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SCIENTIFIC OPINION

Scientific Opinion on Analytical sensitivity of approved TSE rapid tests[1]

EFSA Panel on Biological Hazards (BIOHAZ)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Community Reference Laboratory (CRL) for TSEs studied the analytical sensitivity for all the currently approved TSE rapid tests in order to produce robust analytical sensitivity data and evaluate each test against the same sample sets for the three main types of ruminant TSE: BSE, Classical scrapie and Atypical scrapie. This opinion provides a scientific evaluation of the CRL analytical sensitivity study, based on the requirements as set in the current EFSA protocols for the evaluation of TSE rapid *post mortem* tests. It is concluded that the CRL study findings provide valuable information in determining the continued suitability of tests currently used for TSE monitoring in the EU. Conclusions on the performance of the approved rapid tests within the CRL study are included. On these bases a number of tests cannot be recommended for use for the monitoring of BSE in cattle and TSE in small ruminants in the EU. Finally, the BIOHAZ Panel recommends that a similar study should be conducted with other types of TSE in cattle and small ruminants.

KEY WORDS

BSE, Classical scrapie, Atypical scrapie, analytical sensitivity, TSE rapid tests.

1 On request from the European Commission, Question No EFSA-Q-2009-00687, adopted on 10 December 2009.

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SUMMARY

Following a request from the European Commission, the Panel on Biological Hazards (BIOHAZ Panel) was asked to deliver a scientific opinion on Analytical sensitivity of approved TSE rapid tests.

In 2008 the European Commission asked the Community Reference Laboratory (CRL) for TSEs to assess the analytical sensitivity for all the currently approved TSE rapid tests in order to produce robust analytical sensitivity data and evaluate each test against the same sample sets for the three main types of ruminant TSE: BSE, Classical scrapie and Atypical scrapie. The European Commission forwarded the final report of the study (CRL study) to EFSA and requested EFSA to provide a scientific evaluation of it and, if needed, based on the information available in the CRL report, reconsider and amend previous recommendations related to the approval of each of those rapid tests, based on the requirements as set in the current EFSA protocols for the evaluation of TSE rapid *post mortem* tests.

The current scientific opinion gives an overview of the methodology and results of the CRL study. The CRL study investigated the analytical sensitivity of all the approved TSE rapid tests and also investigated the stability of Atypical scrapie positive samples when stored frozen at -80°C. The BIOHAZ Panel acknowledges that for the first time all the tests were evaluated against the same sample set, allowing a direct comparison of their analytical sensitivity and concludes that the study findings provide valuable information in determining the continued suitability of tests currently used for TSE monitoring in the EU.

With regard to the BSE analytical sensitivity study performed by the CRL, the BIOHAZ Panel concludes that AJ Roboscreen BetaPrion®, Bio-Rad TeSeE™ SAP, Enfer TSE v2, Enfer TSE v3, IDEXX HerdChek Standard, IDEXX HerChek Short, IDEXX HerdChek Ultra Short, Roche Prionscreen and Prionics®-Check Western performed within a maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system. Prionics®-Check LIA and Prionics®-Check PrioSTRIP gave unexplained and unresolved specificity problems which hamper the interpretation of their analytical sensitivity and the comparison with other approved tests. Therefore the BIOHAZ Panel recommends that the analytical sensitivity of Prionics®-Check LIA and Prionics®-Check PrioSTRIP should be re-assessed by appropriate experiments under the supervision of the CRL. Excluding Prionics®-Check LIA and Prionics®-Check PrioSTRIP, for all other tests no potential differences in field detection performance can be inferred on the sole basis of the difference in analytical sensitivity reported in this study.

With regard to the Classical scrapie analytical sensitivity study performed by the CRL, the BIOHAZ Panel concludes that all tests (Bio-Rad TeSeE™ SAP, Bio-Rad TeSeE™ Sheep/Goat, Enfer TSE v2, Enfer TSE v3, IDEXX HerdChek Standard, IDEXX HerdChek Short, IDEXX HerdChek Ultra Short, Prionics®-Check LIA SR, Prionics®-WB Check Western SR) performed within a maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system. Marginal specificity problems were observed with Prionics®-Check LIA SR and Enfer TSE v3 with sheep samples, which did not compromise the estimation of their analytical sensitivity. No potential differences in field detection performance can be inferred on the sole basis of the difference in analytical sensitivity reported in this study.

With regard to the Atypical scrapie stability study, the BIOHAZ Panel concludes that an apparent decrease in the detected signal could be observed during the stability study and that this was taken into account in the study.

With regard to the Atypical scrapie analytical sensitivity study performed by the CRL, the BIOHAZ Panel concludes that Bio-Rad TeSeE™ SAP, Bio-Rad TeSeE™ Sheep/Goat, IDEXX HerdChek Standard, IDEXX HerdChek Short and IDEXX HerdChek Ultra Short performed within the maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system. It is also concluded that Enfer

TSE v2, Enfer TSE v3, Prionics®-Check LIA SR and Prionics®-WB Check Western SR could fail in identifying field Atypical scrapie cases that other validated tests would detect. The EFSA protocol for the evaluation of rapid *post mortem* tests to detect TSE in small ruminants states that tests that are not able to meet requirements for all types of TSE agents on known positive samples should not be considered for testing in the field. Consequently, and based on the information obtained from the CRL study, Enfer TSE v2, Enfer TSE v3, Prionics®-Check LIA SR and Prionics®-WB Check Western SR cannot be recommended for use for TSE monitoring in small ruminants.

The BIOHAZ Panel recommends that a similar study should be conducted with samples of Atypical BSE (BSE-L, BSE-H) and of sheep BSE, if material is made available to the CRL for TSE. Finally it is also recommended that, if feasible, samples of Atypical BSE, sheep BSE, Classical scrapie and Atypical scrapie should be included in the batch release testing procedure.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

According to Regulation (EC) No 999/2001 on the prevention, control and eradication of certain transmissible spongiform encephalopathies (TSEs) each Member State has to develop an annual TSE monitoring programme which includes a screening procedure using rapid tests. Rapid tests shall be approved for that purpose and listed in Annex X to Regulation (EC) No 999/2001.

The original rapid test evaluations were carried out between 1999 and 2005. They involved assessment of analytical and diagnostic sensitivity criteria, but different sample panels were utilised resulting in potential difficulties to directly compare all the tests.

In 2008 DG SANCO asked the Community Reference Laboratory for TSEs (CRL for TSE), Weybridge (UK) to assess analytical sensitivity for all the currently approved TSE rapid tests. The scope of this study was to produce robust analytical sensitivity data for the current EU-approved rapid *post mortem* tests designed to detect TSEs. The key design principle of this study was to evaluate each test against the same sample sets for the three main types of ruminant TSE: BSE, Classical scrapie and Atypical scrapie and to allow an inter-assay comparison of analytical sensitivity which has not been possible before. The final report of this study, which is enclosed, was issued by CRL for TSE in April 2009[4].

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Food Safety Authority is requested to provide a scientific evaluation of the above mentioned study and, if needed, based on the information available in the CRL report, reconsider and amend previous recommendations related to the approval of each of those rapid tests, based on the requirements as set in the current EFSA protocols for the evaluation of TSE rapid *post mortem* tests[5].

The deadline for delivering the opinion was agreed for 31 December 2009.

4 A revised, final version of the report was submitted to EFSA in December 2009.

5 Protocol for the evaluation of new rapid BSE *post mortem* tests (adopted by EFSA on 7 June 2007) and Protocol for the evaluation of rapid *post mortem* tests to detect TSE in small ruminants (adopted by EFSA on 7 June 2007).

ASSESSMENT

1. Introduction

Tests used in the EU for the surveillance of Transmissible Spongiform Encephalopathies (TSEs) in ruminants are subject to prior approval by the European Commission and need to go through a specific evaluation procedure. So far, three evaluations have been completed (in 1999, 2002 and 2004) and an additional one is currently ongoing (launched in 2007). After being successfully evaluated, the approved tests are listed in Annex X to Regulation (EC) No 999/2001 (EC, 1999b). The evaluation procedure is based on criteria established and periodically revised by the European Commission, its former Scientific Steering Committee (SSC) and, since its establishment, the European Food Safety Authority (EFSA).

The first two evaluations were intended to evaluate rapid *post mortem* tests for the detection of BSE in cattle. The first one, completed in 1999, was designed and performed by an expert group set up by the European Commission and the Institute for Reference Materials and Measurements (IRMM). A second one was completed in 2002. Compared to the protocols followed during the first evaluation, an additional field trial was designed by the Scientific Steering Committee (SSC) of the European Commission and managed by IRMM. A third evaluation was completed in 2004. This evaluation involved rapid tests for the detection of BSE in cattle and of TSE in small ruminants, including possible *ante mortem* rapid tests. EFSA took over the role of the SSC and updated the existing protocols used for the evaluation. Finally, a fourth evaluation was launched at the end of 2007 by the European Commission, based on protocols recently updated by EFSA. The call for expression of interest for this fourth evaluation, intended for both *ante mortem* and *post mortem* rapid tests, is currently ongoing and will remain open until 2012.

Some of the requirements foreseen by the evaluation protocols used during the four evaluation procedures will be described, where relevant to this opinion, in Section 3.

2. The CRL study

2.1. Origin and aims of the study

During the three past evaluation procedures for TSE rapid tests, the analytical sensitivity of the tests was investigated in accordance with the requirements established by the relevant evaluation protocols established by the European Commission, the SSC and EFSA, as mentioned above. However, different starting samples were used to assess the analytical sensitivity, which did not allow for a direct and definite comparison of the performance of the different tests in this regard. For that reason the European Commission requested the Community Reference Laboratory for TSEs (CRL for TSE) to assess the analytical sensitivity of all the currently approved TSE rapid tests. Therefore a study (CRL study) was designed and performed in a way that all the approved tests were evaluated for their analytical sensitivity (detection limit) against a common panel of samples. This allowed a direct comparison of the different tests and a ranking of the tests by their detection limit.

The aims of the CRL study are reported below, as indicated in the final report of the CRL study submitted to EFSA:

- *"To assess the lowest detection limit of rapid tests approved for the detection of TSE's in bovines using 3 pools (A, B and C) of bovine positive brain material.*

- *To compare CRL pre-prepared dilution series comprising 216 aliquots of 50% water homogenates of pools A, B and C, with the dilution series prepared by the manufacturers in their own laboratories.*

- *To compare CRL pre-prepared dilution series of 50% water homogenates of bovine negative brain material, with the dilution series prepared by the manufacturers in their own laboratories (pool D, negative pool).*

- *To assess the lowest detection limit of rapid tests approved for the detection of TSE's in small ruminants using 3 pools (X, Y and Z) of classical scrapie positive ovine brain material.*

- *To compare CRL pre-prepared dilution series of 50% water homogenates of pools X, Y and Z, with the dilution series prepared by the manufacturers in their own laboratories.*

- *To compare CRL pre-prepared dilution series of 50% water homogenates of ovine negative brain material with the dilution series prepared by the manufacturers in their own laboratories (pool W, negative pool).*

- *To perform a small stability study to establish whether dilution series prepared from homogenates of ovine brain material, which is positive for atypical scrapie, may be stored frozen at –80°C prior to issue to testing laboratories.*

- *To conduct an analytical sensitivity study for atypical scrapie using CRL pre-prepared dilution series of 50% water homogenates.*

- *To conduct a further analytical sensitivity study for atypical scrapie using CRL neat tissue samples."*

2.2. Structure, methodology and performance of the study

As described above, the main aim of the study was to produce contemporary robust analytical sensitivity data for the current EU-approved rapid *post-mortem* tests designed to detect one or all of BSE, Classical scrapie and Atypical scrapie.

The rapid tests evaluated in the CRL study are listed in Table 1.

Table 1: List of rapid tests evaluated by the CRL study.

Cattle BSE study	Sheep Classical scrapie and Atypical scrapie studies
AJ Roboscreen BetaPrion®	Bio-Rad TeSeE™ SAP
Bio-Rad TeSeE™ SAP	Bio-Rad TeSeE™ Sheep/Goat
Enfer TSE v2	Enfer TSE v2
Enfer TSE v3	Enfer TSE v3
IDEXX HerdChek Standard (bovine conjugate)	IDEXX HerdChek Standard (scrapie conjugate)
IDEXX HerdChek Short (bovine conjugate)	IDEXX HerdChek Short (scrapie conjugate)
IDEXX HerdChek Ultra Short (bovine conjugate)	IDEXX HerdChek Ultra Short (scrapie conjugate)
Roche Prionscreen	Prionics®-WB Check Western SR
Prionics®-Check PrioSTRIP	Prionics®-Check LIA SR
Prionics®-Check Western	
Prionics®-Check LIA	

In the sections below, information on the structure and methodology of the study is reported as extracted from the CRL study.

2.2.1. BSE analytical sensitivity study

"Analytical sensitivity was assessed for BSE tests using tissue samples originating from cattle infected with classical (C-type) BSE. […] *The CRL prepared 3 tissue pools (A, B and C) from BSE-positive CNS tissue, and one tissue pool (D) prepared from BSE negative CNS tissue. The pools were prepared by chopping tissue finely and then treating portions of tissue in a Seward Stomacher 80 Biomaster for 120 seconds for 3 successive treatments. Positive tissue was from confirmed BSE cases.* […] *Each pool was divided and one part used at the CRL to prepare dilution series using the CRL standard method. The second part of each pool was divided into aliquots. These aliquots were issued blind to manufacturers together with negative tissue to prepare their own dilution series in negative brain tissue to match the CRL samples. All negative tissue (bovine CNS) came from samples tested negative by Bio-Rad TeSeE™ ELISA was obtained from the laboratory of the Government Chemist (LGC)* […]. *All CRL dilution series of homogenates consisted of doubling dilutions from a positive sample pool mixed with an equal volume of nuclease-free water down to 1 part positive tissue in 4096 parts negative sample (50% negative tissue/50% nuclease-free water). Sufficient material was prepared for each pool to allow testing of 2 aliquots of the first 2 dilutions and 5 aliquots from the subsequent dilutions for each test. Samples were blind coded and put into a panel by CRL representatives. The panel for each test comprised 216 aliquots, 54 samples per pool.* […] *Each manufacturer undertook sample preparation and testing at their nominated testing laboratories under observation of CRL representatives."*

Following a request for clarification, the CRL for TSE confirmed that all test batches used within the study were industrially produced and intended for diagnostic use. It was also clarified by the CRL for TSE that BSE and Classical scrapie tissue pools made by the CRL were all prepared in the same manner and that the pools consisted of 100% tissue.

Three test manufacturers (AJ Roboscreen, IDEXX and Roche) opted to test only the CRL pre-prepared dilution series and not to prepare and test additional manufacturer prepared dilution series. The CRL study reports that these manufacturers chose this because they "*considered that the production method employed by the CRL for generating test samples had no negative impact on their test performance and/or due to constraints in manufacturer resources*".

It is noted that in the case of the evaluation of two tests (Prionics®-Check Western and Prionics®-Check LIA), during the visit of the company by the CRL the tests did not perform satisfactorily. None of the BSE negative samples gave a negative result when tested with Prionics®-Check LIA and some results did not match the expected results in the case of Prionics®-Check Western. Therefore the CRL agreed with the company to perform a second visit, during which the Prionics®-Check Western was re-run successfully. In the case of Prionics®-Check LIA, valid results were obtained when testing the manufacturers' prepared dilution series, while no meaningful data were obtained for the CRL pre-prepared dilution series.

2.2.2. Classical scrapie analytical sensitivity study

"The CRL prepared 3 positive tissue pools (X, Y and Z) from ovine classical scrapie-positive CNS tissue, and one tissue pool (W) prepared from classical scrapie-negative CNS tissue. The pools were prepared by chopping tissue finely and then treating portions of tissue in a Seward Stomacher 80 Biomaster for 120 seconds for 3 successive treatments. Positive tissue originated from confirmed classical scrapie cases. […] *All CRL dilution series of homogenates consisted of doubling dilutions from a positive sample pool mixed with an equal volume of nuclease-free water down to 1 part positive tissue in 4096 parts negative sample (50% negative tissue/nuclease free 50% water). Sufficient material was prepared for each pool to allow testing of 2 aliquots of the first 2 dilutions and 5 aliquots from subsequent dilutions for each test. Samples were blind coded by CRL representatives. The panel for each test comprised 216 aliquots, 54 samples per pool.* […] *Each*

manufacturer undertook sample preparation and testing at their nominated testing laboratories under observation of CRL representatives."

Following a request for clarification, the CRL for TSE confirmed that all test batches used within the study were industrially produced and intended for diagnostic use. It was also clarified by the CRL for TSE that BSE and Classical scrapie tissue pools made by the CRL were all prepared in the same manner and that the pools consisted in 100% tissue.

One test manufacturer (IDEXX) opted to test only the CRL pre-prepared dilution series and not to prepare and test additional manufacturer prepared dilution series. The CRL study reports that this manufacturer chose this because it "*considered that the production method employed by the CRL for generating test samples had no negative impact on their test performance and/or due to constraints in manufacturer resources*".

2.2.3. Atypical scrapie samples stability study

Analytical sensitivity of TSE rapid tests approved for the detection of TSEs in small ruminants was also assessed against Atypical scrapie samples. However, the report of the CRL study indicates that this was more difficult, principally because less is known about how Atypical scrapie tissue behaves when prepared as homogenates and stored at low temperatures and because the availability of material is limited. As a consequence, the Atypical scrapie study was not designed in the same way as for Classical scrapie and BSE.

"A stability study was undertaken to establish whether dilution series prepared from homogenates of ovine brain material positive for atypical scrapie could be stored frozen at -80° for several months[6] *prior to issue to testing laboratories without compromising the level of analyte within the sample. Several atypical cases had been selected to provide candidate tissue for this aspect of the study. The CRL selected the sample with the strongest signal using the Bio-Rad TeSeE™ Sheep/Goat test and showing widespread IHC staining in fixed sections from adjacent brain sections. Several small samples of this material were removed and frozen at –80°C to act as controls for testing at specific time points in the stability study. The remainder of the tissue was processed by chopping tissue finely and then macerating portions of tissue in a Seward Stomacher for 120 seconds for 3 successive treatments as a 1/2 tissue/nuclease free water homogenate. The portions were then mixed together and a subsequent dilution series made and aliquotted. The dilution series used for the study was as follows: 1/5, 1/10, 1/50, 1/200, 1/500, 1/750, 1/1000. This sequence differed from the dilution series proposed in the original protocol due to scarcity of suitable material. The material was divided into aliquots. One set of samples was tested immediately by the CRL using the Bio-Rad Western blot. The finely chopped tissue was used as a control (this was diluted at the time of testing 1/1 with nuclease free water). In order to be economical with tissue, once each dilution series had been made, the remaining samples were distributed, as detailed in both the Bio-Rad TeSeE™ SAP and Bio-Rad TeSeE™ Sheep/Goat test kit instructions, into the grinding tubes of each rapid test to be used. They were then stored at -80°C and tested after various periods of storage using the coarsely chopped tissue as a control, as described above."*

2.2.4. Atypical scrapie analytical sensitivity study

"The CRL prepared an analytical sensitivity dilution series from stomached atypical scrapie-positive CNS tissue of known provenance, from two atypical scrapie cases. A sample from the animal used in the stability study mentioned previously […] *was also used in the first atypical scrapie sensitivity*

6 It is noted that the study was run for four weeks instead of several months. However, the CRL for TSE has clarified that the study was performed for several months but that only results concerning the first four weeks are discussed in the report of the CRL study because of their relevance for the specific purposes of the study.

study. The second sample used in the first part of the sensitivity study originated from an animal that had been specifically challenged […]. *The coded dilution series were despatched blind to the manufacturers testing laboratories with recommendations to test within 1 day of receipt and report the results back to the CRL within one week. Negative tissue (ovine CNS) from samples tested negative by Bio-Rad TeSeE™ SAP were obtained from VLA Shrewsbury. All tissue samples used to produce tissue pools and CRL dilution series had originally been tested positive or negative with the approved Bio-Rad TeSeE™ test.* […] *The positive tissue was mixed 1/2 with nuclease free water. The dilution series consisted of doubling dilutions from a positive sample down to 1 part positive tissue in 1024 parts negative sample (produced as negative tissue/water homogenate). Sufficient material was prepared for each pool to allow testing of 2 aliquots for each dilution step for each test. The samples were coded at the CRL and issued as a blind panel. A further atypical scrapie study was conducted in February 2009 as two manufacturers (Enfer and Prionics) failed to detect the atypical samples in the analytical sensitivity part of the atypical scrapie study. Consequently the additional study was conducted using a larger panel of atypical scrapie samples from different animals. Twelve neat tissue samples prepared as a duplicate series of chopped material were blinded by the CRL and despatched to Prionics and Enfer for testing in February 2009. The samples were also be tested by Bio-Rad TeSeE™ and Bio-Rad Western Blot. The CRL received atypical scrapie results from the manufacturers on 17th February 2009. The resultant data sets were analysed by the CRL. All manufacturers agreed the protocols for this work with the CRL prior to commencing the study. All manufacturers were instructed to undertake testing according to their current version of Instructions for Use."*

Following a request for clarification, the CRL for TSE confirmed that all test batches used within the study were industrially produced and intended for diagnostic use.

2.2.5. Main differences of the design of the studies on the different TSE agents

While comparing the different analytical sensitivity studies performed by the CRL, it is noted that:

- The design of the analytical sensitivity studies performed for Classical BSE and Classical scrapie are similar, including the nature of the sample.
- The design of the analytical sensitivity study performed for Atypical scrapie is different and in particular:
    - A stability study was performed for homogenates of ovine brain material positive for Atypical scrapie.
    - The samples to be tested were only provided to the manufacturers as a CRL dilution panel and the manufacturers were not given the opportunity to make their own dilution panel for testing. Therefore a comparison of results obtained after testing samples prepared by CRL and the manufacturers was not possible.
    - The number of samples provided was limited compared to the study performed for Classical BSE and Classical scrapie.
    - Following the failure of two manufacturers to detect the positive samples provided, a second assessment of the tests was performed by providing a set of neat samples for testing.

3. Requirements in past and current protocols for the evaluation of TSE rapid tests

3.1. Analytical sensitivity of rapid tests for the detection of BSE in cattle

3.1.1. First evaluation (completed in 1999)

Information on the requirements of the evaluation protocol and on the testing performed is reported in the final report of the European Commission (EC, 1999a).

As part of the evaluation, test detection limits were assessed in order to obtain an indication of the capability of the test to detect the presence of pre-clinical BSE. The test detection limit was defined as the smallest detectable amount of the analyte. Because of the nature of the assay, this determination was relative. This parameter was assessed by supplying each candidate with specially prepared samples made up from central nervous tissue, containing positive tissue diluted in negative tissue. The positive tissue came from six clinically affected animals and the negative tissue came from twenty negative animals. In order to achieve acceptable viscosity, a 20% aqueous solution containing 5% sucrose was added to the central nervous positive tissue and this was homogenised with an Ultraturrax mixer. The same procedure was used in the preparation of the negative tissue. Various dilutions of the positive tissue, down to 10^{-5}, were used. The 10^{-1} and the $10^{-1.5}$ dilutions were prepared by gravimetrical mixing of the pooled negative and positive material. The lower dilutions were each prepared by 1 in 10 dilution of the corresponding higher concentrated homogenate. The positive tissue had been titrated in mice, yielding a titre of $10^{3.1}$ mouse i.c./i.p LD50/g of tissue. The number of samples examined by each test is set out in Table 2.

Table 2: Samples and dilutions series examined during the first evaluation (BSE cattle).

Dilution	Number of samples	Dilution	Number of samples
Undiluted	6	$10^{-3.0}$	20
$10^{-1.0}$	20	$10^{-3.5}$	20
$10^{-1.5}$	20	$10^{-4.0}$	20
$10^{-2.0}$	20	$10^{-4.5}$	20
$10^{-2.5}$	20	$10^{-5.0}$	20

Three of the rapid tests currently approved for detection of BSE in cattle were evaluated through the first evaluation procedure: Prionics®-Check Western, prior version of Enfer TSE v2, prior version of Bio-Rad TeSeE™ SAP.

3.1.2. Second evaluation (completed in 2002)

Information on the requirements of the evaluation protocol and on the testing performed is reported in the final report of the European Commission (EC, 2002).

The protocol used to assess the analytical sensitivity of the tests under evaluation was largely similar to the one used for the previous evaluation. The positive homogenate was part of the material prepared for the 1999 study described above. The material that was not used in the 1999 study was stored at -70 ºC. In 2001, it was used for the production of a new series of diluted homogenates. Various dilutions of the positive tissue, down to 10^{-3}, were used. The number of samples examined by each test is set out in Table 3.

Table 3: Samples and dilutions series examined during the second evaluation (BSE cattle).

Dilution	Number of samples	Dilution	Number of samples
Undiluted	1	$10^{-2.5}$	4
$10^{-1.0}$	4	$10^{-3.0}$	4
$10^{-1.5}$	4	Negative	4-5
$10^{-2.0}$	4		

In addition to testing a dilution series prepared by IRRM, the manufacturers were also asked to test a dilution series prepared by themselves and produced according to their protocol. This facilitated an assessment of the impact of homogenisation of brain tissue. The starting material was the same as that used in the dilution series prepared by IRRM. It did not contain any buffer.

One of the rapid tests currently approved for detection of BSE in cattle was evaluated through the second evaluation procedure: Prionics®-Check LIA.

3.1.3. Third evaluation (completed in 2004)

Information on the requirements of the evaluation protocol and on the testing performed is reported in the final report of the IRMM (IRMM, 2004a).

To evaluate the detection limit, a common pool of brainstem tissue of six confirmed BSE positive animals was produced and distributed to each participant in equal parts. In order to achieve acceptable viscosity, homogenates of 80% tissue and 20% water were provided. The material did not contain any buffers or sugars. The material was not titrated as was the positive pool for the former BSE test evaluations. The test developers were asked to prepare dilutions on site of 1:5, 1:50, 1:100 and 1:200 of the positive brain homogenate in fresh brain homogenate of non-infected cattle. The dilutions from 1:5 to 1:200 were mandatory, at least two replicates were analysed on three different microtiter plates. The objective of this exercise was predominantly to determine the test detection limits and to gain a perspective on the behaviour of the respective test in highly heterogeneous samples and in pre-clinical animals. The number of samples examined by each test is set out in Table 4. To better determine the real detection limits, some manufacturers were asked to prepare and test a second dilution series.

Table 4: Samples and dilutions series examined during the third evaluation (BSE cattle).

