final report

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Rapid Response Surveillance Capability Development for TSEs

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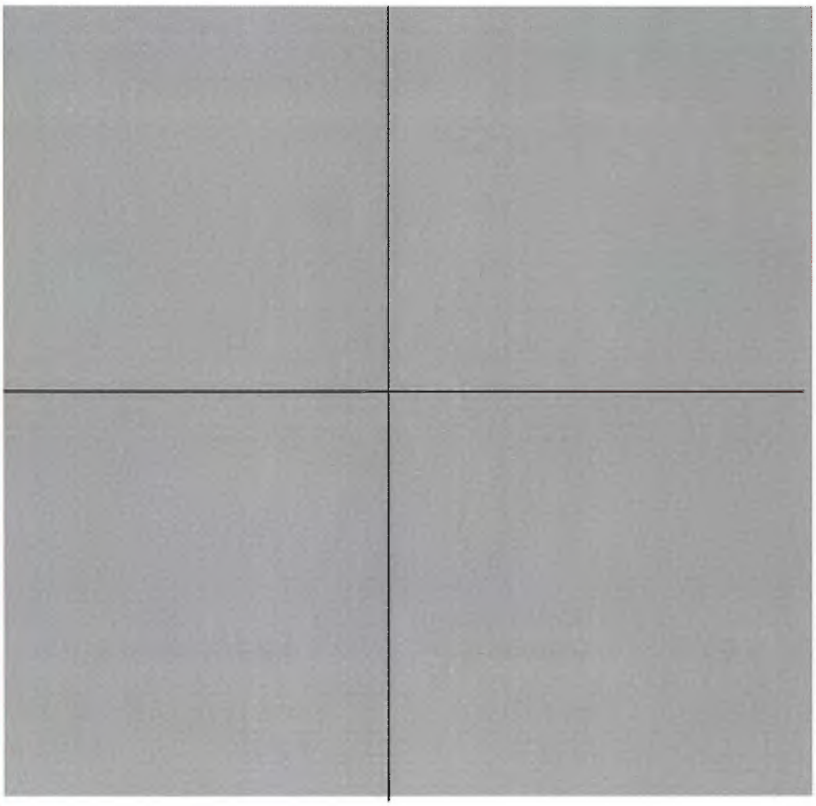
MLA PROJECT TSE 008

Establishment of the laboratory facility for enhanced TSE surveillance at the Animal Disease Surveillance Laboratory, Toowoomba



Final Report to Animal Health Australia.
September 30, 2006.

Queensland Government
Department of **Primary Industries and Fisheries**

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APPENDICES

GLOSSARY

AAHL	Australian Animal Health Laboratory
ADSL	Animal Disease Surveillance Laboratory (formerly Toowoomba Veterinary Laboratory)
AHA	Animal Health Australia
AHC	Animal Health Council
AQIS	Australian Quarantine Inspection Service
BSE	Bovine Spongiform Encephalopathy
Derogated PC3	Physical Containment between Levels 2 & 3
ELISA	Enzyme Linked Immunosorbent Assay
HEPA filter	High Efficiency Particulate Air
LIMS	Laboratory Information Management System
MLA	Meat and Livestock Australia
NATA	National Association of Testing Authorities (Australia)
NGSP	National Granuloma Submission Program
NLIS	National Livestock Identification System
PC2	Physical Containment Level 2
PC3	Physical Containment Level 3
PrP^c	Cellular prion protein – normal isoform
PrP^{res}	Resistant prion protein - abnormal protease resistant isoform (generic nomenclature)
PrP^{sc}	Scrapie prion protein - abnormal protease resistant isoform
QDPI&F	Queensland Department of Primary Industries and Fisheries
RFID	Remote Frequency Identification Device
RO water	Reverse Osmosis Water
SOP	Standard Operating Procedure
TeSeE	The Bio-Rad ELISA method for determination of PrP^{sc}
TSE	Transmissible Spongiform Encephalopathy
WHO	World Health Organisation

INTRODUCTION

This document is the final report on the establishment and operation of the enhanced Transmissible Spongiform Encephalopathy (TSE) surveillance capacity at the Animal Disease Surveillance Laboratory (ADSL), Toowoomba. It contains a critical objective analysis of the process and issues identified in the establishment of such a facility in a regional laboratory in Australia.

BACKGROUND

The aim of the project was to evaluate Australia's ability to rapidly implement enhanced TSE surveillance by targeting specific post-mortem cases.

ADSL was subcontracted to adopt technology and establish a testing capability for TSE's using the Bio-Rad TeSeE ELISA kit method for purification and detection of resistant prion proteins (PrP^{sc}). The Australian Animal Health Laboratory (AAHL) validated this method for use under Australian conditions and selected it for use in this Project.

The facility at ADSL was completed in 2004 to Physical Containment Level 2 (PC2) standard. It was not purpose built for TSE testing. Modifications were made to the laboratory to bring it to derogated Physical Containment Level 3 (PC3) standard. This level of containment was considered adequate for testing targeted surveillance animals under current Australian conditions i.e. in the absence of the detection of prion diseases in production animals.

Initial validation and verification of the test method was based on testing 200 samples analysed and provided by AAHL. Surveillance followed with the target of 2000 samples tested per year. These samples were to be approximately 90% bovine and 10% ovine. Test protocols and testing regimes were geared towards delivery of results for this level of testing. 42 samples were processed most weeks to maximise reagent use and keep abreast of samples as received at the laboratory.

The Bio-Rad TeSeE method has been approved within the European Union as a rapid test for the Bovine Spongiform Encephalopathy (BSE) and Scrapie testing programmes on cattle, sheep and goats.

This test is of high sensitivity and used for screening purposes only. Samples must be sent to AAHL for confirmatory testing using more specific methods before a positive diagnosis can be made.

LABORATORY STANDARDS REQUIRED FOR TSE TESTING USING BIORAD TEST INCLUDING PHYSICAL CONTAINMENT REQUIREMENTS IN THE CURRENT AUSTRALIAN CLIMATE.

1. Laboratory facility at ADSL, Toowoomba is derogated PC3.

TSE agents are classified as either Hazard Level 2 (e.g. Scrapie) or Level 3 (e.g. BSE) by the UK Advisory Committee on Dangerous Pathogens based on the consequences of exposure. Australian Standards do not give a rating for these particular TSE's since they are exotic to Australia. The level of physical containment of the facility where the work is conducted is derived from the hazard group of the agent. The examples given would require a minimum of Physical Containment Level 2 (PC2) for Scrapie and Physical Containment Level 3 (PC3) for BSE.

A PC3 facility would meet safety requirements for dealing with all known Classes of TSE's. Consideration was given to eliminating some of the requirements for this level of containment due to the properties of TSE's, the nature of transmission and the risk of exposure under current Australian conditions. TSE's are resistant to many commonly used methods of decontamination such as formaldehyde fumigation. They are unlikely to be transmitted via aerosols and the chance of exposure under current Australian conditions is considered negligible.

The major differences between PC2 and PC3 facilities are given in Table 1. The adopted procedures and the rationale for each for the facility at ADSL are also given. The decisions were influenced by existing infrastructure and efficient use of available resources. They were made in consultation with Australian and UK Standards and on recommendations given by AAHL and Bio-Rad.

The facility at ADSL is a derogated PC3 facility with containment features between PC2 and PC3.

Table 1. Comparison of facilities and procedures used at ADSL with PC2 and PC3 facilities.

Containment issue	PC2	PC3	ADSL
Laboratory Facilities:			**The facility was built to meet PC2 requirements and subsequently upgraded to derogated PC3.**
• Surfaces able to withstand heat and resistant to commonly used reagents and disinfectants.	**YES**	**YES**	**YES** All surfaces are of laboratory standard.
• Hands free basin provided near the exit.	**YES**	**YES**	**YES** One sink was originally installed in the laboratory area designed for both laboratory use and for hand washing. An additional hand basin located just outside the restricted area of the laboratory, near the exit, was installed for hand washing on exit. A suitable tap was difficult to source. Laboratory and personal hygiene use is separated. The hand basin drains back into the laboratory plumbing and can be diverted for collection.

Containment issue	PC2	PC3	ADSL
• Emergency shower and eyewash provided.	**YES**	**YES**	**YES** An emergency shower and eyewash was incorporated in the original design and is located just outside the restricted area of the laboratory. The waste from this system goes directly into the drain.
• Ability to seal area for formaldehyde fumigation.	**NO**	**YES**	**NO** TSE's are resistant to formaldehyde and other forms of decontamination by fumigation. The facility does not have and does not require the capacity to be sealed for fumigation.
• Spaces under benches and equipment accessible for cleaning.	**YES**	**YES**	**YES** The laboratory design included movable cabinets under the benches which can easily be rolled out of the way to assist with cleaning.
• Emergency two way communication or alarm system installed.	**NO**	**YES**	**NO** Note: The facility can be viewed from an adjoining laboratory.
• Requirement for liquid waste containment.	**NO**	**YES**	**YES** The laboratory sink is fitted with a bypass valve so that liquid waste can be collected.
• Pressure steam steriliser available.	**YES**	**YES**	**YES** PC3 facilities preferably have the steriliser housed within the laboratory. Where this is not possible wastes are bagged and placed in an unbreakable container with a secured lid which has the provision for penetration of steam. ADSL designed and had manufactured stainless steel boxes to transport material to the general laboratory autoclave.
Air Handling:			The risk of transmission of TSE's by aerosol is considered minimal. Consideration was given to Australia's TSE free status.
• Negative Air Pressure in the laboratory.	**NO**	**YES**	**NO** The facility does not have this PC3 requirement.
• Extract air filtered via HEPA filter or equivalent.	**NO**	**YES**	**NO** The facility does not have this PC3 requirement.

Containment issue	PC2	PC3	ADSL
Security and Access:			.
• Access to the laboratory limited to laboratory personnel and those authorised by the management.	**YES**	**YES**	**YES** The facility at ADSL is separated from the rest of the laboratory by a key padlock operated glass door. The combination for the keypad is known only to those staff working on the project. The clean area housing the safety shower, hand basin and adjacent office is separated from the restricted laboratory area by a visual barrier and cordoned off by a chain when the room is in use.
• Laboratory physically separated from other areas by a double door system. Entry gained through an air-lock.	**NO**	**YES**	**NO** There is no air-lock. The air is not separate from the rest of the laboratory. The facility has its own air conditioner.
Personal Protective Equipment (PPE):			
• Protective clothing provided to give protection to the front part of the body.	**YES**	**YES**	**YES** Back-closing gowns are worn in the facility. Disposable gowns are recommended during the purification process so that any potentially contaminated material can be disposed of.
• Closed footwear worn.	**YES**	**YES**	**YES** Disposable overshoes and dedicated footwear are used in the facility.
• Protective Eyewear worn to protect eyes and face from splash hazards.	**YES**	**YES**	**YES** Safety glasses are worn in the facility. Visors are not used but would offer extra protection
• Gloves worn.	**YES**	**YES**	**YES** Double gloves are worn in the facility. The inner glove is worn at all times. The second glove is used at all times when working in the Biohazard Hood and handling samples. This glove is removed and placed into an autoclave bag in the hood for disposal. A third transparent polystyrene glove is used to examine tissues during the extraction process and is replaced between samples.

Containment issue	PC2	PC3	ADSL
• Respiratory protection equipment available.	**NO**	**YES**	**NO** All procedures with exposed samples are performed in a Class II Biological Safety Cabinet. While this does not filter odours it eliminates the possibility of aerosol contaminants.
Work Practices:			
• Laboratory safety precautions and standard operating procedures adhered to at all times.	**YES**	**YES**	**YES** All staff familiar with the laboratory safety manual. Induction document read and signed before commencing duties at ADSL. Standard operating procedures have been documented for safe handling and testing within the TSE facility.
Containment Equipment:			
• Cabinets to be used when aerosols are produced and when working with specimens containing micro-organisms transmissible by the respiratory route. 1. Biological Safety Cabinet Class I or II. 2. Cytotoxic Drug Cabinet.	**1.**	**2.**	**1.** Class II Biological Safety Cabinets used in the TSE facility. The Australian/NZ STD stipulates the use of Cytotoxic Drug Cabinets for aerosol containment of prion materials. The Class II Biological Safety Cabinets offer the same protection for technical staff carrying out the test procedure. The prepositioning of the filter in Cytotoxic Drug Cabinets offers protection to cabinet service technicians. Under current Australian conditions and in the absence of detection of prions in domestic livestock in Australia the Class II cabinets have been employed. In the unlikely event of the detection of TSE's **this procedure must be reviewed with particular reference to future servicing and maintenance of the cabinets.**

3. The major differences from full PC3 requirements are:

- Filtration of exit air via HEPA filters is not essential.
- Negative air pressure in the laboratory is not essential.
- The ability to seal the area for formaldehyde fumigation is not essential.

4. **The elements incorporated into this facility include:**

- Testing is carried out in a separate laboratory specifically intended for this use and access is restricted to staff involved in the work
- All equipment used for the test is contained within this laboratory. This particularly pertains to centrifuges or other aerosol generating equipment. Also included are refrigerators and freezers used for sample and homogenate storage.
- All procedures with unsealed samples are performed within a Class II Biological Safety Cabinet.
- Personal protective equipment (PPE) including disposable gowns and overshoes are available at the entrance to the laboratory and must be put on at the entry point.
- Staff are directed to wear a gown, double disposable gloves, safety glasses and mask and overshoes or dedicated laboratory shoes when directly handling samples and purified products.
- A designated area for washing and disinfection is available on exit from the laboratory.
- Staff members receive specific training on the risks associated with prions and their decontamination.
- Solid waste (sample tissues, homogenates, tubes etc) is retained within the laboratory in autoclavable bags placed in autoclavable discard stands or containers.
- Liquid waste is stored within the laboratory and diluted 1:1 with 2M sodium hydroxide.
- The nature of exit of liquid and solid waste from the laboratory is dependant on the test result and the associated risk.
- Surfaces and equipment are decontaminated as required with 2M sodium hydroxide or 2% sodium hypochlorite.
- Staff members are competent laboratory technologists familiar with the theory and practice of ELISA technology.
- Class II Biological Safety Cabinets are used for all procedures involving direct handling of samples or products. The Australian Standard relates to work pertaining to the handling of prion material of types Class II and Class III. With these prion materials it stipulates that cabinets for aerosol containment and gloves must be used. Cytotoxic Drug Cabinets are stipulated due to the prepositioning of the filters as protection for cabinet maintenance staff. Under current Australian conditions and in the absence of detection of prions in domestic livestock in Australian the Class II cabinets have been employed. In the unlikely event of the detection of TSE's **this procedure must be reviewed with particular reference to future servicing and maintenance of the cabinets.**

- The autoclave facilities are located in a separate part of ADSL. Waste is transferred to the autoclaves in enclosed stainless steel boxes (Figure 1). The outside of the box can be wiped down with 2M NaOH for disinfection. They have a sliding cover with ventilation holes to allow steam penetration. The cover is moved sideways to close the holes for transportation to the autoclave.

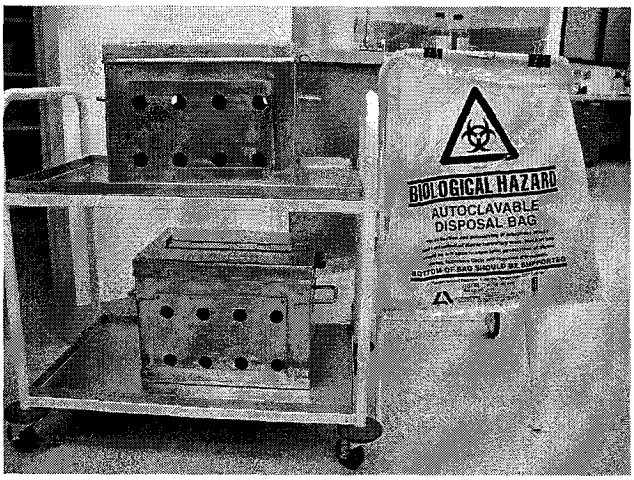


Figure 1

PHYSICAL ALTERATIONS TO BE MADE TO MEET THE LABORATORY STANDARDS

The laboratory extension at ADSL was completed in July 2004. It was designed to meet PC2 requirements and is an extension to the existing infrastructure of the laboratory. Surveillance to date has indicated that there are no TSE's in domestic livestock in Australia. This was taken into consideration when determining the physical requirements of the facility for TSE surveillance.

Alterations were made to the laboratory to take it to derogated PC3 level. These were:

- Securing the facility with the addition of a keypad lock on the door. The code is known only by those trained personnel directly involved with the project (Figure 2);

Figure 2

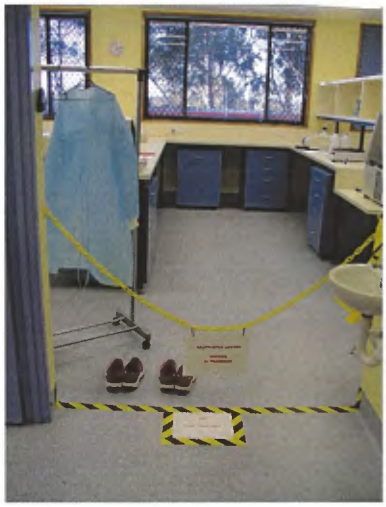


Figure 3

- Demarcation of the clean and the dirty sections of the facility by a visual barrier (Figure 3);
- The addition of a hand basin in the clean section for routine hygiene on exit. (The facility had an existing safety shower and eyewash in this area);
- Installation of a by-pass valve under the sink to divert liquids into a storage receptacle until the test result is known (Figure 4); and
- Installation of plumbed Reverse Osmosis (RO) water (Figure 4).

