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FIXATION, TISSUE PROCESSING, HISTOLOGY AND IMMUNOHISTOCHEMISTRY PROCEDURES FOR DIAGNOSIS OF ANIMAL TSE (BSE, SCRAPIE, ATYPICAL SCRAPIE)

Histopathology, Pathology Department, VLA Weybridge

1. Fixation
2. Formic acid decontamination
3. Tissue processing
4. Histological staining
5. PrP immunolabelling & antibodies
6. Histology laboratory technical tips

1. Fixation

Whole Brain: Brains are fixed in a 10 × volume (4 to 6 litres for bovine brain, 1 to 2 litres for ovine brain) of formol saline (supplier VWR) or 10% neutral buffered formalin (Supplier VWR). Fixative is changed after 7 days. After a further 7 days fixation, the brain is sliced transversely through the neuroaxis at intervals of approximately 3mm to 5mm. Areas of the brain routinely processed are the medulla at the level of the obex, the medulla through the caudal cerebellar peduncles and the midbrain. Blocks of tissue are placed back into fresh formol saline for a further 7 days.

Brainstem for diagnosis: The medulla at the level of the obex is removed at post-mortem and fixed in a 10 × volume of the preferred fixative for a minimum of 3 to 5 days. The fixed block is trimmed prior to tissue processing.

Viscera: Fixed in 10% neutral buffered formalin for a minimum of 3 days.

2. Formic Acid Decontamination of Fixed Blocks

For routine diagnostic purposes, thoroughly fixed tissues can be decontaminated prior to tissue processing. Fixed and trimmed tissues are placed in processing cassettes and immersed in 98% formic acid (supplier VWR) for one hour. Blocks are then washed in running tap water for 20 minutes, then returned to fixative prior to tissue processing.

If tissues are not treated prior to processing, they must be treated with formic acid prior to histological (H&E) staining. The immunohistochemistry procedure includes a formic acid epitope demasking step which also acts to decontaminate sections. See section 4.

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3. Tissue Processing

Tissue processing schedules for enclosed, automatic processors are devised according to species and size / type of tissue. Three schedules are illustrated using Shandon Hypercentre XP or Pathcentre.

- Overnight processing for bovine and ovine medulla obex
- Overnight processing for ovine viscera
- Extended processing (3 days) for large CNS blocks

Position	Reagent	Overnight schedule for bovine and ovine medulla obex				Overnight schedule for bovine and ovine viscera				Extended processing for large CNS blocks			
		Time (hours / mins)	Vacuum (Yes / No)	Temperature (°C / Ambient)	Drain (seconds)	Time (hours / mins)	Vacuum (Yes / No)	Temperature (°C / Ambient)	Drain (seconds)	Time (hours / mins)	Vacuum (Yes / No)	Temperature (°C / Ambient)	Drain (seconds)
1	Fixative	3 h	Y	37	30	1 m *	N	A	30	1 m *	N	A	30
2	70% Alcohol	0.5 h	Y	37	30	1.5 h	N	A	30	2 h	N	A	30
3	70% Alcohol	0.75 h	Y	37	30	1.75 h	N	A	30	3 h	N	A	30
4	90% Alcohol	1 h	Y	37	30	1h	Y	A	30	6 h	N	A	30
5	90% Alcohol	1 h	Y	37	30	1h	Y	A	30	6 h	N	A	30
6	100% Alcohol	1 h	Y	37	30	1h	Y	A	30	3 h	N	A	30
7	100% Alcohol	1 h	Y	37	30	1h	Y	A	30	3 h	N	A	30
8	100% Alcohol	1 h	Y	37	30	1h	Y	A	30	6 h	N	A	30
9	Chloroform	2.5 h	Y	37	30	2.5 h	Y	A	30	8 h	N	A	60
10	Chloroform	3 h	Y	37	60	3 h	Y	A	60	16 h	N	A	60
11	Wax	1.5 h	Y	62	120	1.5 h	Y	62	120	8 h	N	62	120
12	Wax	3 h	Y	60	120	3 h	Y	60	120	8 h	Y	60	120

* or set machine delay

Blocks are routinely embedded and sectioned at 4µm. Sections are floated onto purified water at 10°C below the melting point of the wax used, and picked up on charged or Vectabond (Vector Laboratories) treated slides, drained upright, dried at 37°C for a minimum of 20 minutes (but preferably overnight) and then melted on at 60°C for 30 minutes to improve adhesion. Do not leave unstained sections in the 60°C oven for longer than 30 minutes, nor store unstained sections for lengthy periods before immunolabelling.