Dilution	Number of samples	Dilution	Number of samples
Undiluted	2	1:100	6
1:5	6	1:200	6
1:50	6	Negative	2

Five of the rapid tests currently approved for detection of BSE in cattle were evaluated through the third evaluation procedure: Enfer TSE v2 automated sample preparation, IDEXX HerdChek (bovine conjugate), Prionics®-Check PrioSTRIP, AJ Roboscreen BetaPrion®, Roche PrionScreen. Following to the approval of changes made to the approved Enfer TSE v2, a new test was listed in Regulation (EC) No 999/2001 and is currently approved: Enfer TSE v3.

3.1.4. Current fourth evaluation (launched in 2007)

Information on the requirements of the evaluation protocol is reported in the EFSA protocol for the evaluation of new rapid BSE *post mortem* tests (EFSA, 2007a).

In the framework of the pre-evaluation stage of the evaluation, the manufacturer will be required to test a typical panel of 20 proficiency test samples as issued by the CRL for TSE and this set will also include a panel of dilution series. All samples will be prepared as macerates. The relative detection limit for each test will be analyzed using serial dilutions of macerate. The dilution series will be made from macerates of Classical BSE positive brain stem at clinical stage diluted with macerates of negative brain material. Tests should be able to detect at least 5% positive tissue in negative tissue (dilution series prepared by the manufacturer from macerates already validated with the highest sensitive test during previous evaluations). Equivalent samples will have been subjected to prior testing with an ELISA test having shown high analytical sensitivity performance on previous evaluations. For all positive samples, a confirmatory WB aiming at profile identification will have been carried out (using 0.5 grams tissue using anti-PrP antibody with at least equivalent sensitivity as with Sha31 mAb as anti PrP antibody). Closure of the pre-evaluation and entry into the full evaluation requires, among others, 100% performance on the proficiency test panel[7] and the limit of detection of the test to be better than, similar to or no more than 2 logs poorer than the most sensitive test.

In the framework of the subsequent laboratory evaluation stage of the evaluation, each manufacturer will test in their own or in a chosen laboratory a panel of samples for evaluation. This testing will be supervised by an EFSA/IRMM approved person. Among others, the samples will include an analytical sensitivity series (prepared from macerates and further processed according to the manufacturer protocol). The protocol foresees that analytical sensitivity must not be lower than a difference of two $\log_{10}$ from the highest sensitivity assay of existing approved tests.

3.2. Analytical sensitivity of rapid tests for the detection of TSE in small ruminants

No evaluation of rapid tests for the detection of TSE in small ruminants took place during the first and second evaluation.

3.2.1. Third evaluation (completed in 2004)

Information on the requirements of the evaluation protocol and on the testing performed is reported in the final report of the IRMM (IRMM, 2004b) and in its Addendum (IRMM, 2005).

To assess the test detection limits of each test under evaluation, each participant was supplied with tissue from scrapie positive animals. This was supplied in the form of a homogenate of 50% tissue and 50% water produced at IRMM. Homogenates of brainstem, lymph nodes and spleen were prepared. Since it was unknown if the assays would show different performances with material from different geographical regions, two pools of positives homogenates were prepared with tissues from Cyprus and the United Kingdom, respectively. These were usually analysed independently. Each homogenate contained a mixture of tissues from at least six different animals. Various dilutions of the positive homogenate were prepared by the participant following the test specific protocol. The negative diluent was produced freshly by the test developer with tissue slices from uninfected animals. None of the homogenates were titrated, but all tissues derived from scrapie affected animals with clear clinical symptoms. The participants were requested to analyse from two to six replicates of each dilution (various dilutions from 1:5 to 1:16,000 depending on the test and tissue). Aliquots of each dilution were coded by Commission staff present on site.

7 This criterion should be more precisely defined in the EFSA protocol, especially if a dilution series is included in the samples.

In addition to Classical scrapie samples, cerebral tissue samples from three cases of Atypical scrapie in sheep were included in the dilution testing panel. The participants were requested to analyse from two to six replicates of each dilution (various dilutions from 1:5 to 1:16,000 depending on the test). Finally, all the tests were re-evaluated against dilutions (1:5, 1:10, 1:25, 1:50, 1:100) of experimental BSE in sheep brain homogenates to provide information on the analytical sensitivity, similarly to what was done for scrapie.

Six of the rapid tests currently approved for detection of TSE in small ruminants were evaluated through the third evaluation procedure: Bio-Rad TeSeE™ SAP, Bio-Rad TeSeE™ Sheep/Goat, Enfer TSE v2, IDEXX HerdChek (scrapie conjugate), Prionics®-WB Check Western SR, Prionics®-Check LIA SR.

Following to the approval of changes made to the approved Enfer TSE v2, a new test was listed in Regulation (EC) No 999/2001 and is currently approved: Enfer TSE v3.

3.2.2. Current fourth evaluation (launched in 2007)

Information on the requirements of the evaluation protocol is reported in the EFSA protocol for the evaluation of rapid *post mortem* tests to detect TSE in small ruminants (EFSA, 2007b).

In the framework of the pre-evaluation stage of the evaluation, the manufacturer will be required to test a typical panel of 20 proficiency test samples as issued by the CRL for TSE and this set will also include a panel of dilution series to determine the analytical sensitivity of the assay. The dilution series will be made from macerates of Classical scrapie positive brain stem at clinical stage diluted with macerates of negative brain material. Tests should be able to detect at least 5% positive tissue in negative tissue. Equivalent samples will have been subjected to prior testing with a test having shown high analytical sensitivity performance during previous evaluations. For all positive samples, a confirmatory WB aiming at profile identification will have been carried out (using 0.5 grams tissue using anti-PrP antibody with at least equivalent sensitivity as with Sha31 mAb as anti PrP antibody). Closure of the pre-evaluation and entry into the full evaluation requires, among others, 100% accuracy on testing of the proficiency test panel[8] and the limit of detection (i.e. detection limit as determined by bioassay)[9] of the test to be better than, similar to or no more than two $\log_{10}$ poorer than the most sensitive test identified during previous evaluations.

In the framework of the subsequent laboratory evaluation stage of the evaluation, among others, the detection limit of each diagnostic test (analytical sensitivity/bioassay which is considered as a gold standard) will be evaluated. The detection limit of each test will be determined for BSE in sheep, Classical scrapie isolates and Atypical scrapie, as detailed in the EFSA protocol. All samples will be collected and prepared as macerates by IRMM and the relative detection limit will be analysed using serial dilutions of these macerates. The EFSA protocol further details bioassay titration procedures, which will allow a comparison of test performance with reference to biological gold standard. Use of the bioassay data will be at discretion of the experts evaluating test results. A new test will be approved if the limit of detection (i.e. detection limit as determined by bioassay) against all the classes of material used is better than, similar to or no more than two $\log_{10}$ poorer than the most sensitive (using bioassay as an external reference).

8 This criterion should be more precisely defined in the EFSA protocol, especially if a dilution series is included in the samples.

9 The requirement for a bioassay titration in the framework of the pre-evaluation stage of the evaluation seems, although scientifically justified, not to be realistic. The EFSA protocol should be amended accordingly and a specific acceptance criterion should be indicated on how to compare results on the analytical sensitivity obtained with rapid tests rather than with bioassay. In the frame of the present opinion, the results obtained in the biochemical tests are used as a proxy for the bioassay.

3.3. Other current requirements for rapid tests for the detection of BSE in cattle

A number of requirements other than on analytical sensitivity of tests are foreseen by the current evaluation protocol (EFSA, 2007a) during the different steps of the evaluation procedure (assessment of the dossiers, pre-evaluation, laboratory evaluation, field trial). The main aspects involve diagnostic sensitivity and diagnostic specificity.

With regard to diagnostic sensitivity, during the laboratory evaluation no false negatives in 50 confirmed positive samples should be detected and during the field trial no more than one false negative in 200 confirmed positive samples should be detected by the tests.

With regard to diagnostic specificity, during the laboratory evaluation no more than one false positive in 200 negative samples should be detected and during the field trial no more than 5 false positives in 10,000 negative samples should be detected by the tests.

However, these aspects were out of the scope of the CRL study and will not be discussed further within this opinion.

3.4. Other current requirements for rapid tests for the detection of TSE in small ruminants

A number of requirements other than on analytical sensitivity of tests are foreseen by the current evaluation protocol (EFSA, 2007b) during the different steps of the evaluation procedure (assessment of the dossiers, pre-evaluation, laboratory evaluation, alternative approach to the field trial). The main aspects involve diagnostic sensitivity and diagnostic specificity.

With regard to diagnostic sensitivity, during the laboratory evaluation a total of 450 positive Classical scrapie samples (200 slices, 200 macerates and 50 autolysed samples), a number of sheep BSE samples, preclinical scrapie cases and Atypical scrapie cases (minimum 10) will be tested. No false negatives in the clinical BSE and Classical scrapie brainstem slices samples tested should be detected[10], as well as in the samples from Atypical scrapie cases.

With regard to diagnostic specificity, during the laboratory evaluation a total of 1,250 negative samples (1,000 slices, 200 macerates and 50 autolysed samples) will be tested. No more than 4 false positives in the brainstem slice samples tested should be detected[10].

With regard to the alternative approach to the field trial, initial approval after the full laboratory evaluation of the tests will be subject to completion of a satisfactory evaluation of raw data from a minimum of two testing laboratories in which the test has been introduced, totalling 10,000 negative samples. Following evaluation of the data, by the CRL for TSE, provided that there is no evidence of problems with respect to performance, the approval process will be validated by the EFSA TSE testing expert group.

Tests that can detect positives in pre-clinical cases should be preferred for approval.

However, these aspects were out of the scope of the CRL study and will not be discussed further within this opinion.

10 Additional specific rules apply to lymph node tissue.

4. Scientific evaluation of the CRL study

The CRL study (designed and carried out from November 2007 to April 2009) provides a useful evaluation of the current *post mortem* tests used in cattle and sheep approved for detection of one or all of BSE, Classical scrapie and Atypical scrapie.

For the first time all of the tests were evaluated against the same sample set (including cattle BSE, sheep Classical scrapie and sheep Atypical scrapie), allowing a direct comparison of the analytical sensitivity of the rapid tests to be made. The study findings provide valuable information in determining the continued suitability of the tests currently used for TSE monitoring in the EU. There is some lack of consistency between the CRL study and the EFSA protocols for the evaluation of new TSE rapid tests (EFSA, 2007a, 2007b) in some areas. In particular, the dilution series pre-prepared by CRL (2 step dilutions including 1:16 and 1:32) did not allow to verify exactly the requirements of the EFSA protocols in terms of abnormal PrP analytical sensitivity ("*Tests should be able to detect at least 5% positive tissue in negative tissue*"). There are also some imprecisions in the EFSA protocols themselves, which require revision. However, the EFSA Panel on Biological Hazards (BIOHAZ Panel) considers that the methodologies used in the CRL study are scientifically sound and provide a solid basis for comparing the analytical sensitivity of the *post mortem* rapid TSE tests currently approved.

Assessment of specificity was not within the scope of the CRL study. However, false positive results were obtained for a negative sample by some of the assays. The frequency of false positive results obtained with testing a negative sample in replicates was quite different between those assays. Such results make the assessment of the limit of detection (analytical sensitivity) by replicate testing of serial dilutions for some of the assays difficult or even impossible. These results may also allow some conclusion about the diagnostic specificity of these assays. Nevertheless, real specificity figures of the assays would need to be addressed on the basis of testing high numbers of different negative samples under field conditions.

4.1. BSE analytical sensitivity study

4.1.1. Samples

Three positive central nervous system (CNS) tissue pools (pool A, B, C) were prepared from confirmed Classical (C-type) BSE cases. The tissue was finely chopped and homogenized in a Stomacher. The same procedure was applied to a negative tissue pool (pool D) prepared from negative CNS tested by Bio-Rad TeSeE^{TM} ELISA.

Each pool was divided and one part used at the CRL to prepare dilution series from 1:2 to 1:4096. Two aliquots were sent to the manufacturers of each dilution step 1:2 and 1:4, while 5 aliquots of the further dilutions were distributed. The second part of each pool was divided into aliquots then sent to the manufacturers together with negative tissue to prepare their own dilution series. Concerning the negative tissue pool, 54 aliquots were tested in all test sessions. This adds up to 216 sample aliquots sent to each manufacturer for testing.

4.1.2. Performance on manufacturer prepared dilutions

Table 5 summarises the detection limits obtained for the different rapid tests on the manufacturer prepared three positive pools and negative samples and reports the conclusions of the EFSA BIOHAZ Panel on the overall detection limit of the rapid tests.

Table 5: Detection limits of the rapid tests for detection of BSE in cattle (manufacturer prepared dilutions).

Test	Detection limit pool A	Detection limit pool B	Detection limit pool C	Number of false positives/ number of negative samples tested	Conclusion of the EFSA BIOHAZ Panel on the detection limit
AJ Roboscreen BetaPrion®	n.d.	n.d.	n.d.	n.d.	--
Bio-Rad TeSeE™ SAP	1:256 3/5	1:512 1/5	1:128 5/5	0/54	1:512
Enfer TSE v2	1:128 5/5	1:256 1/5	1:128 4/5	0/54	1:256
Enfer TSE v3	1:128 1/5	1:128 4/5	1:64 5/5	0/54	1:128
IDEXX HerdChek Standard	n.d.	n.d.	n.d.	n.d.	--
IDEXX HerdChek Short	n.d.	n.d.	n.d.	n.d.	--
IDEXX HerdChek Ultra Short	n.d.	n.d.	n.d.	n.d.	--
Roche Prionscreen	n.d.	n.d.	n.d.	n.d.	--
Prionics®-Check PrioSTRIP	1:128 5/5	1:256 3/5	1:128 5/5	1/54	1:256
Prionics®-Check Western	1:512 4/5	1:512 4/5	1:512 3/5	0/54	1:512
Prionics®-Check LIA (Visit 1)	1:4096 5/5	1:4096 5/5	1:4096 5/5	54/54	c.b.i.
Prionics®-Check LIA (Visit 2)	1:256 1/5	1:256 1/5	1:256 2/5	1/54	c.b.i.

n.d.: not done, since the manufacturers opted for testing only the CRL pre-prepared dilution series
c.b.i.: cannot be interpreted because of the presence of false positive results

Analysis of results obtained with manufacturer prepared dilutions

The detection limit varied for the different tests. Bio-Rad TeSeE™ SAP and Prionics®-CheckWestern displayed the highest analytical sensitivity (1:512). Enfer TSE v3 displayed a lower analytical sensitivity (1:128).

Testing of the 54 reference negative samples using Prionics®-Check PrioSTRIP resulted in one false positive result.

During the first visit of the CRL all the positive and negative samples tested with Prionics®-Check LIA gave positive results. As mentioned in Section 2.2.1 of the opinion, a second visit was organised by the CRL and in this case, while meaningful results were obtained when testing positive samples, it is noted that testing of the 54 reference negative samples resulted in one false positive result.

These results hamper the interpretation of the analytical sensitivity of Prionics®-Check LIA and its comparison with other approved tests.

Performances on manufacturer prepared dilutions haven't been determined on five tests (AJ Roboscreen BetaPrion®, IDEXX HerdChek Standard, IDEXX HerdChek Short, IDEXX HerdChek Ultra Short and Roche Prionscreen).

4.1.3. Performance on CRL pre-prepared dilutions

Table 6 summarises the detection limits obtained for the different rapid tests on the CRL pre-prepared three positive pools and negative samples and reports the conclusions of the EFSA BIOHAZ Panel on the overall detection limit of the rapid tests.

Table 6: Detection limits of the rapid tests for detection of BSE in cattle (CRL pre-prepared dilutions).

Test	Detection limit pool A	Detection limit pool B	Detection limit pool C	Number of false positives/ number of negative samples tested	Conclusion of the EFSA BIOHAZ Panel on the detection limit
AJ Roboscreen BetaPrion®	1:256 5/5	1:256 5/5	1:256 4/5	0/54	1:256
Bio-Rad TeSeE™ SAP	1:32 4/5	1:64 3/5	1:32 5/5	0/54	1:64
Enfer TSE v2	1:64 3/5	1:128 3/5	1:128 1/5	0/54	1:128
Enfer TSE v3	1:64 4/5	1:64 4/5	1:64 4/5	0/54	1:64
IDEXX HerdChek Standard	1:1024 1/5	1:1024 3/5	1:1024 2/5	0/54	1:1024
IDEXX HerdChek Short	1:1024 2/5	1:1024 2/5	1:1024 2/5	0/54	1:1024
IDEXX HerdChek Ultra Short	1:1024 1/5	1:1024 2/5	1:512 5/5	0/54	1:1024
Roche Prionscreen	1:128 5/5	1:128 5/5	1:128 5/5	0/54	1:128
Prionics®-Check PrioSTRIP	1:512 2/5	1:2048* 1/5	1:1024 1/5	7/54	c.b.i.
Prionics®-CheckWestern	1:256 3/4	1:512 1/4	1:256 2/3	0/54	1:512
Prionics®Check LIA (Visit 1)	1:4096 5/5	1:4096 5/5	1:4096 5/5	54/54	c.b.i.
Prionics®-Check LIA (Visit 2)	1:4096 5/5	1:4096 5/5	1:4096 5/5	54/54	c.b.i.

*: all the five 1:1024 dilutions tested negative
c.b.i.: cannot be interpreted because of the presence of false positive results

Analysis of results obtained with CRL pre-prepared dilutions

The detection limit varied for the different tests. The three IDEXX HerdChek protocols displayed the highest analytical sensitivity (1:1024), followed by the Prionics®-Check Western (1:512) and the AJ Roboscreen BetaPrion® (1:256). Enfer TSE v3 and Bio-Rad TeSeE™ SAP displayed a lower analytical sensitivity (1:64).

Testing of the 54 reference negative samples using the Prionics®-Check PrioSTRIP test resulted in 7 false positive results.

During the first visit of the CRL all the positive and negative samples tested with Prionics®-Check LIA gave positive results. As mentioned in Section 2.2.1 of the opinion, a second visit was organised by the CRL and again all the positive and negative samples tested with Prionics®-Check LIA gave positive results.

These results hamper the interpretation of the analytical sensitivity of Prionics®-Check LIA and Prionics®-Check PrioSTRIP and their comparison with other approved tests.

4.1.4. Overall analysis of results

AJ Roboscreen BetaPrion®, Bio-Rad TeSeE™ SAP, Enfer TSE v2, Enfer TSE v3, IDEXX HerdChek Standard, IDEXX HerdChek Short, IDEXX HerdChek Ultra Short, Roche Prionscreen and Prionics®-Check Western performed within a maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system.

Prionics®-Check LIA and Prionics®-Check PrioSTRIP gave unexplained and unresolved specificity problems which hamper the interpretation of their analytical sensitivity and the comparison with other approved tests.

Excluding Prionics®-Check LIA and Prionics®-Check PrioSTRIP, for all other tests no potential differences in field detection performance can be inferred on the sole basis of the difference in analytical sensitivity reported in this study.

4.2. Classical scrapie analytical sensitivity study

4.2.1. Samples

Three positive CNS tissue pools (pool X, Y, Z) were prepared from confirmed Classical scrapie cases. The tissue was finely chopped and homogenized in a Stomacher. The same procedure was applied to a negative sample tissue pool (pool W) prepared from negative CNS.

Using these macerates (positive pools X, Y, Z and a negative macerate), dilutions were prepared from 1:2 to 1:4096. Two aliquots were sent to the manufacturers of each dilution step 1:2 and 1:4, while 5 aliquots of the further dilutions were distributed. In addition, 54 aliquots of a negative pool were tested in all test sessions. This adds up to 216 sample aliquots sent to each manufacturer for testing.

4.2.2. Performance on manufacturer prepared dilutions

Table 7 summarises the detection limits obtained for the different rapid tests on the manufacturer prepared three positive pools and negative samples and reports the conclusions of the EFSA BIOHAZ Panel on the overall detection limit of the rapid tests.

Table 7: Detection limits of the rapid tests for detection of Classical scrapie in small ruminants (manufacturer prepared dilutions).

Test	Detection limit pool X	Detection limit pool Y	Detection limit pool Z	Number of false positives/ number of negative samples tested	Conclusion of the EFSA BIOHAZ Panel on the detection limit
Bio-Rad TeSeE™ SAP	1:64 5/5	1:128 4/5	1:512 5/5	0/54	1:512
Bio-Rad TeSeE™ Sheep/Goat	1:512 2/5	1:512 5/5	1:2048 5/5	0/54	1:2048
Enfer TSE v2	1:512 1/5	1:256 4/5	1:1024 5/5	0/54	1:1024
Enfer TSE v3	1:256 1/5	1:256 1/5	1:512 5/5	1/54	1:512
IDEXX HerdChek Standard	n.d.	n.d.	n.d.	n.d.	--
IDEXX HerdChek Short	n.d.	n.d.	n.d.	n.d.	--
IDEXX HerdChek Ultra Short	n.d.	n.d.	n.d.	n.d.	--
Prionics®-Check LIA SR	1:8 4/5	1:32 2/5	1:64 5/5	0/54	1:64
Prionics®-WB Check Western SR	1:64 2/5	1:256 1/5	1:256 1/5	0/54	1:256

n.d.: not done, since the manufacturer opted for testing only the CRL pre-prepared dilution series
c.b.i.: cannot be interpreted because of the presence of false positive results

Analysis of results obtained with manufacturer prepared dilutions

The detection limit varied for the different tests. Bio-Rad TeSeE™ Sheep/Goat displayed the highest analytical sensitivity (1:2048), followed by Enfer TSE v2 (1:1024). Prionics®-Check LIA SR displayed a lower analytical sensitivity (1:64).

Testing of the 54 reference negative samples using Enfer TSE v3 resulted in one false positive result.

4.2.3. Performance on CRL pre-prepared dilutions

Table 8 summarises the detection limits obtained for the different rapid tests on the CRL pre-prepared three positive pools and negative samples and reports the conclusions of the EFSA BIOHAZ Panel on the overall detection limit of the rapid tests.

Table 8: Detection limits of the rapid tests for detection of Classical scrapie in small ruminants (CRL pre-prepared dilutions).

Test	Detection limit pool X	Detection limit pool Y	Detection limit pool Z	Number of false positives/ number of negative samples tested	Conclusions of the EFSA BIOHAZ Panel on the detection limit
Bio-Rad TeSeE™ SAP	1:64 5/5	1:128 1/5	1:256 5/5	0/54	1:256
Bio-Rad TeSeE™ Sheep/Goat	1:512 4/5	1:512 4/5	1:2048 1/5	0/54	1:2048
Enfer TSE v2	1:128 5/5	1:128 5/5	1:512 2/5	0/54	1:512
Enfer TSE v3	1:128 3/5	1:128 5/5	1:256 5/5	0/54	1:256
IDEXX HerdChek Standard	1:512 2/5	1:512 5/5	1:2048 2/5	0/54	1:2048
IDEXX HerdChek Short	1:512 1/5	1:512 5/5	1:1024 5/5	0/54	1:1024
IDEXX HerdChek Ultra Short	1:256 5/5	1:512 4/5	1:1024 5/5	0/54	1:1024
Prionics®-Check LIA SR	1:8 3/5	1:8 5/5	1:32 3/5	1/54	1:32
Prionics®-WB Check Western SR	1:32 3/5	1:64 1/5	1:128 2/5	0/54	1:128

c.b.i.: cannot be interpreted because of the presence of false positive results

Analysis of results obtained with CRL pre-prepared dilutions

The detection limit varied for the different tests. Bio-Rad TeSeE™ Sheep/Goat and IDEXX HerdChek Standard displayed the highest analytical sensitivity (1:2048), followed by IDEXX HerdChek Short and IDEXX HerdChek Ultra Short (1:1024). The Prionics®-Check LIA SR test displayed a lower analytical sensitivity (1:32).

Testing of the 54 reference negative samples using the Prionics®-Check LIA SR test resulted in one false positive result.

4.2.4. Overall analysis of results

Pool Z seems to have a higher PrP^{Sc} amount than pools X and Y, as all test systems displayed the highest analytical sensitivity with this pool, with a difference of two dilution steps identified for almost all test systems.

All tests (Bio-Rad TeSeE™ SAP, Bio-Rad TeSeE™ Sheep/Goat, Enfer TSE v2, Enfer TSE v3, IDEXX HerdChek Standard, IDEXX HerdChek Short, IDEXX HerdChek Ultra Short, Prionics®-Check LIA SR and Prionics®-WB Check Western SR) performed within a maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system.

Marginal specificity problems were observed with Prionics®-Check LIA SR and Enfer TSE v3 with sheep samples, which did not compromise the estimation of their analytical sensitivity.

No potential differences in field detection performance can be inferred on the sole basis of the difference in analytical sensitivity reported in this study.

4.3. Atypical scrapie samples stability study

One of the considerations of a former EFSA opinion (EFSA, 2007b) was that in case of Atypical scrapie cases, special attention should be paid at the long term stability of PrP^{Sc} when preparing the samples. Indeed, on the basis of former experiences in the frame of evaluation of TSE tests since 2001, it appears that one of the parameters that could impact assay performance to different degrees is the long term stability of PrP^{Sc} in macerates or frozen Atypical scrapie samples. For other types of TSEs similar problems were reported but to an extent which is not likely to impact dramatically on field detection sensitivity (Everest et al., 2006; Gretzschel et al., 2006; Klingeborn et al., 2006; Roels et al., 2002). One of the aims of the CRL study was "*to perform a small stability study to establish whether dilution series prepared from homogenates of ovine brain material, which is positive for atypical scrapie, may be stored frozen at -80°C prior to issue to testing laboratories*", as early results suggested degradation of Atypical scrapie homogenates over time.

Atypical scrapie isolates originated from one field case of Atypical scrapie (ARQ/AHQ 6 year old sheep). The animal was detected positive following to active surveillance (fallen stock), therefore was not optimally collected, but stored at -80°C following initial transportation on card ice.

In the study it was reported that an apparent decrease in the detected signal could be observed with the Bio-Rad Western Blot (longer development time needed) for the 1/50 aliquot of Atypical scrapie homogenates at 4 weeks. For the Bio-Rad TeSeE™ Sheep/Goat a decrease in OD values was observed for the undiluted samples at 2 weeks, reaching a 50% decrease at 4 weeks. A similar approximately 50% decrease was reported for the 1/5 and 1/10 dilution per 2 weeks, reaching the cut-off OD value for the latter at 4 weeks. These findings were taken into account for the determination of the time period for the preparation and the distribution of the samples.

However, some observations can be made on this study:

- No conclusions can be drawn on the stability of Classical scrapie and BSE homogenates.
- The decrease in OD values using the Bio-Rad TeSeE™ Sheep/Goat was more important compared to the decrease of signal with the Bio-Rad Western Blot, so that the signal for the 1/50 aliquot at 4 weeks was still readily detectable with the Bio-Rad Western Blot, but not with the Bio-Rad TeSeE™ Sheep/Goat.