Figure 4

EQUIPMENT SELECTION AND PURCHASE

Equipment selection for all major pieces of equipment was based on recommendations given by Bio-Rad. Branded equipment was strongly recommended to ensure the validity of the test results. The use of alternate equipment could be validated. Selection of minor items such as pipettes and consumables was based on personal choice and local availability.

Bio-Rad branded equipment purchased included:

Product	Code	Quoted Price
Precess 48	35 90 200	$42000
Microplate incubator IPS	35 87 530	$3778
Immunowash Model 1575	170-7009	$9995
Microplate Reader Model 680	168-1000	$9995

Precess 48 (Figure 5)
This homogeniser is purpose built to hold the Bio-Rad grinding tubes used in the purification step and is considered preferential to ensure standardised homogenisation in the Purification step. It holds 48 tubes and is programmed to grind the samples in a standard manner. The equipment unfortunately, was faulty on arrival and had to be taken to Brisbane for repairs. Repair took six weeks from the time of identification of the fault. Preventative maintenance is performed by the Bio-Rad service technician at 12 monthly intervals. Depending on the work needed to be done the machine may have to be sent to Brisbane. Consideration needs to be given to decontamination procedures. Wiping the external surface of the machine with 2% sodium hypochlorite would be adequate while test status is negative.

Microplate incubator IPS (Figure 6)
The incubator holds four ELISA plates and maintains a constant temperature of 37°C. It is a convenient alternative to the standard cabinet type incubators when testing few plates.

Figure 5

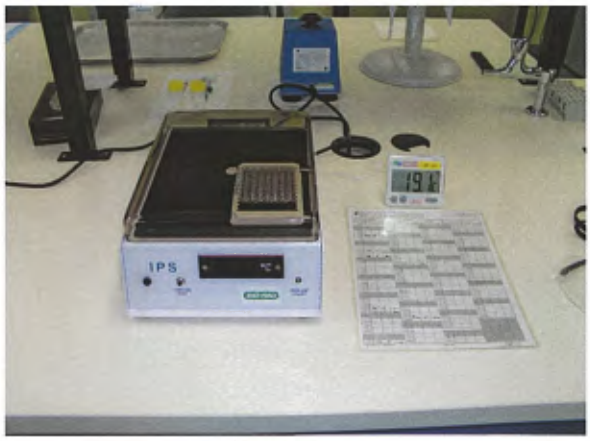


Figure 6

Immunowash Model 1575 (Figure 7)
The plate washer is a strip washer (ie washes one row at a time). This could be a rate limiting step if numerous plates were run. The wash buffer quantity contained in the Detection Kit is inadequate when using the kits on numerous occasions with small batches of samples. This may have significant cost implications if whole kits are purchased just to obtain the wash buffer. Bio-Rad does not supply extra reagents for their kits. Other plate washers may be substituted following validation.

Microplate Reader Model 680 (Figure 8)
The plate reader software is programmed to run the TeSeE ELISA test. It produces an Excel spreadsheet Screening Report. It is possible to download the sample numbers from LIMS into the report after the plate has been read. Further work will be required to transfer the results back into LIMS. All calculations, flagging of results and validation of controls are processed by the software. This is advantageous to minimise calculation or transcription errors. One difficulty experienced with the plate reader concerns its ongoing calibration. The Bio-Rad service technician calibrates the machine as no effective alternative has been found to date. Calibration plates must be externally recalibrated every two years. Bio-Rad does not recalibrate the plates they provide and they are costly to replace ($4546). In addition calibration plates used for other readers are not suitable because they have a black line reading at 4.000 absorbance units and the Bio-Rad plate reader gives a numerical result to a maximum of 3.500 units. Above this limit the result is given as '*'. Therefore no comparison with the expected result can be made.


Figure 7


Figure 8

Non Bio-Rad branded equipment purchased from Bio-Rad included:

Product	Code	Quoted Price
Eppendorf Centrifuge 5804R	35 89 190 plus accessories	$31,350

Eppendorf Centrifuge 5804R (Figure 9)
This same item could have been purchased from other suppliers, possibly at reduced prices. If purchased elsewhere it would not have come under the original service agreement.

Figure 9

Bio-Rad Cold Tray for ELISA incubation at +2-8°C (Figure 10)
The cold tray was developed and made by Bio-Rad specifically for this test and given to the Project free of charge. The ELISA plate is placed on the tray in the refrigerator. This ensures that the plate is held at +2-8°C during the incubation of the conjugate in the Detection Method. It is shown below in Figure 10.

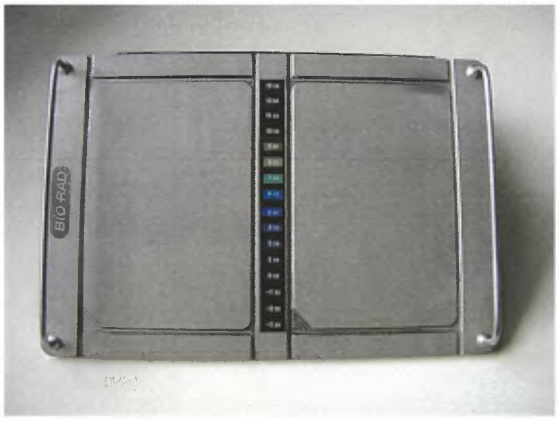


Figure 10

Refrigerators and Freezers
While domestic refrigerators have proved satisfactory in a laboratory setting the same cannot be said for cyclic defrost freezers. Chest freezers are not cyclic defrost and therefore can be useful particularly for bulk storage. For ease of use and to maintain constant temperature (a minimum of -20°C is required for this method), an upright biomedical freezer is required.

Service and Preventative Maintenance Contracts:

Service, calibration and repair of the equipment supplied by Bio-Rad is conducted by a Bio-Rad service technician based in Brisbane. The service contract includes all parts, labour and travel for breakdowns, calibrations and preventative maintenance. This service is free under warranty for the first year with charges applied thereafter. Quoted prices from Bio-Rad were:

Year 1: Free
Year 2: $19,305
Year 3: $14,807

A preventative maintenance contract and extended warranty has been negotiated with Bio-Rad to service the equipment for the purposes of maintaining NATA accreditation. The following options are being considered:

Option 1 – All breakdowns, calibrations and preventative maintenance for Precess homogeniser, plate reader and washer $7,500/year (plus GST) including four quarterly visits to calibrate the plate reader. (Preferred option)

Option 2 -All breakdowns and preventative maintenance. $5,500/year including one annual visit for maintenance and calibration of the plate reader. ADSL would calibrate the reader at the end of the other three quarters using a Checkmark plate from Bio-Rad costing $4546. The plate must be recalibrated every two years. Bio-Rad does not calibrate these plates and no alternative is currently known but is being investigated. (Option not considered viable)

Option 3 – One preventative maintenance visit per year and four validation visits to calibrate the plate reader. $5,700 per year with 30% discount on all parts, labour and travel for breakdowns paid in addition to service fee (Possible cheaper alternative to option one above).

National Association of Testing Authority, Australia (NATA) stipulate quarterly calibration of plate readers. The Bio-Rad service technician has to date undertaken this task. Preventative maintenance of the plate washer is recommended for optimal performance and the Precess mandatory to minimise rotor damage. This should be done at least annually again by the service technician.

The level of service from Bio-Rad has been extremely high. However, given the time taken to repair the Precess (see above) there may be considerable delays in testing in the event of equipment breakdowns.

The centrifuge is speed checked by Crown Scientific annually as a NATA requirement.

The pipettes are calibrated in house in a separate part of the laboratory. They can be autoclaved for 121°C for 20 minutes for routine decontamination. Filter tips are used to minimise the risk of contamination. In the event of a positive sample further precautions would be instigated before the pipettes could leave the TSE facility. This would include wipe down with sodium hypochlorite, autoclaving at higher temperatures (134°C for at least 18 minutes) and mandatory use of PPE during calibration procedures.

The Class II Biological Safety Cabinets are serviced by Contamination Control Laboratories annually. These would have to be decontaminated with 2M NaOH, decommissioned and upgraded to Cytotoxic Drug Cabinets in the event of a positive sample.

All service staff travel from Brisbane. Travel time is added to the service charge.

Summary of purchased items:

Requirements to establish TSE laboratory and run 2000 samples for the first year (excl. GST)

PRODUCT	CODE	SUPPLIER	UNIT SIZE	UNIT PRICE $	# UNITS
Capital Items in red					
Purification Reagents					
Purification kit	35 51 144	Bio-Rad	Kit for 192 samples	1200	15
Calibration syringe needle	35 51 174	Bio-Rad	200 syringes	130	15
Test sample syringe	35 51 175	Bio-Rad	200 syringes	180	20
1.5mL tube (no cap)	72.607	Sarstedt	1000	152	4
Caps	65.716.999	Sarstedt	1000	78	4
Gripper rack	93.1428	Sarstedt	Each	66	4
Storage boxes	95.064.997	Sarstedt	Each	7.84	30
Marking pens	95.954	Sarstedt	Each	3.79	10
Purification Equipment and Accessories					
Precess 48	35 90 200	Bio-Rad	Each	42000	1
Heating block	35 89 046	Bio-Rad	Each	4000	2
Rack for heating block	35 89 199	Bio-Rad	Each	100	6
5804R centrifuge	35 89 190	Bio-Rad	Each	25000	1
Drum rotor	35 89 189	Bio-Rad	Each	5500	1
Adaptors for centrifuge	35 89 191	Bio-Rad	X6	850	1
Vortex	166-061edu	Bio-Rad	Each	399	1
Pipettes and tips					
Multistepper 8 channel (black)	L/4540500	Pathtech	Each	1195	2
Finnpipette (focus) 10-100uL	4600030	Pathtech	Each	350	2
Finnpipette (focus) 100-1000uL	4600050	Pathtech	Each	350	2
Pipette stand for focus	L/9420340	Pathtech	Each	180	1
Pipette stand for multistepper (grey)	L/9420290	Pathtech	Each	65	1
Pipette tips filtered/boxed	CLPBT1000	Pathtech	Box of 480	70	20
Pipette tips filtered/boxed	CLPBT200	Pathtech	Box of 1000	85	15
Multistepper tips bulk	L/9401300	Pathtech	Box of 400	56	5
Multistepper tips racked	L/9401330	Pathtech	1x96 rack	20	2
10mL disposable graduated pipettes	A6-1254	Sarstedt	Box of 1000	135	1

PRODUCT	CODE	SUPPLIER	UNIT SIZE	UNIT PRICE $	# UNITS
Capital Items in red					
Detection Reagents					
Detection Kit	355-1145	Bio-Rad	Kit for 192 tests	6400	15
Detection Equipment and Accessories					
Model 1575 Immunowash	170-7009	Bio-Rad	Each	9995	1
Microplate incubator IPS	35 87 530	Bio-Rad	Each	3778	1
Microplate reader model 680 (450nm)	168-1000	Bio-Rad	Each	9995	1
620nm filter	168-1054	Bio-Rad	Each	495	1
Microplate manager software	170-9520	Bio-Rad	Each	1560	1
PC serial cable for plate reader	168 1005	Bio-Rad	Each	240	1
PC for lab with printer		SOA bill to AHA	Each	2500	1
Cold tray for incubation		Bio-Rad	Each	Free for QDPI&F	1
Spare bulb	1681006	Bio-Rad	Each	268.40	1
Miscellaneous					
Class II cabinet repair		Contamination Control Labs	Each	2500	1
Class II cabinet		Contamination Control Labs	Each	14000	1
Double Glass door refrigerator		Orford Refrigeration	Each	3500	1
Refrigerator	372L	Wilsonton Betta Electrical	Each	959	1
-20C Medical Grade Freezer (upright)	333L	Quantum	Each	3697	1
Chest Freezer	360L	Wilsonton Betta Electrical	Each	890	1
Min/Max Thermometers		Quantum	Each	39.50	5
37- 100C glass thermometer		Bio-Rad	Each	300	2
Electronic balance – 3 digit to 620g		Quantum	Each	1850	1
Minor Items					
Glass Schott bottles	2L	ARI	Each	17.41	4
carboys	4L	Interpath	Each	60	2
	10L		Each	88.30	2
Measuring cylinders	10mL	ARI	Each	4.62	2
	20mL			4.74	2
	100mL			5.58	2
	1000mL			39.42	2

PRODUCT	CODE	SUPPLIER	UNIT SIZE	UNIT PRICE $	# UNITS
Capital Items in red					
Disposable:					
-Gloves Latex inner		Labtek	Box of 100	7.60	40
-Gloves Nitrile outer		Brady's	Box of 100	10.50	40
-Gloves Clear polythene		Brady's	Box of 100	1.40	10
-masks (ties and loop)	MIA2442 & SM10120132	Brady's	Box of 50	8.90	10
-gowns	29-382	Brady's	Carton of 20	55	10
-overshoes	GUOSN001	Brady's	Pack of 100	16.50	10
-underpads absorbent, plastic backed	CEAEU	Brady's	Box of 200	43	2
Safety glasses		various			
Skin Cleanser Bioprep		Ecolab	Carton of 12	268	1
Dispenser		Ecolab	Each	40	1
High heat autoclave bags	112001	BioCorp	200	147	10
Biohazard bags yellow (30x 60cm)	BAGAUT	Livingstone	500	240	500
Support Stand for autoclave bags		Livingstone	Each	250	2
Plastic snap-lock bags	RSV 250x 150	QIS Packaging	1000	36	100
Disposable Sharps Containers 5.1L	301272	Becton Dickinson	Carton of 20	135.80	3
NaOH (2M)	BDH104384F-2.5	Livingstone	2.5kg	140	12
Na hypochlorite (10% diluted to 2%)		Pool Supplies	5L		2
Squirt bottles		ARI	Each		
Stainless Steel Trolley	138339	SDS	Each	451	2
Stainless Steel autoclavable containers		QDPI&F	4 boxes	2500	1
Plastic containers for liquid waste		Hardware	Each	10	4
Paper towel holder		Hardware			
Paper towel rolls		Hardware			
Kimwipes			each	4	5
Absorbent Paper Towel					
5, 10 and 20mL specimen containers for reagent dilutions					

DEVELOPMENT OF SOP'S/QA MANUAL/REPORTING MECHANISMS

Standard Operating Procedures (SOP's) and Quality Assurance:

SOP's have been developed and documented. The scope of accreditation at ADSL was extended to include "20.15.02 Prions: Detection of prion protein by immunological methods (*including ELISA, Western Blots, immunohistochemistry*)"in March, 2006. This was assessed by NATA by desktop evaluation of the methods. Accreditation was confirmed at ADSL's biennial audit in August, 2006. The laboratory was successfully assessed to ISO/IEC 17025: 2005 *General requirements for the competence of testing and calibration laboratories* and ISO/IEC 17025 Application document – *Supplementary requirements for accreditation in the field of veterinary testing* (2005).

Only minor observations were recorded and included the following;

- The tracking and organisation of sample storage was noted as excellent.
- Staff were commended for their knowledge and experience. A reminder was given to record temperatures of fridges and freezers daily.
- The list of MLA owned equipment was recommended to be cross referenced to the laboratory data base.
- The laboratory was reminded that on-going competency in the performance of the test must be demonstrated in the event that requests for testing become infrequent. NATA would not recommend a minimum number of samples to be tested as the onus is on the laboratory to assess this.

Consideration is currently being given to requirements for the maintenance of NATA accreditation.

The documents that have been prepared for this Project are attached as Appendices. A summary of each is given below:

1. **TSE-001 TeSeE – The Bio-Rad ELISA method for *in vitro* purification of PrP^{sc} (Appendix A).**

   This method details the procedure for the extraction and homogenisation of material from the obex region of the brain stem. This is followed by purification, concentration and solubilisation of PrP^{sc} (Proteinase K resistant prion proteins) from the homogenate.

   The method is taken from the Bio-Rad TeSeE Purification Kit Cat. No. 355 1144.

   The appendix to this method details the sampling method for Bio-Rad TSE Screening Assays.

2. **TSE-002 TeSeE – The Bio-Rad ELISA method for *in vitro* detection of PrP^{sc} (Appendix B).**

   This method details the procedure for the detection of PrP^{sc} from the purified sample obtained from Method TSE-001. It is an immuno-enzymatic technique (sandwich format) using 2 monoclonal antibodies. The first is bound to the polystyrene wells of the detection plate and the second is bound to peroxidise. Positive and negative controls are added to each plate.

   The method is taken from the Bio-Rad TeSeE Detection Kit Cat. No. 355 1145.

3. **TSE-003 TeSeE – Procedure for handling and disposal of material associated with the use of the Bio-Rad ELISA method for *in vitro* purification and detection of PrP^{sc} (Appendix C).**

This method outlines the requirements for the handling and disposal of materials associated with the Bio-Rad TeSeE kit method for the purification and detection of resistant prion proteins.