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4. Histological Staining – Haematoxylin & Eosin (BSE Modification)

A modified H&E is employed. The reduced differentiation increases contrast of the sections, allowing easier identification of vacuolar pathology.

NOTE: If the tissue was not decontaminated prior to processing, then sections should be treated prior to H&E (BSE) staining by immersion in *98% formic acid (*undiluted) for 5 minutes after dewaxing and rehydration. Sections should then be washed in running tap water for a minimum of 10 minutes prior to staining.

H&E (BSE) Staining Protocol (Shandon Varistain)

Duration of staining is established by the use of a suitable control, e.g. bovine obex and microscopic examination to evaluate staining intensity, on a daily basis.

Step	Reagent	Time
1	Xylene	3 min
2	Absolute Alcohol	3 min
3	Harris' Haematoxylin (batch no.)	Variable – check daily
4	Absolute Alcohol	5 min
5	0.1% Acid Alcohol	1 min
6	Tap Water	5 min
7	1.5% Lithium Carbonate	30 sec
8	Purified Water	2 min
9	2% Eosin	3 min
10	Tap Water	5 min
11	Absolute Alcohol	1 min
12	Absolute Alcohol	3 min
13	Xylene	5 min

Slides are then mounted in DPX either by hand (for large slides) or by use of an automated coverslipper.

Reagent	Notes
Harris' Haematoxylin acidified. Clin Tech Limited, Clacton-On-Sea, Essex, CO15 4XA. UK	Each new batch of haematoxylin is started at 25 minutes duration, with the time varied according to the staining intensity desired. Progressive oxidation of haematoxylin as the batch ages, decreases the time required to achieve a given intensity
Eosin yellowish C.I. 45380 Gurr. Certistain – Merck	Used as a 2% solution in tap water with 0.2% formaldehyde added as preservative
0.1% Acid Alcohol	0.1% 1M HCl in absolute ethanol
Water Washes	Water washes are changed every run. Steps 6 and 10 are static tap water

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5. PrP Immunohistochemistry for BSE and Scrapie Diagnosis

Details of the VLA statutory IHC protocol, reagents used and technical advice are provided. A generic PrP IHC protocol worksheet is also provided and may be adapted for use in your own laboratory.

Protocols need to be optimised for each antibody in each laboratory to give best performance. The protocol used for TSE diagnosis at the VLA is given below. Several commercially available and research antibodies, both monoclonal (both rat and mouse) and polyclonal have been tested and have given satisfactory results in this protocol. Care must be taken to select an appropriate species-specific biotinylated secondary antibody.

Sections can either be stained flat in trays (by hand), using a capillary gap format such as the Shandon Sequenza or by using automated immunostainers. Appropriate protocol modifications may be required to suit the immunodetection system routinely used. For immunolabelling in trays (for example for large slides) larger reagent volumes need to be applied and sections coverslipped after primary antibody application to prevent drying out of sections and subsequent background problems. All hand stained slides must also be washed and drained effectively between reagent application.

Slide and Reagent Preparation

Prepare sections at 4µm from formalin-fixed, paraffin wax embedded tissues, float onto purified water at 10°C below the melting point of the wax used. Pick up on charged or Vectabond (Vector Laboratories) coated microscope slides. Drain thoroughly, and air-dry sections at 37°C overnight, then melt on to improve adhesion by incubating until the wax melts (60°C for 30 minutes). It is always preferable to use freshly cut sections for immunohistochemistry, although control sections can be stored (before melting on at 60°C) for up to 4 weeks at 4°C in a sealed container with desiccant. Long-term storage of cut sections is not recommended.