4.4. Atypical scrapie analytical sensitivity study

4.4.1. Samples

A first dilution series from stomached Atypical scrapie-positive CNS tissue (ovine cerebrum) originating from two Atypical scrapie cases was prepared by the CRL, together with two samples from negative ovine CNS tissue. Dilutions were prepared from 1:2 to 1:1024. Two aliquots were sent to the manufacturers of each dilution step.

A further study was conducted later on as tests of two manufacturers (Enfer and Prionics) failed to detect the above Atypical scrapie samples. Twelve neat tissue samples were prepared and dispatched in duplicate to the manufacturers.

4.4.2. Performance on the first CRL set of samples

Table 9 summarises the detection limits obtained for the different rapid tests on the CRL pre-prepared dilution samples and the conclusions of the EFSA BIOHAZ Panel on the detection limit of the rapid tests. Samples were prepared on 10 November 2008 and dispatched to the manufacturers. Results were received by the CRL from the manufacturers on 17 November 2008. The CRL also tested the same samples by Bio-Rad Western Blot on 21 November 2008. Details of the results of this last confirmatory testing can be found in Appendix A.

Table 9: Detection limits of the rapid tests for detection of Atypical scrapie in small ruminants (first CRL set of samples).

Test	Detection limit observed	Number of false positives/number of negative samples tested		Conclusion of the EFSA BIOHAZ Panel on the detection limit
Bio-Rad TeSeE™ SAP	1:128 2/2	0/2	0/2	1:128
Bio-Rad TeSeE™ Sheep/Goat	1:128 1/2	0/2	0/2	1:128
Enfer TSE v2	no dilutions were detected as positive	0/2	0/2	unable to detect a positive signal in any of the positive samples
Enfer TSE v3	no dilutions were detected as positive	0/2	0/2	unable to detect a positive signal in any of the positive samples
IDEXX HerdChek Standard	1:16 2/2*	0/2	0/2	1:16
IDEXX HerdChek Short	1:64 1/2	0/2	0/2	1:64
IDEXX HerdChek Ultra Short	1:16 2/2	0/2	0/2	1:16
Prionics®-Check LIA SR	no dilutions were detected as positive	0/2	0/2	unable to detect a positive signal in any of the positive samples
Prionics®-WB Check Western SR	1:2 1/2**	2/2	1/2	c.b.i.

*: one 1:2 sample scored a high negative OD value
**: one 1:8 sample scored positive
c.b.i.: cannot be interpreted because of the presence of false positive results

Analysis of results

Major discrepancies were observed in the detection limit of the different tests.

Bio-Rad TeSeE™ SAP, Bio-Rad TeSeE™ Sheep/Goat, IDEXX HerdChek Standard, IDEXX HerdChek Short and IDEXX HerdChek Ultra Short gave consistent results with positive detection limits varying between 1:16 and 1:128.

Enfer TSE v2, Enfer TSE v3 and Prionics®-Check LIA SR gave negative results for all the dilutions of the positive samples tested (from 1:2 to 1:1024).

Prionics®-WB Check Western SR detected one out of the two replicates at 1/2 and 1/8 dilution but failed to detect any positive at 1/4, 1/32 and further dilution steps. Moreover this test gave a false

positive result with one of the two negative control replicates and two false positives with the other two negative control replicates.

4.4.3. Performance on the second CRL set of samples

Considering the problem met by Enfer TSE v2, Enfer TSE v3, Prionics®-Check LIA SR and Prionics®-WB Check Western SR, the CRL decided to have a panel of confirmed Atypical scrapie cases tested neat by the manufacturers. The samples aliquots were dispatched on 10 February 2009 and all tests were performed within one week following the shipment. Table 10 summarises the results obtained for the different rapid tests on the second series of CRL samples. At the same moment the CRL tested (12 February 2009) one aliquot of each sample by confirmatory WB to ensure that Atypical scrapie associated PrP^{Sc} was detectable in samples. Details of the results of these confirmatory samples can be found in Appendix B.

Analysis of results

Enfer TSE v2, Enfer TSE v3 and Prionics®-Check LIA SR failed to detect any of the positive samples tested. Prionics®-WB Check Western SR allowed detection of both replicates corresponding to two positive cases and one of the replicates from a third one.

4.4.4. Overall analysis of results

Bio-Rad TeSeE™ SAP, Bio-Rad TeSeE™ Sheep/Goat, IDEXX HerdChek Standard, IDEXX HerdChek Short and IDEXX HerdChek Ultra Short performed within the maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system.

Enfer TSE v2, Enfer TSE v3, Prionics®-Check LIA SR and Prionics®-WB Check Western SR could fail in identifying field Atypical scrapie cases that other validated tests would detect.

Table 10: Detection limits of the rapid tests for detection of Atypical scrapie in small ruminants (second CRL set of samples).

CRL sample reference	Sample	Brain area	Dilution	Test results Enfer v2 (received 17 Feb 2009)	Test results Enfer v3 (received 17 Feb 2009)	Test results Prionics®-Check LIA SR (received 17 Feb 2009)	Test results Prionics®-WB Check Western SR (received 17 Feb 2009)	Confirmatory CRL test Bio-Rad WB (12 Feb 2009)
OH0140	ovine Atypical scrapie positive	cerebellum	neat	negative 0/2	negative 0/2	negative 0/2	positive 2/2	positive (Atypical)
OH0139	ovine Atypical scrapie positive	cerebellum	neat	negative 0/2	negative 0/2	negative 0/2	positive 1/2	positive (Atypical)
OH0138	ovine Atypical scrapie positive	rostral medulla	neat	negative 0/2	negative 0/2	negative 0/2	negative 0/2	positive (Atypical)
OH0137	ovine Atypical scrapie positive	cerebellum	neat	negative 0/2	negative 0/2	negative 0/2	negative 0/2	negative
OH0136	ovine Atypical scrapie positive	cerebellum	neat	negative 0/2	negative 0/2	negative 0/2	positive 2/2	positive (Atypical)
OH0135	ovine Atypical scrapie positive	cerebellum	neat	negative 0/2	negative 0/2	negative 0/2	negative 0/2	positive (Atypical)
OH0134	ovine Atypical scrapie positive	rostral medulla	neat	negative 0/2	negative 0/2	negative 0/2	negative 0/2	inconclusive
OH0133	ovine Classical scrapie positive	cerebellum and rostral medulla	neat	positive 2/2	positive 2/2	positive 2/2*	positive 2/2*	positive
OH0132	ovine Classical scrapie positive	cerebellum and rostral medulla	neat	positive 2/2	positive 2/2	positive 2/2	positive 2/2	positive
OH0129	ovine negative	cerebellum and rostral medulla	neat	negative 0/2	negative 0/2	negative 0/2	negative 0/2	negative
OH0130	ovine negative	cerebellum and rostral medulla	neat	negative 0/2	negative 0/2	positive 1/2*	positive 1/2*	negative
OH0131	ovine negative	cerebellum and rostral medulla	neat	negative 0/2	negative 0/2	negative 0/2	negative 0/2	negative

*: the two marked samples were accidentally mixed at the time of test preparation, leading to potential contamination of tissue for analysis

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

- For the first time, all the tests were evaluated against the same sample set (including cattle BSE, sheep Classical scrapie and sheep Atypical scrapie), allowing a direct comparison of the analytical sensitivity of the rapid tests to be made. The study findings provide valuable information in determining the continued suitability of tests currently used for TSE monitoring in the EU.

- There is some lack of consistency between the CRL study and the EFSA protocols for the evaluation of new TSE rapid tests; however, the Panel on Biological Hazards considers that the methodologies used in the CRL study are scientifically sound and provide a solid basis for comparing the analytical sensitivity of the *post mortem* rapid TSE tests currently approved.

- In the framework of the CRL study, a stability study was performed for Atypical scrapie, but not for Classical scrapie and BSE homogenates.

With regard to cattle BSE:

- AJ Roboscreen BetaPrion®, Bio-Rad TeSeE™ SAP, Enfer TSE v2, Enfer TSE v3, IDEXX HerdChek Standard, IDEXX HerdChek Short, IDEXX HerdChek Ultra Short, Roche Prionscreen and Prionics®-Check Western performed within a maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system.

- Prionics®-Check LIA and Prionics®-Check PrioSTRIP gave unexplained and unresolved specificity problems which hamper the interpretation of their analytical sensitivity and the comparison with other approved tests.

- Excluding Prionics®-Check LIA and Prionics®-Check PrioSTRIP, for all other tests no potential differences in field detection performance can be inferred on the sole basis of the difference in analytical sensitivity reported in this study.

With regard to sheep Classical scrapie:

- All tests (Bio-Rad TeSeE™ SAP, Bio-Rad TeSeE™ Sheep/Goat, Enfer TSE v2, Enfer TSE v3, IDEXX HerdChek Standard, IDEXX HerdChek Short, IDEXX HerdChek Ultra Short, Prionics®-Check LIA SR and Prionics®-WB Check Western SR) performed within a maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system.

- Marginal specificity problems were observed with Prionics®-Check LIA SR and Enfer TSE v3 with sheep samples, which did not compromise the estimation of their analytical sensitivity.

- No potential differences in field detection performance can be inferred on the sole basis of the difference in analytical sensitivity reported in this study.

With regard to the Atypical scrapie stability study:

- An apparent decrease in the detected signal could be observed during the stability study and this was taken into account in the study.

With regard to sheep Atypical scrapie:

- Bio-Rad TeSeE™ SAP, Bio-Rad TeSeE™ Sheep/Goat, IDEXX HerdChek Standard, IDEXX HerdChek Short and IDEXX HerdChek Ultra Short performed within the maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system.

- Enfer TSE v2, Enfer TSE v3, Prionics®-Check LIA SR and Prionics®-WB Check Western SR could fail in identifying field Atypical scrapie cases that other validated tests would detect.

RECOMMENDATIONS

- The analytical sensitivity of Prionics®-Check LIA and Prionics®-Check PrioSTRIP with cattle BSE samples should be re-assessed by appropriate experiments under the supervision of the CRL. Based on the result of the CRL study these tests cannot currently be considered to perform within a maximal 2 $\log_{10}$ inferiority range as compared to the most sensitive test system. Currently they cannot be recommended for use for BSE monitoring in cattle.

- The EFSA protocol for the evaluation of rapid *post mortem* tests to detect TSE in small ruminants (EFSA, 2007b) states that tests that are not able to meet requirements for all types of TSE agents on known positive samples should not be considered for testing in the field. Consequently, and based on the information obtained from the CRL study, Enfer TSE v2, Enfer TSE v3, Prionics®-Check LIA SR and Prionics®-WB Check Western SR cannot be recommended for use for TSE monitoring in small ruminants.

- A similar study should be conducted with samples of Atypical BSE (BSE-L, BSE-H) and of sheep BSE, if material is made available to the CRL for TSE.

- With regard to the differences in performance of the different assays according to the different types of TSE agents considered, samples of Atypical BSE, sheep BSE, Classical scrapie and Atypical scrapie should be included in the batch release testing procedure, if feasible.

DOCUMENTATION PROVIDED TO EFSA

1. Determination of analytical sensitivity (detection limit) for currently approved TSE rapid tests. Final report. Kath Webster, Mike Flowers, Claire Cassar and Daniele Bayliss. For the TSE Community Reference Laboratory. Veterinary Laboratories Agency Weybridge, United Kingdom. Revised December 2009. Submitted by the European Commission. (See Annex to the opinion)

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APPENDICES

A. BIO-RAD WESTERN BLOT RESULTS OBTAINED BY THE CRL ON THE SAMPLES USED FOR THE FIRST ATYPICAL SCRAPIE ANALYTICAL SENSITIVITY STUDY

Two ovine cerebrum samples positive for Atypical scrapie by immunohistochemistry (IHC), confirmed with Bio-Rad Western Blotting, were used for the study. Post-homogenate samples were also tested by the CRL with Bio-Rad Western Blot (on 21 November 2008) (see Table 1 and Figure 1) and some of them also with Bio-Rad TeSeE.

Table 1: Results of the Western Blot performed on post-homogenate samples (21 November 2008).

Number (see figure below)	Sample	Dilution	Result with Bio-Rad Western Blotting*
1	Ovine Atypical scrapie positive	1:2	positive (Atypical)
2	Ovine Atypical scrapie positive	1:4	positive (Atypical)
3	Ovine Atypical scrapie positive	1:8	weak positive (Atypical)
4	Ovine Atypical scrapie positive	1:16	weak positive (Atypical)
5	Ovine Atypical scrapie positive	1:32	negative
6	Ovine Atypical scrapie positive	1:64	negative
7	Ovine Atypical scrapie positive	1:128	negative
8	Ovine Atypical scrapie positive	1:256	negative
9	Ovine Atypical scrapie positive	1:512	negative
10	Ovine Atypical scrapie positive	1:1024	negative
11	Negative	-	negative
12	Negative	-	negative
13	Negative	-	negative
14	Bovine positive control	-	
15	Ovine positive control (Classical)	-	

* With contrast enhancement the low molecular mass band is visible in all the samples but this band alone is not sufficient to classify the samples as positive therefore samples 5-13 are classed as negative.

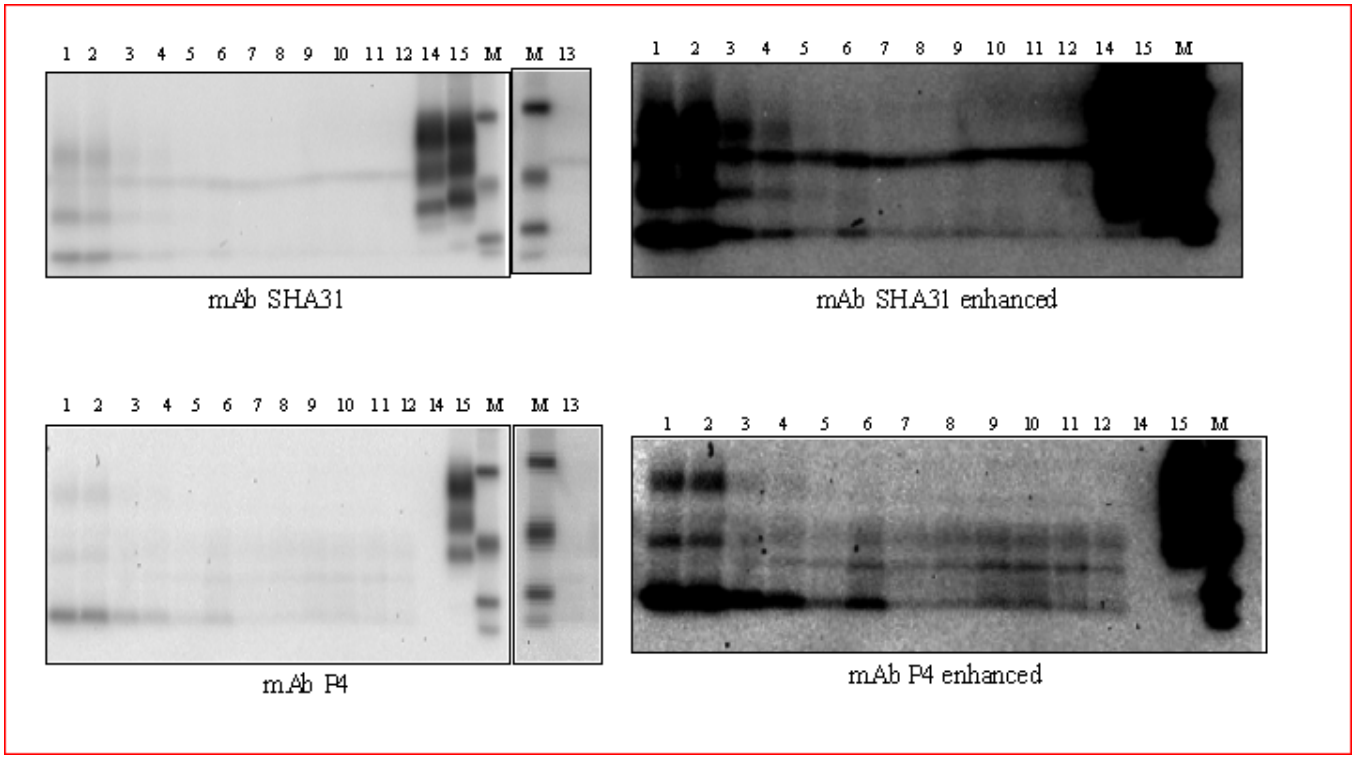

Figure 1: Results of the Western Blot performed on post-homogenate samples (21 November 2008).

B. Bio-Rad Western Blot results obtained by the CRL on the samples used for the second Atypical scrapie analytical sensitivity study

Seven ovine samples from different areas of the brain (five from cerebellum and two from rostral medulla) positive for Atypical scrapie by immunohistochemistry (IHC), two ovine samples from different areas of the brain positive for Classical scrapie and three negative whole brain samples were used for the study. Samples dispatched to the manufacturers for testing were also tested by the CRL with Bio-Rad Western Blot (on 12 February 2009) (see Table 2 and Figure 2).

Table 2: Results of the Western Blot performed on post-homogenate samples (12 February 2009).

CRL sample reference	Number (see figure below)	Sample	Dilution	Result with Bio-Rad Western Blotting
-	1	sigma marker	-	
-	2	other test samples	-	
-	3	other test samples	-	
OH0129	4	Ovine negative	neat	negative
OH0130	5	Ovine negative	neat	negative
OH0131	6	Ovine negative	neat	negative
OH0132	7	Ovine Classical scrapie positive	neat	positive
OH0133	8	Ovine Classical scrapie positive	neat	positive
OH0134	9	Ovine Atypical scrapie positive	neat	inconclusive
OH0135	10	Ovine Atypical scrapie positive	neat	positive (Atypical)
OH0136	11	Ovine Atypical scrapie positive	neat	positive (Atypical)
OH0137	12	Ovine Atypical scrapie positive	neat	negative
OH0138	13	Ovine Atypical scrapie positive	neat	positive (Atypical)
OH0139	14	Ovine Atypical scrapie positive	neat	positive (Atypical)
OH0140	15	Ovine Atypical scrapie positive	neat	positive (Atypical)
-	16	Bovine positive control	-	positive
-	17	Ovine positive control	-	positive
-	18	sigma marker	-	

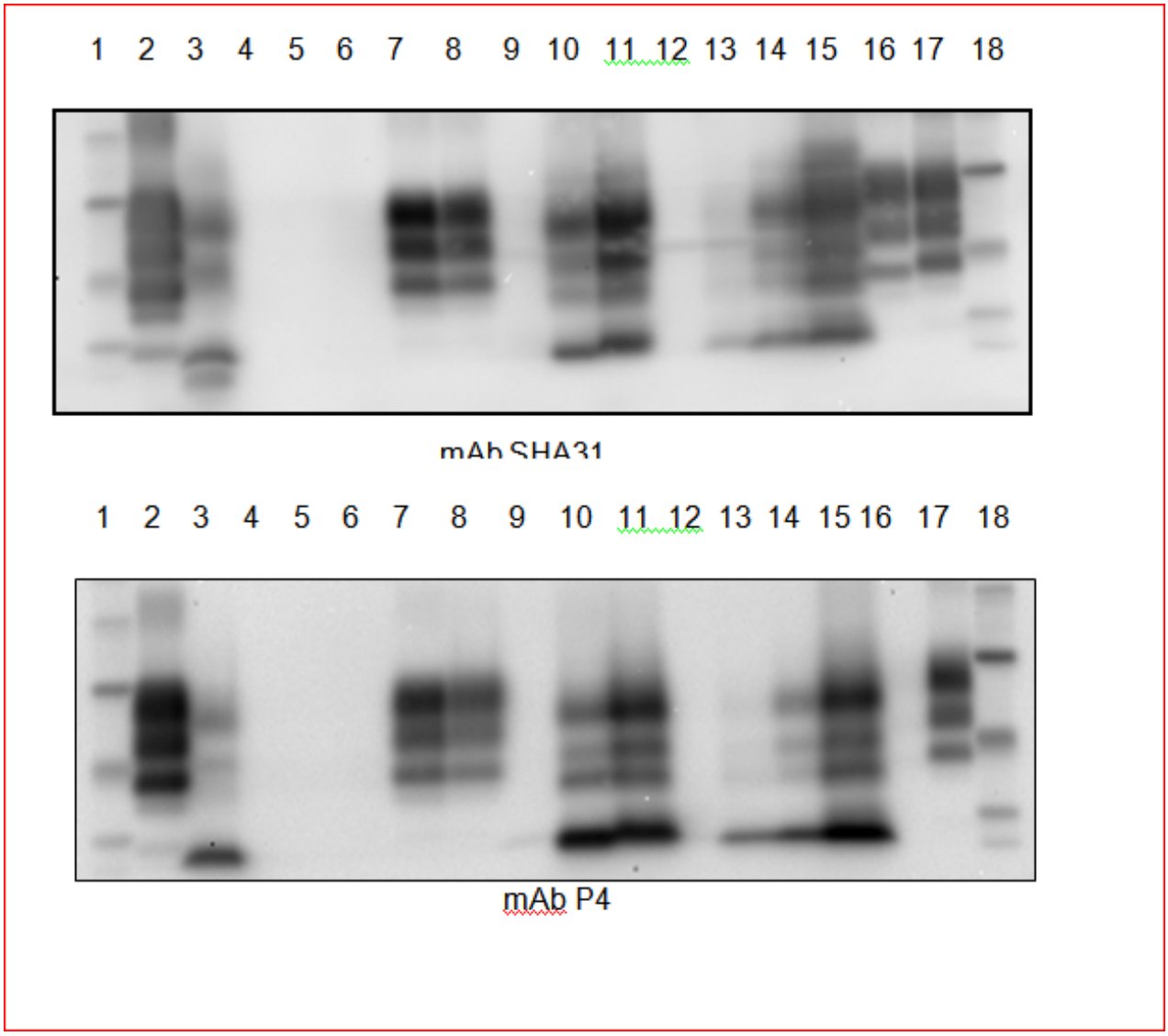

Figure 2: Results of the Western Blot performed on post-homogenate samples (12 February 2009).

DETERMINATION OF ANALYTICAL SENSITIVITY (DETECTION LIMIT) FOR CURRENTLY APPROVED TSE RAPID TESTS

FINAL REPORT

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Revised December 2009

LIST OF ACRONYMS	
EU	European Union
TSE	Transmissible Spongiform Encephalopathies
BSE	Bovine Spongiform Encephalopathy
SSC	Scientific Steering Committee
EFSA	European Food Safety Authority
CRL	Community Reference Laboratory
DG SANCO	Directorate General Health and Consumer Affairs
CNS	Central Nervous System
LIA	Luminescent Immuno Assay
ELISA	Enzyme Linked Immunosorbent Assay
LGC	Laboratory of the Government Chemist
VLA	Veterinary Laboratories Agency
EIA	Enzyme Immunoassay
OD	Optical Density
SAP	Short Assay Protocol
NSP	New Sample Preparator
PrP	Prion Protein
RLU	Relative Light Units
LU	Light Units
WB	Western Blot
SR	Small Ruminant
S&G	Sheep and Goat
IRMM	Institute of Reference Materials and Measurements
A	Sample Absorbance
AREF	Reference Absorbance
NCC	Negative Control Cutoff
SC	Sample Cutoff
NCM	Negative Control Mean
SM	Sample Mean
IHC	Immunohistochemistry

1. BACKGROUND

1.1 Scope

Provisional reported figures for 2007 indicate that over 10 million bovine, ovine and caprine TSE tests were undertaken within the 27 countries of the EU according to the framework of the EU TSE monitoring programmes [1].

According to Regulation European Commission (EC) No 999/2001 the prevention, control and eradication of certain transmissible spongiform encephalopathies (TSEs) in each Member State will be developed through an annual programme for monitoring BSE and scrapie that includes a screening procedure using rapid tests. Rapid tests shall be approved for that purpose in accordance with the approved procedure (Commission proposal put for an opinion to the Standing Committee on the Food Chain and Animal Health) and listed in Annex X of Regulation (EC) No 999/2001[2].

The European Commission (EC) originally undertook an evaluation exercise of rapid *post mortem* TSE tests in 1999 [3]. Several tests were assessed using brain tissue from clinical cases of BSE in cattle. As a consequence, three tests (Prionics®-Check Western, Enfer Version 2 and Bio-Rad Platelia-BSE® (later to be called TeSeE™) were approved under Regulation (EC) No 999/2001 [2].

A subsequent laboratory evaluation commissioned by the EC examined the performance of five *post mortem* rapid tests using brain tissue from clinical cases of Bovine Spongiform Encephalopathy (BSE) in cattle and a smaller sample size strategy. In response to the study outcome, the Scientific Steering Committee (SSC) recommended that the tests should be successfully trialed with field samples prior to approval. Two of the tests (Prionics®-Check LIA and InPro CDI-5) passed the field trial and were approved according to Regulation (EC) No 999/2001 in 2003 [4].

In 2003 the European Food Safety Authority (EFSA) and its Scientific Expert Working Group on Transmissible Spongiform Encephalopathy (TSE) Testing were asked by the EC to take over the mandate of the former Scientific Steering Committee (SSC) for the scientific evaluation of rapid TSE/BSE tests. At that point 5 rapid BSE test kits were approved by the EC for the post mortem testing of slaughtered cattle in accordance with the TSE Regulation (EC) No 999/2001[4]. Following an EC call for expression of interest, a laboratory evaluation of selected rapid post mortem BSE tests was divided in two phases: a phase I laboratory evaluation and a field trial. The laboratory evaluation was organised, carried out and analysed by the EC Institute of Reference Materials and Measurements (IRMM) and the results were assessed by EFSA's Working Group on TSE Testing. Only those candidates that had successfully passed the phase I laboratory evaluation were allowed to enter into the field trial. Based on an overall assessment on the application information, the phase I laboratory evaluation, the field trials and the approval of the package inserts, the EFSA Working Group on TSE Testing expressed their favourable opinion on 7 new tests and recommended these tests

for approval by the European Commission in the framework of regulation (EC) No 999/2001[5].