The objective is to establish safe guidelines for the handling and disposal of materials within and from the TSE laboratory to minimise risk to laboratory personnel and the environment. The procedure has been developed to address these issues in the derogated PC3 facility at ADSL taking into consideration current Australian conditions.

4. **TSE-004 – Quality Assurance/Processing (Appendix D)**

This method outlines the procedure to be followed for processing samples in the TSE laboratory. Also included are quality assurance issues and the procedures associated with the use of the Laboratory Information Management System (LIMS) to check-in samples, produce worksheets, enter and authorise results and check samples out for disposal.

5. **MU TSE Measurement Uncertainty Estimation TeSeE – The Bio-Rad ELISA Method for *In Vitro* Detection of PrP^{sc} (Appendix E)**

This document records the estimation of measurement uncertainty for the TeSeE test. With the limited data set available to date the uncertainty was estimated as 52% for samples with absorbance values less than 0.150 and 7% for samples with absorbances greater than 0.150. The main component of the total uncertainty is the precision of absorbance determinations. Protocols for retests and confirmatory tests minimize the chance of false positive or false negative results.

6. **Addendum**

Documents from the LIMS team will be forwarded as an addendum to this report when they are finalised. They detail the process by which samples are registered, tracked and disposed of, how worksheets are generated, results entered and authorised and reports produced. A summary of LIMS issues with regards this project will be presented.

Reporting Mechanism:

The following reporting mechanism extracted from the November 2004 Operations manual for the project as agreed to by AHC (BSE Testing- Confidentiality Arrangements Item 2.7 Meeting 04 26-28 October 2004) was followed by the laboratory throughout the project.

The reporting of test results by the laboratories forms an integral part of the project. The reporting of data needs to follow 1 of 2 pathways. The pathways are either (a) negative results or (b) false positive/positive results.

The Jurisdictional representative is responsible for maintaining a record of all details required for the analysis of the data obtained during the R & D Project. The data should include but is not limited to information provided in the table below (Table 1). Information collated by the TSEFAP NTC jurisdictional coordinator will be made available to AHA in an approved format for the reporting against project milestones. The information is required for the tracing of cohorts of animals if required during the trial.

Table 1: Data collected by TSEFAP NTC coordinator for Post Mortem R & D Project.

Specimen Number	*Collection site*	*Collection Date*	*Owner name*	*Owner Address*	*Owner RLPB Shire LGA*	*Owner PIC*	*Species*	*Age*	*Sex*	*Clinical signs*	*Comments*	*Laboratory result*

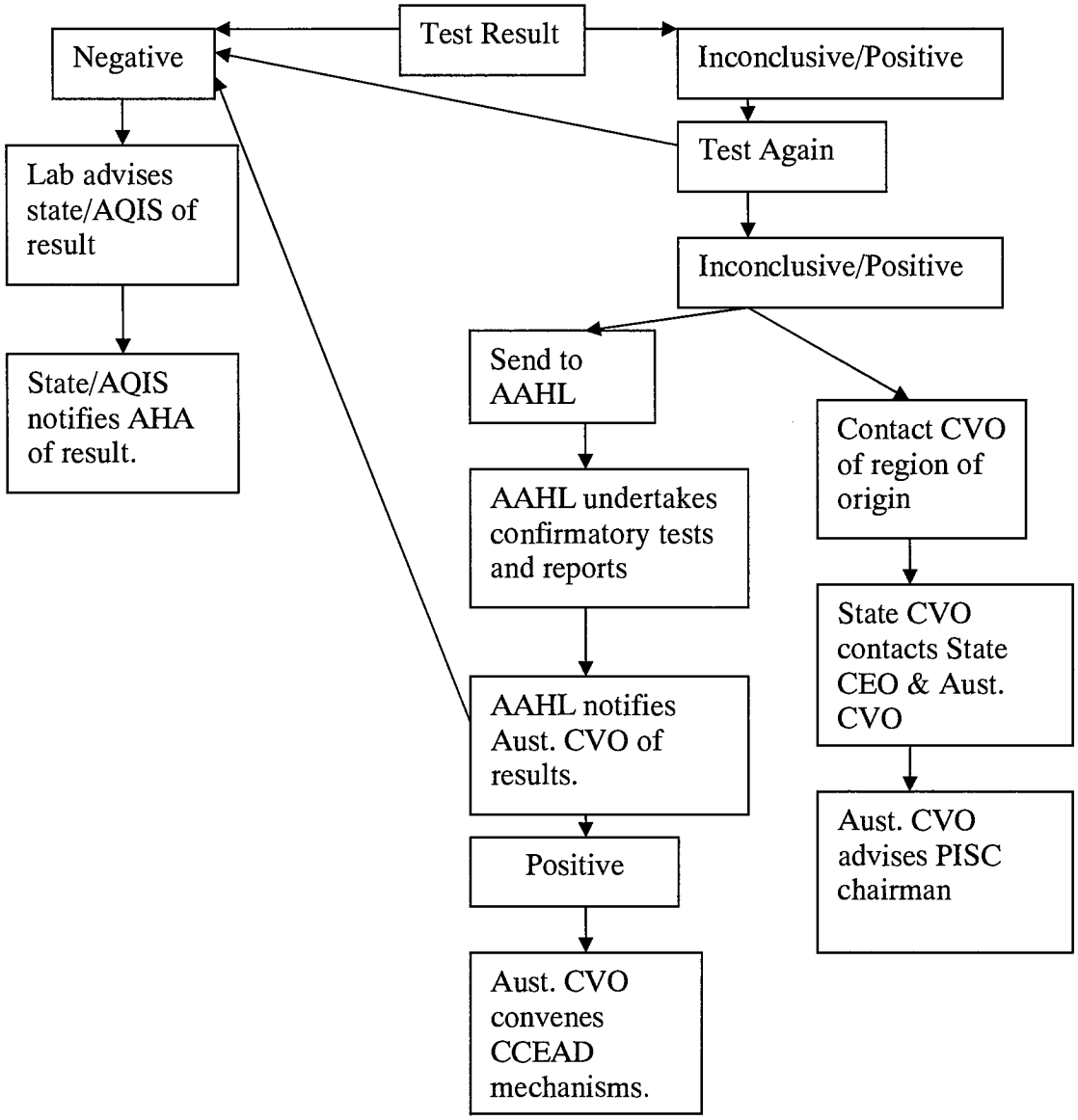
NEGATIVE RESULTS:
For ALL negative results, the laboratory will report the result to the TSEFAP NTC representative of the jurisdiction of the veterinarian who submitted the sample, and the AQIS Veterinary Officer at submitting export abattoirs, the latter in accordance with the SAFEMEAT decision on a test and hold policy at export abattoirs for carcases and carcase parts that pass ante and post mortem inspection.

INCONCLUSIVE/POSITIVE RESULTS:
For ALL inconclusive/positive results the following steps will be followed (these steps have been developed and ratified by the AHC):

- *Bio-Rad's recommendations for positive screen tests should be followed (i.e. an initial positive screen test results in a retest, and the sample is only considered positive to screen testing if both tests return a positive result).*
- *If AAHL is not the screen testing laboratory, suitable samples would then be despatched to AAHL.*
- *the screen testing laboratory would advise the CVO from the submitting State/Territory.*
- *the State/Territory CVO would advise the State/Territory CEO and Australian CVO*
- *the Australian CVO would advise the PISC Chairman*
- *no further advice or action would be undertaken until AAHL advise the results/preliminary results of confirmatory testing (expected to take no more than 24 hours from sample receipt)*
- *if AAHL advise the Australian CVO of one or more positive confirmatory tests (e.g. Prionics, immunohistochemistry), normal CCEAD mechanisms involving governments and affected industries would be invoked*

- *no public announcement would be made until CCEAD has considered the matter, and a case specific communication strategy agreed following NMG reference, as necessary.*

The following flow chart depicts the reporting mechanism:



The only alteration, agreed at the project Steering Committee Meeting at Toowoomba in April 2005, was the inclusion of notification of CVO Qld for TSE suspect material originating from ADSL for confirmatory testing at AAHL.

Results throughout the project were negative, so reporting pathway for inconclusive and positive samples was not tested.

STAFF TRAINING

Staff training was essential for the successful implementation of the method. The training undertaken was considered to be suitable and worthwhile. The hands on experience at AAHL before commencement of the Project, was invaluable. It provided an insight into workflow, equipment and reagents, time considerations, safety issues and background. Discussions at this stage were extremely helpful and provided information which circumvented possible difficulties that may have arisen.

The system in place at ADSL is geared towards testing our target number of samples (2000 per year). There has been no consideration to date for a ramp up of testing if required. Further training and development of procedures would be required to consider this.

The protocol in the event of a positive or inconclusive ELISA result has not been trialled to date.

The following training was undertaken:

23 September 2004 - Introductory visit from Bio-Rad representative (0.5 day)

This was a brief introduction to the Bio-Rad TeSeE test. A Power Point presentation covered the history of the test, its sensitivity and specificity, technical aspects of its use and sampling methods employed by the EU. It was a good introduction and was followed by discussion. This resulted in the suggestion of the development of a synthetic peptide which could be used as a positive control in the purification step. The use of a positive control at this step would increase confidence in the lack of false negatives from over purification. This suggestion was taken on board by Bio-Rad but to date has not eventuated.

26-27 October, 2004 – Prion Handling Techniques and TSE purification and detection by ELISA at AAHL (2 days)

One pathologist and two technical officers from QDPI & F spent two days at AAHL. Participating in the exercise were representatives from AAHL, MLA, AHA and Bio-Rad. An overview of the Project was given along with background information about prions and associated diseases. The technician responsible for TSE testing at AAHL gave her perspective on the test. This was followed by hands-on experience with the test. This training was extremely important and gave staff an insight into requirements before any work commenced at ADSL. Meeting the staff at AAHL facilitated communication further into the Project when advice was sought on a range of safety and technical issues.

2-4 March, 2005 – Installation and Demonstration of equipment by Bio-Rad technical representative (3 days)

The Bio-Rad technical representative installed the equipment in the TSE facility. He was experienced with the test and gave useful information. For example he suggested using samples not subject to Proteinase K digestion as a control measure. Unfortunately the homogeniser was faulty and so we were unable to run through the procedure at that time.

14-15 July, 2005 – In house testing and discussion (1 day)

The technical officer from AAHL responsible for TSE testing visited the ADSL facility to observe and comment on the procedure. This was a valuable exercise to confirm that the operating procedures that had been implemented were acceptable.

Up to end of September, 2006 – LIMS training and implementation (14 days)

The equivalent of 14 full days was spent by ADSL staff on LIMS training. This included technical and specimen receipt staff and pathologists. Initially this was to familiarise them with the system and then to develop worksheets and reports suitable to requirements. Fine tuning is still required to allow the facility to be fully autonomous from the LIMS team.

THE USE OF THE BIORAD TEST

The Bio-Rad test presents no particular difficulties for trained and experienced technical staff.

The purification procedure is labour intensive and time consuming. The steps in this part of the procedure are time dependent. As a consequence samples are best run in batches to fit into the time frames for each step. Of the 42 samples that were prepared each week for the ELISA 20 and 22 samples respectively were run in one of two purification batches. A polystyrene foam tray was made to hold the tubes thus allowing all tubes to be emptied at once when the supernatant was eliminated as part of the procedure. This was done simply by pressing a pencil through a piece of foam in the same pattern as the samples were placed in the racks.

Purification is a rate limiting step that determines the maximum number of samples that can be handled at one time by a single operator.

The detection procedure is a standard sandwich ELISA easily performed by technical staff with training in this area. Running 42 samples per week made the most efficacious use of reagents and reflected the receipt of samples. One batch was run on a full plate (i.e.90 samples). While this produced valid results transferring the samples from the purification tubes onto the ELISA plate was difficult, but achievable, in the given 15 minute time frame.

A summary of test performance for the verification phase follows:

Variable	Result
ELISA results	
Number of samples tested	200
Samples tested per week	42
Number of ELISA plates run	5
Test runs within acceptable parameters	Yes
Number of negative control values eliminated due to variance from the mean (homogeneity)	2 out of 20 negative control results
All negative controls < 0.150	Yes
Mean negative control	0.007 (absorbance)
Range of negative control	0.002 to 0.020 (absorbance)
Mean positive control	1.832 (absorbance)
Range of positive control	1.673 to 2.030 (absorbance)
All positive controls > 1.000	Yes
Sample Weights	
Number of samples weighed	95 of 200 total
% of samples weighed	48%
Average weight before adjustment	353 (grams)
Range of weights before	241 to 517 (grams)
Average weight after adjustment	361 (grams)
Range of weights after adjustment	317 to 439 (grams)

Parameters in all these tests were within acceptable limits.

Acceptable homogeneity for the negative controls is the mean of the negative controls + 40%. Two out of a total of 20 negative controls were invalid due to variance from the mean (homogeneity). These were eliminated in accordance with the Bio-Rad protocol without affecting the test result. The cut-off point for a reactive sample is based on the mean negative control result (i.e. mean negative control + 0.210). By eliminating outliers from the mean calculation a more precise cut-off is derived. All sample absorbance values were less than 0.037 which is well below the cut-off. Negative controls were well below the acceptable upper limit of 0.150 absorbance units.

All ten positive control results were valid. They were well above the lower acceptable limit of 1.000 absorbance units.

One sample was included in each test run which had no proteinase K added during the purification step. These samples returned positive results with an average absorbance of 0.951. This was used as an internal control to determine the efficacy of the purification step.

A portion of samples were weighed when extracted. Most were extracted using the Bio-Rad syringe, however a small proportion were excised with a scalpel because of the quality of the sample.

It was recommended that 10% of all samples are weighed to determine if they are in the acceptable range of 350mg +/- 40mg. Also weighed are any samples that appear insufficient and those extracted using a scalpel.

Numerous runs of small numbers of samples resulted in a limited supply of wash buffer, since the buffer expires two weeks from preparation. This was not an issue when the testing assumed the rate of 42 samples per week. Bio-Rad will not supply extra reagents for their kits.

A summary of test performance for the sample testing follows:

Variable	Result
ELISA results	
Number of samples tested	2165 (1901 bovine, 262 ovine, 1 goat, 1 unspecified)
Samples tested per week	42
Number of ELISA plates run	50
Test runs within acceptable parameters	Yes
Number of negative control values eliminated due to variance from the mean (homogeneity)	20 out of 196 negative control results
All negative controls < 0.150	Yes
Mean negative control	0.007 (absorbance)
Range of negative control	0.000 to 0.032 (absorbance)
Mean positive control	2.2667 (absorbance)
Range of positive control	1.263 to greater than 3.500 (absorbance)
All positive controls > 1.000	Yes
Sample Weights	
Number of samples weighed	567 of the 2165 total
% of samples weighed	26%
Number of samples requiring adjustment	100 of the 567
Average weight before adjustment	361 (grams)

Range of weights before	230 to 498 (grams)
Average weight after adjustment	362 (grams)
Range of weights after adjustment	321 to 419 (grams)

One ELISA plate was repeated because the plate was bumped prior to the addition of the substrate. Reagent (conjugate) splashed into adjacent wells resulting in colour development in some wells. All samples were clearly negative on retest. Any samples with colour development from the initial plate were retested in duplicate.

One sample was initially reactive due to an unknown cause. It was retested in duplicate from the homogenate and was clearly negative on retest.

The Bio-Rad method underwent some minor changes during the period of testing. They included:

- A documented procedure for sampling which came as an insert with the sampling syringes. It clearly stated the recommendation for weighing at least 10% of the homogenate samples. This had been done since the start of testing and was continued.

- The change in nomenclature for the resistant prion proteins. This changed from PrP^{res} (resistant Prion protein) to PrP^{sc} (scrapie prion protein) to reflect current terminology. (The ADSL methods will also be changed).

- Reagent B was flagged as not autoclavable. On obtaining the MSDS for this product it was found to contain Butanol and therefore flammable. The disposal procedure was amended for this reagent from autoclaving to addition of equal parts of 2M NaOH and then incineration by a licensed waste disposal company. If larger quantities of this Reagent were used (i.e. in the event of escalation of testing) an alternate arrangement would need to be considered.

On one occasion the positive control values were above 0.350 absorbance values. The actual value was not recorded by the Bio-Rad plate reader software which showed a result of '*'. Bio-Rad advised that the test run was valid and that the results could be accepted since the positive control was greater than the acceptable level of 0.100 yet beyond the detectable limit of the reader. There appeared to be batch variation in the positive controls.

ADMINISTRATIVE/OPERATIONAL ISSUES.