VLA uses Elga purified water (Elgastat Option 3: purified and sterilised by reverse osmosis, deionisation and ultra-violet irradiation) for preparation of all reagents. Water can be highly variable and local conditions can influence antibody binding and immunolabelling. Replacement of tap water washes with purified water can be helpful.

R145 is a rat monoclonal antibody to PrP produced by VLA. The immunogen was a synthetic peptide corresponding to the sequence YQRESQAYYQRGA (221-233) of bovine PrP. It is supplied as 1mg of freeze dried product, which should be stored in the dark at 4°C until reconstitution with 1ml of sterile distilled water, giving a stock concentration of 1mg/ml. Once reconstituted, the stock should be aliquotted into suitable volumes (minimum 50µl) and stored at -80°C. One of the stored aliquots should be thawed and titrated to determine optimal working dilution in each laboratory. Do not refreeze. For TSE diagnosis, VLA uses R145 at 1/500. Most other monoclonal antibodies

are produced in mice. Care should be taken in selecting the correct biotinylated secondary antibody i.e. use anti-rat for R145, anti-mouse for most other monoclonal antibodies and anti-rabbit for polyclonal antibodies raised in rabbits. Antibodies should be reconstituted following the manufacturers' directions.

PrP monoclonal antibodies for confirmatory TSE diagnosis by IHC

Commercially available antibodies successfully used at VLA and other laboratories are tabulated below:

Antibody	Species raised in	Binding region	Species Reactivity	Supplier and product code
R145	Rat	221-233	Bovine, ovine including atypical scrapie, cervine	http://www.defra.gov.uk/vla/services/ser_products.htm
2G11	Mouse	146-R154R171-182	Ovine including atypical scrapie, cervine, feline. Not suitable for bovine	http://www.institut-pourquier.fr P0610
F99	Mouse	217-231	Bovine,ovine including atypical scrapie, cervine	http://www.vmrd.com F99/97.6.1
F89	Mouse	146-159	Bovine,ovine including atypical scrapie, cervine	http://www.vmrd.com F89/160.1.5
12F10	Mouse	142-160	Bovine, not suitable for ovine	http://www.caymanchem.com 189710
L42	Mouse	145-163	Bovine,ovine including atypical scrapie, cervine	http://www.r-biopharm.com R8005
6H4	Mouse	144-152	Bovine,ovine	http://www.prionics.com
KG9	Mouse	140-180	Bovine,ovine	http://www.iah.bbsrc.ac.uk
SAF 84	Mouse	160-170 (human) 175-180 (bovine)	Bovine,ovine including atypical scrapie	http://www.caymanchem.com 189775

Note – N terminal derived antibodies such as P4, 12B2 and FH11 **ARE NOT** suitable for routine TSE diagnosis

Care should be taken in selecting the correct biotinylated secondary antibody. Use anti-mouse for the antibodies raised in mouse and anti-rat for R145