No evaluation of rapid TSE tests on material from small ruminants by the Commission was possible before February 2004. In the absence of such data, tests performing satisfactorily on bovine tissues were provisionally approved for small ruminants and used for active and passive surveillance for TSE in sheep and goats during 2002-2004 (Bio-Rad TeSeE™ Sheep and Goat, Enfer Version 2, InPro CDI-5, Prionics®-Check LIA and Prionics®-Check Western). Subsequently in 2005 an EU evaluation exercise of rapid *post mortem* TSE tests intended for small ruminants was undertaken. IRMM carried out an evaluation of diagnostic and analytical sensitivity, and diagnostic specificity and repeatability of six rapid post mortem tests (Bio-Rad TeSeE®, Bio-Rad Sheep/Goat, Enfer TSE V2.0, Institut POURQUIER LIA Scrapie, Prionics®-Check Western Small Ruminant test and Prionics®-Check LIA Small Ruminants) on samples from natural scrapie cases. Additionally the capability of these tests and their diagnostic sensitivity for the detection of "atypical" scrapie strain (Nor98) in sheep tissue were evaluated. During August 2004, further brain samples from three clinical cases of sheep orally challenged with BSE-affected cattle brain homogenate were screened using each of the six rapid tests. In March 2005, in response to concerns of the EFSA Working Group on TSE Testing following confirmation of a case of BSE in a French goat, the six rapid tests were re-evaluated against dilutions of brain homogenates from experimentally BSE infected sheep to provide analytical sensitivity for this material comparable to that previously obtained for scrapie.

In 2005, EFSA received IRMM reports on the laboratory evaluation of three additional post mortem TSE tests intended for small ruminants. Based on an overall assessment the experts of the EFSA Working Group on TSE Testing recommended two tests (IDEXX HerdChek, InPro CDI-5) for approval by the European Commission to be used in the field to assess the prevalence of classical scrapie and BSE in brainstem samples of sheep. Both tests are also recommended for the detection of "atypical" scrapie (Nor98) using cerebrum or cerebellum samples. In addition the IDEXX HerdChek test was also recommended for brainstem samples to detect "atypical" scrapie (Nor98). The Fujirebio FRELISA post mortem Test, (Fujirebio Inc.) was not recommended for approval on small ruminant tissue. Subsequently further rapid BSE test kits were approved by the EC for the post mortem testing of slaughtered small ruminants in accordance with the TSE Regulation (EC) No 999/2001 [6].

Further modifications were made to Annex X of Regulation (EC) No 999/2001 in April 2008 [7]. Annex X defined 12 approved tests for use in the rapid testing of BSE in bovine animals. The approved tests included: Prionics®-Check Western test, Prionics®-Check LIA test, Enfer TSE Kit version 2.0, automated sample preparation, Enfer TSE Version 3, Bio-Rad TeSeE™ test, Beckman Coulter InPro CDI kit, CediTect BSE test, IDEXX HerdChek BSE Antigen Test Kit, EIA, Prionics®-Check PrioSTRIP, Roboscreen Beta Prion BSE EIA Test Kit, Roche Applied Science PrionScreen, Fujirebio FRELISA BSE post-mortem rapid BSE Test. Annex X (April 2008) also defined 9 approved tests for use in the rapid testing of TSE in ovine and caprine animals. The approved tests included: Beckman Coulter InPro CDI kit, Bio-Rad Te-SeE test, Bio-Rad TeSeE™ Sheep/Goat test, Enfer TSE Kit version 2.0,

Enfer TSE Version 3, IDEXX HerdChek BSE-Scrapie Antigen Test Kit, EIA, POURQUIER'S-LIA Scrapie, Prionics®-Check Western Small Ruminant test and Prionics®-Check LIA Small Ruminants.

The scope of this particular study was to produce contemporary robust analytical sensitivity data for the current EU-approved rapid *post mortem* tests designed to detect one or all of BSE, scrapie and atypical scrapie. Importantly, the design strategy of this study involved all tests being evaluated against the same sample set. There was an assessment of analytical sensitivity within these previous studies but different starting samples were used. Consequently it was challenging to draw conclusions as direct comparisons of the data were difficult. DG SANCO has requested that the CRL assess analytical sensitivity for all the currently approved TSE rapid tests**.** The motivation behind this request came from recommendations in EFSA opinions [13,14] that currently approved tests should be required to confirm their robustness and their ability to fulfil the additional performance requirements (eg detection of atypical scrapie cases and analytical sensitivity). The CRL has proposed that an analytical sensitivity study should be carried out on a regular basis.

The resulting information will enable the European Commission to mandate EFSA for a scientific evaluation of the reports and continued suitability for the currently approved rapid tests to maintain EU approval.

1.2. Aims of the Study.

- To assess the lowest detection limit of rapid tests approved for the detection of TSE's in bovines using 3 pools (A, B and C) of bovine positive brain material.
- To compare CRL pre-prepared dilution series comprising 216 aliquots of 50% water homogenates of pools A, B and C, with the dilution series prepared by the manufacturers in their own laboratories.
- To compare CRL pre-prepared dilution series of 50% water homogenates of bovine negative brain material, with the dilution series prepared by the manufacturers in their own laboratories (pool D, negative pool).
- To assess the lowest detection limit of rapid tests approved for the detection of TSE's in small ruminants using 3 pools (X, Y and Z) of classical scrapie positive ovine brain material.
- To compare CRL pre-prepared dilution series of 50% water homogenates of pools X, Y and Z, with the dilution series prepared by the manufacturers in their own laboratories.
- To compare CRL pre-prepared dilution series of 50% water homogenates of ovine negative brain material with the dilution series prepared by the manufacturers in their own laboratories (pool W, negative pool).
- To perform a small stability study to establish whether dilution series prepared from homogenates of ovine brain material, which is positive for atypical scrapie, may be stored frozen at –80^{o}C prior to issue to testing laboratories.
- To conduct an analytical sensitivity study for atypical scrapie using CRL pre-prepared dilution series of 50% water homogenates.
- To conduct a further analytical sensitivity study for atypical scrapie using CRL neat tissue samples.

1.3. Project Plan

The project was undertaken according to the following stages as detailed in Table 1.1.

Key stages	Time Period
Establishment of protocol	November 2007
Confirmation of manufacturer participation	June –August 2008
Confirmation of tests for study inclusion	June –September 2008
Sourcing of TSE negative ovine and bovine tissue	January- July 2008
Sourcing of BSE positive bovine tissue	January- June 2008
Sourcing of classical & atypical scrapie positive ovine tissue	January-November 2008
Atypical scrapie stability study	April –October 2008
Interim Report -preparation & submission to EC	June 2008
Preparation and blinding of tissue pools	August & November 2008
Preparation of CRL dilution series	August & November 2008
Consignment and shipment	September & November 2008
Testing of BSE & Classical Scrapie samples by participants under supervision of CRL	September- October 2008
Testing of Atypical Scrapie samples by participants	November 2008
Analysis of results	November 2008
Manufacturers invited to submit comments	January 2009
Testing of neat Atypical Scrapie samples by 2 participants	February 2009
Final Report submitted	April 2009

Table 1.1 Analytical sensitivity study project plan.

1.4. Manufacturers and rapid tests involved in the study

Analytical sensitivity evaluation was undertaken for tests listed according to manufacturer. All tests listed were approved TSE rapid tests (according to EC Regulation 999/2001 at the time of testing [7]) and therefore their inclusion as part of the study was mandatory (see Table 1.02).

Bio-Rad withdrew the TeSeE™ Standard Protocol from the study as it has now been replaced by the TeSeE™ Short Assay Protocol (SAP) for distribution in the 27 countries of the EC. Written confirmation of Bio-Rad's intent to withdraw this product from the EU market was received prior to commencement of the laboratory exercise (see Annex 8).

Enfer tested both the version 2 and the version 3 using the automated method of sample preparation. The manual method was not included as part of the evaluation. The sole difference between the manual and automated method is in the method of sample preparation and this has been accepted as equivalent. Consequently there was no requirement to perform both methods as there would be no overall effect on the performance of the tests.

The POURQUIER-LIA and CediTect BSE test have now both been withdrawn by the manufacturers.

Two manufacturers listed in Annex X of Regulation (EC) No 999/2001 failed to respond to the invitation to participate in this study, namely Fujirebio Inc. and InPro. As a consequence these manufacturers' tests were not evaluated within the scope of this study. The EU have withdrawn these tests from the approved test list in Annex X of the EU regulations (Regulation 162/2009 [15]).

Manufacturer	Test name	Test Target		
		BSE	Classical Scrapie	Atypical Scrapie
AJ Roboscreen	BetaPrion®	√		
Bio-Rad	TeSeE™ SAP	√	√	√
Bio-Rad	TeSeE™ Sheep /Goat		√	√
Enfer	TSE v2 (manual homogenisation, automated sample handling)	√	√	√
Enfer	TSE v3 (manual homogenisation, automated sample handling)	√	√	√
IDEXX	HerdChek- Standard (bovine conjugate)	√		
IDEXX	HerdChek- Short (bovine conjugate)	√		
IDEXX	HerdChek- Ultra Short (bovine conjugate)	√		
IDEXX	HerdChek- Standard (scrapie conjugate)		√	√
IDEXX	HerdChek- Short (scrapie conjugate)		√	√
IDEXX	HerdChek- Ultra Short (scrapie conjugate)		√	√
Prionics®	Prionics®-Check LIA	√		
Prionics®	Prionics®-Check LIA SR		√	√
Prionics®	Prionics®-Check PrioSTRIP	√		
Prionics®	Prionics®- Check Western	√		
Prionics®	Prionics®-WB Check Western SR		√	√
Roche	Prionscreen	√		

Grey shading	Represents test not approved for listed target
√	Represents test that may be used for detection of listed target

Table 1.02. Summary of manufacturers, rapid tests and test targets involved in the study

Samples were prepared by the CRL standard method of homogenate preparation (detailed in Protocol for the Evaluation of rapid post mortem tests to detect TSE in small ruminants, Annex 2 of the EFSA Journal (2007) [14]. These samples from the same four pools were tested by each test manufacturer enabling comparison between CRL and manufacturer-prepared dilution series. Several manufacturers (AJ Roboscreen, IDEXX and Roche) chose to opt out of preparing and testing a

manufacturer-prepared dilution series as they either considered that the production method employed by the CRL for generating test samples had no negative impact on their test performance and/or due to constraints in manufacturer resources, primarily availability of people hours. All other manufacturers (Bio-Rad, Enfer and Prionics) opted to test both the CRL pre-prepared samples and to prepare their own dilution series from the raw material provided by the CRL, which was re-coded by CRL representatives prior to commencement of testing to ensure a blind study. Prior to commencement of the laboratory phase these manufacturers (Bio-Rad, Enfer and Prionics) advised the CRL specifically that the CRL sample set prepared as 50% water homogenates was likely to compromise their test performance. These manufacturers considered that compromised test performance would yield sub-optimal analytical sensitivity data for the CRL sample set.

2. METHODOLOGY

2.1 Preparation of Test Material

Analytical Sensitivity was assessed for BSE tests using tissue samples originating from cattle infected with classical (C-type) BSE. Only 40-50 atypical BSE cases have been described worldwide, and samples from these rare field cases were not available within the scope of this study.

Tests approved for the detection of scrapie in small ruminant samples were assessed with material prepared from classical scrapie samples. The evaluation of analytical sensitivity for atypical scrapie was also undertaken, but this was more difficult, principally because less is known about how atypical scrapie tissue behaves when prepared as homogenates and because the availability of material is limited.

As a consequence, the atypical scrapie study was not designed in the same way as described below for classical scrapie and BSE. Before embarking on an analytical sensitivity study for atypical scrapie, a stability study was undertaken to show whether homogenates could be prepared and stored frozen at – 80 ºC, prior to issue to testing labs/manufacturers. Additionally, because of the scarcity of atypical scrapie material it was not feasible to work with three pools.

The samples for this study were prepared by the CRL using standard methods for TSE QA sample production and issued to test manufacturers for testing, thus providing greater reassurance that the samples would be homogeneous. The participating manufacturers were also given the opportunity to make their own dilution sets of samples for testing, except in the Atypical Scrapie study where samples were prepared by the CRL and distributed to all participating manufacturers in a QA proficiency testing style exercise.

2.1.1 Preparation of BSE Material

The CRL prepared 3 tissue pools (A, B and C) from BSE-positive CNS tissue, and one tissue pool (D) prepared from BSE negative CNS tissue. The pools were prepared by chopping tissue finely and then treating portions of tissue in a Seward Stomacher 80 Biomaster for 120 seconds for 3 successive treatments. Positive tissue was from confirmed BSE cases. All details pertaining to the sample provenance were recorded (See Annex 6).

Each pool was divided and one part used at the CRL to prepare dilution series using the CRL standard method. The second part of each pool was divided into aliquots. These aliquots were issued blind to manufacturers together with negative tissue to prepare their own dilution series in negative brain tissue to match the CRL samples. All negative tissue (bovine CNS) came from samples tested negative by Bio-Rad TeSeE™ ELISA was obtained from the Laboratory of the Government Chemist (LGC). All details pertaining to the samples were recorded. (Annex 6).

All CRL dilution series of homogenates consisted of doubling dilutions from a positive sample pool mixed with an equal volume of nuclease-free water down to 1

part positive tissue in 4096 parts negative sample (50% negative tissue/50% nuclease-free water). Sufficient material was prepared for each pool to allow testing of 2 aliquots of the first 2 dilutions and 5 aliquots from the subsequent dilutions for each test. Samples were blind coded and put into a panel by CRL representatives. The panel for each test comprised 216 aliquots, 54 samples per pool.

2.1.2. Preparation of Classical Scrapie Material

The CRL prepared 3 positive tissue pools (X, Y and Z) from ovine classical scrapie-positive CNS tissue, and one tissue pool (W) prepared from classical scrapie-negative CNS tissue The pools were prepared by chopping tissue finely and then treating portions of tissue in a Seward Stomacher 80 Biomaster for 120 seconds for 3 successive treatments. Positive tissue originated from confirmed classical scrapie cases. All details pertaining to the sample provenance were recorded (See Annex 6).

All CRL dilution series of homogenates consisted of doubling dilutions from a positive sample pool mixed with an equal volume of nuclease- free water down to 1 part positive tissue in 4096 parts negative sample (50% negative tissue/ nuclease free 50% water). Sufficient material was prepared for each pool to allow testing of 2 aliquots of the first 2 dilutions and 5 aliquots from subsequent dilutions for each test. Samples were blind coded by CRL representatives. The panel for each test comprised 216 aliquots, 54 samples per pool.

2.1.3. Preparation of Atypical Scrapie Material

2.1.3.1. Stability Study

A stability study was undertaken to establish whether dilution series prepared from homogenates of ovine brain material positive for atypical scrapie could be stored frozen at –80^{o}C for several months prior to issue to testing laboratories without compromising the level of analyte within the sample.

Several atypical cases had been selected to provide candidate tissue for this aspect of the study. The CRL selected the sample with the strongest signal using the Bio-Rad TeSeE™ Sheep/Goat test and showing widespread IHC staining in fixed sections from adjacent brain sections. Several small samples of this material were removed and frozen at –80°C to act as controls for testing at specific time points in the stability study.

The remainder of the tissue was processed by chopping tissue finely and then macerating portions of tissue in a Seward Stomacher for 120 seconds for 3 successive treatments as a 1/2 tissue/nuclease free water homogenate. The portions were then mixed together and a subsequent dilution series made and aliquotted. The dilution series used for the study was as follows: 1/5, 1/10, 1/50, 1/200, 1/500, 1/750, 1/1000. This sequence differed from the dilution series proposed in the original protocol due to scarcity of suitable material. The material was divided into aliquots.

One set of samples was tested immediately by the CRL using the Bio-Rad Western blot. The finely chopped tissue was used as a control (this was diluted at the time of testing 1/1 with nuclease free water). In order to be economical with tissue, once each dilution series had been made, the remaining samples were distributed, as detailed in both the Bio-Rad TeSeE™ SAP and Bio-Rad TeSeE™ Sheep/Goat test kit instructions, into the grinding tubes of each rapid test to be used. They were then stored at -80°C and tested after various periods of storage using the coarsely chopped tissue as a control, as described above.

2.1.3.2. Sensitivity Study

The CRL prepared an analytical sensitivity dilution series from stomached atypical scrapie-positive CNS tissue of known provenance, from two atypical scrapie cases. A sample from the animal used in the stability study mentioned previously (sample ref.SS00564687) was also used in the first atypical scrapie sensitivity study. The second sample used in the first part of the sensitivity study originated from an animal that had been specifically challenged (sample ref. PG1077/08) – see Annex 6 Table 2 of the final report for sample information.

The coded dilution series were despatched blind to the manufacturers testing laboratories with recommendations to test within 1 day of receipt and report the results back to the CRL within one week.

Negative tissue (ovine CNS) from samples tested negative by Bio-Rad TeSeE™ SAP were obtained from VLA Shrewsbury. All tissue samples used to produce tissue pools and CRL dilution series had originally been tested positive or negative with the approved Bio-Rad TeSeE™ test (see Annex 6).

The positive tissue was mixed 1/2 with nuclease free water. The dilution series consisted of doubling dilutions from a positive sample down to 1 part positive tissue in 1024 parts negative sample (produced as negative tissue/water homogenate). Sufficient material was prepared for each pool to allow testing of 2 aliquots for each dilution step for each test. The samples were coded at the CRL and issued as a blind panel.

A further Atypical Scrapie study was conducted in February 2009 as two manufacturers (Enfer and Prionics) failed to detect the atypical samples in the analytical sensitivity part of the Atypical Scrapie study. Consequently the additional study was conducted using a larger panel of atypical scrapie samples from different animals. Twelve neat tissue samples prepared as a duplicate series of chopped material were blinded by the CRL and despatched to Prionics and Enfer for testing in February 2009. The samples were also be tested by Bio-Rad TeSeE™ and Bio-Rad Western Blot. The CRL received atypical scrapie results from the manufacturers on 17th February 2009. The resultant data sets were analysed by the CRL.

All manufacturers agreed the protocols for this work with the CRL prior to commencing the study. All manufacturers were instructed to undertake testing according to their current version of Instructions for Use.

2.2. Laboratory Exercise

Following CRL preparation, those manufacturers participating in the BSE phase of the evaluation received 3 BSE positive tissue pools (Pools A, B,C), 1 BSE negative pool (blinded as a positive pool, Pool D), 4 negative tissue pools and CRL pre-prepared aliquots of a BSE dilution series. Manufacturers participating in the scrapie analytical sensitivity study received 3 classical scrapie positive (Pools X,Y,Z), 1 Classical Scrapie negative pool (blinded as a positive pool, Pool W), 4 negative pooled tissues and 216 CRL pre-prepared aliquots of a classical scrapie dilution series. The laboratory evaluation was undertaken between 8th September and 10th October 2008. Retesting of some tests took place between 27th October and 29th October 2008. Each manufacturer undertook sample preparation and testing at their nominated testing laboratories under observation of CRL representatives. All homogenates and dilution series were delivered in sealed containers. All recipients were advised to check the contents of their consignment on delivery but for the contents to be stored at -70°C until required for the commencement of the supervised laboratory exercise.

Due to the scarcity of atypical scrapie material it was not feasible to produce negative and positive pooled tissues in the same manner. Consequently the atypical scrapie study took the form of a proficiency-test circulation exercise rather than tissue delivery as in the BSE and scrapie exercise. A “date of test” element was introduced such that no manufacturer was disadvantaged by any delays between time of sample production and time of testing.

Consequently, a dilution series comprising 12 samples as a duplicate series (24 samples in total), were dispatched to each manufacturer of scrapie detecting rapid test kits. The samples were prepared on 10th November 2008. The CRL requested atypical scrapie results from the manufacturers by 17th November 2008. The resultant data sets produced by the manufacturers were analysed by the CRL.

A further atypical scrapie study was conducted in February 2009 as two manufacturers (Enfer and Prionics) were unable to detect the atypical samples in the analytical sensitivity part of the Atypical Scrapie study. Consequently the additional study was conducted using a larger panel of atypical scrapie samples from different animals. Twelve neat tissue samples prepared as a duplicate series blinded by the CRL, were despatched to Prionics and Enfer for testing in February 2009.

2.3. Statistical Analysis

End-point calculation

The 50% end-point for each set of tests was estimated by the trimmed Spearman-Karber method[16] as implemented in the software 'tsk'. This program originated at Montana State University and was modified by the Duluth and Athens National Exposure Research Laboratories.

Statistical analysis

For each species a two-way analysis of variance was done on samples on the 50% end-points expressed as –log2 (dilution) with sample and test as main effects. The test means were then compared by Tukeys HSD test based on the studentized range at the 5% significance level.

3. RESULTS

The laboratory evaluation for BSE, classical scrapie and atypical scrapie was undertaken between 8th September and 29th October 2008, this period included second retesting visits to manufacturers where necessary. The analytical sensitivity results are presented according to the manufacturers interpretation of their test.

3.1 Analytical sensitivity for BSE rapid tests

3.1.1 AJ Roboscreen, Leipzig, Germany

3.1.1.1. BetaPrion® BSE EIA Test -Test Principle

The following information is extracted from the BetaPrion® BSE EIA Test Kit insert:

'The BetaPrion® BSE EIA Test Kit consists of two modules, the BetaPrion® BSE Purification Kit, which includes the purification tools and the BetaPrion® BSE Detection Kit, which is based on a sensitive ELISA.

The BetaPrion® BSE EIA Test Kit is a continuous 100 min test for the detection of PrP_{res} in bovine brain samples. Specimens of bovine brain are homogenized and incubated with Proteinase K. Solubilised PrP_{res} is captured by a specific monoclonal anti-PrP antibody coated to the wells of a microtitre strip. Bound PrP_{res} is detected with a HRP-conjugated anti-PrP antibody. The wells are washed and a substrate solution is added. The developed colour indicates the existence of PrP_{res} in the specimen in comparison to a negative and a positive control in case of overshoot of the declared cut-off.'

3.1.1.2. Interpretation and validation of results

The cut-off value is defined as 0.2 optical density $OD_{450/620mn}$ and must be used for the discrimination of BSE positive samples from negative samples. The interpretation of data is based on all samples having an OD < 0.2 are classified as BSE –negative. Samples with $OD_{450/620mn}$ values of $\geq$ 0.2 are classified as initially reactive and must be retested in duplicate using the original homogenate. If one of the two replicate readings is $\geq$ 0.2 the sample is classified as BSE positive and must be dealt with according to the national guidelines. The $OD_{450/620mn}$ value of the positive control must be at least 1.0 and for the negative control the OD value must be below 0.1. The plate must be repeated if both of the negative controls have an $OD_{450/620mn} \geq 0.1$.

3.1.1.3. Period of Assessment

The testing of BSE samples by AJ Roboscreen at their laboratory in Leipzig, Germany took place between 23 – 25th September 2008 under observation by a CRL representative.

3.1.1.4. Rapid Test Kit information

Purification kit Lot number 0208-01, expiry date 28/02/09, was used throughout the testing. The current manufacturer user instructions Version 2.3/2008 were used throughout the testing period. CRL representatives observed that the Roboscreen syringe used for transfer of homogenate from CRL tubes to test measured volumes of approximately 0.4g, according to the Roboscreen IFU, the brain sample to be tested should weight 350 ± 50 mg. The Precellys 24 was used for ribolysing. The Tecan (run on Programme 1) replaced the Columbus plate washer (programme BSE-5) as listed in the IFU. It was observed that sample tubes were pre-loaded into the centrifuge before the 15 minute incubation was completed, thus potentially lowering the temperature during incubation.

3.1.1.5. Problem Samples and Testing Issues

No problem samples or repeated samples were encountered during the course of testing.

3.1.1.6. BetaPrion® BSE EIA Analytical Sensitivity Test Results

AJ Roboscreen opted to undertake testing of CRL pre-prepared sample dilution series only.

3.1.1.6.1. BetaPrion® BSE EIA CRL pre-prepared dilution series

With reference to Table 3.01, a replicate dilution series from three BSE positive pools (Pools A,B,C) previously prepared by the CRL were tested using the BetaPrion® BSE EIA test and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/256. This threshold of detection was observed for all three pools. For Pools A & B a total number of 5 of 5 positive replicates were recorded. For Pool C a total number of 4 of 5 positive replicates were recorded.

All 54 negative samples from Pool D tested negative using the BetaPrion® BSE EIA test.

When data from all 3 pools were compared it was observed that there was a consistent trend among the pools with positive signals detected down to a dilution factor of 1/256.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive Replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive Replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive Replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	5/5	**Pool B**	1/256	**POS**	5/5	**Pool C**	1/256	**POS**	4/5
Pool A	1/512	**Neg**	0/5	**Pool B**	1/512	**Neg**	0/5	**Pool C**	1/512	**Neg**	0/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**Neg**	0/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.01. BetaPrion® BSE EIA CRL pre-prepared dilution series

3.1.2 Bio-Rad, Marnes-la-Coquette, France

3.1.2.1. Bio-Rad TeSeE™ Short Assay Protocol Test -Test Principle

The following information is extracted from Bio-Rad TeSeE™ Short Assay Protocol Kit insert:

The Bio-Rad TeSeE™ Short Assay Protocol (SAP) Detection Kit is an immuno-enzymatic microplate method (sandwich format) using 2 monoclonal antibodies for the detection of the abnormal prion protein, resistant to proteinase K, in tissues collected from infected animals. Tissue homogenates are prepared by grinding in a preparation buffer using small glass beads and a homogeniser stage. Tissue homogenates are then purified prior to treatment with proteinase K. Samples aliquots then undergo antibody capture stages and a final antibody detection stage. Visualisation of the sample signal takes place via addition of peroxidase-labelled antibody that is incubated with the test samples. Addition of a peroxidase substrate enables a colorimetric reaction to be measured. The result is read as optical density value at a wavelength of 450 nm - 620 nm.

3.1.2.2. Interpretation and validation of results

The kit set up requires that each experimental plate contains four negative controls and two positive controls. The cut –off values are calculated for every experimental plate by addition of the mean value of the four negative controls with a fixed value of 0.210.
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 ODs) is lower than this value.

Samples with an optical density lower than the cut-off value are considered to be negative according to the TeSeE™ SAP Detection Kit. However, results located just below the cut-off value (cut-off value - 10%) must be interpreted carefully. The manufacturers advise that in such cases the samples should be retested in duplicate, starting from the original homogenate. Samples with an optical density greater than or equal to the cut-off value are considered to be initially reactive according to the TeSeE™ SAP Detection Kit and should be retested in duplicate, starting from the original homogenate, before a final interpretation of the results can take place.

3.1.2.3. Period of Assessment

CRL representatives visited Bio-Rad at their laboratory in Marnes-la-Coquette, France. The testing of BSE samples took place between 29th September – 3rd October 2008 under observation by the CRL.

3.1.2.4. Rapid Test Kit information

Purification kit Lot number 8G 0064 and SAP Detection kit Lot Number 8F 0022 was used throughout the testing. The current manufacturer instructions for use Version Rev. A.4 - 12/2006 were used throughout the testing period.

All samples were processed using the the semi-automatic processing stage of the purification protocol, the Bio-Rad New Sample Preparator (NSP) was used to undertake the purification stage according to IFU Rev. A.4 - 12/2006. Version 2.0f (Ref.91460) of the NSP Manual.