The issues encountered were as follows:

- Laboratory Information Management System (LIMS) implementation:
    - LIMS is not currently used at ADSL for other disciplines. The system being implemented is a prototype and is process driven. It is not simply a reporting mechanism. Sample tracking and operational details are captured on-line where previously this was done on paper. Consequently the process has been examined step by step to ensure accuracy and a smooth flow. LIMS was adopted for this Project to maximise security of results. Only staff associated with the Project can access the system and only in those areas that they require to complete their specific jobs.
    - The plate reader software has been interfaced with LIMS to download the sample numbers. Further refinement of this process and in addition automatic download of results back to LIMS would be advantageous. *Automatic downloads was not considered a priority by the LIMS team particularly since all results to date were negative and therefore transcription errors negligible.*
    - Currently a procedure for capturing the preparation of the homogenate on the system is being developed. Also developed is a means of entering comments in results. *This is available but not trialled by the time of completion of the Project.*
    - Familiarisation of laboratory staff with the system has taken some time (14 days spent so far with LIMS staff in Toowoomba). Implementation requires significant input from IT personnel and laboratory staff who are familiar with each others needs. The LIMS team in Brisbane have been working closely with staff at the laboratory to attain operational status. The system must be tailored to suit the laboratory procedure.
    - LIMS is a system requiring considerable training and experience for competence.
- Determination of containment practices using guidelines from UK Advisory Committee on Dangerous Pathogens – TSE Agents, WHO Infection Control Guidelines for TSE, Australian/NZ Standard (AS/NZS 2243.3:2002), AAHL and Bio-Rad recommendations.
    - To determine the procedures for this facility the above sources were taken into consideration. A combination of their recommendations was adopted in consultation with AAHL. Since AAHL is a PC3 facility it is not possible to replicate their procedures. Due attention was paid to current Australian conditions regarding TSE status and the nature of the surveillance while not compromising Workplace Health and Safety and containment requirements.
- Selection and Purchase of equipment
    - This phase of the project took approximately five months. Following the AAHL training initial equipment needs were assessed, quotes and technical details obtained and purchases made.
    - Selection was based on a combination of recommendations from AAHL, Bio-Rad, local availability and price.

- Acquisition of computers and payment

    - The QDPI&F lease arrangement for computers was complicated by the external funding of the project. We now have a computer in the office funded by QDPI&F and one in the laboratory funded by the Project.

    - Delivery of computers took months and was delayed by the lack of monitors at the time.

- Purchase of printer

    - Printer requirements for the laboratory are at odds with general QDPI&F standards. At the time of implementation of the project the Department was changing over to multifunction devices for all its printing needs and there was a moratorium placed on the purchase of stand alone printers. A multifunction device would be useful for faxing information out of the restricted area but are bulky and considerably more expensive. A special case needs to be made to purchase printers. *A printer was found in the laboratory that was surplus to requirements.*

- Staff recruitment

    - One staff member was recruited to a temporary position through the QDPI&F recruitment and selection process. This position was filled on April 1, 2005 some 6 months after the training at AAHL.

    - Permanent QDPI&F staff members also work on the project to maintain continuity and to provide adequate staff numbers for back-up arrangements.

    - Staff arrangements for the maintenance of accreditation will be dependant on allocation of resource and is currently being negotiated.

- Calibration of equipment in contained facility

    - By purchasing most of the equipment through Bio-Rad the majority of the servicing and calibrations is done under one service contract.

    - Items which are not under this banner but require regular calibration include pipettes, balance and biohazard hoods. Pipettes are autoclaved out of the facility and calibrated in house. External calibrations are required for the balance and biohazard hoods. Service technicians (with their equipment) are given access to the facility provided they wear PPE as outlined in the SOP's.

    - If a positive sample was detected Class II cabinets would be decommissioned (see below). All equipment would be decontaminated with sodium hypochlorite (plastics) or sodium hydroxide (stainless steel) before service technicians were allowed access to them.

- Manufacture of stainless steel boxes

    - Three private companies were approached to manufacture the boxes. None of these were able to produce them. The boxes were made on site by QDPI&F engineers. The final product is excellent.

- Choice of Class II Biological Safety Cabinets versus Cytotoxic Drug Cabinet

    - The Australian/NZ STD stipulates the use of Cytotoxic Drug Cabinets for aerosol containment of prion materials. The Class II Biological Safety Cabinets offers the same protection for technical staff carrying out the experimental procedure. The prepositioning of the filter in Cytotoxic Drug Cabinets offers protection to cabinet service technicians. Under current Australian conditions and in the absence of prion diseases in domestic livestock in Australia the Class II cabinets have been employed. In the unlikely event of the detection of TSE's **this procedure must be reviewed with particular reference to future servicing and maintenance of the cabinets.**

    - The choice was based on the ergonomics of the work station and price in light of the above (Class II cabinets approximately $14,000 each and Cytotoxic Drug Cabinets approximately $18,000 each. ADSL already had one Class II cabinet on site which needed minor repairs).

    - Class II cabinets would require decontamination, decommission and replacement with two Cytotoxic Drug Cabinets.

- Delivery time and expiry dates on kits

    - Delivery time has ranged from one month from order date for the first kit ordered to ten days. Bio-Rad have undertaken to maintain adequate kits in Australia for our use which has been estimated to be one kit every three weeks. We are able to increase or decrease this by notifying them.

    - Shelf life of kits has varied from three months to eight months. Bio-Rad recommended requesting (on the order) kits with at least six months to expiry resulting in most kits having at least six months useful life left. The maximum shelf life of the kits is twelve months.

- Change over of staff at Bio-Rad and response time for technical queries

    - The technical representative who was our contact from the initiation of this project left Bio-Rad just prior to commencement of testing of the 2000 annual samples. It took approximately one month to re-establish communication with the Company. During that time a number of technical queries were left unanswered.

- Inadequacy of wash buffer volume supplied.

    - 250mL of wash buffer is supplied with the TeSeE detection kit. This is diluted 1/10 for use and can be stored for two weeks before disposal. When running the ELISA in batches, as is practical to meet turnaround times, the wash buffer is of insufficient quantity. This is partly due to the amount used by the automated plate washer. The washer requires a certain minimum volume to operate and uses some buffer to prime it. There is no allowance for any error in operation eg spillage. To date Bio-Rad has

not come up with a solution. *This did not present any difficulties once routine testing of 42 samples per week commenced.*

- Extraction of calibrated amount of obex tissue.
    - Identified as an area where ongoing calibration is required. This is a necessary quality procedure outlined in the Bio-Rad method.
- Sample quality and identification
    - Some samples did not contain an identifiable obex due to damage or fragmentation of the sample (Figure 11). Where obvious stem material was provided testing was performed and the lack of discernable obex noted. Occasionally no stem material was provided and the sample was designated as unsuitable (18 of 2165 samples received). Many of these cases consisted of cerebellum indicating incorrect sampling technique. *This was corrected upon discussion with AQIS.*

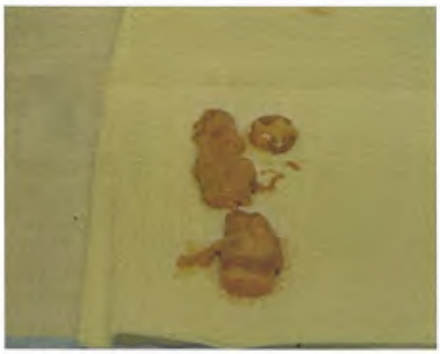


Figure 11.

Figure 12

    - Some samples were badly autolysed and liquefied (23 of 2165 samples received) and designated as unsuitable (Figure 12). This was probably associated with inappropriate sample storage or prolonged transportation.
    - Sample containers leaked necessitating clean up in specimen receipt, greatly increasing processing times and also creating health and safety issues. The problem appeared to be the use of polycarbonate containers. *Discussions with manufacturers and some trial work indicated that this can be overcome by the use of 50 ml polypropylene containers with polyethylene screw cap lids with an internal sealing flange.*
    - There was an inconsistent approach to sample identification that could lead to traceability problems in the event of a positive. *Consideration should be given to a consistent approach to sample id such as one sample per submission form with each form having a unique identifier similar to the AQIS forms used for the National Granuloma Submission Program. The use of NLIS as a means of identification could be improved by the use of bar coding to prevent transcription errors.*

- Documentation

    - Some documentation lacked specific crucial information or was illegible. This could again create problems with sample identification and tracking in the event of a positive. *Consideration should be given to the use of online submission forms which are completed by typing and field advancement and form completion in dependant on provision of crucial information.*

- Reporting

    - Reporting was carried out as per agreed procedures. As no positive or inconclusive samples were detected this procedure was not tested. All participants need to be aware of the reporting procedures as requests were received to provide reports outside of the agreed procedures. Contact lists and details need to be upgraded in order to maintain confidence in procedures. A number of contact details for AQIS meatwork officers were incorrect resulting in reports going to company main offices instead of AQIS.

- Freight charging arrangements to the laboratory

    - These need to be streamlined. ADSL paid freight on samples delivered to the laboratory and was reimbursed by AHA. This was inefficient and there are costs with processing reimbursements. Freight charges can vary depending on the proximity to the testing laboratory. *A maximum freight charge can be incorporated into the cost of testing or alternatively, freight charges could be paid by the submitter.*

- Turn-around times on test and hold.

    - These were discussed with AQIS and ADSL gave an undertaking that most test and hold samples should be cleared within ten working days from receipt at the laboratory. Problems generally arose where samples were not clearly identified as "Test and Hold" on the submission form. *Consideration needs to be given to a specific tick box on any future development of submission forms.*

FUTURE WORK AND ISSUES HIGHLIGHTED

- Refinement of LIMS system. *Ongoing*

- A synthetic peptide for use as a purification control is being investigated by Bio-Rad. *Control not available to date.*

- Recommend use of electronic multistep pipette ($1200 – two required) to dispense 25-250µL of reagents in the purification step to reduce time and operator fatigue. Two minutes is allowed for addition of reagents in this step. Multistep pipettes would allow more samples to be run in each batch. *Purchase dependant on resource allocation*

- Maintenance of capability currently being considered. Points for consideration include:

    1. Staff retention. The temporary position associated with this Project has been terminated.
    2. Throughput of testing to maintain accreditation currently being considered.
    3. Participation by ADSL staff in AAHL proficiency testing program.

- Exploration of alternative kit methods. The IDEXX kit for example eliminates the purification step which is a rate limiting factor in the method. If large numbers of samples were tested there would be an advantage in considering this as an alternative.

- Exploration of throughput capabilities. How many samples could be handled under differing staff levels in the event of an escalation of testing (wartime options)?

- Ensuring systems for reporting ELISA inconclusive or positive results are trialled and efficient.

CONCLUSION

The Animal Disease Surveillance Laboratory was able to successfully renovate an existing PC2 lab to derogated PC3. Equipment was installed and procedures and methods implemented to test bovine and ovine brain stem material sourced from around the country for TSE using the TeSeE Bio-Rad ELISA. The laboratory's scope of NATA accreditation was extended to include prion detection by immunological methods in production animals in July 2006. This was followed by successful completion of a NATA technical audit in August 2006.

Test validation was completed on 200 samples provided by AAHL in July 2005 with testing of the target 2000 annual samples commencing in September of that year. A total of 2165 samples were completed by September 2006. Results were provided on 2124, 41 samples were unsuitable. Of the total, 1901 were bovine and 262 sheep reaching the target of 90% bovine and 10% ovine. One goat and one unspecified sample were also received.

An interim project report was provided to AHA in September 2005 and two progress reports on testing were provided to the steering committee in March and June 2006. The National TSE Steering Committee met on site at Toowoomba in April 2005 to review the facility and the progress. A technical representative from AAHL visited the facility in July 2005 to review the procedures, the facility and containment aspects.

Maintenance of testing capability at ADSL including ongoing accreditation by NATA is currently being negotiated.

Under the conditions outlined in this report and with a single dedicated full time laboratory technician, maximum testing capabilities are estimated at 180 samples a week (i.e. two ELISA plates). This level of testing would present difficulties in other sections of the laboratory especially specimen receipt and the autoclave facility which would need to be addressed. The main rate limiting step identified in the test is the purification process. Semi-automation using multistep pipettes would alleviate this difficulty.

The requirements for any escalated level of testing were not investigated or tested in this component of the project and would require further work.

TeSeE – The Bio-Rad ELISA method for *in vitro* purification of PrP^{sc}.

APPENDIX A

REVISION REGISTER

Issue No.	Date of Issue	Amendment Details
1	9\5\05	All pages
2	15\12\05	All pages
3	3\10\06	All pages

Controlled Copy No: 26 **Controlled:** ☑

Authorised: ..... **Uncontrolled:** ❑

Authorised Signatory

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1 SCOPE AND APPLICATION

1.1 This method has been implemented at the Animal Disease Surveillance Laboratory, Toowoomba (ADSL) to fulfil the laboratory component of project 'TSE 008'. The project is co-ordinated by Animal Health Australia (AHA) and funded by Meat and Livestock Australia (MLA).

1.2 The aim of the project is to evaluate Australia's ability to rapidly implement enhanced Transmissible Spongiform Encephalopathy (TSE) surveillance by targeting specific post-mortem cases.

1.3 ADSL has been subcontracted to adopt technology and establish a testing capability for TSE's using the BioRad TeSeE ELISA kit method for purification and detection of resistant prion proteins (PrP^{sc}). The Australian Animal Health Laboratory (AAHL) has validated this method for use under Australian conditions.

1.4 Initial validation and verification of the test method will be based on testing 200 samples analysed and provided to us by AAHL. Surveillance will follow with the testing of approximately 2000 samples per year. These samples will be approximately 90% bovine and 10% ovine.

1.5 The BioRad TeSeE method has been approved within the European Union as a rapid test for the BSE and scrapie testing programmes on cattle, sheep and goats.

1.6 Surveillance to date has indicated that there are no TSE's in domestic livestock in Australia.

1.7 This method is designed for screening purposes. Samples must be sent to AAHL for confirmatory testing using more specific methods before a positive diagnosis can be made.

1.8 The steps involved are for the purification of samples of nervous tissue to prepare them for the detection step described in Method TSE-002.

2 PRINCIPLE

GENERAL

2.1 Transmissible spongiform encephalopathies are fatal degenerative brain diseases occurring in a range of animal species including sheep, cattle, goats, deer and elk. Humans are also susceptible to various forms of TSE's. A common feature of all TSE's is the appearance of microscopic vacuoles in the grey matter of the brain induced by unconventional transmissible agents routinely called prions.

2.2 The associated prions are an abnormal isoform of the naturally occurring prion protein (PrP) called PrP^{sc}. This isoform is characterised by particular biochemical properties and especially by an increased resistance to protease.

2.3 Iatrogenic, familial and sporadic cases of TSE have been described. Transmission can occur through inoculation or ingestion.

PURIFICATION

2.4 The TeSeE purification kit allows purification, concentration and solubilisation of PrP^{sc} from samples of nervous tissues.

2.5 Processing samples comprises the following steps:

- Grinding of samples in a homogeniser
- Treatment of samples with Proteinase K
- Concentration of PrP^{sc} by precipitation and centrifugation
- Solubilisation of PrP^{sc} for immunoenzymatic assay using the reagents of the TeSeE Detection Kit

3 REAGENTS

3.1 Bio-Rad TeSeE Purification Kit Ref. 355 1144. The Kit comprises the following:

LABELLING	TYPE OF REAGENTS	PRESENTATION
Grinding Tube	Grinding tube containing ceramic beads in a buffer solution Preservative: Proclin 300 (0.1%)	2 bags - (2 x 96 tubes)
Reagent A	Denaturing solution (To dilute Proteinase K and denature prion protein)	1 bottle - (55ml)
Reagent B	Clarifying solution (For concentration of PrP^{sc} by precipitation and centrifugation) Colouring: bromophenol blue	1 bottle – (55ml)
Reagent C	Resolving buffer (For solubilisation of PrP^{sc})	1 bottle – (7ml)
PK	Proteinase K Colouring: Phenol Red	1 bottle – (0.5ml)

Caution:

Never autoclave solutions containing sodium hypochlorite or Reagent B.

Note:

The TeSeE Purification Kit is stored refrigerated (+2-8°C). All reagents are stable at this temperature until the expiry date indicated on the kit (before and after opening of the bottles).

After dilution, the reconstituted proteinase K solution when stored at room temperature (+18-30°C) must be used within 6 hours (i.e. make fresh each day).

Calculate the required volumes of Reagent B and C and decant into a plastic container for use. Do not return any to the original reagent bottle.

Reagents A and B and grinding tubes are generic components. They can be used with all batches of the purification kits.

3.2 2M NaOH (Prepared according to instructions in Appendix B)

3.3 2% Na Hypochlorite (Prepared according to instructions in Appendix C)

3.4 RO water

4 APPARATUS

4.1 A derogated PCIII laboratory is required. (See Method TSE-003)

4.2 Test sample syringe (Cat. # 35 51 175 from BioRad)

4.3 Calibration syringe (Cat. # 35 51 174 from BioRad)

4.4 1.5ml plastic screw top tubes and caps (Cat. # 72.607 and 65.716.999 from Sarstedt)

4.5 Gripper rack for tubes (Cat. # from Sarstedt)

4.6 Precess 48 homogenizer (Cat. # 35 90 200 from BioRad)

4.7 Heating blocks for 37°C and for 100°C (Cat. # 35 89 046 from BioRad)

4.8 Rack for heating block (Cat. # 35 89 199 from BioRad)

4.9 5804R centrifuge (Cat. # 35 89 190 from BioRad)

4.10 Drum rotor (Cat. # 35 89 189 from BioRad)

4.11 Adaptors for centrifuge (Cat. # 35 89 191 from BioRad)

4.12 Vortex (Cat. # 166-061 edu from BioRad)

4.13 Multistepper pipettes 50-300uL

4.14 10-100uL and 100-1000uL micropipettes and stands

4.15 Pipette tips

4.16 10mL disposable graduated pipettes and bulbs

4.17 PC and printer

4.18 2 x Class II biohazard hoods

4.19 Laboratory grade freezer

4.20 Refrigerator

4.21 Min/max thermometers for calibrating heating blocks, fridges and freezers

4.22 Electronic top loading 3 digit balance to 620g

4.23 Glass Schott bottles (2L)

4.24 Carboys (4L)

4.25 Measuring cylinders (10mL, 20mL, 100mL and 1000mL)

4.26 Absorbent paper

4.27 Disposable gloves, masks, gowns and booties

4.28 Slip-on boots

4.29 Autoclave bags (high heat)

4.30 Squirt bottles

4.31 Stainless steel trolley

4.32 Stainless steel autoclavable containers

4.33 Plastic bins for liquid waste

4.34 Sample trays/containers

4.35 Autoclave facility

5 SAMPLING AND SAMPLES

PURIFICATION

5.1 Purification of PrP^{sc} is performed on samples of nervous tissues. Distribution of PrP^{sc} is heterogeneous in central nervous system with the obex area from the brainstem producing optimal detection.