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Reagent	Preparation
Formic Acid 98%	Undiluted, concentrated formic acid
Epitope Demasking Solution Citrate Buffer pH 6.1	2.55g Tri-sodium citrate dihydrate 0.255g Citric acid 1 litre Purified water Adjust pH of working solution to 6.1 using 10mM citric acid solution (2.10g citric acid in 100ml purified water)
Endogenous Peroxidase Block (1% concentration H_2O_2)	Prepare fresh 3% stock (30% aqueous solution or 100vols) hydrogen peroxide in methanol 15ml of hydrogen peroxide stock solution plus 485ml methanol to give final hydrogen peroxide concentration of 1% in methanol
Tris Buffered Saline-Tween 20 (TBST) 5mM Tris, 0.85% NaCl, 0.05% Tween-20, pH 7.6	0.605g Tris 8.5g NaCl 500µl Tween-20 Dissolve reagents in 800ml purified water, adjust pH to 7.6 with 100mM HCl and adjust final volume to 1 litre with purified water
Normal serum block	Normal serum from the species the secondary antibody was raised in, diluted to 1/66 in buffer (150µl in 10ml TBST)
Primary Antibody Diluent	1% Bovine serum albumin (BSA) in TBST 0.1g BSA (molecular biology grade) in 10ml TBST
Overnight Holding Solution	Purified water. This stage is optional
Biotinylated secondary (Link) Antibody	Appropriate biotinyated secondary antibody dilute in TBST supplemented with normal serum (1/66) from the species the secondary antibody was raised in For mouse mab: 50µl goat anti Mouse, plus 150µl normal goat serum in 10ml TBST
Conjugate	Vector Elite ABC 100µl A, 100ul B in 5ml TBST. Mix and stand for 30 minutes. Do not vortex mix
Chromogen Diaminobenzidine (DAB)-Tween-20 DABT	20 ml McIlvanes citrate buffer (see below) + 0.05% Tween-20 1 x 10mg tablet Sigma DAB tablet Ensure dissolved thoroughly and filter before use. H_2O_2 undiluted (30%solution) Add 12µl just before use and mix well
McIlvanes Citrate Buffer plus 0.05% Tween-20	Stocks kept for maximum one month at room temperature *Solution A - 200mM disodium hydrogen orthophosphate* 14.125g Disodium hydrogen orthophosphate 500ml Purified water 250µl Tween-20 *Solution B - 100mM citric acid* 10.5g Citric acid 500ml Purified water 250µl Tween-20 Mix 35ml of solution A with 15ml of solution B and adjust pH to 6.4 (use solution B if pH too high and solution A if pH too low)
Ambient Temperature	19°C-24°C
Reagent Volumes	190µl for standard sized microscope slides. Use 380µl for chromogen

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VLA PrP Immunolabelling Protocol

Step	Method
1	Immerse slides in xylene (two containers, 5 minutes each) followed by absolute alcohol (two containers, 5 minutes each) to deparaffinise. Agitate twice in each reagent and drain between reagents
2	Wash in running tap water for 5 minutes. Agitate slide rack when slides are immersed
3	Epitope demasking step 1 and decontamination – immerse sections in undiluted 98% formic acid for 30 minutes
4	Immerse sections in container of tap water. Tip off water immediately and replace with slowly running tap water. Leave for 15 minutes. If tap water is unsuitable, ensure that flowing purified water is used
5	Rinse twice in purified water
6	Epitope demasking step 2 – citrated autoclaving (pH 6.1). Fill glass container with citrate buffer pH6.1 Add anti-bump glass beads to form a single layer below the slides. Immerse sections, and leave container uncovered. Autoclave using a liquid cycle at a holding temperature of 121°C for 30 minutes. The procedure should take at least 1.5 hours
7	Once autoclave is safe to open, remove the container and replace the hot buffer slowly with water (tap or purified water). Wash thoroughly for 10 minutes, with several water changes
8	Endogenous peroxidise block: Immerse sections in freshly prepared 3% hydrogen peroxide in methanol for 20 minutes
9	Wash in tap water for 10 minutes
10	Immerse sections in purified water, clip slides into Sequenza system or lay out in humid chamber. Check slide drainage and that there are no bubbles present. Slides may be held in purified water overnight if required (room temperature or at 4°C)
11	Apply TBST for 5 minutes. Do not allow sections to dry from this point
12	Normal serum block: Apply normal serum for 20 minutes at room temperature. Do not wash off
13	Apply diluted primary antibody for 60 minutes at room temperature
14	Wash 3 times in TBST for 5 minutes each
15	Apply appropriate biotinylated secondary antibody for 30 minutes at room temperature
16	Wash 3 times in TBST for 5 minutes each
17	Apply Vector Elite AB Complex for 30 minutes at room temperature. This reagent must be prepared 30 minutes before use
18	Wash 3 times in TBST for 5 minutes each
19	Apply DABT chromogen* to sections for 10 minutes at room temperature.
20	Wash slides in purified water 5 minutes & un-clip slides
21	Wash slides in running tap water for 5 minutes
22	Counterstain in Mayer's haematoxylin (using established time for each batch)
23	Dehydrate through two containers of absolute alcohol for 5 minutes each, followed by clearing in two containers of xylene for 5 minutes each. Agitate twice in each reagent and drain between reagents
24	Mount slides in DPX or equivalent, allow to dry and label

Note – if an alcohol soluble chromogen is used, slides must not be dehydrated and cleared, and an aqueous mountant is required.