CRL representatives observed that two different operators prepared the manufacturers bovine dilution series. On the first preparation day the CRL representatives observed that at the sample preparation stage > 400mg of the starting material was initially being dispensed (IFU criteria state 350 mg ± 40mg), by a single operator, before comment was made and the operator was changed. CRL representatives observed that pipette tips were not changed when making the dilution series for the tissue pools, consequently there may have been inestimable carryover of tissue down the dilution series.

3.1.2.5. Problem Samples and Testing Issues

Bio-Rad - prepared bovine samples 84 and 85 (Pool B 1:256 dilution in duplicate) were pipetted into same well at position 12C on plate 16. The doubled sample was removed from the deep well plate and there were no OD readings for these two dilution samples. The NSP report for this plate reflected this situation with an error message indicating 2 empty wells.

The test results for several sample dilutions were identified as requiring cautious interpretation (i.e. according to the manufacturers kit instructions) inferring that the samples required retesting in duplicate.

3.1.2.6. Bio-Rad TeSeE™ SAP Test Analytical Sensitivity Results

3.1.2.6.1 Bio-Rad Short Assay Protocol Manufacturer prepared dilution series

Bio-Rad opted to undertake testing of CRL pre-prepared sample dilution series and preparation and testing of their own dilution series prepared from 4 tissue pools provided by the CRL. The results presented demonstrate test performance on CRL prepared sample dilution series and manufacturer prepared dilution series using CRL material.

With reference to Table 3.02, a replicate dilution series from three BSE positive pools (Pools A,B,C) prepared by the manufacturers representatives were tested using the Bio-Rad Short Assay Protocol test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution

down to 1/512. This threshold of detection was observed for Pool B dilution series with 1 of 5 replicates testing positive. For Pools A & C a positive signal was detected at 1:256 and 1/128 dilution with a total number of 3 of 5 and 5 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool D (negative pooled homogenate) prepared by the manufacturers representatives tested negative using the Bio-Rad Short Assay Protocol test.

When data from all 3 pools were compared it was observed that there was some variability among the 3 pools with positive thresholds ranging from a dilution factor of 1/128 to 1/512.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	4/4*
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	3/5**	**Pool B**	1/256	**POS**	5/5	**Pool C**	1/256	**neg**	0/5**
Pool A	1/512	**neg**	0/5	**Pool B**	1/512	**POS**	1/5**	**Pool C**	1/512	**neg**	0/5
Pool A	1/1024	**neg**	0/5	**Pool B**	1/1024	**neg**	0/5	**Pool C**	1/1024	**neg**	0/5
Pool A	1/2048	**neg**	0/5	**Pool B**	1/2048	**neg**	0/5	**Pool C**	1/2048	**neg**	0/5
Pool A	1/4096	**neg**	0/5	**Pool B**	1/4096	**neg**	0/5	**Pool C**	1/4096	**neg**	0/5
* Replicate No. 5 could not be tested due to mixing of two separate samples				** 6 Results recorded as requiring cautious interpretation according to manufacturers IFU							

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.02 Bio-Rad Short Assay Protocol Manufacturer prepared dilution series

3.1.2.6.2. Bio-Rad Short Assay Protocol CRL pre-prepared dilution series

With reference to Table 3.03, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the Bio-Rad Short Assay Protocol test and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1:64. This threshold of detection was observed for Pool B dilution series with 3 of 5 replicates testing positive. For Pools A & C a positive signal was detected at 1:32 dilution with a a total number of 5 of 5 positive replicates recorded.

All 54 negative samples from Pool D pre-prepared by the CRL tested negative using the Bio-Rad Short Assay Protocol test.

When data from all 3 pools were compared it was observed that there was some variability among the 3 pools with positive thresholds ranging from a dilution factor of 1/32 to 1/64.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Total Number of Positive replicates	Total Number of Positive replicates
Pool A	1/2	POS	2/2	Pool B	1/2	POS	2/2	Pool C	1/2	POS	2/2
Pool A	1/4	POS	2/2	Pool B	1/4	POS	2/2	Pool C	1/4	POS	2/2
Pool A	1/8	POS	5/5	Pool B	1/8	POS	5/5	Pool C	1/8	POS	5/5
Pool A	1/16	POS	5/5	Pool B	1/16	POS	5/5	Pool C	1/16	POS	5/5
Pool A	1/32	POS	4/5	Pool B	1/32	POS	5/5	Pool C	1/32	POS	5/5
Pool A	1/64	Neg	0/5	Pool B	1/64	POS	3/5*	Pool C	1/64	Neg	0/5
Pool A	1/128	Neg	0/5	Pool B	1/128	Neg	0/5	Pool C	1/128	Neg	0/5
Pool A	1/256	Neg	0/5	Pool B	1/256	Neg	0/5	Pool C	1/256	Neg	0/5
Pool A	1/512	Neg	0/5	Pool B	1/512	Neg	0/5	Pool C	1/512	Neg	0/5
Pool A	1/1024	Neg	0/5	Pool B	1/1024	Neg	0/5	Pool C	1/1024	Neg	0/5
Pool A	1/2048	Neg	0/5	Pool B	1/2048	Neg	0/5	Pool C	1/2048	Neg	0/5
Pool A	1/4096	Neg	0/5	Pool B	1/4096	Neg	0/5	Pool C	1/4096	Neg	0/5

** 1 Result recorded as requiring cautious interpretation according to manufacturers IFU

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	Neg	54/54

Table 3.03 Bio-Rad Short Assay Protocol CRL pre-prepared dilution series

3.1.3 Enfer TSE Kit V2 & V3, Enfer Scientific Limited, Naas, Co. Kildare, Ireland

3.1.3.1. Enfer TSE Version 2 & Version 3 Test Kit -Test Principle

The following information is extracted from Enfer TSE Version 2 & Version 3 Test Kits insert:

The Enfer TSE Kit Version 2.0 is an immunological method for the detection of the prion protein PrP from central nervous tissue of cattle, sheep and goats. A sample of central nervous tissue is homogenized and treated with Proteinase K under defined conditions and centrifuged. The supernatant is incubated in prepared microplate wells: during this incubation any PrPSc in the sample is bound to the wells. After a washing step the wells are treated with Enfer Buffer 3. After a second washing step rabbit anti-PrP is added to the well and incubated; if any PrPSc is present on the well this antiserum will specifically bind to it. After a third washing step goat anti-rabbit IgG conjugated to horseradish peroxidase is added to the wells and incubated; if any rabbit anti-serum is present on the well the conjugate will be bound. After a fourth wash any bound conjugate is detected using a luminogenic substrate for peroxidase

The Enfer TSE Kit Version 3.0 is also an immunological method for the detection of the prion protein PrP from central nervous tissue of cattle, sheep and goats. A sample of central nervous tissue is homogenized and treated with Proteinase K under defined conditions and centrifuged. The supernatant is incubated in prepared microplate wells: during this incubation any PrPSc in the sample is bound to the wells. After a washing step the wells are treated with Enfer Buffer 3. After a second washing step rabbit anti-PrP is added to the well and incubated; if any PrPSc is present on the well this antiserum will specifically bind to it. After a third washing step goat anti-rabbit IgG conjugated to horseradish peroxidase is added to the wells and incubated; if any rabbit anti-serum is present on the well the conjugate will be bound. At this stage in the protocol, for Enfer TSE Version 3 unbound secondary conjugate is washed away and a solution containing 3,3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide is added to the wells. Wells with bound secondary conjugate develop a purple colour, which is converted to an orange colour when the reaction is stopped with sulphuric acid, the colour is read spectrophotometrically at 450nm. The amount of secondary conjugate, and hence colour, in the wells is directly related to the concentration of PrPSc in the sample.

3.1.3.2. Interpretation and validation of results for Enfer TSE Version 2 and Enfer TSE Version 3

When using *Enfer TSE Version 2 (V2)*, the control results must be validated before the sample results can be interpreted. The mean luminescence of Peptide Indicator Wells and positive and negative controls is determined and the median value for the Blank Control Reagent calculated. The values given are for measurements made on an Enfer recommended chemiluminometer. To calculate the median value of the Blank Control Reagent, the four Light Unit values are arranged in ascending

numerical order. The median is the arithmetic average of the two middle values. The median of Blank Control Reagent replicates must be below 4.0 LU.

To calculate the mean value for the Peptide Indicator Wells, the mean value must be equal to or above the limit (as supplied for the individual lot) for the Peptide Indicator Wells (after subtraction of the median blank value). If negative control is run, the mean must be less than 5.5 LU after subtraction of the median blank reading. If the above criteria are not met, the EIA run is invalid and must be repeated. The threshold light signal for a suspect positive determination is 5.5 LU (after subtraction of the median blank reading) as measured on an Enfer recommended chemiluminometer. All samples giving signals greater than or equal to 5.5 LU (after subtraction of the median blank reading) in one or both duplicate wells must be considered initially reactive and must be retested in duplicate, starting from the tissue. A sample is considered positive when the retesting results give a positive signal in one or both wells.

When using Enfer Version 3 (V3*)*, each plate must be considered separately when calculating and interpreting results of the assay. Approved software may be used for calculation and interpretation of results. The control results must be validated before the sample results can be interpreted. The Blank Control is calculated by calculating the median absorbance of the four replicates of the Enfer Buffer 1 blank control wells. The median is the arithmetic average of the two middle values when the data are arranged in numerical order; the median absorbance of the Blank Control replicates must be less than 0.2. The cut-off value is calculated by adding 0.3 to the Blank Control. The mean absorbance of the replicates of the Peptide Indicator Wells minus the Blank Control is calculated with the mean absorbance minus the Blank Control of the Peptide Indicator Wells, must be greater than 0.8. If a Negative Control is run calculate the mean absorbance of the replicates, the mean minus the Blank Control must have a value of 0.3 or less. Samples giving an absorbance less than or equal to the Cut-off value are considered non-reactive in Enfer TSE Version 3. Samples giving an absorbance in one or both wells greater than the Cut-off value, are considered initially reactive in the assay (see limitations of the procedure). Such samples must be retested in duplicate, starting from the tissue. A sample is considered positive when the retesting results give a signal greater than the Cut-off value in one or both wells.

3.1.3.3. Period of Assessment

CRL representatives visited Enfer at their laboratory in Naas, Ireland. The testing of BSE & Scrapie samples took place between 6th October– 10th October 2008 under observation by the CRL.

3.1.3.4. Rapid Test Kit information

Enfer TSE Version 2 kit Lot number K08I08A (Manuf. Date 2008/05/29, Exp. Date 2008/11/29) and Enfer TSE Version 3 kit Lot number K09I08A (Manuf. Date 2008/09/09, Exp. Date 2009/01/07) were used throughout the testing. The current manufacturer user instructions for Version 2 (C104J06GB September 2007) or Version 3 (C016L72GB November 2007) were used throughout the testing period.

3.1.3.5. Problem Samples and Testing Issues

Plate 5 (Bovine CRL sample dilution series) was loaded in an inverted orientation. With this in mind the data were reinterpreted in the correct orientation. The manufacturers prepared a common set of samples which were then split for evaluation of both tests (Enfer Version 2 and Enfer Version 3).

3.1.3.6. Enfer TSE Version 2 Test Analytical Sensitivity Results

Enfer undertook testing of the CRL pre-prepared sample dilution series and preparation and testing of their own dilution series prepared from tissue pools provided by the CRL. The results presented demonstrate test performance on both sets of material.

3.1.3.6.1. Enfer TSE V2 Manufacturer prepared dilution series

With reference to Table 3.04, a replicate dilution series from three BSE positive pools (Pools A, B, C) prepared by the manufacturers representatives were tested using the Enfer TSE V2 test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/256. This threshold of detection was observed for pool B with 1 of 5 replicates testing positive at a dilution factor of 1/256. The threshold of detection observed for pools A and C was a dilution factor of 1/128 with 5 of 5 replicates testing positive for Pool A and 4 of 5 replicates testing positive for Pool C at 1/128.

All 54 negative samples from Pool D (negative pooled homogenate) prepared by the manufacturers representatives tested negative using the Enfer V2 test.

When data from all 3 pools were compared it was observed that there was a consistent trend among the pools A and C with positive signals at a dilution of 1/128 with; overall a positive signal was detected down to a dilution factor of 1/256 for Pool B.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	12	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	4/5
Pool A	1/256	**Neg**	0/5	**Pool B**	1/256	**POS**	1/5	**Pool C**	1/256	**Neg**	0/5
Pool A	1/512	**Neg**	0/5	**Pool B**	1/512	**Neg**	0/5	**Pool C**	1/512	**Neg**	0/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**Neg**	0/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.04 Enfer TSE V2 Manufacturer prepared dilution series

3.1.3.6.2. Enfer TSE V2 CRL pre- prepared dilution series

With reference to Table 3.05, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the Enfer TSE V2 test and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/128. This threshold of detection was observed for Pool B and Pool C dilution series with 3 of 5 replicates and 1 of 5 replicates testing positive respectively. For Pool A a positive signal was detected at 1/64 dilution with a total number of 3 of 5 positive replicates recorded.

All 54 negative samples from Pool D pre-prepared by the CRL tested negative using the Enfer V2 test.

When data from all 3 pools were compared it was observed that there was a consistent trend between Pool B & C with positive signals detected down to a dilution factor of 1/128. The positive threshold for Pool A was one dilution less at 1/64.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	3/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	4/5
Pool A	1/128	**Neg**	0/5	**Pool B**	1/128	**POS**	3/5	**Pool C**	1/128	**POS**	1/5
Pool A	1/256	**Neg**	0/5	**Pool B**	1/256	**Neg**	0/5	**Pool C**	1/256	**Neg**	0/5
Pool A	1/512	**Neg**	0/5	**Pool B**	1/512	**Neg**	0/5	**Pool C**	1/512	**Neg**	0/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**Neg**	0/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.05 Enfer V2 TSE CRL pre-prepared dilution series

3.1.3.6.3. Enfer TSE V3 Manufacturer produced dilution series

With reference to Table 3.06, a replicate dilution series from three BSE positive pools (Pools A, B, C) prepared by the manufacturers representatives were tested using the Enfer TSE V3 test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/128. This threshold of detection was observed for 2 dilution series (pools A and B) with 1 of 5 replicates and 4 of 5 replicates testing positive for Pools A & B. A positive signal was recorded from a 1/2 dilution down to 1/64 for the Pool C dilution series with 5 of 5 replicates testing positive.

All 54 negative samples from Pool D (negative pooled homogenate) prepared by the manufacturers representatives tested negative using the Enfer V3 test.

When data from all 3 pools were compared it was observed that there was a consistent trend among all three pools with positive signals detected down to a dilution factor of 1/128.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	1/5	**Pool B**	1/128	**POS**	4/5	**Pool C**	1/128	**Neg**	0/5
Pool A	1/256	**Neg**	0/5	**Pool B**	1/256	**Neg**	0/5	**Pool C**	1/256	**Neg**	0/5
Pool A	1/512	**Neg**	0/5	**Pool B**	1/512	**Neg**	0/5	**Pool C**	1/512	**Neg**	0/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**Neg**	0/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.06 Enfer TSE V3 Manufacturer produced dilution series

3.1.3.6.4. Enfer TSE V3 CRL pre-prepared dilution series

With reference to Table 3.07, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the Enfer TSE V3 test and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/64. This threshold of detection was observed for Pools A, B and Pool C dilution series with 4 of 5 replicates testing positive for all pools.

All 54 negative samples from Pool D pre-prepared by the CRL tested negative using the Enfer V3 test.

When data from all 3 pools were compared it was observed that there was a consistent trend among all three pools with positive signals detected down to a dilution factor of 1/64.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	4/5	**Pool B**	1/64	**POS**	4/5	**Pool C**	1/64	**POS**	4/5
Pool A	1/128	**Neg**	0/5	**Pool B**	1/128	**Neg**	0/5	**Pool C**	1/128	**Neg**	0/5
Pool A	1/256	**Neg**	0/5	**Pool B**	1/256	**Neg**	0/5	**Pool C**	1/256	**Neg**	0/5
Pool A	1/512	**Neg**	0/5	**Pool B**	1/512	**Neg**	0/5	**Pool C**	1/512	**Neg**	0/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**Neg**	0/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.07 Enfer TSE V3 CRL pre-prepared dilution series

3.1.4 IDEXX Laboratories, Maine, USA

3.1.4.1. IDEXX HerdChek Standard, Short and Ultrashort Assay -Test Principle

The following information is extracted from the IDEXX HerdChek Standard, Short and Ultrashort Assay Test Kit insert:

The IDEXX HerdChek Bovine Spongiform Encephalopathy (BSE) Antigen Test Kit is an antigen capture enzyme immunoassay (EIA) for detection of the abnormal conformer of the prion protein (PrPSc) in post-mortem brain (obex preferred) tissues from bovines affected by BSE. It is designed to rapidly identify samples containing disease-associated PrPSc with minimal sample handling and can be automated for high throughput applications. This kit uses a proprietary method that allows detection of abnormal prions. A PrPSc-specific ligand is immobilized on the surface of the BSE antigen-capture plate. Test samples are prepared by homogenizing the tissues and then diluting the sample with working plate diluent. After the sample is applied to the plate, the disease-associated conformer binds to the immobilized ligand with high affinity. The plates are washed to remove unbound materials, including the normal conformer of the PrP protein. Following incubation with conditioning buffer, the captured antigen is then detected using a PrP-specific antibody that has been conjugated to horseradish peroxidase (HRPO). The plate is washed to remove unbound conjugate and a peroxidase substrate is added. Colour development is related to the relative amounts of PrPSc captured by the ligand immobilized in the microtitre plate well.

IDEXX offer three approved variations in BSE methodology for their HerdChek test kit, the Standard, Short and Ultrashort Assay. The three protocols have equivalent performance and the variation in conditions consists of different incubation conditions (temperature, duration of incubation & agitation conditions) for critical stages in the assay protocol, namely the initial incubation, capture plate incubation and conjugate incubation.

3.1.4.2. Interpretation and validation of results

Interpretation of sample results is based on the sample absorbance (A). A sample with A450–AREF (reference wavelength value read at 620-650 nm) less than the cut-off value is considered to be negative by the IDEXX HerdChek BSE Antigen Test Kit. Samples whose A450–AREF is greater than or equal to the cut-off are classified as positive for PrPSc. Retesting can be done from the original tissue homogenate or from homogenate prepared using the optional heat treatment protocol, described below. If either retest value is equal to or greater than the test cut-off, the sample is considered positive. The sample is considered negative when both retest replicates are less than the test cut-off.

3.1.4.3. Period of Assessment

IDEXX representatives undertook this evaluation at the Molecular Pathogenesis Group facilities (MPG4) based at the Veterinary Laboratories Agency, Weybridge, UK. The testing of BSE samples took place between 22 – 25th September 2008 under observation by CRL representatives.

3.1.4.4. Rapid Test Kit information

IDEXX HerdChek BSE-scrapie test kit Lot number KC 561 (Expiry date 30 Oct, 2008) was used throughout the testing. The current manufacturer user instructions 06-04813-1106-08519-05 Version #11#5 were used throughout the testing period. The 'CC' conjugate concentrate for bovine tissue, was used for all testing.

3.1.4.5. Problem Samples and Testing Issues

No problem samples or repeated samples were encountered during the course testing.

3.1.4.6. IDEXX HerdChek Standard, Short and Ultrashort Assay - Analytical Sensitivity Results

IDEXX opted to undertake testing only of CRL pre-prepared sample dilution series. The results presented demonstrate test performance on CRL prepared sample dilution series.

3.1.4.6.1. IDEXX Bovine HerdChek Standard Assay Protocol CRL pre-prepared dilution series

With reference to Table 3.08, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the IDEXX HerdChek Standard Assay Protocol and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/1024. This threshold of detection was observed for Pools A, B and C with 1 /5, 3/ 5 and 2/5 replicates testing positive for all pools respectively.

All 54 negative samples from Pool D pre-prepared by the CRL tested negative using the IDEXX HerdChek Standard Assay Protocol.

When data from all 3 pools were compared it was observed that there was a consistent trend among pools A, B and C with positive signals detected down to a dilution factor of 1/1024.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	5/5	**Pool B**	1/256	**POS**	5/5	**Pool C**	1/256	**POS**	5/5
Pool A	1/512	**POS**	5/5	**Pool B**	1/512	**POS**	5/5	**Pool C**	1/512	**POS**	5/5
Pool A	1/1024	**POS**	1/5	**Pool B**	1/1024	**POS**	3/5	**Pool C**	1/1024	**POS**	2/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.08 IDEXX Bovine HerdChek Standard Assay Protocol CRL pre-prepared dilution series

3.1.4.6.2. IDEXX Bovine HerdChek Short Assay Protocol CRL pre-prepared dilution series

With reference to Table 3.09, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the IDEXX HerdChek Short Assay Protocol and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/1024. This threshold of detection was observed for Pools A, B and BC with 2/5 replicates testing positive for bothall pools respectively. A positive signal was detected from a 1/2 dilution down to 1/512 for Pool C dilution series with 5 / 5 replicates testing positive.

All 54 negative samples from Pool D pre-prepared by the CRL tested negative using the IDEXX HerdChek Short Assay Protocol.

When data from all 3 pools were compared it was observed that there was a consistent trend between pools A &, B and C with positive signals detected down to a dilution factor of 1/1024. The positive threshold for Pool C was one dilution less at 1/1024.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	5/5	**Pool B**	1/256	**POS**	5/5	**Pool C**	1/256	**POS**	5/5
Pool A	1/512	**POS**	5/5	**Pool B**	1/512	**POS**	5/5	**Pool C**	1/512	**POS**	5/5
Pool A	1/1024	**POS**	2/5	**Pool B**	1/1024	**POS**	2/5	**Pool C**	1/1024	**POS**	2/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.09 IDEXX Bovine HerdChek Short Assay Protocol CRL pre-prepared dilution series

3.1.4.6.3. IDEXX Bovine HerdChek Ultrashort Assay Protocol CRL pre-prepared dilution series

With reference to Table 3.10, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the IDEXX HerdChek Ultrashort Assay Protocol and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/1024. This threshold of detection was observed for Pools A and B with 1/5 and 2/5 replicates testing positive respectively. A positive signal was detected from a 1/2 dilution down to 1/512 for Pool C dilution series with 5 of 5 replicates testing positive.

All 54 negative samples from Pool D pre-prepared by the CRL tested negative using the IDEXX test.

When data from all 3 pools were compared it was observed that there was a consistent trend between pools A & B with positive signals detected down to a dilution factor of 1/1024. The positive threshold for Pool C was one dilution less at 1/512.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	POS	2/2	Pool B	1/2	POS	2/2	Pool C	1/2	POS	2/2
Pool A	1/4	POS	2/2	Pool B	1/4	POS	2/2	Pool C	1/4	POS	2/2
Pool A	1/8	POS	5/5	Pool B	1/8	POS	5/5	Pool C	1/8	POS	5/5
Pool A	1/16	POS	5/5	Pool B	1/16	POS	5/5	Pool C	1/16	POS	5/5
Pool A	1/32	POS	5/5	Pool B	1/32	POS	5/5	Pool C	1/32	POS	5/5
Pool A	1/64	POS	5/5	Pool B	1/64	POS	5/5	Pool C	1/64	POS	5/5
Pool A	1/128	POS	5/5	Pool B	1/128	POS	5/5	Pool C	1/128	POS	5/5
Pool A	1/256	POS	5/5	Pool B	1/256	POS	5/5	Pool C	1/256	POS	5/5
Pool A	1/512	POS	5/5	Pool B	1/512	POS	5/5	Pool C	1/512	POS	5/5
Pool A	1/1024	POS	1/5	Pool B	1/1024	POS	2/5	Pool C	1/1024	Neg	0/5
Pool A	1/2048	Neg	0/5	Pool B	1/2048	Neg	0/5	Pool C	1/2048	Neg	0/5
Pool A	1/4096	Neg	0/5	Pool B	1/4096	Neg	0/5	Pool C	1/4096	Neg	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	Neg	54/54

Table 3.10 IDEXX Bovine HerdChek Ultrashort Assay Protocol CRL pre-prepared dilution series

3.1.5 Roche Diagnostics, Penzberg, Germany

3.1.5.1. Prionscreen -Test Principle

The following information is extracted from the Prionscreen -Test Kit insert:

The Prionscreen test is designed for the *in vitro* determination of the pathological form of Prion Protein (PrP^{SC}) in brain tissue sample of cattle as marker for Bovine Spongiform Encephalopathy (BSE). The Prionscreen test uses Proteinase K for the digestion and removal of common form of Prion Protein (PrP^{c}) and an Enzyme-Linked ImmunoSorbent Assay (ELISA) for the detection of the remaining PrP (27–30) in streptavidin-coated microplates.

Briefly, a sample of the brain tissue is homogenized to solubilise PrP^{C} and PrP^{SC}. Proteinase K completely digests PrPc, whereas PrP^{SC} resists the protease and remains available for the detection. Proteinase K digestion is stopped and PrP^{SC} is dissociated and unfolded to PrP (27-30) which can be recognized by specific antibodies.

PrP (27-30) is simultaneously bound by the biotin-labelled capture antibody and by the peroxidase-conjugated detection antibody. This complex binds via the biotin-labelled antibody to the streptavidin-coated surface of the microplate. After some washing steps tetramethylbenzidine (TMB) as substrate for the peroxidase function of the detection antibody is added and the developed colour is measured photometrically. The colour is proportional to the concentration of PrP (27–30).

3.1.5.2. Interpretation and validation of results

The absorbance values of the negative control are used to calculate the medians used for setting the cut-off value. The median for the samples and the positive control are used to verify the test function. For data interpretation at least 8 samples have to be analyzed on one microplate. The median for the positive controls has to be equal to, or above OD 1.2. Only 2 values out of 8 positive controls are accepted with a deviation from the median of more than 20 %. The median for the negative controls has to be equal to, or below OD 0.2. Only 2 values out of 8 negative controls are accepted above OD 0.2. If the validity requirements have not been met, the test has to be repeated. For evaluation purposes the number of expected negative samples must be higher than the expected number of positive samples. Over-readings are interpreted as OD 4.0.The validity of the cut-off is justified by using the median of the samples and the cut-off value.

For the purposes of this particular study the sample set was tested as presented by the CRL to the manufacturers, consequently the number of expected negative samples was not higher in number than the expected number of positive samples

3.1.5.3. Period of Assessment

A CRL representative visited Roche at their laboratory in Munich, Germany. The testing of BSE samples took place between 23rd and 25th September 2008 under observation by the CRL.

3.1.5.4. Rapid Test Kit information

Purification kit Lot number 1479200, Expiry date 31.08.09, was used throughout the testing. The current manufacturer user instructions Version July 2007 were used throughout the testing period.

3.1.5.5. Problem Samples and Testing Issues

No problem samples or repeated samples were encountered in the course of this test study.

