic sample is inconclusive, e.g. as signals are:
 - Very faint (repeat SAF preparation with a higher amount of brain material).
 - Banding pattern does not match the positive control (repeat procedure, use other diagnostic methods in addition).

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Protocol 3. SCRAPIE ASSOCIATED FIBRIL (SAF) DETECTION

Reagents

Solution A

10g N-Lauroyl Sarcosine
100ml de-ionised water
Adjust to pH 7.4 with 1M Sodium Dihydrogen Orthophosphate

Solution B

1g N-Lauroyl Sarcosine
10g Sodium Chloride
100ml de-ionised water
Adjust to pH 7.2 with 1M Sodium Dihydrogen Orthophosphate or 0.1N Sodium Hydroxide

Solution C

Proteinase K – working strength 100ug/ml

2% Phosphotungstic Acid (PTA)

2g phosphotungstic Acid
100ml deionised water
Adjust to pH 6.6 with 10N and 1N potassium Hydroxide

• Sample Extraction

1. Take 1g of appropriate CNS tissue, place in a glass tissue grinder and add 2.5ml of **Solution A**. Cut resistant gloves are worn under latex disposable gloves.
2. Homogenise with at least 20 strokes of the piston, or until smooth suspension is obtained, and pour off into polycarbonate centrifuge tube. The glass tissue grinder is held within pipe lagging during homogenisation. If the homogenate has too much foam, add 2-3 drops of n-octanol. Leave homogenate at room temperature for 30 minutes.
3. Centrifuge at 22,000g average (20,000rpm) for 10 minutes.
4. Gently recover the supernatant by pipetting into a clean polycarbonate tube and discard pellet.
5. Top up the supernatant with **Solution A** and mix by aspiration with a pipette. Centrifuge at 540,000g average (100,000 rpm) for 20 minutes.

6. Discard the supernatant and re-suspend the pellet by aspiration with a pipette in 2.5 ml of **Solution B**.
7. Centrifuge at 540,000g average (100,000 rpm) for 25 minutes.
8. Discard the supernatant and re-suspend the pellet, by aspiration with a pipette in 1.5 ml **Solution C**. Stir for 1 hour at 37°C using a Teflon coated magnet.
9. Centrifuge at 22,000g average (20,000 rpm) for 10 minutes using the Beckman microcentrifuge tubes (Eppendorf type) and the TLA.45 rotor.
10. Discard supernatant and re-suspend pellet by aspiration with a pipette in 50µl of sterile, deionised water.
11. Mix for a minimum 30 seconds using a vortex mixer, ensure that the pellet is fully resuspended.
12. Pipette one drop (25µl) of the final extract onto a strip of dental wax and using the Dumont EM forceps place one 300 mesh formvar/carbon/plasma glo treated EM grid onto this drop (coated side down). Leave for approximately 10 seconds then blot, using the edge of the grid, on the fine grade filter paper.
13. Float the grid on 25µl of 2% potassium phosphotungstic acid at pH 6.6 (coated side down). Leave for approximately 10 seconds then blot, using the edge of the grid, on the fine grade filter paper. Place on a grid mat (coated side up) within a clean Petri dish. Cover with lid and allow to dry.
14. Examine the grid in a transmission electron microscope at magnifications between 19K and 30K with an accelerating voltage of approximately 80Kv.