There is a PrP IHC protocol worksheet available which provides a generic template for PrP IHC using mouse monoclonal antibodies. We strongly recommend using a worksheet protocol to record each step.

6. Histology laboratory Technical advice

Fixation

Formaldehyde forms cross links with proteins, stops autolysis and bacterial degradation.

- Sample containers must be clearly labelled with the animal identification
- Fixative should be ten times the volume of tissue and in a container with a wide opening to allow removal of the rigid fixed sample
- 10% buffered formalin or 10% formol saline fixative should be purchased ready made if possible.
- Fixative must be changed if contaminated with blood
- Sample must not be refrigerated during or after fixation
- Do not carry out any further work until tissue is completely fixed (no pinkness)

Blocking and cassetting

- Allocate unique number to case and provide paperwork to follow sample through the process
- Use very sharp blade using a single smooth cut & cut tissue so that the area required for sectioning is flat

Formic acid decontamination

- Do not decontaminate blocks with formic acid until the tissue is fully fixed
- Formic acid must be thoroughly washed off the tissues. Transfer cassettes to a container of water, discard the water and then wash blocks thoroughly in running tap water for 30 minutes

Tissue Processing

Tissue processing & embedding in wax provides a support matrix for the tissue to enable thin sections to be cut

- Tissues must be completely fixed before processing
- Each cassette should contain tissues from a single animal
- Processors either have many containers of reagents into which the samples are transferred sequentially (Carousel) or have one chamber into which reagents are pumped in or out (Enclosed)
- Tissues are immersed in alcohol in increasing concentrations to remove water from the tissue. Poor dehydration results in poor processing. Too long in alcohol can cause artefacts such as vacuolation and shrinkage
- Organic solvents such as xylene or chloroform (known as clearing agents) enable removal of alcohol and allow wax infiltration. Too long in clearing agents can harden tissue and make sectioning difficult
- Sample are immersed in molten paraffin wax (different formulations and melting points available) which when solidified provides a support matirx
- ‘Agitation’ option on processors is useful as it improves penetration of reagents and speed of processing. Several baths of each reagent are required to ensure complete processing
- ‘Vacuum’ option on processors is useful for the wax step as it helps speed up penetration of this slowly permeating reagent

- The length of time in each reagent is dependent upon the size and structure of the tissue
- Reagents must be changed in accordance with an appropriate schedule (to account for the number of cycles or number of cassettes or length of time) as each will become contaminated with the previous one and contamination can cause incomplete processing of the block. This can make sectioning difficult as the block may become sunken and soft and the resulting stained sections may be of very poor quality. Wax must be changed if the odour of the clearing agent can be detected

Embedding tissue to produce wax block

- Embed tissues as soon as processing is finished. Excess time in molten wax will harden tissue and lead to poor sectioning
- Open one cassette at a time to prevent misidentification of tissues
- Select an embedding base mould (rubber, metal or plastic, or use L pieces) large enough to give a clear rim of wax all around the tissue
- Place the surface to be sectioned face down in the embedding mould. Gently press flat into solidifying wax onto a cool surface to ensure a flat and even orientation for sectioning
- Fill cassette with wax and cool rapidly
    - We use electrically heated forceps, embedding centres and refrigerated cooling plates. Simpler systems include forceps warmed on a hotplate, a heated container of molten wax, a hot plate with molten wax in a tray to contain samples and a tray of ice to cool the block
- Once completely cooled, remove the solid block from the mould & trim or melt away excess wax from cassette edge (otherwise block will slip in microtome)

Sectioning

Produces a section of tissue approximately one cell thick to include the areas required for diagnosis