3.1.5.6. Prionscreen Test Results

Roche opted to undertake testing only of the CRL pre-prepared sample dilution series. The results presented therefore demonstrate test performance on CRL prepared sample dilution series.

3.1.5.6.1. Prionscreen Test CRL pre-prepared dilution series

With reference to Table 3.11, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the Prionscreen Test and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/128. This threshold of detection was observed for Pools A, B and Pool C dilution series with 5 of 5 replicates testing positive for all pools.

All 54 negative samples from Pool D pre-prepared by the CRL tested negative using the Prionscreen test.

When data from all 3 pools were compared it was observed that there was a consistent trend among the three pools (A,B & C) with positive signals detected down to a dilution factor of 1/128.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive Replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive Replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive Replicates
Pool A	1/2	POS	2/2	Pool B	1/2	POS	2/2	Pool C	1/2	POS	2/2
Pool A	1/4	POS	2/2	Pool B	1/4	POS	2/2	Pool C	1/4	POS	2/2
Pool A	1/8	POS	5/5	Pool B	1/8	POS	5/5	Pool C	1/8	POS	5/5
Pool A	1/16	POS	5/5	Pool B	1/16	POS	5/5	Pool C	1/16	POS	5/5
Pool A	1/32	POS	5/5	Pool B	1/32	POS	5/5	Pool C	1/32	POS	5/5
Pool A	1/64	POS	5/5	Pool B	1/64	POS	5/5	Pool C	1/64	POS	5/5
Pool A	1/128	POS	5/5	Pool B	1/128	POS	5/5	Pool C	1/128	POS	5/5
Pool A	1/256	Neg	0/5	Pool B	1/256	Neg	0/5	Pool C	1/256	Neg	0/5
Pool A	1/512	Neg	0/5	Pool B	1/512	Neg	0/5	Pool C	1/512	Neg	0/5
Pool A	1/1024	Neg	0/5	Pool B	1/1024	Neg	0/5	Pool C	1/1024	Neg	0/5
Pool A	1/2048	Neg	0/5	Pool B	1/2048	Neg	0/5	Pool C	1/2048	Neg	0/5
Pool A	1/4096	Neg	0/5	Pool B	1/4096	Neg	0/5	Pool C	1/4096	Neg	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	Neg	54/54

Table 3.11. Prionscreen Test CRL pre-prepared dilution series

3.1.6. Prionics AG, Schlieren-Zurich, Switzerland

3.1.6.1. Prionics®-Check PrioSTRIP -Test Principle & Interpretation/Validation of results

The following information is extracted from the *Prionics®-Check PrioSTRIP* Kit insert:

The Prionics®-Check PrioSTRIP follows a four step protocol, consisting of Homogenization, Protease Digestion, Pre-incubation and Detection. After sample cutting, defined sections of tissue (medulla oblongata) of the obex region in the brain stem test samples are homogenized. Treatment with Proteinase K degrades PrP^{c} completely while PrP^{Sc} is reduced to the 27 – 30 kD fragment. The proteolytic reaction is stopped, and PrP^{Sc} is detected in the Prionics®-Check PrioSTRIP assay. Digested homogenates are incubated with the antibody conjugate. PrP^{Sc} present in the homogenates binds to the conjugate, which is a latex bead-labelled monoclonal antibody. By dipping the PrioSTRIP® into the sample-conjugate-mixture, the flow through the membrane is started. PrP-Conjugate complexes are retained at the test line by the second (capture) antibody. Uncomplexed conjugate is bound at the control line serving as a control for the proper performance of the immunochromatographic assay.

Interpretation of results is visual. A PrioSTRIP® Visual Interpretation Sheet is supplied for each PrioSTRIP® Test Plate. The instructions on the Visual Interpretation Sheet indicate that a negative result is characterized by the observation of control line only in the result window. An initial reactive result is characterized by two lines i.e. both the Control line and the Test line (1-2 mm below the Control line) are visible. The test is invalid if no lines or only Test line are visible. The control line must appear in all samples.

Interpretation of results is either visual or using the PrioSCAN® software. For the analytical sensitivity study the PrioSCAN® software was used to interpret the results. The PrioSCAN® converts the blue lines on the strips into digital data. The values obtained with the Prio-SCAN® are given as Relative Density Units (RDU). The cutoff is lot dependent and provided with each new lot, encoded on the lot calibration sheet. The sample is:

Negative, if the value of the test line is below cut-off and the control line is present
Initial reactive, if the value of the test line is above cut-off and the control line is present
Invalid, if no control line is present

If the Negative Control or the functional Positive Control or both do not show the correct result, the plate is invalid and all samples on the plate have to be retested from the corresponding homogenates.
Results:

- All samples found to be initial reactive need to be retested in duplicate starting from their corresponding homogenates. In case one or both results are detected as positive or invalid, the result needs to be indicated to the National Reference Laboratory.
- All samples found to be invalid need to be retested (single) starting from their corresponding homogenates.

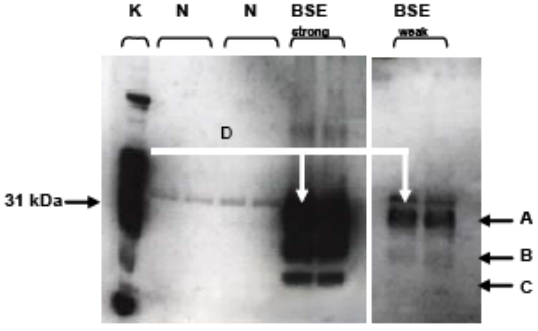
3.1.6.2. Prionics®-Check WESTERN BSE -Test Principle & Interpretation/Validation of results

The following information is extracted from the Prionics®-Check Western BSE Kit insert:

The Prionics®-Check WESTERN follows a five step protocol, consisting of Homogenization, Protease Digestion, Gel Electrophoresis, Blotting and Immunological Detection. After sample cutting, defined sections of tissue are homogenized from a defined piece of brain tissue. Treatment with Proteinase K degrades PrP^{c} completely while PrP^{Sc} is reduced to the 27 - 30 kD fragment. The proteolytic reaction is stopped, and PrP^{Sc} is detected in the Prionics®-Check WESTERN assay. Digested homogenates are subjected to gel electrophoresis and Western blotting. The blot membranes are incubated with a monoclonal antibody – with high affinity for PrP – for the detection of protease resistant PrP^{Sc}. The signal is visualized using the secondary antibody-alkaline phosphatase (AP) conjugate.

The following figure shows the expected band patterns of BSE-negative, BSE-positive and control samples, respectively. The control sample (K) contains the normal isoform of the prion protein (PrP^{c}) that is visualized via immunological detection. The corresponding diffuse band is spread from 25-35 kD due to glycosylation of PrP^{c} which causes a heterogeneous distribution. Negative samples (N) do not show a specific signal.

The 31 kD band (not always visible) results from unspecific binding of the secondary antibody to Proteinase K and can be used as an orientation aid. Positive samples (BSE strong; BSE weak) exhibit a signal consisting of three bands, the top one (A) corresponding to a protein with an approximate molecular weight of 30 kD. The signal intensity of all bands (in particular that of the lower bands B and C) can be weaker than depicted here, but the top band (A) should be clearly visible. The arrow (D) illustrates the difference in molecular weight between digested, pathological prion protein and the undigested, normal protein.



3.1.6.3. Prionics®-Check LIA BSE -Test Principle & Interpretation/Validation of results.

The following information is extracted from the Prionics®- Check LIA BSE Kit insert:

The Prionics®-Check LIA is a microplate based immunoassay (ELISA) which detects protease-resistant PrP^{Sc} in brain tissue homogenates. Prionics®-Check LIA achieves its high precision and reliability through the unique properties of the buffer solutions and the high affinity of the two monoclonal antibodies directed against the prion protein.

The values obtained by the plate luminometer are given as Relative Light Units (RLU) and calculated by the Prionics®-Check LIA Analysis Software for identification of positive and negative results. Alternatively, cut-off values may be calculated manually, following the same calculation protocol. The cut-off is calculated in five steps for each plate: This process allows both the general characteristics of the negative control and the individual characteristics of the particular plate into account. Step 1: The mean value of the Negative Controls (plate positions E1, E2, F1, F2, G1, G2, H1, H2) is calculated (NCM). Step 2: The mean value of the Negative Controls is multiplied by 10. This calculation defines the Negative Control Cut-off (NCC). Step 3: The mean of all sample values (plate positions A3 through H12) below NCC is calculated (SM). Step 4: The SM is multiplied by 10 to obtain the Sample Cut-off (SC). Step 5: Samples with values below the SC are identified negative. Samples with values above the SC are identified initially reactive. To ensure statistical representation, at least 8 samples have to be below the NCC. If less than 8 samples (per plate) are below the NCC in step 3, the NCC is taken as cut-off and samples above the NCC are identified as initially reactive.

For the analytical sensitivity study a set of known negative samples was used to calculate the SC. Otherwise low dilutions would artificially raise the cut-off.

3.1.6.4. Period of Assessment

CRL representatives visited Prionics AG at their laboratory in Zurich, Switzerland. The testing of BSE samples took place between 9th and 13th September 2008 under observation by the CRL. A second visit to Prionics AG was conducted between 27th and 30th October 2008, during which a CRL representative observed a repeat exercise of Western Blot and LIA tests for bovine samples.

3.1.6.5. Rapid Test Kit information

The Prionics®-Check PrioSTRIP BSE Kit Lot no. S80606A, the Prionics®-Check Western Kit Lot no W080808B Exp Date 20/05/2009 (Visit 1 and Visit 2), Prionics®–Check LIA BSE Kit Lot no JE118361 Exp date: 16/04/09 (1st Visit) JH122044 Exp Date 27/09/09 (2nd Visit) were used for the testing.

The current manufacturer user instructions for Prionics®-Check PrioSTRIP were Version 3.0_e, Prionics®-Check Western were Version 10.0 e and Prionics®–Check

LIA BSE were Version 4.20e. These versions were used throughout the testing period.

3.1.6.6. Repeated Samples & Testing Issues

During the first visit to Prionics, the manufacturers prepared a common set of samples which were then split for evaluation of all tests (Prionics®-Check PrioSTRIP, Prionics®-Check WESTERN, Prionics®-Check LIA).

In the course of the first visit to Prionics, it was observed that the Prionics®-Check LIA for BSE was not performing satisfactorily. In summary, for the Prionics®-Check LIA results it was observed that the background signal was high for the sample set prepared by Prionics AG (≈ 5,000 RLU) and very high for the CRL sample set (20,000 RLU). None of the samples tested scored a negative result. The investigation into sub-optimal outcome for the Prionics®-Check LIA testing during the first visit addressed several issues which may have contributed, including contamination of the laboratory environment and that CRL samples constitute a 50% water homogenate which may contribute to high background readings when tested with the Prionics®-Check LIA (see Annex 9 Prionics AG – Interim Report). The potential for contamination of the laboratory environment is still under investigation by Prionics.

Additionally, for the Prionics®-Check WESTERN it was observed that the results from membrane 8 and 9 did not match the expected results. Prionics conducted an investigation into the outcome of this testing and concluded that, for the Prionics®-Check WESTERN, samples were mixed up at the time of testing.

A second set of CRL pre-prepared samples and bulk positive tissue to make the manufacturer dilution series were despatched to Prionics. During the second visit to Prionics, the Prionics®-Check WESTERN was re-run successfully for all samples originally tested on membranes 8 and 9. The Prionics®-Check LIA was evaluated against both CRL and manufacturer dilution series. A complete data set was obtained for the Prionics®-Check LIA using the manufacturers' dilution series. No meaningful data were obtained for the CRL prepared dilution series.

During the first visit it was noted that the CRL pre-prepared, blinded dilution series were prepared differently from the protocol set out in the IFU. In summary, the CRL samples were diluted 1/5 with homogenisation working solution and not 1/10 as per the manufacturers instructions for use. The reason given for this adjustment was for the test to take account of the 50% homogenate starting point, rather than 100 % tissue as required by the protocol. By contrast, the manufacturers dilution series was made according to the IFU as the samples were pure tissue but, as recommended by the CRL, the starting material for each pool was diluted 1/1 with negative tissue to enable subsequent data points to be compared directly to the CRL series. Due to the deviation from the protocol during preparation of these blinded CRL sample series, there are data points present in the results table for these manufacturers tests that have a result for what constitutes a neat sample.

Additionally, the strategy described above, was only used during the first visit. During the second visit the IFU was followed for both CRL and manufacturer prepared

samples. Consequently, the results presented for the Prionics®-Check WESTERN constitute a combination of data presented for the first and second visit. As different dilution factors need to be accounted for depending on the visit, the resulting table of data comprises of dilution factors that have been adjusted according to their original preparation method. Hence the number of replicates listed for the Prionics®-Check WESTERN is different from the number listed for all other tests.

As stated in section 3.1.6.3, with reference to the Prionics®-Check LIA, for both BSE and Scrapie sample sets, the cut-off used for testing during the first visit was the NCC as it could not be guaranteed that each plate contained more than 8 samples which were true negatives. This is the method that Prionics always use when they are to test samples that are part of a dilution series, this strategy prevents the possibility of any low positive reactors on the plate influencing the calculation of the Sample Mean (SM) and thus causing an elevated sample cut-off (SC). This method was successful for the LIA Scrapie, but as noted below high background signals prevented results being gained for the LIA BSE.

During the second visit to Prionics, the high background values for the Prionics®-Check LIA BSE persisted. In order to gain an appropriate cut-off value, Prionics therefore repeated testing and included in-house BSE Negative QC samples for each plate. The cut-off value was therefore calculated using the average value of the QC samples multiplied by 10. There was not sufficient material from the CRL set for repeated testing, which is why only results from the manufacturers set are presented for the Prionics®-Check LIA BSE.

3.1.6.7. Test Results

Prionics opted to undertake testing of CRL pre-prepared sample dilution series and preparation and testing of a dilution series prepared from 4 tissue pools provided by the CRL. The results presented demonstrate test performance on CRL prepared sample dilution series and manufacturer prepared dilution series.

3.1.6.7.1. Prionics®-Check PrioSTRIP

3.1.6.7.1.1. Prionics®-Check PrioSTRIP Manufacturer produced dilution series

With reference to Table 3.12, a replicate dilution series from three BSE positive pools (Pools A, B, C) prepared by the manufacturers were tested using the Prionics®-Check PrioSTRIP and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1:256. This threshold of detection was observed for Pool B dilution series with 3 of 5 replicates testing positive. A positive signal was detected from a 1/2 dilution down to 1/ 128 was observed for Pools A and C with 5 of 5 replicates testing positive for both pools.

53 of 54 negative samples from Pool D prepared by the manufacturer tested negative using the Prionics®-Check PrioSTRIP test. One false positive result was recorded with a test line reading of 241 and Test Cut-off of 125.

When data from all 3 pools were compared it was observed that there was a consistent trend between pools A & C with positive signals detected down to a dilution factor of 1/128. The positive threshold for Pool B was one dilution more at 1/256.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**Neg**	0/5	**Pool B**	1/256	**POS**	3/5	**Pool C**	1/256	**Neg**	0/5
Pool A	1/512	**Neg**	0/5	**Pool B**	1/512	**Neg**	0/5	**Pool C**	1/512	**Neg**	0/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**Neg**	0/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	*53/54*

Table 3.12 Prionics®-Check PrioSTRIP Manufacturer produced dilution series

3.1.6.7.1.2. Prionics®-Check PrioSTRIP CRL produced dilution series

With reference to Table 3.13, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the Prionics®-Check PrioSTRIP and the current version of the manufacturers instructions for use. A positive signal was detected from a neat preparation down to 1/1024, with a single potentially spurious result detected at 1/2048. This threshold of detection was observed for Pool C dilution series with 1 of 5 replicates testing positive. A positive signal was detected from a 1/2 dilution down to 1/ 512 was observed for Pools A and B with 2 of 5 and 4 of 5 replicates testing positive for both pools.

47 of 54 negative samples from Pool D pre-prepared by the CRL tested negative using the Prionics®-Check PrioSTRIP test. Seven false positive results were recorded with OD readings ranging from 129 up to 2063, the Test Cut-off was 125.

When data from all 3 pools were compared it was observed that there was some variability among pools A, B & C with positive signals detected down to a dilution factor of 1/1024 for Pool C. The positive threshold for Pool A and B was one dilution less at 1/512. A positive result for Pool B was recorded for 1 of 5 samples at a dilution factor 1/2048.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	Neat	**POS**	2/2	**Pool B**	Neat	**POS**	2/2	**Pool C**	Neat	**POS**	2/2
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	5/5	**Pool B**	1/4	**POS**	5/5	**Pool C**	1/4	**POS**	5/5
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	3/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	4/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	2/5	**Pool B**	1/256	**POS**	3/5	**Pool C**	1/256	**POS**	1/5
Pool A	1/512	**POS**	2/5	**Pool B**	1/512	**POS**	4/5	**Pool C**	1/512	**POS**	3/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**POS**	1/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**POS**	1/5	**Pool C**	1/2048	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	*47/54*

Table 3.13 Prionics®-Check PrioSTRIP CRL produced dilution series

3.1.6.7.2. Prionics®-Check WESTERN

3.1.6.7.2.1. Prionics®-Check WESTERN Manufacturer produced dilution series

With reference to Table 3.14, a replicate dilution series from three BSE positive pools (Pools A, B, C) prepared by the manufacturers were tested using the Prionics®-Check WESTERN and the current version of the manufacturers instructions for use. A positive signal was detected from a 2 dilution down to 1/512. This threshold of detection was observed for Pools A, B and C dilution series with 4 of 5 replicates testing positive for pools A and B and 3 of 5 replicates testing positive for pool C.

All 54 negative samples from Pool D prepared by the manufacturers tested negative using the Prionics®-Check WESTERN test.

When data from all 3 pools were compared it was observed that there was a consistent trend among all pools with positive signals detected down to a dilution factor of 1/512.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	5/5	**Pool B**	1/256	**POS**	5/5	**Pool C**	1/256	**POS**	4/5
Pool A	1/512	**POS**	4/5	**Pool B**	1/512	**POS**	4/5	**Pool C**	1/512	**POS**	3/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**Neg**	0/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	54/54

Table 3.14 Prionics®-Check WESTERN Manufacturer produced dilution series

3.1.6.7.2.2. Prionics®-Check WESTERN CRL produced dilution series

With reference to Table 3.15, a replicate dilution series from three BSE positive pools (Pools A, B, C) previously prepared by the CRL were tested using the Prionics®-Check WESTERN and the current version of the manufacturers instructions for use. A positive signal was detected from a neat, and 1/2 dilution down to 1/ 512. This threshold of detection was observed for Pool B with 1 of 4 replicates testing positive. A positive signal was detected down to a dilution factor of 1/256 for Pools A and C.

All 54 negative samples from Pool D pre-prepared by the CRL tested negative using the Prionics®-Check WESTERN test.

When data from all 3 pools were compared it was observed that there was a consistent trend among all pools with positive signals detected down to a dilution factor of 1/512.

The number of replicates listed in Table 3.15 for the CRL samples - Prionics®-Check WESTERN Blot results is different to the number listed for all other tests due to variation in sample preparation during visit 1 and visit 2. The results as presented in Table 3.15 enable direct comparisons to now be made among the results for the different pools and with western blot results produced from manufacturer – prepared dilution series.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates §	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates §	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates §
								Pool C	Neat	POS	1/2
Pool A	1/2	POS	2/2	Pool B	1/2	POS	2/2	Pool C	1/1	POS	2/2
Pool A	1/4	POS	7/7	Pool B	1/4	POS	7/7	Pool C	1/4	POS	3/3
Pool A	1/8	POS	2/2	Pool B	1/8	POS	3/3	Pool C	1/8	POS	6/6
Pool A	1/16	POS	6/6	Pool B	1/16	POS	5/5	Pool C	1/16	POS	5/5
Pool A	1/32	POS	6/6	Pool B	1/32	POS	6/6	Pool C	1/32	POS	4/4
Pool A	1/64	POS	5/5	Pool B	1/64	POS	3/3	Pool C	1/64	POS	6/6
Pool A	1/128	POS	4/4	Pool B	1/128	POS	7/7	Pool C	1/128	POS	5/5
Pool A	1/256	POS	3/4	Pool B	1/256	POS	4/4	Pool C	1/256	POS	2/3
Pool A	1/512	Neg	0/6	Pool B	1/512	POS	1/4	Pool C	1/512	Neg	0/5
Pool A	1/1024	Neg	0/6	Pool B	1/1024	Neg	0/5	Pool C	1/1024	Neg	0/8
Pool A	1/2048	Neg	0/4	Pool B	1/2048	Neg	0/5	Pool C	1/2048	Neg	0/3
Pool A	1/4096	Neg	0/2	Pool B	1/4096	Neg	0/3	Pool C	1/4096	Neg	0/3

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	Neg	54/54

§ The sampling number of replicates listed for the Prionics Western Blot tests is different to the number listed for all other tests due to variation in sample preparation during visit 1 and visit 2.

Table 3.15 Prionics®-Check WESTERN CRL produced dilution series

3.1.6.7.3. Prionics®-Check LIA

3.1.6.7.3.1. Prionics®-Check LIA Manufacturer produced dilution series Visit 1.

With reference to Table 3.16, a replicate dilution series from three BSE positive pools (Pools A, B, C) prepared by the manufacturer were tested using the Prionics®-Check LIA and the current version of the manufacturers instructions for use. A positive signal was detected for all samples tested including the samples representing the negative pool (Pool D). No meaningful data was collected for this test during the first visit to Prionics.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	5/5	**Pool B**	1/256	**POS**	5/5	**Pool C**	1/256	**POS**	5/5
Pool A	1/512	**POS**	5/5	**Pool B**	1/512	**POS**	5/5	**Pool C**	1/512	**POS**	5/5
Pool A	1/1024	**POS**	5/5	**Pool B**	1/1024	**POS**	5/5	**Pool C**	1/1024	**POS**	5/5
Pool A	1/2048	**POS**	5/5	**Pool B**	1/2048	**POS**	5/5	**Pool C**	1/2048	**POS**	5/5
Pool A	1/4096	**POS**	5/5	**Pool B**	1/4096	**POS**	5/5	**Pool C**	1/4096	**POS**	5/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**POS**	***0/54***

Table 3.16 Prionics®-Check LIA Manufacturer produced dilution series Visit 1.

3.1.6.7.3.2. Prionics®-Check LIA CRL produced dilution series Visit 1.

With reference to Table 3.17, a replicate dilution series from three BSE positive pools (Pools A, B, C) pre-prepared by the CRL were tested using the Prionics®-Check LIA and the current version of the manufacturers instructions for use. A positive signal was detected for all samples tested including the samples representing the negative pool (Pool D). No meaningful data was collected for this test during the first visit to Prionics.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	5/5	**Pool B**	1/2	**POS**	5/5	**Pool C**	1/2	**POS**	5/5
Pool A	1/4	**POS**	5/5	**Pool B**	1/4	**POS**	5/5	**Pool C**	1/4	**POS**	5/5
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	5/5	**Pool B**	1/256	**POS**	5/5	**Pool C**	1/256	**POS**	5/5
Pool A	1/512	**POS**	5/5	**Pool B**	1/512	**POS**	5/5	**Pool C**	1/512	**POS**	5/5
Pool A	1/1024	**POS**	5/5	**Pool B**	1/1024	**POS**	5/5	**Pool C**	1/1024	**POS**	5/5
Pool A	1/2048	**POS**	5/5	**Pool B**	1/2048	**POS**	5/5	**Pool C**	1/2048	**POS**	5/5
Pool A	1/4096	**POS**	5/5	**Pool B**	1/4096	**POS**	5/5	**Pool C**	1/4096	**POS**	5/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**POS**	***0/54***

Table 3.17 Prionics®-Check LIA CRL produced dilution series – Visit 1

3.1.6.7.3.3. Prionics®-Check LIA Manufacturer produced dilution series Visit 2

With reference to Table 3.18 and Table 3.19, the Prionics®-Check LIA BSE data presented for CRL and manufacturer samples were generated during a second visit by the CRL to Prionics. A second CRL visit took place after no meaningful results were obtained for all samples tested with Prionics®-Check LIA BSE during the first visit.

With reference to Table 3.18 a replicate dilution series from three BSE positive pools (Pools A, B, C) prepared by the manufacturer were tested using the Prionics®-Check LIA and the current version of the manufacturers instructions for use.

A positive signal was detected from a 1/2 dilution down to 1/ 256. This threshold of detection was observed for Pools A, B and C dilution series with 1 of 5 replicates testing positive for pools A and pool B and 2 of 5 replicates testing positive for Pool C. A positive signal was detected from a 1/2 dilution down to 1/128 for Pool A samples with 1 of 5 replicates testing positive.

53 of 54 negative samples from Pool D prepared by the manufacturers tested negative using the Prionics®-Check LIA BSE test. One sample gave a false positive result with an RLU value of 11715 and Test Cut-off of 5470. It was observed that the the majority of the negative samples in pool D had high negative values.