5.2 For bovine animals, the BioRad sampling syringe allows easy and rapid sampling of obex area while minimizing operator risk of sharps injuries. Samples not easily obtained in this way (e.g. too small) may be cut from the designated sample site using a scalpel.

5.3 Samples are stored refrigerated (+2-8°C) if purification is performed within 24 hours or can be stored frozen (-15-30°C) for several months. **They can only be submitted to 3 freezing/thawing cycles.**

5.4 Difficulties can be encountered during the grinding step when using dehydrated samples. If necessary, the grinding step may need to be repeated several times for this type of sample.

5.5 Unsuitable samples are brought to the attention of the assigned pathologist. Reasons for unsuitability include extremely autolysed samples or samples that do not represent the target site (obex). Insufficient samples are assessed by the technical staff.

6 PROCEDURE FOR PURIFICATION

PREPARATION OF REAGENTS:

6.1 All reagents of the TeSeE Purification Kit except proteinase K are ready for use.

6.2 Reagent A is the dilution buffer for Proteinase K. The solution is prepared in the following way. (e.g. 4uL of proteinase K is added to 1mL of reagent A)

NUMBER OF SAMPLES	REAGENT A (x)	PROTEINASE K
2	1mL	4uL
10	3mL	12uL
18	5mL	20uL
26	7mL	28uL
34	9mL	36uL
42	11mL	44uL
50	13mL	52uL
58	15mL	60uL
66	17mL	68uL
74	19mL	76uL
82	21mL	84uL
90	23mL	92uL

6.3 **The volumes must be pipetted exactly.** The tip containing the PK must be correctly rinsed by successive aspiration/distribution cycles in buffer A.

6.4 After reconstitution, mix the solution by successive inversions until a red homogenous solution is obtained.

PROCEDURE:

CAUTION:

PPE detailed in the method TSE-003 must be worn at all times.

All solid and liquid waste is disposed of according to the procedures detailed in the method TSE-003.

All parts of this procedure where direct contact is made with the sample are performed in a Class II Biohazard Hood.

Enclosed samples ONLY are transferred to and from storage, homogeniser, balance, vortex and centrifuge, which are all located in the TSE laboratory.

SAMPLING

Label one homogenising tube for each sample with a LIMS label. Label a corresponding 1.5mL screw capped tube with the corresponding tube number as allocated by the LIMS ELISA Worksheet.

A mass of 350mg from the brainstem is taken using either method A (by weight) or B (by calibrated syringe) below:

CAUTION: RISK OF SHARPS INJURY.

A – BY WEIGHT

6.5 All samples extracted by syringe are weighed to ensure they are within the required range.

6.6 Tare a clean empty plastic tube and lid on the balance.

6.7 Take a mass equivalent to 350mg +/- 40mg of nervous tissue from the brainstem (preferably obex) using a scalpel. Deposit the sample into the tube, close the lid and check the weight of the sample on the balance.

6.8 Transfer the tissue to a labelled BioRad grinding tube, close the lid firmly and proceed to the grinding step in the homogenizer (Precess 48).

B – BY CALIBRATED SYRINGE (Refer Appendix 10.1)

6.9 The Bio-Rad calibrated syringe has a bevelled end. Push the end of the syringe up into the brainstem twisting the outer sleeve to facilitate cutting while pulling back on the plunger. Withdraw the syringe when it is full of tissue up to the second of any of the mark types (i.e. square, triangle or line). The distance between any two marks of the same type denotes tissue equivalent to 350mg.

6.10 Transfer the tissue to a labelled BioRad grinding tube by pushing the trigger in from mark to mark, close the lid firmly and proceed to the grinding step in the homogeniser (Precess 48).

6.11 10% of the samples are weighed and weights recorded on the Excel Spreadsheet at: U:\Biosecurity...\TSE 008\weights. For an ELISA run of half a plate (40 samples) therefore at least 4 samples are monitored to ensure samples are within the required weight range.

SAMPLE GRINDING

6.12 Place the Bio-Rad grinding tubes in the crown of the homogenizer. Perform one agitation cycle in the Precess 48 set on Mode 1.

6.13 Samples are visually inspected to ascertain sufficient homogenisation.

6.14 When grinding is insufficient, another 1 or 2 agitation cycles can be performed, by ensuring that the temperature of the tube returns to room temperature (+18-30°C) between each cycle. The 5-minute warm down cycle of the Precess is sufficient to allow this.

SAMPLE STANDARDISATION

6.15 Remove the grinding tubes from the homogeniser.

6.16 **Resuspend the homogenate by inversion before opening the tubes** and take 250µl with the calibration syringe and needle taking care to immerse the needle in the pellet of beads to avoid sampling poorly homogenised tissue fragments. (The beads act as a filter).

6.17 Transfer each 250µL sample into a labelled 1.5 mL screw top micro test-tube. Remove the lid from only one grinding tube at a time to avoid sampling error.

Note:
If the sample is to be tested in duplicate in the ELISA two tubes must be prepared at this step.

Note:
At this stage, both grinding tubes and micro test tubes can be stored, closed at:

- **Room temperature (+18-30°C) for 8 hours.**
- **+2-8°C (in ice or in the refrigerator) for 15 hours**
- **–20°C minimum for 1 year.**
    - **Frozen samples must be thawed at room temperature (+18-30°C) and mixed by inversion before use.**
    - **Samples can be submitted to a maximum of 3 freezing/thawing cycles.**

PK TREATMENT

6.18 Make 'x' amount of Proteinase K solution according to the table in Section 6.2 of this method.

6.19 Distribute 250µL of reconstituted proteinase K solution into each micro test-tube and mix immediately by inversion of the closed tubes 10 times.

6.20 Incubate at 37°C +/- 2°C in a heating block incubator for 10± 1 minute.

Note:

To reduce the possibility of reagent contamination estimate the quantity of reagents required for each step and dispense this volume into another container. Do not pour any of this back into the original vial.

Set up a series of several micro test tubes and adapt the number of micro test tubes to the equipment used (automatic pipette/racks) to avoid exceeding intervals of 5 minutes for distribution of reconstituted proteinase K between the first and last micro test-tube.

Do not exceed 2 minutes between the mixing and the incubation at 37°C.

PRECIPITATION OF PrP^{sc} WITH BUFFER B

6.21 Aliquot 'x' amount of Buffer B into a plastic vial. Do not pour this back into the original Reagent Bottle when finished.

6.22 Remove the micro test tubes from the heating block incubator. Open them and distribute 250µL of reagent B into each micro test-tube.

6.23 Mix until a homogenous blue colour is obtained by inversion 10 times.

Note:
Follow the same order of distribution as described for the PK Treatment.

Do not exceed intervals of 2 minutes between taking the sample from the incubator and the mixing with Reagent B.

CONCENTRATION OF THE PrP^{sc}

6.24 Within 30 minutes from the addition of buffer B, centrifuge the micro test tubes for 7 minutes at 15,000g at 20°C.

SOLUBILISATION

6.25 Discard the supernatant by inverting over a waste container and dry the micro test tubes by inverting onto absorbent, plastic backed paper for 5 minutes.

6.26 Aliquot 'x/10' of Reagent C into a plastic vial. Do not pour this back into the original Reagent Bottle when finished.

6.27 Distribute 25uL of Reagent C into each micro test-tube.

Note:
Do not exceed an interval of 10 minutes between the end of the drying operation and distribution of Reagent C.

Do not exceed 2 minutes between the reagent C distribution and the beginning of the incubation.

6.28 Incubate in a heating block for 5 (+/- 1) minutes at 100°C +/- 5°C.

6.29 Remove the micro-tubes from the incubator and mix with a Vortex (5+/- 2 seconds)

Note:
Samples can be stored if required:

- **Refrigerated for 5 hours at +2-8°C**
- **Frozen for 72 hours at a minimum of -20°C.**
- **Frozen samples must be thawed at room temperature (+18°C to +30°C) and mixed with a vortex (5+/- 2 seconds) after thawing and before use.**

DETECTION

6.30 Purified samples are used in the TeSeE detection method (TSE-002)

6.31 The Class II cabinet must be completely cleaned using 2M NaOH between the purification and detection steps.

7 CALCULATION AND EXPRESSION OF RESULTS

7.1 Refer to the detection method (TSE-002)

8 TEST REPORT

8.1 Refer to the detection method (TSE-002) and the Quality assurance/Processing document (TSE-004)

9 REFERENCES

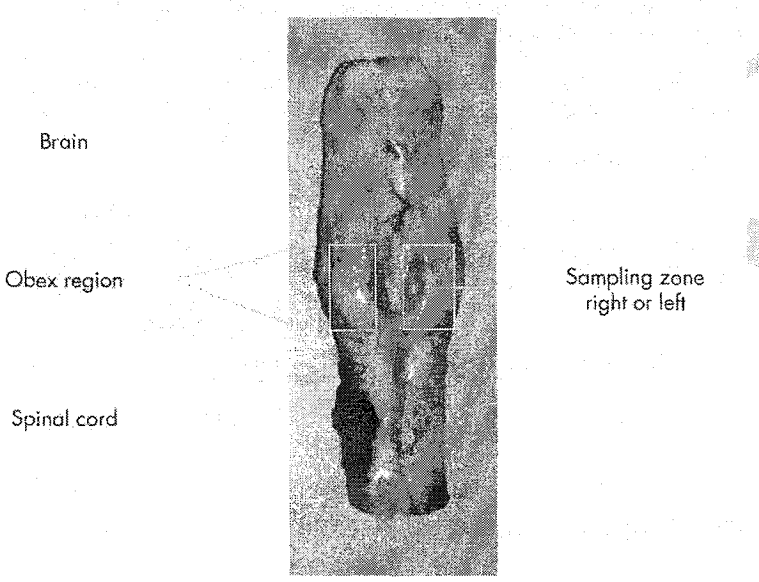
9.1 Bio-Rad User's manual for TeSeE Reagents kits for *in vitro* purification and detection of PrP^{sc} Version Rev. E7 -01/2006.

9.2 Bio-Rad Sample Syringe Manual – Sampling method for Bio-Rad TSE Screening Assays Version Rev. B 10/2005.

10 APPENDICES

A Sampling Method for Bio-Rad TSE Screening Assays (Taken from Sample Syringe Kit insert - Cat. # 355 1175)

1 The Bio-Rad TSE screening assays are performed on a sample of 350+/- 40mg of central nervous tissue (CNS). The specific anatomical region for detecting PrP^{sc} in infected animals is the brain stem, more precisely in the area of the vagal nerve nucleus, in the obex region. This is the area of the brainstem where PrP^{sc} is most concentrated.



2 Sample collection at the abattoir: The brain stem is easily and quickly collected with an appropriate tool or sample collection spoon, via the occipital foramen, without opening the cranial cavity.

Sample collection with the Bio-Rad collection spoon

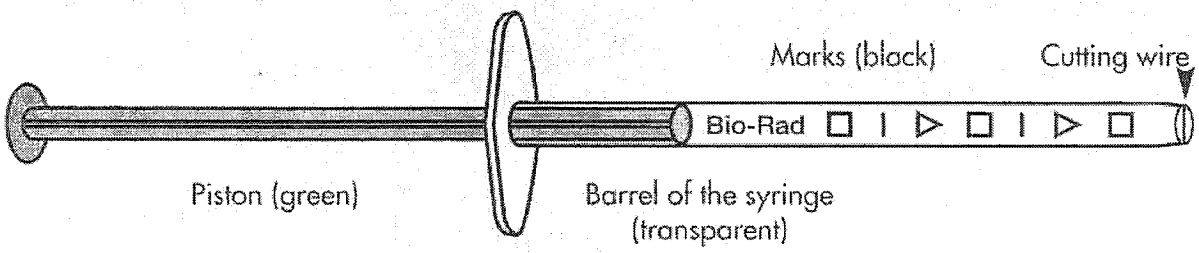
3 Sampling procedure at the laboratory

The whole brain stem sample is sent to the laboratory where the appropriate amount of cerebral material is cut from the obex region using a scalpel blade or collected with the Bio-Rad sample syringe. The latter procedure makes it possible to sample the required amount of the appropriate area quickly and safely, without the risk of sharps injury.

The following describes the procedure to effectively collect the sample from the obex region using the Bio-Rad sample syringe without damaging the tissue.

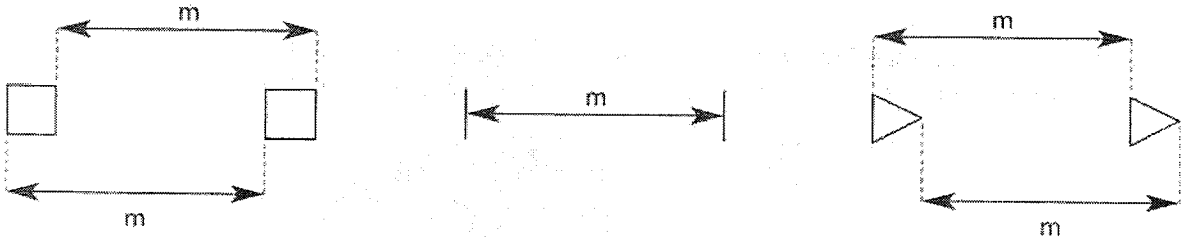
4 Bio-Rad Sample Syringe

The Bio-Rad sample syringe consists of a green piston and a transparent syringe barrel. The syringe barrel is labelled with a series of geometric shapes:



5 Sample Mass Required For the Test

The sample mass should occupy the space between two symbols of the sample shape which corresponds to a mass (m) of 350+/- 40mg.



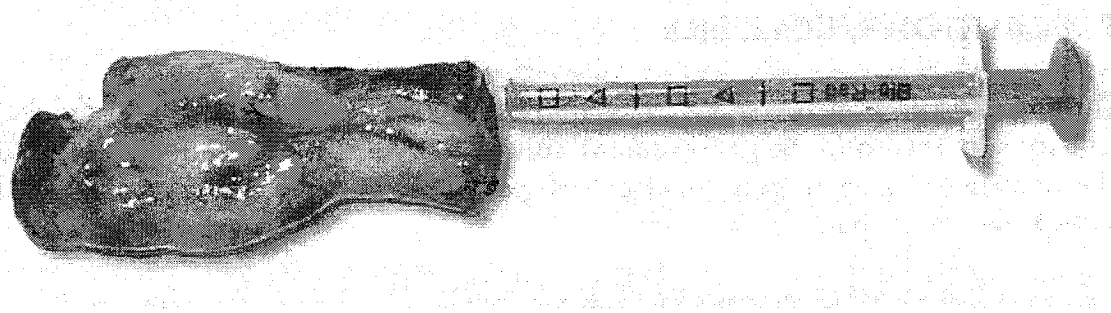
6 Operating Procedure

6.1 Take a sample syringe and pull out the green piston to approximately 1cm from its home position then push home again

6.2 Firmly grasp the brain stem in one hand, using a disposable wrapper (plastic bag, glove etc.) in order to avoid possible cross-sample contamination. The end of the brain stem should remain accessible.

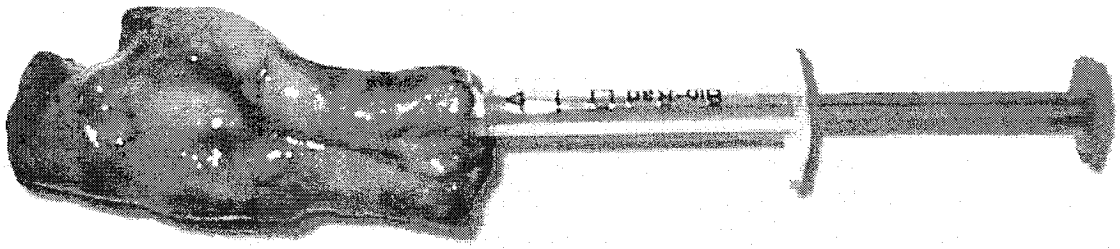
6.3 Use the other hand to position the open end of the sampling syringe on the right or left side of the base of the brain stem.

Note: a complete hemi-section of brain stem with an intact obex region must remain available after sample collection for confirmatory testing.



6.4 Insert the syringe barrel gradually into the brain stem whilst holding the green piston stationary.

Note: While collecting the sample from the obex region, take care that the syringe barrel remains within the selected side of the brain stem.