- Remove wax from surface of the block to expose the tissue (trim blocks at room temperature. Avoid trimming holes by using no greater than 10µm steps)
- Chill block on refrigerated plate or covered ice tray for 10 minutes before sectioning at 5µm. Blocks kept on ice for too long will crack and absorb water.
- Clean the surface of the water bath by ‘skimming’ with paper towel
- Float sections onto water that is hot enough to allow section to flatten completely but not expand too much or disintegrate. This will vary with the wax used and is typically 10ºC less than the melting point of the wax.
- Pick up sections on to charged slides. These improve adhesion of tissue to the glass and help stop washing off during staining
    - Use wax rolls to remove bubbles from under section. Use only clean wax to prepare wax rolls
- Ensure that sections are clearly labelled and dry properly
    - Dry upright at 37ºC for several hours
    - Before immunostaining, incubate slides at 60ºC to melt wax and improve adhesion. This is NOT part of the dewaxing process

Haematoxylin and Eosin staining (H&E)

- Use an automatic staining machine, series of Coplin jars or glass troughs
- Agitate the slide rack upon immersion in each reagent to ensure that the reagent quickly reaches the section surface

- Drain the slide rack thoroughly between reagents to reduce carry over and contamination
- Change reagents regularly in accordance with a schedule which takes into account the number of slide racks, number of slides and length of time
- Use separate sets of xylene and alcohol for the steps before and after staining (one for dewaxing slides to water and a separate set for taking slides from water back to xylene)
- Wax must be fully removed otherwise staining will not work or will be of poor quality. Ensure that the lab temperature is above 19ºC
- The time in haematoxylin should be chosen to give good detail within the nucleus and good Nissl staining (blue). Cytoplasm of the neurones and other structures should not be stained. Mayer's haematoxylin is recommended and should be purchased ready prepared.
- After staining the section must be "blued" This means that the stain turns from red to blue due to the pH of a weak alkaline solution. This can be tap water if slightly alkaline (hard water) or Scott's tap water substitute or saturated Lithium carbonate or ammoniated water can be used
- Permanent mountants give the best resolution

PrP Immunohistochemistry

- Always include a known positive control with test cases to ensure that the procedure has worked.
- Never use xylene if ambient temperature is less than 19ºC otherwise wax may not be fully removed and method will not work properly.
- Do not dilute formic acid used for epitope demasking. It is supplied at 96 - 98% solution.
- Use heat resistant glass (Pyrex), not metal containers for autoclaving and do not use a lid
    - Place a layer of glass beads in bottom of vessel to reduce bubbling of the buffer (as the liquid boiling point is reached before the chamber pressure is raised)
    - Ensure that the autoclave heats the liquid to the required temperature, for sufficient time (around 1hour 30 mins)
- Use pH probe designed for Tris if using TBS based buffer diluent or wash buffer. (PBS can also be used).
- If using the Shandon Sequenza system, a detergent such as Tween 20 should be added to reagents. This reduces surface tension, improves reagent flow and also reduces background non-specific staining
    - If using the Shandon Sequenza system make sure bubbles are not trapped and that liquids do not drain either too slowly or too quickly when slides are clipped with coverplates
- If staining flat in a humid chamber tray, make sure that the slide is kept level to ensure that the section is covered with reagent and does not dry out. The tray should be covered with a lid during incubations in order to maintain a humid atmosphere
- If using a normal serum block do not wash prior to the primary antibody as the bonds formed are weak
- If the primary antibody is to be frozen for storage, aliquot volumes of a minimum of 10µl into sterile Eppendorf tubes and freeze. Defrost and use one aliquot at a time. A titration should be performed on one of the frozen aliquots and the optimal antibody concentration applied to all of the sub-aliquots. Do not freeze and thaw antibodies

- Primary antibody dilution should be established by titration; the concentration to give strongest labelling with lowest background is required
    - Do not pipette total volumes smaller than 1µl
    - Use sterile pipette tips for aliquotting and diluting all antibodies in order to prevent microbial contamination
- Time each incubation from when reagent is added to the first section
- At the end of each incubation time, apply wash at the same speed as the previous step to keep incubation times constant on all slides.
- Save a little of the conjugate to test the chromogen before applying it to the section.
- Apply the haematoxylin counterstain (Mayer's) for sufficient time to identify the tissue structure but must not mask fine PrP labelling