When data from all 3 pools were compared it was observed that there was a consistent trend between pools A, B & C with positive signals detected down to a dilution factor of 1/256.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	2/2	**Pool B**	1/2	**POS**	2/2	**Pool C**	1/2	**POS**	2/2
Pool A	1/4	**POS**	2/2	**Pool B**	1/4	**POS**	2/2	**Pool C**	1/4	**POS**	2/2
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	3/5	**Pool B**	1/128	**POS**	4/5	**Pool C**	1/128	**POS**	3/5
Pool A	1/256	**POS**	1/5	**Pool B**	1/256	**POS**	1/5	**Pool C**	1/256	**POS**	2/5
Pool A	1/512	**Neg**	0/5	**Pool B**	1/512	**Neg**	0/5	**Pool C**	1/512	**Neg**	0/5
Pool A	1/1024	**Neg**	0/5	**Pool B**	1/1024	**Neg**	0/5	**Pool C**	1/1024	**Neg**	0/5
Pool A	1/2048	**Neg**	0/5	**Pool B**	1/2048	**Neg**	0/5	**Pool C**	1/2048	**Neg**	0/5
Pool A	1/4096	**Neg**	0/5	**Pool B**	1/4096	**Neg**	0/5	**Pool C**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	*53/54*

Table 3.18 Prionics®-Check LIA Manufacturer produced dilution series Visit 2

3.1.6.7.3.4. Prionics®-Check LIA CRL produced dilution series – Visit 2

With reference to Table 3.19, a replicate dilution series from three BSE positive pools (Pools A, B, C) pre-prepared by the CRL were tested using the Prionics®-Check LIA BSE and the current version of the manufacturers instructions for use. A positive signal was detected for all samples tested including the samples representing the negative pool (Pool D). No meaningful data was collected for this test during the second visit to Prionics.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool A	1/2	**POS**	5/5	**Pool B**	1/2	**POS**	5/5	**Pool C**	1/2	**POS**	5/5
Pool A	1/4	**POS**	5/5	**Pool B**	1/4	**POS**	5/5	**Pool C**	1/4	**POS**	5/5
Pool A	1/8	**POS**	5/5	**Pool B**	1/8	**POS**	5/5	**Pool C**	1/8	**POS**	5/5
Pool A	1/16	**POS**	5/5	**Pool B**	1/16	**POS**	5/5	**Pool C**	1/16	**POS**	5/5
Pool A	1/32	**POS**	5/5	**Pool B**	1/32	**POS**	5/5	**Pool C**	1/32	**POS**	5/5
Pool A	1/64	**POS**	5/5	**Pool B**	1/64	**POS**	5/5	**Pool C**	1/64	**POS**	5/5
Pool A	1/128	**POS**	5/5	**Pool B**	1/128	**POS**	5/5	**Pool C**	1/128	**POS**	5/5
Pool A	1/256	**POS**	5/5	**Pool B**	1/256	**POS**	5/5	**Pool C**	1/256	**POS**	5/5
Pool A	1/512	**POS**	5/5	**Pool B**	1/512	**POS**	5/5	**Pool C**	1/512	**POS**	5/5
Pool A	1/1024	**POS**	5/5	**Pool B**	1/1024	**POS**	5/5	**Pool C**	1/1024	**POS**	5/5
Pool A	1/2048	**POS**	5/5	**Pool B**	1/2048	**POS**	5/5	**Pool C**	1/2048	**POS**	5/5
Pool A	1/4096	**POS**	5/5	**Pool B**	1/4096	**POS**	5/5	**Pool C**	1/4096	**POS**	5/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool D (Negative Pool)	**Neg**	***0/54***

Table 3.19 Prionics®-Check LIA CRL produced dilution series – Visit 2

3.2 Analytical sensitivity for Classical Scrapie rapid tests

3.2.1 Bio-Rad, Marnes-la-Coquette, France

3.2.1.1. Bio-Rad TeSeE™ Short Assay Protocol Test & Bio-Rad TeSeE™ Sheep & Goat -Test Principle

The following information is extracted from the Bio-Rad TeSeE™ Short Assay Protocol Kit insert:

The Bio-Rad TeSeE™ Short Assay Protocol (SAP) Detection Kit is an immuno-enzymatic microplate method (sandwich format) using 2 monoclonal antibodies for the detection of the abnormal prion protein, resistant to proteinase K, in tissues collected from infected animals. Tissue homogenates are prepared by grinding in a preparation buffer using small glass beads and a homogeniser stage. Tissue homogenates are then purified prior to treatment with proteinase K. Samples aliquots then undergo antibody capture stages and a final antibody detection stage. Visualisation of the sample signal takes place via addition of peroxidase-labelled antibody that is incubated with the test samples. Addition of a peroxidase substrate enables a colorimetric reaction to be measured. The result is read as optical density value at a wavelength of 450 nm - 620 nm.

The following information is extracted from the Bio-Rad TeSeE™ Sheep & Goat (S&G) Detection Kit insert:

The Bio-Rad TeSeE™ Sheep/ Goat Detection Kit is an immuno-enzymatic microplate method (sandwich format) using 2 monoclonal antibodies for the detection of the abnormal prion protein from ovine and caprine samples, resistant to proteinase K, in tissues collected from infected animals. Tissue homogenates are prepared by grinding in a preparation buffer using small glass beads and a homogeniser stage. Tissue homogenates are then purified prior to treatment with proteinase K. Samples aliquots then undergo antibody capture stages and a final antibody detection stage. Visualisation of the sample signal takes place via addition of peroxidase-labelled antibody that is incubated with the test samples. Addition of a peroxidase substrate enables a colorimetric reaction to be measured. The result is read as optical density value at a wavelength of 450 nm - 620 nm.

3.2.1.2. Interpretation and validation of results

The Bio-Rad TeSeE™ Short Assay Protocol (SAP) Detection Kit set up requires that each experimental plate contains four negative controls and two positive controls. The cut –off values are calculated for every experimental plate by addition of the mean value of the four negative controls with a fixed value of 0.210. The mean of the positive control optical densities (R4 ODs) must be higher or equal to 1.000 when using the Bio-Rad SAP kit. The test must be repeated if the mean of the positive control optical densities (R4 ODs) is lower than this value.

Samples with an optical density lower than the cut-off value are considered to be negative. However, results located just below the cut-off value (cut-off value - 10%)

must be interpreted carefully. The manufacturers advise that in such cases the samples should be retested in duplicate, starting from the original homogenate. Samples with an optical density greater than or equal to the cut-off value are considered to be initially reactive according to the TeSeE™ Detection Kit and should be retested in duplicate, starting from the original homogenate, before a final interpretation of the results can take place.

The Bio-Rad TeSeE™ Sheep/Goat Detection Kit set up requires that each experimental plate contains four negative controls and two positive controls. The cut –off values are calculated for every experimental plate by addition of the mean value of the four negative controls with a fixed value of 0.14. The mean of the positive control optical densities (R4 ODs) must be higher or equal to 0.800 when using the Bio-Rad TeSeE™ Sheep/Goat kit. The test must be repeated if the mean of the positive control optical densities (R4 ODs) is lower than this value.

Samples with an optical density lower than the cut-off value are considered to be negative. However, results located just below the cut-off value (cut-off value - 10%) must be interpreted carefully. The manufacturers advise that in such cases the samples should be retested in duplicate, starting from the original homogenate. Samples with an optical density greater than or equal to the cut-off value are considered to be initially reactive according to the TeSeE™ Detection Kit and should be retested in duplicate, starting from the original homogenate, before a final interpretation of the results can take place.

3.2.1.3. Period of Assessment

CRL representatives visited Bio-Rad at their laboratory in Marnes-la-Coquette, France. The testing of BSE samples took place between 29th September – 3rd October 2008 under observation by the CRL.

3.2.1.4. Rapid Test Kit information

SAP Purification kit Lot number 8G 0064 and SAP Detection kit Lot Number 8F 0022 was used throughout the testing. Sheep/Goat Purification kit Lot number 8F 0017 and Sheep/Goat Detection kit Lot Number 8F 0017 was used throughout the testing. The current manufacturer user instructions Version Rev. A.4 - 12/2006 were used throughout the testing period.

All samples were processed using the semi-automatic processing stage of the purification protocol, the Bio-Rad New Sample Preparator (NSP) was used to undertake the purification stage according to IFU Rev. A.4 - 12/2006 and Version 2.0f (Ref.91460) of the NSP Manual.

3.2.1.5. Problem Samples and Testing Issues

The manufacturers decided to prepare a common set for each of the 4 scrapie negative pools that could then be used for both Sheep/Goat and SAP testing as this approach saved time and reagents.

It was noted that sample tissue OH0107-05, the negative pool X was a different colour to rest of negative sample pools. This sample was not used in the study and a duplicate negative sample from pool X bottle (SAP) was used instead.

The test results for several sample dilutions were identified as requiring cautious interpretation (i.e. according to the manufacturers kit instructions) inferring that the samples required retesting in duplicate.

3.2.1.6. Bio-Rad TeSeE™ SAP Test Analytical Sensitivity Results

Bio-Rad opted to undertake testing of CRL pre-prepared sample dilution series and preparation and testing of dilution series prepared from 4 tissue pools provided by the CRL. The results presented demonstrate test performance on CRL prepared sample dilution series and manufacturer prepared dilution series using CRL material.

3.2.1.6.1. Bio-Rad Short Assay Protocol Manufacturer prepared dilution series

With reference to Table 3.20 a replicate dilution series from three Scrapie positive pools (Pools X, Y, Z) prepared by the manufacturers were tested using the Bio-Rad Short Assay Protocol test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/512. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/64 and 1/128 dilution with a total number of 5 of 5 and 4 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) prepared by the manufacturers representatives tested negative using the Bio-Rad Short Assay Protocol test.

When data from all 3 pools were compared it was observed that there was some variability among the pools with positive thresholds ranging from a dilution factor of 1/64 to 1/512.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**Neg**	0/5	**Pool Y**	1/128	**POS**	4/5**	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**Neg**	0/5	**Pool Y**	1/256	**Neg**	0/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**POS**	5/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

** 1 Result recorded as requiring cautious interpretation according to manufacturers IFU

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.20 Bio-Rad Short Assay Protocol Manufacturer prepared dilution series

3.2.1.6.2. Bio-Rad Short Assay Protocol CRL pre-prepared dilution series

With reference to Table 3.21 a replicate dilution series from three Scrapie positive pools (Pools X, Y, Z) pre-prepared by the CRL were tested using the Bio-Rad Short Assay Protocol test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/256. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/64 and 1/128 dilution with a total number of 5 of 5 and 1 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) prepared by the manufacturers representatives tested negative using the Bio-Rad Short Assay Protocol test.

When data from all 3 pools were compared it was observed that there was some variability among the pools with positive thresholds ranging from a dilution factor of 1/64 to 1/256.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**Neg**	0/5	**Pool Y**	1/128	**POS**	1/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**Neg**	0/5	**Pool Y**	1/256	**Neg**	0/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**Neg**	0/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.21 Bio-Rad Short Assay Protocol CRL pre-prepared dilution series

3.2.1.6.3. Bio-Rad TeSeE™ Sheep/Goat Protocol Manufacturer prepared dilution series

With reference to Table 3.22 a replicate dilution series from three Scrapie positive pools (Pools X, Y, Z) prepared by the manufacturers were tested using the Bio-Rad TeSeE™ Sheep/Goat Protocol test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/2048. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X and Y a positive signal was detected at 1/512 dilution with a total number of 2 of 5 and 5 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) prepared by the manufacturers representatives tested negative using the Bio-Rad TeSeE™ Sheep/Goat test.

When data from all 3 pools were compared it was observed that there was some variability among the pools with positive thresholds ranging from a dilution factor of 1/512 to 1/2048 in the case of Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**POS**	5/5	**Pool Y**	1/128	**POS**	5/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**POS**	5/5	**Pool Y**	1/256	**POS**	5/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**POS**	2/5**	**Pool Y**	1/512	**POS**	5/5	**Pool Z**	1/512	**POS**	5/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5**	**Pool Z**	1/1024	**POS**	5/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**POS**	5/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5**

**** 5 Results recorded as requiring cautious interpretation according to manufacturers IFU**

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.22 Bio-Rad TeSeE™ Sheep/Goat Protocol Manufacturer prepared dilution series

3.2.1.6.4. Bio-Rad TeSeE™ Sheep/Goat Protocol CRL prepared dilution series

With reference to Table 3.23 a replicate dilution series from three Scrapie positive pools (Pools X, Y, Z) pre-prepared by the CRL were tested using the Bio-Rad Sheep/Goat test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/2048. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/512 dilution with a total number 3 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) prepared by the manufacturers representatives tested negative using the Bio-Rad TeSeE™ Sheep/Goat test.

When data from all 3 pools were compared it was observed that there was some variability among the pools with positive thresholds ranging from a dilution factor of 1/512 to 1/2048 in the case of Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**POS**	5/5	**Pool Y**	1/128	**POS**	5/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**POS**	5/5	**Pool Y**	1/256	**POS**	5/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**POS**	4/5	**Pool Y**	1/512	**POS**	4/5	**Pool Z**	1/512	**POS**	5/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**POS**	5/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**POS**	1/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

**** 1 Result recorded as requiring cautious interpretation according to manufacturers IFU**

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.23 Bio-Rad TeSeE™ Sheep/ Goat CRL pre-prepared dilution series

3.2.2 Enfer TSE Kit V2 & V3, Enfer Scientific Limited, Naas, Co.Kildare, Ireland

3.2.2.1. Enfer TSE Version 2 & Version 3 Test Kit -Test Principle

The following information is extracted from the Enfer TSE Version 2 & Version 3 Test Kit insert:

The Enfer TSE Kit Version 2.0 is an immunological method for the detection of the prion protein PrP from central nervous tissue of cattle, sheep and goats. A sample of central nervous tissue is homogenized and treated with Proteinase K under defined conditions and centrifuged. The supernatant is incubated in prepared microplate wells: during this incubation any PrPSc in the sample is bound to the wells. After a washing step the wells are treated with Enfer Buffer 3. After a second washing step rabbit anti-PrP is added to the well and incubated; if any PrPSc is present on the well this antiserum will specifically bind to it. After a third washing step goat anti-rabbit IgG conjugated to horseradish peroxidase is added to the wells and incubated; if any rabbit anti-serum is present on the well the conjugate will be bound. After a fourth wash any bound conjugate is detected using a luminogenic substrate for peroxidase

The Enfer TSE Kit Version 3.0 is also an immunological method for the detection of the prion protein PrP from central nervous tissue of cattle, sheep and goats. A sample of central nervous tissue is homogenized and treated with Proteinase K under defined conditions and centrifuged. The supernatant is incubated in prepared microplate wells: during this incubation any PrPSc in the sample is bound to the wells. After a washing step the wells are treated with Enfer Buffer 3. After a second washing step rabbit anti-PrP is added to the well and incubated; if any PrPSc is present on the well this antiserum will specifically bind to it. After a third washing step goat anti-rabbit IgG conjugated to horseradish peroxidase is added to the wells and incubated; if any rabbit anti-serum is present on the well the conjugate will be bound. At this stage in the protocol, for Enfer TSE Version 3 unbound secondary conjugate is washed away and a solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide is added to the wells. Wells with bound secondary conjugate develop a purple colour, which is converted to an orange colour when the reaction is stopped with sulphuric acid, the colour is read spectrophotometrically at 450nm. The amount of secondary conjugate, and hence colour, in the wells is directly related to the concentration of PrPSc in the sample.

3.2.2.2. Interpretation and validation of results for Enfer TSE Version 2 and Enfer TSE Version 3

When using Enfer TSE Version 2 (V2), the control results must be validated before the sample results can be interpreted. The mean luminescence of Peptide Indicator Wells and positive and negative Controls is determined and the median value for the Blank Control Reagent calculated. The values given are for measurements made on an Enfer recommended chemiluminometer. To calculate the median value of the Blank Control Reagent, the four Light Unit values are arranged in ascending numerical order. The median is the arithmetic average of the two middle values. The median of Blank Control Reagent replicates must be below 4.0 LU.

To calculate the mean value for the Peptide Indicator Wells, the mean value must be equal to or above the limit (as supplied for the individual lot) for the Peptide Indicator Wells (after subtraction of the median blank value). If negative control is run, the mean must be less than 5.5 LU after subtraction of the median blank reading. If the above criteria are not met, the EIA run is invalid and must be repeated. The threshold light signal for a suspect positive determination is 5.5 LU (after subtraction of the median blank reading) as measured on an Enfer recommended chemiluminometer. All samples giving signals greater than or equal to 5.5 LU (after subtraction of the median blank reading) in one or both duplicate wells must be considered initially reactive and must be retested in duplicate, starting from the tissue. A sample is considered positive when the retesting results give a positive signal in one or both wells.

When using Enfer Version 3 (V3) each plate must be considered separately when calculating and interpreting results of the assay. Approved software may be used for calculation and interpretation of results. The control results must be validated before the sample results can be interpreted. The Blank Control is calculated by calculating the median absorbance of the four replicates of the Enfer Buffer 1 blank control wells. The median is the arithmetic average of the two middle value when the data are arranged in numerical order. The median absorbance of the Blank Control replicates must be less than 0.2. The cut-off value was calculated by adding 0.3 to the Blank Control. The mean absorbance of the replicates of the Peptide Indicator Wells minus the Blank Control was calculated. The mean absorbance minus the Blank Control, of the Peptide Indicator Wells, must be greater than 0.8. If a Negative Control is run calculate the mean absorbance of the replicates, the mean minus the Blank Control must have a value of 0.3 or less. Samples giving an absorbance less than or equal to the Cut-off value are considered non-reactive in Enfer TSE Version 3. Samples giving an absorbance in one or both wells greater than the cut-off value, are considered initially reactive in the assay (see limitations of the procedure). Such samples must be retested in duplicate, starting from the tissue. A sample is considered positive when the retesting results give a signal greater than the cut-off value in one or both wells.

3.2.2.3. Period of Assessment

CRL representatives visited Enfer at their laboratory in Naas, Ireland. The testing of BSE & Scrapie samples took place between 6th October– 10th October 2008 under observation by the CRL.

3.2.2.4. Rapid Test Kit information

Enfer TSE Version 2 kit Lot number K08I08A (Manuf. Date 2008/05/29, Exp. Date 2008/11/29) and Enfer TSE Version 3 kit Lot number K09I08A (Manuf. Date 2008/09/09, Exp. Date 2009/01/07) were used throughout the testing. The current manufacturer user instructions for Version 2 (C104J06GB September 2007) or Version 3 (C016L72GB November 2007) were used throughout the testing period.

3.2.2.5. Problem Samples and Testing Issues

There were no problem samples or repeated samples. The manufacturers prepared a common set of samples which were then split for evaluation of both versions of the test (Enfer Version 2 and Enfer Version 3).

3.2.2.6. Enfer TSE Version 2 Test Analytical Sensitivity Results

3.2.2.6.1. Enfer TSE V2 Manufacturer prepared dilution series

Enfer opted to undertake testing of CRL pre-prepared sample dilution series and preparation and testing of dilution series prepared from 4 tissue pools provided by the CRL. The results presented demonstrate test performance on CRL prepared sample dilution series and manufacturer prepared dilution series using CRL material.

With reference to Table 3.24, a replicate dilution series from three classical scrapie positive pools (Pools X, Y, Z) prepared by the manufacturers representatives were tested using the Enfer TSE V2 test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/1024. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/512 and 1/256 dilutions respectively with a 1 of 5 and 4 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) prepared by the manufacturers tested negative using the Enfer V2 test.

When data from all 3 pools were compared it was observed that there was some variability among the pools with positive thresholds ranging from a dilution factor of 1/256 to 1/1024 in the case of Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	POS	2/2	Pool Y	1/2	POS	2/2	Pool Z	1/2	POS	2/2
Pool X	1/4	POS	2/2	Pool Y	1/4	POS	2/2	Pool Z	1/4	POS	2/2
Pool X	1/8	POS	5/5	Pool Y	1/8	POS	5/5	Pool Z	1/8	POS	5/5
Pool X	1/16	POS	5/5	Pool Y	1/16	POS	5/5	Pool Z	1/16	POS	5/5
Pool X	1/32	POS	5/5	Pool Y	1/32	POS	5/5	Pool Z	1/32	POS	5/5
Pool X	1/64	POS	5/5	Pool Y	1/64	POS	5/5	Pool Z	1/64	POS	5/5
Pool X	1/128	POS	5/5	Pool Y	1/128	POS	5/5	Pool Z	1/128	POS	5/5
Pool X	1/256	POS	4/5	Pool Y	1/256	POS	4/5	Pool Z	1/256	POS	5/5
Pool X	1/512	POS	1/5	Pool Y	1/512	Neg	0/5	Pool Z	1/512	POS	5/5
Pool X	1/1024	Neg	0/5	Pool Y	1/1024	Neg	0/5	Pool Z	1/1024	POS	5/5
Pool X	1/2048	Neg	0/5	Pool Y	1/2048	Neg	0/5	Pool Z	1/2048	Neg	0/5
Pool X	1/4096	Neg	0/5	Pool Y	1/4096	Neg	0/5	Pool Z	1/4096	Neg	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	Neg	54/54

Table 3.24 Enfer V2 Manufacturer prepared dilution series

3.2.2.6.2. Enfer TSE V2 CRL pre- prepared dilution series

With reference to Table 3.25, a replicate dilution series from three classical scrapie positive pools (Pools X, Y, Z) prepared by the CRL were tested using the Enfer TSE V2 test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/512. This threshold of detection was observed for Pool Z dilution series with 2 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/128 dilution with a total number of 5 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) pre-prepared by the CRL tested negative using the Enfer V2 test.

When data from all 3 pools were compared it was observed that there was some variability among the pools with positive thresholds ranging from a dilution factor of 1/128 to 1/512 in the case of Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	4/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**POS**	5/5	**Pool Y**	1/128	**POS**	5/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**Neg**	0/5	**Pool Y**	1/256	**Neg**	0/5	**Pool Z**	1/256	**POS**	4/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**POS**	2/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.25 Enfer V2 CRL pre-prepared dilution series

3.2.2.6.3. Enfer TSE V3 Manufacturer produced dilution series

With reference to Table 3.26, a replicate dilution series from three classical scrapie positive pools (Pools X, Y, Z) prepared by the manufacturers representatives were tested using the Enfer TSE V3 test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/512. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/128 dilution with a total number of 1 of 5 positive replicates recorded respectively.

53 negative samples from Pool W (negative pooled homogenate) prepared by the manufacturers representatives tested negative using the Enfer V3 test. One sample gave a false positive result with an OD reading of 1.762 with a test cut-off value of 0.300.

When data from all 3 pools were compared it was observed that there was some variability among the pools with positive thresholds ranging from a dilution factor of 1/2561/128 for pools A & BX & Y to 1/512 in the case of Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**POS**	5/5	**Pool Y**	1/128	**POS**	5/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**POS**	1/5	**Pool Y**	1/256	**POS**	1/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**POS**	5/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	53/54

Table 3.26 Enfer V3 Manufacturer prepared dilution series

3.2.2.6.4. Enfer TSE V3 CRL pre-prepared dilution series

With reference to Table 3.27, a replicate dilution series from three classical scrapie positive pools (Pools X, Y, Z) pre-prepared by the CRL were tested using the Enfer TSE V3 test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/256. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/128 dilution with a total number of 3 of 5 and 5 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) pre-prepared by the CRL tested negative using the Enfer V3 test.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**POS**	3/5	**Pool Y**	1/128	**POS**	5/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**Neg**	0/5	**Pool Y**	1/256	**Neg**	0/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**Neg**	0/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.27 Enfer V3 CRL pre-prepared dilution series

3.2.3 IDEXX Laboratories, Maine, USA

3.2.3.1. IDEXX HerdChek Standard, Short and Ultrashort Assay -Test Principle

The following information is extracted from the IDEXX HerdChek Standard, Short and Ultrashort Assay Test Kit insert:

The IDEXX HerdChek Bovine Spongiform Encephalopathy (BSE) Scrapie Antigen Test Kit is an antigen capture enzyme immunoassay (EIA) for detection of the abnormal conformer of the prion protein (PrPSc) in post-mortem brain (obex preferred) tissues from small ruminants (sheep and goats) affected by scrapie. It is designed to rapidly identify samples containing disease-associated PrPSc with minimal sample handling and can be automated for high throughput applications. This kit uses a proprietary method that allows detection of abnormal prions. A PrPSc-specific ligand is immobilized on the surface of the scrapie antigen-capture plate. Test samples are prepared by homogenizing the tissues and then diluting the sample with working plate diluent. After the sample is applied to the plate, the disease-associated conformer binds to the immobilized ligand with high affinity. The plates are washed to remove unbound materials, including the normal conformer of the PrP protein. Following incubation with conditioning buffer, the captured antigen is then detected using a PrP-specific antibody that has been conjugated to horseradish peroxidase (HRPO). The plate is washed to remove unbound conjugate and a peroxidase substrate is added. Colour development is related to the relative amounts of PrPSc captured by the ligand immobilized in the microtitre plate well.

IDEXX offers three approved variations in Scrapie methodology for their HerdChek test kit, the Standard, Short and Ultrashort Assay. The variation in conditions consists of different incubation conditions (temperature, duration of incubation & shaking conditions) for critical stages in the assay protocol, namely the initial incubation, capture plate incubation and conjugate incubation.

3.2.3.2. Interpretation and validation of results

Interpretation of sample results is based on the sample absorbance. A sample whose A450–AREF is less than the cut-off value is considered to be negative by the IDEXX HerdChek Scrapie Antigen Test Kit. Samples whose A450–AREF is greater than or equal to the cut-off are classified as positive for PrPSc. Retesting is undertaken from the original tissue homogenate or from homogenate prepared using the optional heat treatment protocol, described below. If either retest value is equal to or greater than the test cut-off, the sample is considered positive. The sample is considered negative when both retest replicates are less than the test cut-off value.

3.2.3.3. Period of Assessment

IDEXX representatives undertook this evaluation at the Molecular Pathogenesis Group facilities (MPG4) based at the Veterinary Laboratories Agency, Weybridge,

UK. The testing of scrapie samples took place between 22 – 25th September 2008 under observation by CRL representatives.

3.2.3.4. Rapid Test Kit information

IDEXX HerdChek BSE-scrapie kit Lot number KC 561 (Expiry Date 30 Oct, 2008) was used throughout the testing. The current manufacturer user instructions 06-08519-05 Version #05 were used throughout the testing period. The ''SRB-CC'' conjugate concentrate for small ruminant brain tissue, was used for all testing.

3.2.3.5. Problem Samples and Testing Issues

No problem samples or repeated samples were encountered in the course of this test study.

3.2.3.6. IDEXX HerdChek Standard, Short and Ultrashort Assay - Analytical Sensitivity Results

IDEXX opted to undertake testing only of CRL pre-prepared sample dilution series. The results presented demonstrate test performance on CRL prepared sample dilution series.

3.1.4.6.1. IDEXX HerdChek Standard Assay Protocol CRL pre-prepared dilution series

With reference to Table 3.28, a replicate dilution series from three scrapie positive pools (Pools X, Y, Z) previously prepared by the CRL were tested using the IDEXX Standard Protocol test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/2048. This threshold of detection was observed for Pool Z dilution series with 2 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/512 dilution with a total number of 2 of 5 positive replicates and 5 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) prepared by the CRL tested scrapie negative using the IDEXX HerdChek Standard Protocol.

When data from all 3 pools were compared it was observed that there was good comparability between pools A & B with positive thresholds recorded at a dilution factor of 1/512. A positive threshold was recorded at a dilution factor of to 1/2048 in the case of Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**POS**	5/5	**Pool Y**	1/128	**POS**	5/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**POS**	5/5	**Pool Y**	1/256	**POS**	5/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**POS**	2/5	**Pool Y**	1/512	**POS**	5/5	**Pool Z**	1/512	**POS**	5/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**POS**	5/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**POS**	2/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.28 IDEXX Standard Protocol CRL pre-prepared dilution series

3.1.4.6.2. IDEXX HerdChek Short Assay Protocol CRL pre-prepared dilution series

With reference to Table 3.29, a replicate dilution series from three classical scrapie positive pools (Pools X, Y, Z) pre-prepared by the CRL were tested using the IDEXX HerdChek Short Protocol and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/1024. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/512 dilution with a total number of 1 of 5 positive replicates and 5 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) prepared by the CRL tested negative using the IDEXX HerdChek Short Protocol.