6.5 Stop this movement when the top of the syringe barrel has reached the upper limit of the sampling zone.

6.6 Cut the sample core by twisting the syringe barrel through one complete turn.

6.7 Slowly remove the sample syringe from the brain stem, taking care not to damage surrounding tissues. The remaining brain stem can be placed in its original sample container.

6.8 Check for air gaps in the core sample collected. If needed, compress the sample core by closing the top of the syringe barrel and pushing the green piston until the air gaps have been eliminated. At the same time ensure that the tissue nearest the opening of the syringe barrel is retained.

6.9 Holding the top of the syringe barrel still, move the green piston to the nearest symbol

6.10 Check that the sample core covers at least one zone corresponding to "m", as described previously.

6.11 Take a grinding tube and remove the lid, with the sample syringe carefully depress the green piston to the next identical symbol to ensure that the correct mass of tissue ("m") is dispensed in the grinding tube. Remember that you must move the piston to the corresponding position of the next symbol as indicated in "Sample mass required for the test".

6.12 Cut the sample core by gripping the top of the sample syringe against the inner edge of the grinding tube.

7 Precautions

7.1 As for any pipetting device, Bio-Rad recommends that operators using the sample syringe should be periodically monitored, for a representative statistical population of samples taken (This laboratory has adopted a 10% weighing policy). This ensures that sample weights are within range.

7.2 If the sample core does not fill the entire syringe barrel within the marked area for use, despite carrying out the procedure correctly, it is also advisable to weigh the sample.

7.3 Sample syringes are to be used only once and discarded according to the procedures in TSE-003.

B Preparation of 2M NaOH

Caution

Hazardous Substance. Dangerous Goods.
Always wear laboratory gown, enclosed shoes, gloves and safety glasses when handling NaOH.
Store in plastic trays and avoid contact with acids, ammonium salts, strong oxidisers and organic materials/compounds.
Rinse well with water.

1 Molecular Weight of NaOH = 40.00g/mol
2 Weigh out the required amount of NaOH using the 3 digit balance in TSE lab
3 Slowly add it to the measured amount of RO water according to the table below. Since this is used for disinfecting only it is not necessary to adjust the final volume for accuracy or titrate this reagent.

Required Volume (L)	NaOH (g)	RO Water (L)
1	80	1
2	160	2
3	240	3
4	320	4

C Preparation of 2% NaHypochlorite

Caution

10% is Hazardous Substance. Dangerous Goods.
2% is Non-hazardous. Non-Dangerous Goods
Always wear laboratory gown, enclosed shoes, gloves and safety glasses when handling NaHypochlorite.
Store in plastic trays and avoid contact with acids.
Rinse well with water.

1 Na Hypochlorite comes as a 10%w/v solution (available chlorine). This is diluted to 2% for use (1/5)
2 Measure the required volumes of Na Hypochlorite and RO water using the table below.
3 Slowly add the Na Hypochlorite to the water.

Required Volume (L)	Na Hypochlorite (L)	RO Water (L)
1	0.2	0.8
2	0.4	1.6
3	0.6	2.4
4	0.8	3.2

TeSeE – The BioRad ELISA method for *in vitro* detection of PrP^{sc}.

APPENDIX B

REVISION REGISTER

Issue No.	Date of Issue	Amendment Details
1	9\5\05	All pages
2	15\12\05	All pages
3	3\10\06	All pages

Controlled Copy No: 26

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1 SCOPE AND APPLICATION

1.1 As for method TSE-001.

2 PRINCIPLE

GENERAL

2.1 As for method TSE-001.

DETECTION

2.2 The TeSeE Detection Kit is an immuno-enzymatic technique (sandwich format) using 2 monoclonal antibodies for the detection of the abnormal prion protein, resistant to proteinase K in CNS (Central Nervous System) of infected animals. The kit contains sufficient reagents to assay 192 tests.

2.3 The solid phase is composed of 12 strips of 8 polystyrene wells coated with the first monoclonal antibody. The second monoclonal antibody is bound to peroxidase.

2.4 The assay comprises the following reactive steps:

- Distribution of negative (R3) and positive controls (R4), samples prepared with the reagents of the TeSeE purification kit in the wells of the micro plate coated with the first monoclonal antibody. This distribution can be visually controlled, as there is a marked colour difference between an empty well and a well containing a sample.
- Incubation
- Washing, then distribution of the peroxidase-labelled antibody. This distribution can also be visually controlled by the colour difference between an empty well and a well containing the conjugate solution.
- Incubation
- Washing, then revelation of enzymatic activity bound to the solid phase by addition of the substrate.
- Stopping of the colour development, determination of optical density at 450nm – 620nm (bichromatism mode) and interpretation of the results.

3 REAGENTS

3.1 BioRad TeSeE Detection Kit Ref. 355 1145 comprising the following:

LABELLING	TYPE OF REAGENT	PRESENTATION
R1	**Micro plate:** 12 strips of 8 wells coated with an anti-PrP monoclonal antibody	2 plates
R2	**Wash solution:** 10-fold concentrated Tris-NaCl buffer pH 7.4 Preservative: Sodium merthiolate (0.01%)	1 vial (250ml)
R3	**Negative Control:** PBS Buffer pH 7.4 supplemented with BSA Preservative: Proclin 300 (0.1%)	1 vial (4ml)
R4	**Positive Control:** PBS buffer pH 7.4 supplemented with non-infectious synthetic peptide. Lyophilised. Preservative: Proclin 300 (0.1%)	1 vial
R6	**Sample diluent:** PBS buffer pH 7.2 supplemented with BSA and phenol red Preservative: Proclin 300 (0.1%)	1 vial (35 ml)
R7	**Conjugate:** 10-fold concentrated peroxidase-labelled anti-PrP monoclonal antibody in Tris-NaCl buffer pH 7.4 solution supplemented with bovine proteins and coloured with phenol red Preservative: Proclin 300 (0.1%)	1 vial (2.5ml)
R8	**Peroxidase Substrate Buffer:** Solution of citric acid and sodium acetate pH 4.0 containing 0.015% H_2O_2 and 4% dimethylsulfoxide (DMSO)	1 vial (60ml)
R9	**Chromogen:** Tetramethylbenzidine (TMB) solution	1 vial (5ml)
R10	**Stop solution:** 1N sulphuric acid	1 vial (28ml)
	Adhesive films	

3.2 2M NaOH (See TSE-001 Appendix B)

3.3 2% Na Hypochlorite (See TSE-001 Appendix C)

3.4 RO water

4 APPARATUS

4.1 **A derogated PCIII laboratory is required. (See Method TSE-003)**

4.2 Samples purified by the method TSE-001.

4.3 Multistepper pipettes 50-300uL

4.4 10-100uL and 100-1000uL micropipettes and stands

4.5 Pipette tips

4.6 Model 1575 Immunowash (Cat. # 170-7009 from BioRad)

4.7 Micro plate incubator IPS (Cat. # 35 87 530 from BioRad)

4.8 Cold tray for incubation

4.9 Micro plate reader model 680 (450nm) (Cat. # 168-1000 from BioRad)

4.10 620nm filter (Cat. # 168-1054 from BioRad)

4.11 Micro plate Manager Software (Cat. # 170-9520 from BioRad)

4.12 PC and printer

4.13 2 x Class II biohazard hoods

4.14 Laboratory grade freezer

4.15 Refrigerator

4.16 Min/max thermometers for calibrating heating blocks, fridges and freezers

4.17 Glass Schott bottles (2L)

4.18 Carboys (4L)

4.19 Measuring cylinders (10mL, 20mL, 100mL and 1000mL)

4.20 10mL disposable graduated pipettes and bulbs

4.21 Absorbent paper

4.22 Disposable gloves, masks, gowns and booties

4.23 Slip-on boots

4.24 Autoclave bags

4.25 Squirt bottles

4.26 Stainless steel trolley

4.27 Stainless steel autoclavable containers

4.28 Plastic bins for liquid waste

4.29 Autoclave facility

5 SAMPLING AND SAMPLES

DETECTION

5.1 The assay can only be performed on samples obtained from nervous tissue prepared with the reagents and under the conditions of use of the TeSeE Purification Kit (Refer method TSE-001).

5.2 Purified samples must be diluted with 125 µL of reagent R6 of the TeSeE Detection Kit.

6 PROCEDURE

CAUTION:

PPE detailed in the method TSE-003 must be worn at all times.

All solid and liquid waste is disposed of according to the procedures detailed in the method TSE-003.

All parts of this procedure where direct contact is made with the sample are performed in a Class II Biohazard Hood.

Covered micro titre plates are transferred through the TSE laboratory on trays.

PREPARATION OF REAGENTS

6.1 **Kit** - Before use, let the reagents of the TeSeE Detection Kit adjust to room temperature (+18-30°C) for 30 minutes.

6.2 Turn on the incubator and plate washer on 30 minutes before they are required.

Ready to Use:

6.3 **Micro plates (R1):**

- Before opening the vacuum packed bag, let the micro plate adjust to room temperature (+18°C to +30°C) in its protective packaging to avoid any water condensation in the wells.
- Open at the solder point and immediately return the unused rows to the sachet.
- Tightly close the bag after expelling any air, then store at +2-8°C

6.4 **Negative Control (R3)** – ready for use

6.5 **Sample Dilution Solution (R6)** – ready for use

6.6 **Stop Solution (R10)** – ready for use

Reagents to reconstitute:

6.7 **Wash solution (R2)** – Dilute wash solution R2 to 1/10 in RO water according to the following table (example 100ml of reagent R2 + 900ml of distilled water). A final volume of 1000-1250mL is sufficient for one plate with an expiry date of two weeks.

Wash buffer preparation:

Final Volume (mL)	Concentrated wash R2 (mL)	RO water (mL)
500	50	450
625	62.5	562.5
1000	100	900
1250	125	1125
2500	250	2250

6.8 **Positive control (R4)** – Gently tap the bottle of positive control (R4) on the laboratory bench to detach any substance adhering to the rubber stopper. Open the bottle and dissolve the content in 4ml of diluent R6. Reseal the bottle and let stand for approximately 1 minute, homogenizing gently and occasionally to facilitate dissolution. Aliquot into 0.5mL lots and store frozen -20°C.

6.9 **Conjugate (R7)** – Dilute reagent R7 to 1/10 in the freshly reconstituted wash solution according to the following table (example: 0.1ml of reagent R7 plus 0.9ml of reconstituted wash solution). 1ml of ready-for-use conjugate is sufficient for 1 strip. Homogenise gently. Avoid using a vortex.

Conjugate preparation:

Number of strips	Conjugate R7 (mL)	Reconstituted Wash Solution (mL)
1	0.1	0.9
2	0.2	1.8
3	0.3	2.7
4	0.4	3.6
5	0.5	4.5
6	0.6	5.4
7	0.7	6.3
8	0.8	7.2
9	0.9	8.1
10	1.0	9.0
11	1.1	9.9
12	1.2	10.8

6.10 **Enzymatic development solution (R8 + R9)** – Dilute reagent R9 to 1/11 in reagent R8 according to the following table (e.g. 0.1ml of reagent R9 plus 1ml of reagent R8). 1.1ml of enzymatic revelation solution is sufficient for 1 strip. Homogenise gently. Avoid using a vortex.

Enzymatic Development Solution (R8 + R9) preparation:

Number of strips	Reagent R9 (mL)	Reagent R8 (mL)
1	0.1	1.0
2	0.2	2.0
3	0.3	3.0
4	0.4	4.0
5	0.5	5.0
6	0.6	6.0
7	0.7	7.0
8	0.8	8.0
9	0.9	9.0
10	1.0	10.0
11	1.1	11.0
12	1.2	12.0

STORAGE/SHELF LIFE

Store the kit at +2-8°C before use. All reagents are stable at this temperature until the expiry date indicated on the kit. The shelf –lives of the reagents after preparation are as follows:

LABELLING	REAGENT	SHELF-LIFE
R1	Micro plate in tightly closed sachet	1 month at +2-8°C
R2	Diluted wash solution	24 hours at room temperature (+18-30°C 2 weeks at +2-8°C
R4	Reconstituted positive control	2 hours at room temperature (+18-30°C 4 hours at +2-8°C 6 months at –20°C **Note:** **It is recommended to divide the reconstituted solution into 0.5ml aliquots and to store them immediately at –20°C.** **Can be submitted to 3 successive freezing/thawing cycles**
R7	Reconstituted conjugate solution (with diluted wash solution)	8 hours at room temperature (+18-30°C)
R8 + R9	Development solution	6 hours at room temperature (+18-30°C) always protected from light

SAMPLE PREPARATION

6.11 Samples from step 6.29 of the purification method are diluted **(in a Class II Biohazard hood)** with 125µl of reagent R6. (i.e. approx. 25µl sample + 125µl sample diluent).

Note: Estimate the amount of sample diluent required for the number of samples to be tested and dispense this amount into another tube to avoid any contamination in the storage bottle. Do not return any unused portion to the storage bottle.

6.12 Diluted (capped) samples are mixed with a Vortex (5 sec. ± 2 sec.) just before distribution into the plate (R1).

PROCEDURE

6.13 Remove the micro plate rack and the required number of strips (R1) from the protective packaging. Replace the unused strips with the desiccant bag in the micro plate sachet and seal it, expelling as much air as possible.

6.14 If using a new kit prepare the positive control as described in 6.8 above. Alternatively take a previously prepared control from the freezer, defrosted and mixed before use. Mark the bottle to indicate the number of defrosts cycles. (A maximum of three is allowed).

6.15 Distribute **diluted** samples (25µl sample + 125µl sample diluent – see Section 6.11) and controls onto the ELISA plate in the following order:

- Wells A1, B1, C1, D1: 100µl of negative control (R3)
- Wells E1, F1: 100µl of positive control (R4)
- Wells G1, H1, etc: 100µl of sample diluted with reagent (R6)

Note:
Samples are tested in singulate.

6.16 Cover with adhesive film or micro plate lid and incubate for ***75min. ± 15min at 37°C ± 2°C in a micro plate incubator.***

6.17 Prepare wash solution (R2) as described in Section 6.7 above.

6.18 Prepare conjugate solution (R7) as described in Section 6.9 above.

6.19 Remove the plate cover and perform **3 wash cycles** (BioRad 1575 plate washer program TSE 3). The Plate washer automatically primes before each run.

6.20 Dry by inversion on absorbent paper.

Note:
Do not let the micro plate stand for more than 5 minutes after the last wash cycle.

6.21 Distribute 100µl of conjugate solution (R7) into each well.

6.22 Cover the ELISA plate and ***incubate 60min ± 5min at +2-8°C***. Plates are incubated on the Bio-Rad cold plate kept in the refrigerator to ensure a constant incubation temperature. Record the cold-plate temperature on the refrigerator chart in the manner indicated on the chart.

6.23 Prepare the enzymatic revelation solution (R8+R9) as described in Section 6.10 above.

6.24 Remove the plate cover and perform **5 wash cycles** (BioRad 1575 plate washer program TSE 5).

6.25 Dry by inversion on absorbent paper.

Note:
Do not let the micro plate stand for more than 5 minutes after the last wash cycle.

6.26 Distribute 100µl of revelation solution (R8+R9) into each well and ***incubate the plate in darkness and at room temperature (+18°C to +30°C) for 30 min ± 5 min.***

Note:
Allow the plate reader at least 3 minutes to warm up by switching on at this time.
Do not use adhesive film during this incubation.

6.27 Add 100µl of stop solution (R10) to each well in the same sequence and at the same distribution rate as for the revelation solution.

6.28 Thoroughly wipe the bottom of the plate and determine the optical density at 450nm – 620nm (bichromatism mode) within 30 minutes after stopping the reaction (the plate must be protected from light until read).

7 CALCULATION AND EXPRESSION OF RESULTS

7.1 **Calculation of the mean optical density (OD) of the negative control**

- OD R3 = mean of the four OD of R3 wells

7.2 **Calculation of the cut-off value**

- The cut-off value is equal to: OD R3 + 0.210

Eg: OD R3 = 0.020
Cut-off value = 0.020 + 0.210 = 0.230

7.3 **Validation of the Negative Control (R3)**

- **a) - Validation of the individual negative control values:**
  The optical density of each individual negative control must be lower than 0.150. However, a maximum of one individual aberrant value can be eliminated when its optical density is higher or equal to 0.150. The test must be repeated if more than one of the negative controls lies outside this limit.

- **b) - Homogeneity of the negative control values:**
  Calculate the mean of the negative controls with the individual remaining values. Values higher than the mean of the negative +40% (OD R3 + 40%) must be eliminated.
  - If one individual value is eliminated in a) then one additional value can be eliminated in b).
  - If no negative control value is eliminated in a) then two values maximum can be eliminated in b).

- The test must be repeated if more than two values of the negative control are eliminated [(criteria a) + b].

7.4 **Validation of the positive control (R4)**

- The mean of the positive control optical densities (R4 ODs) must be higher or equal than 1.000.

- The test must be repeated if the mean of the positive control optical densities (R4 OD's) is lower than this limit.

8 TEST REPORT

Interpretation of results:

- The cut-off value is calculated as the mean of the negative controls + 0.210.

For the initial test performed in singulate:

- Test values below this cut-off are **negative** and are not repeated. This result is recorded on LIMS (Laboratory Information Management System).
- Test values above this cut-off are **initially reactive.** This result is not recorded on LIMS but is recorded on the laboratory worksheets. These samples are retested in duplicate from the homogenate.
- Test values to 10% below the cut-off value are recorded as **inconclusive** and should be interpreted with caution. This result is not recorded on LIMS but is recorded on the laboratory worksheets. These samples are retested in duplicate from the homogenate.

On retest of initially reactive and inconclusive in duplicate:

- The cut-off is again determined for the retest plate as the mean of the negative controls + 0.210.
- If both of the retest values are below the cut-off the sample is recorded on LIMS as **negative.**
- If both of the retest values are above the cut-off the sample is recorded on LIMS as **positive.**
- If one of the retests is above the cut-off and one below the sample is recorded on LIMS as **positive.** Variation between the duplicates should be no greater than +/- 30% of the mean.
- If either of the retests is within 10% below the cut-off the results should be interpreted with caution. These would be recorded on LIMS as **inconclusive.** Again the variation between the duplicates should be less than specified above.

Samples (the other side of the obex held frozen) of all inconclusive or positive ELISA results are sent to AAHL for confirmatory testing.

All retest results, which pass QC are recorded on LIMS and used for reporting purposes.

Some samples may be **unsuitable** and are recorded as such on LIMS.

Refer to method TSE-004 for Quality Assurance and Processing.

9 REFERENCES

9.1 Bio-rad User's manual for TeSeE Reagents kits for *in vitro* purification and detection of PrP^{sc} Version Rev E.7-01/2006.

10 APPENDICES

TSE – Procedure for handling and disposal of material associated with the use of the Bio-Rad ELISA method for *in vitro* purification and detection of PrP^{sc}.

APPENDIX C

REVISION REGISTER

Issue No.	Date of Issue	Amendment Details
1	9\5\05	All pages
2	15\12\05	All pages
3	3\10\06	All pages

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1 SCOPE AND APPLICATION

1.1 This procedure outlines the requirements for the handling and disposal of materials associated with the BioRad TeSeE ELISA kit method for purification and detection of resistant prion proteins, (abnormal PrP^{sc}).

1.2 This procedure has been developed for use at a regional veterinary laboratory conducting rapid surveillance in a derogated PCIII facility and taking into consideration **current Australian conditions**.

1.3 Surveillance to date has indicated that there are no Transmissible Spongiform Encephalopathies (TSE's) in domestic livestock in Australia.

1.4 The objective is to establish safe guidelines for the handling and disposal of materials within and from the TSE laboratory to minimise risk to laboratory personnel and the environment. These guidelines have been developed in conjunction with recommendations given in the Australian/New Zealand Standard (AS/NZS 2243), by the Australian Animal Health Laboratory, Geelong and guidelines published by the World Health Organisation.

1.5 The reason for the implementation of the TeSeE method at the Animal Disease Surveillance Laboratory, Toowoomba (ADSL) is described in methods TSE-001 and 002.

1.6 An establishment phase using in house histological negative samples and/or not a known source of prion infection (i.e. from young animals) is to be used while installing equipment and procedures.

1.7 Ongoing Surveillance will involve the testing of approximately 2000 samples per year following an initial assessment for evaluation purposes of 200 samples. These samples will be approximately 90% bovine and 10% ovine.

1.8 This method describes added safety precautions that must be adhered to in addition to those outlined in the ADSL Safety Plan and Safety Induction Document.

2 PRINCIPLE

2.1 TSE's, also known as prion diseases, are fatal degenerative brain diseases that occur in humans and certain animal species. They are characterised by microscopic vacuoles and the deposition of prion protein in the grey matter of the brain.

2.2 All forms of TSE are experimentally transmissible.

2.3 The handling of the risk associated with this procedure is dependant on:

- The likelihood of the occurrence of TSE in the representative samples
- The level of infectivity of the samples and associated equipment at various stages of the procedure
- The nature of possible exposure to the infectious agents
- The routes of transmission

2.4 TSE agents exhibit an unusual resistance to conventional chemical and physical decontamination methods and therefore require specific precautions in addition to routine laboratory safety precautions.

2.5 There have been no confirmed cases of occupational transmission of TSE to humans. If TSE's could be transmitted in the occupational setting this would be most likely to occur from exposure to infected tissues or materials by direct inoculation (eg puncture wounds, 'sharps' injuries or contamination of broken skin), by splashing of the mucous membranes or, exceptionally, by ingestion.

2.6 TSE's are unlikely to be transmitted by the aerosol route.

3 REAGENTS

3.1 **BioRad TeSeE Purification Kit Ref. 355 1144.** Refer to method TSE-001 for kit components

3.2 **BioRad TeSeE Detection Kit Ref. 355 1145.** Refer to method TSE-002 for kit components

3.3 2M NaOH (Refer TSE-001 Appendix B).

3.4 2% Na hypochlorite (Refer TSE-001 Appendix C)

3.5 RO water

4 APPARATUS

4.1 **Laboratory facility at the Animal Disease Surveillance Laboratory, Toowoomba (ADSL) is derogated PC3.**

4.2 **The differences from full PC3 requirements are:**

- Filtration of exit air via HEPA filters is not essential.
- Negative air pressure in the laboratory is not essential.
- The ability to seal the area for formaldehyde fumigation is not essential.

4.3 **The elements incorporated into this facility include:**

- The testing is carried out in a separate laboratory specifically intended for this use and access is restricted to staff involved in the work
- All equipment used for the test is contained within this laboratory. This particularly pertains to centrifuges or other aerosol generating equipment. Also included are refrigerators and freezers used for temporary sample and homogenate storage.
- All procedures with unsealed samples are performed within a Class II biosafety cabinet.
- Personal protective equipment including disposable gowns and overshoes are available at the entrance to the laboratory and must be put on at the entry point.
- Staff are directed to wear a gown, double disposable gloves, safety glasses and mask and overshoes or dedicated laboratory shoes when directly handling samples and purified products. The use of this PPE is further elaborated in this method.
- A designated area for washing and disinfection is available on exit from the laboratory.
- Staff must receive specific training on the risks associated with prions and their decontamination.
- Solid waste (sample tissues, homogenates, tubes etc) will be retained within the laboratory in autoclavable bags placed in autoclavable discard stands or containers.
- The autoclave facilities are located in a separate part of ADSL. Waste is transferred to the autoclaves in enclosed stainless steel boxes. These can be decontaminated externally with 2M NaOH.
- Liquid waste is stored within the laboratory and diluted 1:1 with 2M sodium hydroxide.
- The nature of exit of liquid and solid waste from the laboratory is dependant on the test result and is outlined further in this method.

- Surfaces and equipment should be decontaminated as required with 2M NaOH or 2% sodium hypochlorite.

- Staff should be competent laboratory technologists familiar with the theory and practice of ELISA technology.

- Class II Biohazard hoods are used for all procedures involving direct handling of samples or products. The Australian Standard relates to work pertaining to the handling of prion material of types Class II and Class III. With these prion materials it stipulates that cabinets for aerosol containment and gloves must be used. Cytotoxic drug cabinets are stipulated due to the prepositioning of the filters as protection for cabinet maintenance staff. Under current Australian conditions and in the absence of detection of prions of Class II or III in domestic livestock in Australia the Class II cabinets have been employed. In the unlikely event of the detection of TSE's **this procedure must be reviewed with particular reference to future servicing and maintenance of the cabinets.**

4.4 For details of other equipment pertaining to this method refer to methods TSE-001 and TSE-002.

5 SAMPLING AND SAMPLES

5.1 Surveillance samples are obtained from 4D animals. These are dead, disabled, dying and diseased and are targeted because of the increased likelihood of them having TSE.

5.2 The obex region of the brain is targeted to increase the sensitivity of the test.

5.3 During the installation of equipment and while training staff test samples are used which are histological negative for TSE and/or come from young animals.

6 PROCEDURE

6.1 The TSE work area is separated from the main ADSL laboratory area by a keypad locked door. Access is to authorised personnel only.

6.2 The TSE work area is divided into sections, 'clean' and 'dirty'. The clean area comprises the workstation, locker facility with clean gowns and booties, a safety shower and hand-basin. The 'dirty' area comprises the laboratory.

6.3 A yellow and black hazard taped line on the floor clearly separates the 'clean' and 'dirty' areas.

6.4 While testing is in progress a sign is hung at the entrance to the 'dirty' area which reads 'Restricted Access – Testing in Progress' as a further reminder of this barrier.

6.5 The TSE work area has a separate air conditioner to the remainder of ADSL; however air is shared between all sections.

6.6 All liquid waste is held until the completion of a test run. The TSE laboratory is provided with a collection facility under the sink, which allows the diversion of liquid waste in the event of an inconclusive/positive ELISA result. **NB. The diverter is switched to 'Bypass' for the duration of the test procedure.** Disposal is according to test result as outlined below.

6.7 On entry to the 'dirty' area while testing is in progress all staff must put on gowns, disposable boots or designated laboratory shoes and disposable gloves. These items are placed in designated receptacles on the 'dirty' side on exit from the laboratory. Disposal is according to test result as outlined below.

6.8 On entry to the 'dirty' area when testing is completed all staff are requested to wear booties or designated laboratory shoes as a minimum requirement. In addition gowns and gloves are to be worn if in direct contact with equipment, hoods, reagents or samples.

6.9 Gloves are worn in the laboratory area while testing is in progress. Single gloves at least are worn at all times. A second outer glove is worn when handling exposed samples or product and this part of the procedure is

performed in a Class II cabinet. Gloves of different types and colours give a clear reminder to staff that this procedure has been adhered too. Cheaper clear plastic over gloves (i.e. a third glove) are changed between samples to avoid cross contamination.

6.10 Safety glasses must be worn at all times. Masks are worn when dealing with exposed tissues or products. An alternative to masks and glasses is a full face shield.

6.11 All samples, tubes containing purified product and ELISA plates are transported within the laboratory on trays covered in plastic backed absorbent material. All work wherever possible is performed on disposable sheets to avoid environmental contamination.

6.12 The disposal of waste material depends on the risk associated with the test result:

- **Low Risk Waste**
    - Packaging material, paper, plastic bags and boxes not exposed to potentially infectious material is regarded as Low Risk Waste. It is bagged and sent for incineration without autoclaving, providing the laboratory maintains its negative test status.
- **Negative test result (Solid Waste)**
    - Solid waste (including samples, tubes and disposable PPE) is contained in autoclave bags and stainless steel containers and autoclaved for 1 hour at 134°C (AS/NZS 2243.3:2002 is 55 minutes at 132°C). Instruments are soaked overnight in 2M NaOH in plastic tubs on the laboratory sink. The soak fluid is discarded down the sink with plenty of water. Instruments are then rinsed thoroughly with water and re-used.
- **Negative test result (Liquid Waste)**
    - Liquid waste is contained in plastic containers, diluted 1:1 with 2M NaOH to a final concentration of 1M NaOH and held overnight before being flushed down the sink with plenty of water.
    - Reagent B is considered flammable and cannot be autoclaved. The small volumes used in the laboratory are diluted 1:1 with 2M NaOH and sent for incineration with a licensed waste collector
- **Inconclusive/positive result (Solid Waste)**
    - Solid waste is contained in autoclave bags and stainless steel containers and autoclaved for 4.5 hours at 134°C. High heat autoclave bags are required for this purpose. Instruments are soaked overnight in 2M NaOH and both instruments and soak fluid autoclaved for 4.5 hours at 134°C.
- **Inconclusive/positive result (Liquid Waste)**
    - Liquid waste is contained in plastic containers, diluted 1:1 with 2M NaOH to a final concentration of 1M NaOH and held overnight before being autoclaved for 4.5 hours at 134°C.

6.13 The laboratory is regarded as potentially contaminated until all testing is completed and returns a negative result.

6.14 If negative status is retained routine cleaning is:

- Wiping down benches with 70% ethanol at the completion of that days work.
- Dry sweeping floors once per week.
- Class II Biohazard hoods and any work surface exposed to samples or product must be cleaned at the completion of the work with 2M NaOH or sodium hypochlorite at 20,000ppm (2%) available chlorine. The surface is then wiped over with water to minimize damage.

6.15 If an inconclusive or positive result is obtained:

- Exposed surfaces potentially contaminated by TSE agents can be disinfected by flooding, for one hour, with 2M NaOH or 2% sodium hypochlorite.
- Floors and benches must be cleaned with sodium hypochlorite at 20,000ppm (2%) available chlorine.

6.16 The Class II cabinet must be thoroughly cleaned between the purification (Method TSE-001) and detection (Method TSE-002) steps by wiping down with 2M NaOH.

6.17 NaOH is used on stainless steel surfaces while Na hypochlorite is preferable for floors and benches. Surfaces are then rinsed with water to minimise damage and can be dried with ethanol and wiped with absorbent towel.

6.18 Contaminated surfaces containing acids must first be neutralised with NaOH before using Na hypochlorite.

6.19 Never autoclave solutions containing bleach (Na hypochlorite).

7 CALCULATION AND EXPRESSION OF RESULTS

7.1 Refer to the detection method (TSE-002)

8 TEST REPORT

8.1 Disposal of specimens is traced through the Laboratory Sample Manager.

8.2 Workplace health and safety incidents are reported through the departmental procedure.

9 REFERENCES

9.1 Australian/New Zealand Standard. Safety in laboratories part 3: Microbiological aspects and containment facilities. (AS/NZS 2243.3:2002)

9.2 WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies. Report of a WHO consultation Geneva, Switzerland, 23-26 March 1999.

9.3 'Precautions for work with human and animal TSE's' from the UK Advisory Committee on Dangerous Pathogens – TSE Agents, Department of Health, London, UK, 2003

9.4 Personal Communication Peter Le Blanc Smith, Biocontainment Microbiologist, CSIRO Livestock Industries. Australian Animal Health Laboratory (AAHL). Private Bag 24. Geelong Vic. 3220. Australia

9.5 Communication – Biocontainment standards and safety measures as recommended by Prionics and BioRad for laboratories performing "rapid" TSE post mortem surveillance testing

10 APPENDICES

APPENDIX D

REVISION REGISTER

Issue No.	Date of Issue	Amendment Details
1	15/12/05	All pages
2	3/10/06	All pages

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1 SCOPE AND APPLICATION

1.1 This document outlines the procedure to be followed for processing samples in the TSE laboratory for MLA Project TSE-008: the 4-D Project. Also included are quality assurance issues and the use of LIMS.

2 PRINCIPLE

2.1 The method used is the Bio-Rad TeSeE method for *in vitro* purification and detection of PrP^{sc}.

2.2 Sample tracking and result entry is done through the Laboratory Information Management System (LIMS). This process is described in the document 'TSE Processing in LIMS' (Appendix A).

3 REAGENTS

3.1 All reagents and their preparation are described in methods TSE-001 and TSE-002.

3.2 Reagents are discarded when they pass their expiry date. Batch number and expiry date are recorded on the reagent vessels and kits.

3.3 Reagents are stored at 2-8°C and warmed to Room Temperature for 30 minutes before use.

3.4 Proteinase K and Conjugate are kept at 2-8°C where possible.

3.5 The Positive control for the ELISA is reconstituted on first use, aliquoted into 0.5mL amounts and stored frozen at <-20°C.

4 APPARATUS

4.1 All equipment is described in the methods TSE-001 and TSE-002.

4.2 Calibration of equipment is the same as the schedules adopted by the Serology Section at ADSL.

5 SAMPLING AND SAMPLES

5.1 Samples are brain stem tissue in the region of the obex. Technical staff are trained to assess if the tissue contains this site. If determined that this may not be the case a pathologist is asked to inspect the sample. A comment is entered onto the report if the obex is not represented in the sample.

5.2 This method has been approved by the European Union for use on autolyzed tissue. In cases of extreme autolysis a pathologist determines its suitability.

5.3 Samples that require checking are recorded on the LIMS form 'Check-Out TSE Samples from Specimen Receival' (TSE Sample Sheet). The pathologists sign and date this form following their assessment.

5.4 The samples can be subject to three defrost cycles. In practical terms the samples are defrosted once for homogenisation, the homogenates are defrosted once for purification (twice if a retest is required) and the purified samples are either used directly in the test or defrosted once for use.

5.5 10% of the samples are weighed on extraction and weights recorded on the Excel Spreadsheet at: U:\Biosecurity...\TSE 008\Weights. For an ELISA run of half a plate (40 samples) therefore at least 4 samples are monitored to ensure samples are within the required weight range.

5.6 The weights are recorded on the 'TSE Sample Sheet'.

6 PROCEDURE

Check-In

6.1 LIMS is checked daily to determine if there are any samples that have been checked out of Specimen Receipt and are awaiting collection.

6.2 In addition Specimen Receipt places a 'TSE Sample Sheet' in the TSE in-tray.

6.3 Samples are collected from Specimen Receipt by TSE staff. Samples are 'Checked-In' on LIMS in the TSE laboratory. This is done through Section Sample Management>Check in Samples. The samples must be checked against the hardcopy list before they can be checked in.

6.4 LIMS labels are printed for all samples that are to be homogenized through Section Test Planning>Allocate test to worksheet. Select the samples>menu click>print small labels.

6.5 If not processed that day the samples are stored in the freezer.

6.6 Sample lists and labels are placed in the Results folder in the laboratory.

Homogenisation

6.7 Samples are defrosted prior to the homogenisation procedure.

6.8 Homogenates are prepared where possible within one week of receipt.

6.9 LIMS labels are attached to one homogenization tube per sample.

6.10 The extraction of the tissue and the homogenization is performed as per Method TSE-001.

6.11 Homogenate tubes are stored in the TSE Freezer 4 in the box labeled 'Homogenates to be Purified' in Tray 4. The original tissues are returned to the Freezer. Both can be stored for several months. Test times are monitored through LIMS.

6.12 Homogenisation date is recorded on the 'TSE Sample Sheet'

Purification

6.13 When the critical mass of 42 samples is reached the samples are purified. This number of samples equates to six ELISA strips.

6.14 Samples for purification are allocated to a LIMS ELISA Worksheet through Section Test Planning>Allocate Tests to Worksheet. Print the Worksheet.

6.15 In generating this worksheet an Excel spreadsheet containing the sample numbers is generated and sent to the TSE-008\Results folder on U: drive. It is named TSE (worksheet#).csv.

6.16 The LIMS worksheet is in ELISA format and allocates each sample a consecutive purification tube number starting at one.

6.17 One screw capped micro tube per sample is labeled with consecutive numbers starting at one and up to the required number of samples. A marking pen is used to label the lid and side of the tube.

6.18 One homogenate tube and one micro tube is placed in separate racks in corresponding positions and the sample number and tube number checked against the worksheet before sample transfer.

6.19 Homogenates are returned to TSE Freezer 4 in Tray 1 on completion of calibrated sample transfer.

6.20 Purification is performed as per Method TSE-001.

6.21 The Purification Kit #, the date and the initials of the operator are recorded on the LIMS ELISA Worksheet.

6.22 Purified samples can be used immediately in the ELISA, stored refrigerated for 5 hours or frozen at a minimum of -20°C for 72 hours. If frozen they are stored in the Medical Freezer in Tray 4 in the box labeled 'Purified ready for ELISA.

6.23 Storage boxes are labeled with the Worksheet #.

ELISA

6.24 The ELISA is performed as per Method TSE-002.

6.25 Diluted samples are added to the ELISA plate in positions which correlate with the Tube number and according to the LIMS ELISA Worksheet.

6.26 The Detection Kit #, Wash Buffer Batch #, the date and the initials of the operator are recorded on the LIMS ELISA Worksheet.

6.27 The ELISA plate is read using the Microplate Manager Model 680TSE.epr protocol.

6.28 Press 'Run' to read the plate.

6.29 When prompted by the software the following information is given:

- Technician = QDPI@F username
- Detection Plate = the worksheet # only
- Detection Lot = Worksheet name

6.30 Press 'OK' and the plate is read.

6.31 The Bio-Rad report appears on the screen.

6.32 Open the Excel Spreadsheet TSE (worksheet#).csv which is found in u:\TSE-008\Results. This file is generated by LIMS when the Worksheet is created and contains the list of sample numbers being tested. The list of sample numbers is copied from the Excel spreadsheet and pasted into the Microplate Manager spreadsheet in Sheet 2 named 'Sample Information'. (Select All, RMB, copy go to Microplate Manager RMB, paste). This enters the sample number against the result in the correct plate position on the Bio-Rad report.

6.33 To save the results

- Export Text File = u:\TSE-008\Worksheet name_results.txt (This is done from the Sample Information sheet by clicking on the Export Text File box). Change the file # to the current worksheet # and press Save.
- Save the Bio-Rad report = u:\TSE-008\Worksheet name_BioRad.xls (This is done from the Bio-Rad report sheet through the menu File>Save As
- Print the Bio-Rad report
- Close the Bio-Rad report window
- Save the raw data = u:\TSE-008\Worksheet name_plate.mpm (This is done by closing the raw data window by clicking on X. 'Do you want to save as Plate 1, answer yes and then enter the correct save file name).
- The files are all saved in the u:\TSE-008\Results folder. By selecting the last version of each file type and changing the worksheet number this can be done easily.

6.34 **Turn off the Plate Reader!**

7 CALCULATION AND EXPRESSION OF RESULTS

7.1 The Bio-Rad report evaluates the validity of the controls.

7.2 The Bio-Rad report flags negative, inconclusive and positive samples

7.3 Electronic calculations are manually checked once a year.

8 TEST REPORT

8.1 The operator entering the results in LIMS checks the validity of the control values and ensures that the sample results correspond to that seen on the ELISA plate and that the correct samples are represented.

8.2 This operator enters the results on LIMS through Test- Results>Result entry by Worksheet. **Select one sample only in the right hand screen.** If all samples are negative enter the first result as negative and then CTL D to fill the rest. Click on the Save icon and close the window. Sign and Date the printed ELISA worksheet in the Reported By box. A second version of the ELISA worksheet is now printed which is a COPY ONLY but which has the result printed against the sample number. This is done through Test- Results>Result Entry by Worksheet>Select the Worksheet in the right hand screen>Worksheet>Print ELISA. Write Copy Only on this Worksheet.

8.3 A staff member then checks the results on LIMS against the results on the worksheet, ensures that the ELISA performed within all required parameters (as detailed in Method TSE-002) and then signs the Checked By box on the LIMS ELISA Worksheet.

8.4 A staff member other than the one who reported the results then authorises each result individually. This is done through Test- Review>Authorise Tests by Worksheet.

8.5 This second staff member Signs and Dates Authorised box on the printed ELISA worksheet to indicate that they have authorised the electronic record.

8.6 The Bio-Rad Report, LIMS ELISA worksheet and Sample Lists are filed in the Results folder in the laboratory.

8.7 This second staff member then completes the LIMS worksheet on-line through Test-Review>Finalise>Remove Worksheet.

9 REFERENCES

9.1 TSE Processing in LIMS. Version 1.6 03 October, 2006. R. Stewart.

9.2 Bio-Rad User's manual for TeSeE Reagents kits for *in vitro* purification and detection of PrP^{sc} Version Rev.E.7-01/2006.

9.3 Bio-Rad Sample Syringe Manual – Sampling method for Bio-Rad TSE Screening Assays Version Rev.B10/2005.

10 APPENDICES

Appendix A – TSE Processing in LIMS

See attached file TSE008LIMS.doc Version 1.6 03 October, 2006

MEASUREMENT UNCERTAINTY ESTIMATION
TeSeE – THE BIORAD ELISA METHOD FOR *IN VITRO* DETECTION OF PrP^{res}

APPENDIX E

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1 SCOPE AND APPLICATION

1.1 This document records the estimation of measurement uncertainty for TSE-001, 002, 003, 004.

1.2 For methods details, reference is made to the methods TSE-001, 002, 003, 004.

1.3 For procedural details relating to measurement uncertainty, reference is made to QP-15.

2 ESTIMATION OF MEASUREMENT UNCERTAINTY

2.1 Some implications of the test are discussed below.

The Bio-Rad TeSeE test (originally the CEA or Commissariat a l'Energie Atomique sandwich immunoassay for PrPres) was evaluated in terms of its sensitivity, specificity and detection limits by the Directorate General XXIV of the European Union. The report was released in July 1999. This test was one of three validated for use.

Diagnostic sensitivity may be defined as the proportion of known infected reference animals that test positive in the assay. Diagnostic specificity may be defined as the proportion of uninfected reference animals that test negative in the assay. Sensitivity and specificity were calculated on the basis of 300 and 1000 samples respectively. Results indicated that sensitivity and specificity were both 100%.

6 undiluted samples and 20 diluted replicate samples were tested in a dilution series. 20/20 samples were detectable to a dilution of $10^{-2..0}$, 18/20 to a dilution of $10^{-2..5}$ and a single sample was detected at a dilution of $10^{-3..0}$. No samples were detected at a dilution of $10^{-3..5}$.

The cut-off point indicated in the test was considered to be close to optimal with a wide separation between positive and negative populations.

Repeatability of tests was ascertained by testing 36 positive and 64 negative samples in duplicate. All duplicate samples were correctly identified.

The test was subsequently assessed in 2005 as suitable for use on autolysed tissue by analysis of 250 positive samples from fallen stock in various stages of autolysis. All samples were correctly classified as positive for BSE.

Results for the method are expressed as "Positive" or "Negative" or "Inconclusive". This qualitative result is derived from a quantitative absorbance reading. Hence, the measurement uncertainty (MU) is estimated for the absorbance readings of samples.

2.2 Contributors to MU are identified as:

- The accuracy of absorbance readings of samples and controls (2.3 below).
- The precision in absorbance determinations of samples and controls (2.4 to 2.5 below).
- The accuracy of piston operated volumetric apparatus (POVA) for the method volumes used (2.6 below).
- The precision of POVA for the method volumes used – (2.6 below).
- Tip volume variation of POVA (2.7 below).
- The weight of sample (2.8 below).

Other factors (including incubation, temperature and time) were not considered capable of providing a significant contribution to the MU estimation and hence are disregarded in the calculation.

2.3 The accuracy of absorbance readings of samples and controls is estimated as 2%. The figure is based on the VLQMS acceptance criteria for accuracy for ELISA microplate reader checks. Data from three monthly checks conform with this acceptance criteria. The standard uncertainty associated with the accuracy of absorbance readings is derived in Appendix A based on a rectangular distribution of the uncertainty. The relative standard deviation (rsd) is estimated from this value (Appendix A).

2.4 At this stage, the laboratory has only limited replicated absorbance reading data. It is envisaged that the MU estimation will be updated later upon further replicated data being available. The laboratory is not provided with sufficient sample to enable duplicated analysis (Notes; Samples were tested in singulate in accordance with test protocols. Sample may be required later for testing "Inconclusive" results). Hence, the precision is estimated on the data of positive and negative controls. At this stage, the precision estimate is based on the method verification data and from initial testing, tested over a three month period. The variation within this data is considered characteristic of samples received. (Note that characteristic samples are "negative" and the laboratory does not expect to receive any "positive" samples). The data was accumulated over many runs on different days by different analysts. In accordance with the method, the negative control is tested in quadruplicate and the positive control is tested in duplicate. The precision is determined from the normalised difference data (the difference divided by the mean). The standard deviation of the normalised differences is divided by $\sqrt{4}$ in the case of negative control data and by $\sqrt{2}$ in the case of positive control data to transform from a standard deviation for these replicated differences to the standard deviation for single values (Appendices B and C).

2.5 The estimated precision relative standard deviation for single absorbance determinations is calculated as 26 % for negative control data and as 3% for positive control data (Appendices B and C respectively). The negative control data will be used for the MU estimation of samples with absorbances below 0.150 (These will be "Negative" samples). For samples with absorbances above 0.150, the positive control data will be used for the MU estimation (includes "Positive" and "Inconclusive" samples).

2.6 The contribution of POVA to accuracy and precision is considered insignificant in this method since for similar data it is shown to represent less than 1/3 of the total variation (ref. METHOD SER-025 MU).

2.7 The precision component for tip volume variation of POVA used in the TSE methods is covered by the precision of absorbance determinations of samples and controls.

2.8 A subsample is obtained for the method by syringing volumes between designated marks on the Bio-Rad sampling syringe (Appendix A in Method TSE-001). These volumes correspond to sample weights of 0.350 ± 0.040 g. Testing has confirmed that these syringe volumes relate to this weight range (data is available from the laboratory to shown this). A subsample of sample weights is checked against syringe volumes regularly (at least 10 % of samples for each homogenisation run). Sample weights then have an uncertainty of 11.4 %, relating to a standard uncertainty of 5.8 % (95 % confidence interval). The effect on the method MU from the sample weight is reduced given the test procedure. The sample is transferred to a grinding tube in which grinding beads and homogenisation buffer is present. Laboratory data shows that there is a 1 % variation between tube volumes (1.891g) of homogenate and grinding beads (considered insignificant). Testing has shown that the test solution on which absorbance is measured is reduced by a factor of 6.4 ((1.891+0.35)/0.35. Ref. Laboratory data). Hence, the contribution of sample weight on the method MU is estimated at 1.8 % (11.4/6.4%), relating to a standard uncertainty of 1 % (95 % confidence interval).

2.9 The components of MU are then combined in Appendices D and E for samples with absorbances less than 0.150 and with absorbances greater than 0.150 respectively. Without an applicable simple mathematical model, the components are treated as independent variance components. As such, the root sums of squares provides an estimate of the total combined components of MU.

2.10 An expanded uncertainty is determined using a coverage factor of two for a level of confidence of approximately 95%.

2.11 The expanded or total uncertainty of the method is estimated as an rsd of 0.520 (Appendix D) for samples with absorbances less than 0.150 and 0.069 (Appendix E) for samples with absorbances greater than 0.150. Hence the total uncertainty of absorbance readings is estimated as:

52 % for samples with absorbances less than 0.150
7 % for samples with absorbances greater than 0.150

3 CONCLUSION

3.1 The main component of the total uncertainty for the method is the precision of absorbance determinations.

3.2 Consideration of uncertainty associated with false positive results.

3.2.1 Applying the method, the cut-off value for a "Positive" result is the mean absorbance of the negative control plus 0.210. If the mean absorbance of the negative control is 0.010 (typical value), the associated MU is 0.52 * 0.010 = 0.005 ie. the absorbance of the negative control on this occasion is 0.010 ± 0.005 (95% ci). Hence the value with its' associated MU could be as high as 0.015. The cut-off value for a "Positive" result is 0.210 + 0.010 = 0.220. Hence with the derived MU, a negative sample with an absorbance value similar to the negative control could not be reported as "Positive" (95% ci). Hence, there is no chance of a false positive in this case. Furthermore, protocols have been established in the methodology to try to ensure against false positives (Ref. 3.3.2). In particular, all samples with "Positive" results are sent to AAHL for confirmation. Hence, these protocols further inhibit the chance of a false positive.

3.3 Consideration of uncertainty associated with false negative results.

3.3.1 Those absorbance readings within the range of 0.015 and 0.220 for the example above provide an opportunity for a false negative.

3.3.2 For a sample with an absorbance reading at the cut-off value of 0.220, the associated MU is 0.220 * 0.07 = 0.015 ie. the absorbance of the sample on this occasion is 0.22 ± 0.02. A positive sample with an absorbance value similar to the cut-off value of 0.22 could have an absorbance as low as 0.20. Protocols have been established in the methodology to try to ensure against false negatives (and false positives). The method dictates that samples with absorbances within 10 % below the cut-off value are retested in duplicate from the homogenate (The value of 10 % correlates with the estimated MU). Both retest values have to be negative for the sample to be reported as negative (and both positive for the sample to be reported as positive). Duplicate absorbance results should not vary greater than 30 % of their mean. If a variation greater than 30 % occurs, then this is investigated to find the cause and retesting is conducted in duplicate until confidence is established in the final result or samples are sent to AAHL for testing. If a retest gives one "Positive" and one "Negative" result, then the sample is reported in-house as "Inconclusive" and sent to AAHL for testing. Hence, the probability of a false negative is minimal.

3.3.3 Analysts are conscious of MU, particularly in the range around cut-off values (0.20 to 0.24 for the example above).

3.4 The Measurement Uncertainty for the method, TSE-001, 002, 003, 004 is estimated at:

52 % for samples with absorbances less than 0.150
7 % for samples with absorbances greater than 0.150

4 REFERENCES

4.1 Method TSE-001, 002, 003, 004 TeSeE – The BioRad ELISA method for in vitro detection of PrP^{res}.

4.2 Measurement Uncertainty QP-15, Quality Procedures Manual, VLQMS.

4.3 Measurement Uncertainty Training Course Notes, NARL, Australian Government Analytical Laboratories 2001 and 2003.

4.4 Guide to the Expression of Uncertainty in Measurement, BIPM, IEC, IFCC, ISO, IUPAC, IUPAP, OIML. 1995.

4.5 Assessment of Uncertainty of Measurement for Calibration and Testing Laboratories, Cook R R., 2nd ed 2002.

4.6 Quantifying Uncertainty in Analytical Measurement, Eurachem / CITAC Guide, 2nd ed. 2000.

4.7 The Evaluation of tests for the diagnosis of Transmissible Spongiform Encephalopathy in Bovines. European Commission, Directorate General XXIV, Consumer Policy and Consumer Health Protection, 8 July 1999)

4.8 A Comparison of rapid bovine spongiform encephalopathy testing methods on autolysed bovine brain tissue, Angus Wear, Kirstine Henderson, Kath Webster and Indu Patel. J Vet Diagn Invest 17:99-102 (2005)

5 APPENDICES

5.1 Appendix A: Absorbance Accuracy Component of Measurement Uncertainty for TSE-002.

5.2 Appendix B: Absorbance Precision Component of Measurement Uncertainty for samples with absorbances less than 0.1 for TSE methods.

5.3 Appendix C: Absorbance Precision Component of Measurement Uncertainty for samples with absorbances greater than 0.1 for TSE methods.

5.4 Appendix D: Sample Weights relating to Syringe Volumes.

5.5 Appendix E: Total Components of Measurement Uncertainty for samples with absorbances less than 0.1 for TSE methods.

5.6 Appendix F: Total Components of Measurement Uncertainty for samples with absorbances greater than 0.1 for TSE methods.