When data from all 3 pools were compared it was observed that there was good comparability between pools A & B with positive thresholds recorded at a dilution factor of 1/512. A positive threshold was recorded at a dilution factor of to 1/20481/1024 in the case of Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**POS**	5/5	**Pool Y**	1/128	**POS**	5/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**POS**	5/5	**Pool Y**	1/256	**POS**	5/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**POS**	1/5	**Pool Y**	1/512	**POS**	5/5	**Pool Z**	1/512	**POS**	5/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**POS**	5/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.29 IDEXX Short Protocol CRL pre-prepared dilution series

3.1.4.6.3. IDEXX HerdChek Ultrashort Assay Protocol CRL pre-prepared dilution series

With reference to Table 3.30, a replicate dilution series from three classical scrapie positive pools (Pools X, Y, Z) previously pre-prepared by the CRL were tested using the IDEXX Ultrashort Protocol test and the current version of the manufacturers instructions for use. A positive signal was recorded from a 1/2 dilution down to 1/1024. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. For Pools X & Y a positive signal was detected at 1/256 and 1/512 dilutions with a total number of 5 of 5 positive replicates and 4 of 5 positive replicates recorded respectively.

All 54 negative samples from Pool W (negative pooled homogenate) prepared by the CRL tested negative using the IDEXX Ultrashort Protocol test.

When data from all 3 pools were compared it was observed that there was some variability among results for all pools with positive thresholds recorded between a dilution factor of 1/256 and 1/1024.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	5/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**POS**	5/5	**Pool Y**	1/128	**POS**	5/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**POS**	5/5	**Pool Y**	1/256	**POS**	5/5	**Pool Z**	1/256	**POS**	5/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**POS**	4/5	**Pool Z**	1/512	**POS**	5/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**POS**	5/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.30 IDEXX Ultrashort Protocol CRL pre-prepared dilution series

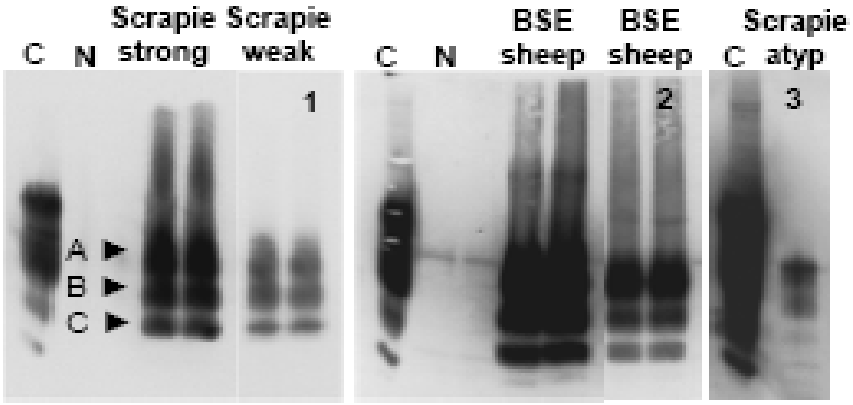
3.2.4. Prionics AG, Schlieren-Zurich, Switzerland

3.2.4.1.Prionics®-Check WESTERN SR -Test Principle & Interpretation/Validation of results

The following information is extracted from the Prionics®- Check WESTERN SR Kit insert:

The Prionics®-Check WESTERN SR follows a five step protocol, consisting of Homogenization, Protease Digestion, Gel Electrophoresis, Blotting and Immunological Detection. After sample cutting, defined sections of tissue are homogenized from a defined piece of brain tissue. Treatment with Proteinase K degrades PrP^c completely while PrP^{Sc} is reduced to the 27 - 30 kD fragment. The proteolytic reaction is stopped, and PrP^{Sc} is detected in the Prionics®-Check WESTERN SR assay. Digested homogenates are subjected to gel electrophoresis and Western blotting. The blot membranes are incubated with monoclonal antibodies – with high affinity for PrP – for the detection of protease resistant PrP^{Sc}. The signal is visualized using the secondary antibody-alkaline phosphatase (AP) conjugate.

The following figure shows the expected band patterns of scrapie-negative, scrapie - positive and control samples, respectively. The control sample (C) contains the normal isoform of the prion protein (PrP^c) that is visualized via immunological detection. The corresponding diffuse band is spread from 25-35 kD due to glycosylation of PrP^c which causes a heterogeneous distribution.



Negative samples (N) do not show a specific signal. The 31 kD band (not always visible) results from unspecific binding of the secondary antibody to Proteinase K and can be used as an orientation aid. Scrapie and BSE in sheep positive samples exhibit a signal consisting of three bands (scrapie $_{strong}$) the top one (A) corresponding to a protein with an approximate molecular weight of just below 30 kD. The signal intensity of all bands (in particular that of the lower bands B and C) can be weaker than depicted here, but the top band (A) should be clearly visible in the case of atypical scrapie/NOR 98 a more diffuse pattern of PrP immunoreactivity is observed.

3.2.4.2. Prionics® –Check LIA SR -Test Principle & Interpretation/Validation of results

The following information is extracted from the Prionics®- Check LIA SR Kit insert:

The Prionics®-Check LIA SR is a microplate based immunoassay (ELISA) which detects protease-resistant PrP^{Sc} in brain tissue homogenates. Prionics®-Check LIA achieves its high precision and reliability through the unique properties of the buffer solutions and the high affinity of the two monoclonal antibodies directed against the prion protein.

The values obtained by the plate luminometer are given as Relative Light Units and calculated by the Prionics®-Check LIA SR Analysis Software for identification of positive and negative results. Alternatively, cut-off values may be calculated manually, following the same calculation protocol. The cut-off is calculated in five steps for each plate: This process allows both the general characteristics of the negative control and the individual characteristics of the particular plate into account. Step 1: The mean value of the Negative Controls (plate positions E1, E2, F1, F2, G1, G2, H1, H2) is calculated (NCM). Step 2: The mean value of the Negative Controls is multiplied by 10. This calculation defines the Negative Control Cut-off (NCC). Step 3: The mean of all sample values (plate positions A3 through H12) below NCC is calculated (SM). Step 4: The SM is multiplied by 10 to obtain the Sample Cut-off (SC). Step 5: Samples with values below the SC are identified negative. Samples with values above the SC are identified initially reactive. To ensure statistical representation, at least 8 samples have to be below the NCC. If less than 8 samples (per plate) are below the NCC in step 3, the NCC is taken as cut-off and samples above the NCC are identified as initially reactive.

For the analytical sensitivity study the NCC was used to interpret the results. Otherwise low dilutions would artificially raise the cut-off.

3.2.4.3. Period of Assessment

CRL representatives visited Prionics AG at their laboratory in Zurich, Switzerland. The testing of scrapie samples took place between 9th and 13th September 2008 under observation by the CRL.

3.2.4.4. Rapid Test Kit information

Prionics®-Check LIA SR Lot No. M080303A and Prionics®-Check WESTERN SR Lot. No. U71205A (Visit 1 and Visit 2) were used throughout the testing. The current manufacturer user instructions for Prionics®-Check WESTERN SR were Version 2.2 e and Prionics®–Check LIA SR were Version 1.7e used throughout the testing period.

3.2.4.5. Problem Samples and Testing Issues

During the first visit the manufacturers prepared a common set of samples which were then split for evaluation of both tests (Prionics®-Check LIA SR and Prionics®-Check WESTERN SR).

During the first visit it was also noted that the CRL pre-prepared dilution series were prepared differently to the protocol set out in the IFU. In summary, the CRL samples were diluted 1/5 with homogenisation working solution and not 1/10 as per the manufacturers instructions. The reason given for this adjustment was for the test to take account of the 50% homogenate starting point, rather than 100 % tissue as required by the protocol. By contrast, the manufacturers dilution series was made according to the IFU but, as recommended by the CRL the starting material for each pool was diluted with 1/1 with negative tissue to enable subsequent data points to be compared directly to the CRL series. Due to the deviation from the protocol during preparation of the CRL sample series, there are data points present in the results table for these manufacturers tests that have a result for what constitutes a neat sample.

3.2.4.6. Test Results

Prionics opted to undertake testing of CRL pre-prepared sample dilution series and preparation and testing of dilution series prepared from 4 tissue pools provided by the CRL. The results presented demonstrate test performance on CRL prepared sample dilution series and manufacturer prepared dilution series using CRL material.

3.2.4.6.1. Prionics®-Check WESTERN *SR*

3.2.4.6.1.1. Prionics®-Check WESTERN SR Manufacturer produced dilution series

With reference to Table 3.31 A replicate dilution series from three scrapie positive pools (Pools X, Y, Z) prepared by the manufacturers were tested using the Prionics®-Check WESTERN SR and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/ 256. This threshold of detection was observed for Pools Y and Z dilution series with 1 of 5 replicates testing positive for both pools. A positive signal was detected from a 1/2 dilution down to 1/ 64 for pool X with a threshold of detection of 2 of 5 replicates testing positive.

All 54 negative samples from Pool W prepared by the manufacturers tested negative using the Prionics®-Check WESTERN SR test.

When data from all 3 pools were compared it was observed that there was good comparability for results from pools Y & Z with positive thresholds recorded at a dilution factor of 1/256. The positive threshold for Pool X was recorded at a dilution factor of 1/64.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	4/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	5/5	**Pool Y**	1/32	**POS**	5/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**POS**	2/5	**Pool Y**	1/64	**POS**	5/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**Neg**	0/5	**Pool Y**	1/128	**POS**	4/5	**Pool Z**	1/128	**POS**	5/5
Pool X	1/256	**Neg**	0/5	**Pool Y**	1/256	**POS**	1/5	**Pool Z**	1/256	**POS**	1/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**Neg**	0/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.31 Prionics®-Check WESTERN SR Manufacturer produced dilution series.

3.2.4.6.1.2. Prionics®-Check Western SR CRL produced dilution series

With reference to Table 3.32A replicate dilution series from three scrapie positive pools (Pools X, Y, Z) pre-prepared by the CRL were tested using the Prionics®-Check WESTERN SR and the current version of the manufacturers instructions for use. A positive signal was detected from neat and 1/2 dilution down to 1/128. This threshold of detection was observed for Pool Z dilution series with 2 of 5 replicates testing positive for this pool. A positive signal was detected from a 1/2 dilution down to 1/32 for pool X and 1/2 dilution down to 1/64 for pool Y with a threshold of detection of 3 of 5 replicates and 1 of 5 testing positive respectively.

All 54 negative samples from Pool W pre-prepared by the CRL tested negative using the Prionics®-Check WESTERN SR test.

When data from all 3 pools were compared it was observed that there some variability among results for all pools with positive thresholds recorded between a dilution factor of 1/32 for Pool X and 1/128 for Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	Neat	**POS**	2/2	**Pool Y**	Neat	**POS**	2/2	**Pool Z**	Neat	**POS**	2/2
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	5/5	**Pool Y**	1/4	**POS**	5/5	**Pool Z**	1/4	**POS**	5/5
Pool X	1/8	**POS**	5/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**POS**	5/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**POS**	3/5	**Pool Y**	1/32	**POS**	4/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**Neg**	0/5	**Pool Y**	1/64	**POS**	1/5	**Pool Z**	1/64	**POS**	4/5
Pool X	1/128	**Neg**	0/5	**Pool Y**	1/128	**Neg**	0/5	**Pool Z**	1/128	**POS**	2/5
Pool X	1/256	**Neg**	0/5	**Pool Y**	1/256	**Neg**	0/5	**Pool Z**	1/256	**Neg**	0/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**Neg**	0/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.32 Prionics®-Check WESTERN SR CRL produced dilution series

3.2.4.6.2. Prionics®- Check LIA SR

3.2.4.6.2.1. Prionics®-Check LIA SR Manufacturer produced dilution series.

With reference to Table 3.33 A, a replicate dilution series from three scrapie positive pools (Pools X, Y, Z) prepared by the manufacturers were tested using the Prionics®-Check LIA SR and the current version of the manufacturers instructions for use. A positive signal was detected from a 1/2 dilution down to 1/ 64. This threshold of detection was observed for Pool Z dilution series with 5 of 5 replicates testing positive. A positive signal was detected from a 1/2 dilution down to 1/ 8 for pool X and 1/32 for Pool Y with a threshold of detection of 4 of 5 and 2/5 replicates testing positive respectively.

All 54 negative samples from Pool W prepared by the manufacturers tested negative using the Prionics®-Check LIA SR test.

When data from all 3 pools were compared it was observed that there was some variability among results for all pools with positive thresholds recorded between a dilution factor of 1/8 and 1/64.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	2/2	**Pool Y**	1/4	**POS**	2/2	**Pool Z**	1/4	**POS**	2/2
Pool X	1/8	**POS**	4/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**Neg**	0/5	**Pool Y**	1/16	**POS**	5/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**Neg**	0/5	**Pool Y**	1/32	**POS**	2/5	**Pool Z**	1/32	**POS**	5/5
Pool X	1/64	**Neg**	0/5	**Pool Y**	1/64	**Neg**	0/5	**Pool Z**	1/64	**POS**	5/5
Pool X	1/128	**Neg**	0/5	**Pool Y**	1/128	**Neg**	0/5	**Pool Z**	1/128	**Neg**	0/5
Pool X	1/256	**Neg**	0/5	**Pool Y**	1/256	**Neg**	0/5	**Pool Z**	1/256	**Neg**	0/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**Neg**	0/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5
Pool X	1/4096	**Neg**	0/5	**Pool Y**	1/4096	**Neg**	0/5	**Pool Z**	1/4096	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	54/54

Table 3.33 Prionics®-Check LIA SR Manufacturer produced dilution series

3.2.4.6.2.2. Prionics®-Check LIA SR CRL produced dilution series

With reference to Table 3.34, a replicate dilution series from three scrapie positive pools (Pools X, Y, Z) pre-prepared by the CRL were tested using the Prionics®-Check LIA SR and the current version of the manufacturers instructions for use. A positive signal was detected from a neat and 1/2 dilution down to 1/ 32, with a single potentially spurious result detected at 1/512. This threshold of detection was observed for Pools Z dilution series with 3 of 5 replicates testing positive for both pools. A positive signal was detected from a 1/2 dilution down to 1/ 8 for pools X and Y with a threshold of detection of 3 of 5 and 5 of 5 replicates testing positive for each pool respectively.

53 negative samples from Pool W pre-prepared by the CRL tested negative using the Prionics®-Check LIA SR test. One sample gave a false positive result with an RLU reading of 1037 and a test cut-off value of 391.0.

When data from all 3 pools were compared it was observed that there some variability among results for all pools with positive thresholds recorded between a dilution factor of 1/8 for Pools X and Y and 1/32 for Pool Z.

Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates	Pool Ref	Dilution	Manufacturer Test Result	Total Number of Positive replicates
Pool X	Neat	**POS**	2/2	**Pool Y**	Neat	**POS**	2/2	**Pool Z**	Neat	**POS**	2/2
Pool X	1/2	**POS**	2/2	**Pool Y**	1/2	**POS**	2/2	**Pool Z**	1/2	**POS**	2/2
Pool X	1/4	**POS**	5/5	**Pool Y**	1/4	**POS**	5/5	**Pool Z**	1/4	**POS**	5/5
Pool X	1/8	**POS**	3/5	**Pool Y**	1/8	**POS**	5/5	**Pool Z**	1/8	**POS**	5/5
Pool X	1/16	**Neg**	0/5	**Pool Y**	1/16	**Neg**	0/5	**Pool Z**	1/16	**POS**	5/5
Pool X	1/32	**Neg**	0/5	**Pool Y**	1/32	**Neg**	0/5	**Pool Z**	1/32	**POS**	3/5
Pool X	1/64	**Neg**	0/5	**Pool Y**	1/64	**Neg**	0/5	**Pool Z**	1/64	**Neg**	0/5
Pool X	1/128	**Neg**	0/5	**Pool Y**	1/128	**Neg**	0/5	**Pool Z**	1/128	**Neg**	0/5
Pool X	1/256	**Neg**	0/5	**Pool Y**	1/256	**Neg**	0/5	**Pool Z**	1/256	**Neg**	0/5
Pool X	1/512	**Neg**	0/5	**Pool Y**	1/512	**Neg**	0/5	**Pool Z**	1/512	**POS**	1/5
Pool X	1/1024	**Neg**	0/5	**Pool Y**	1/1024	**Neg**	0/5	**Pool Z**	1/1024	**Neg**	0/5
Pool X	1/2048	**Neg**	0/5	**Pool Y**	1/2048	**Neg**	0/5	**Pool Z**	1/2048	**Neg**	0/5

Pool Ref	Manufacturer Test Result	Total Number of Negative Replicates
Pool W (Negative Pool)	**Neg**	53/54

Table 3.34 Prionics®- Check LIA SR CRL produced dilution series.

3.3. Atypical Scrapie Stability Study.

A stability study was undertaken to establish whether dilution series of atypical scrapie brain homogenates retain their original positive signal after storage frozen at –80°C. Published data indicates that there are differences in stability observed for atypical versus classical scrapie[17,18,19.] Additionally, it has been suggested that over a time period atypical scrapie sample dilutions stored under low temperature conditions deteriorate in signal intensity, when tested by the CRL with both ELISA and Western blot methodologies [17,18,19]. This part of the study was carried out over a 4-week period and provided essential background information for establishing the CRL approach to the atypical scrapie sensitivity component of the project.

Briefly, samples were made by dividing up tissue derived from a field case (Sample ref: SS00564687, see Annex 6 for sample information). Several small samples of this material were removed and frozen at –70°C to act as controls for testing at specific time points in the stability study. The remainder of the tissue was processed by chopping tissue finely and then macerating portions of tissue in a Seward Stomacher for 120 seconds for 3 successive treatments, as a 1/2 tissue/nuclease free water homogenate. The portions were then mixed together and a subsequent dilution series made and aliquotted. The dilution series used for the study was as follows: 1/5, 1/10, 1/50, 1/200, 1/500, 1/750, 1/1000. The material was divided into aliquots.

One set of samples was tested immediately by the CRL using the Bio-Rad Western blot. The finely chopped tissue was used as a control (this was diluted at the time of testing 1/1 with nuclease free water). In order to be economical with tissue, once each dilution series had been made, the remaining samples were distributed, as detailed in both the Bio-Rad TeSeE™ SAP and Bio-Rad TeSeE™ Sheep/Goat test kit instructions, into the grinding tubes of each rapid test to be used. They were then stored at -80°C and tested after various periods of storage up to and including 4 weeks. The negative control was negative tissue and the positive control was the same positive tissue as used to prepare the dilution series. The positive sample was stored as tissue (rather than a sample prepared in water using the stomacher) and adjusted prior to testing in order to have the same tissue concentration as the 1/5 dilution. Using the western blot, the positive tissue sample and the 1/5 stomacher prepared sample gave similar results and the diluted sample was positive at a dilution of 1/50 when tested at 0, 2 and 4 weeks after preparation.

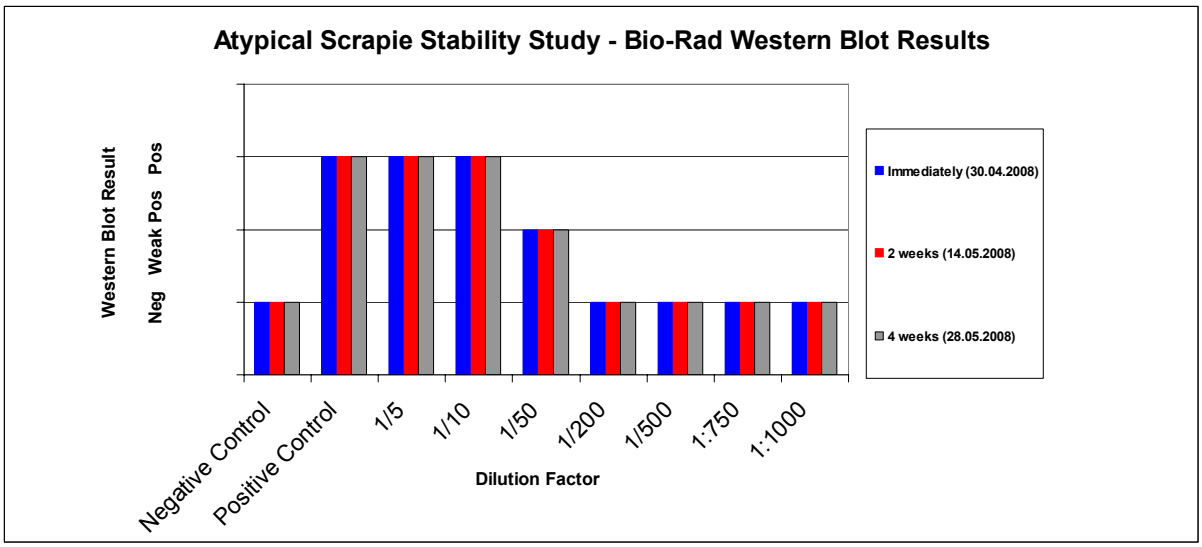


Figure 3.1 Results for Atypical Scrapie sample dilutions as determined with Bio-Rad Western Blot confirmatory test.

With reference to Fig 3.1 and Western Blot images (Annex 1) these data suggested similar results for all sample dilutions at different time points during the study period.

Overall the general trend for western blot analysis indicates that a positive result was observed for dilutions 1/5, 1/10 and 1/50 at each sampling point (0, 2 and 4 weeks). At week 4 the 1/50 dilution signal on the enhanced Sha31 blot image required further contrast to detect the diluted sample than for previous blots (0 and 2 weeks). The contrast of the image was enhanced to visualise the profile for the 1:50 diluted sample. All runs are detected for a fixed time (VLA actually undertake two exposure times for all blots - 1 & 10 min).

Minor variation in signal intensity is commonly seen when repeat WB testing of single samples or when testing different aliquots of the same homogenate. For each sample set throughout the stability/storage study this type of variability could be seen and the comment was made in case this was the starting point of a 'real' diminishing signal which would indicate degradation of the PrP.

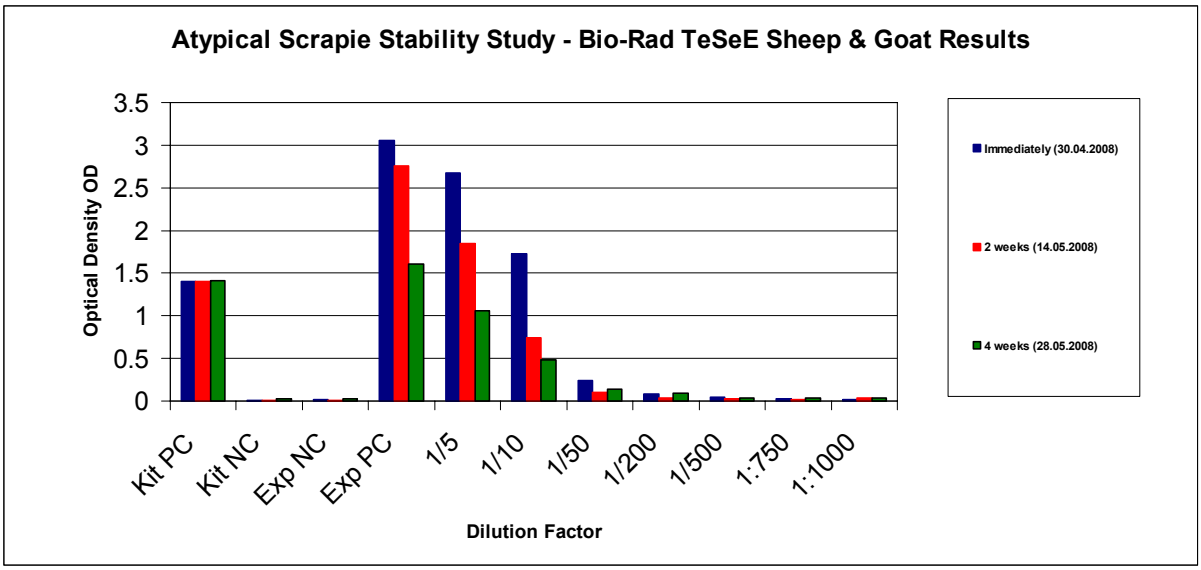


Figure 3.2: Optical Density results for atypical scrapie sample dilutions using Bio-Rad TeSeE™ Sheep & Goat ELISA test kit.

Results from the Bio-Rad TeSeE™ Sheep/Goat ELISA (Fig.3.2) suggested that there had been some degradation of the atypical scrapie samples occurring during the study period. If the results are assessed in terms of diagnostic result, the 1/50 dilution is considered positive at time 0 and the 1/5 and 1/10 dilutions at 2 and 4 weeks after storage. The experimental atypical positive control tissue sample (1/5 dilution) had higher readings at each time point than the 1/5 dilution of the scrapie positive homogenate. The experimental positive control was defined as stored positive tissue from the same source as the homogenates.

All samples in the stability study were also tested by the CRL with the IDEXX HerdChek kit (data not shown) that confirmed the data trends recorded for the Bio-Rad Western blot and Bio-Rad TeSeE™ Sheep/Goat ELISA.

3.4 Analytical sensitivity for Atypical Scrapie rapid tests

It was also important to evaluate analytical sensitivity for atypical scrapie, The atypical scrapie study was not designed in the same way as described for classical scrapie and BSE. Due to limited availability of material, it was not feasible to produce negative and positive pooled tissues for distribution to all manufacturers supporting scrapie/small ruminant rapid tests. Consequently the atypical scrapie study took the form of a proficiency-test circulation exercise rather than tissue delivery as in the BSE and classical scrapie exercise, because of the scarcity of suitable material. This was supported by results of the stability study. A “date of test” element was introduced such that no manufacturer was disadvantaged by any delays between times of sample production and testing.

Consequently a dilution series comprising 12 samples as a duplicate series blinded by the CRL, were despatched to each manufacturer of scrapie rapid detection kits. The panel comprised a dilution series of 10 dilutions and two negative samples prepared in duplicate. The samples were prepared on 10th November 2008. The CRL received atypical scrapie results from the manufacturers on 17th November 2008. The resultant data sets were analysed by the CRL.

Two manufacturers failed to detect the atypical samples (Enfer and Prionics) in the analytical sensitivity part of the atypical scrapie study. Consequently, as additional material had become available, a follow-up study was conducted using a larger panel of atypical scrapie samples from different animals. Twelve neat finely chopped tissue samples prepared as a duplicate series blinded by the CRL, were despatched to Prionics and Enfer for testing on 10th February 2009. The panel consisted of 7 atypical tissue samples from different areas of brain tissue (5 cerebellum samples and two rostral medulla samples), 2 classical scrapie samples and 3 negative whole brain samples. Prionics used the same aliquots for preparation of samples for testing of the Prionics®-Check WESTERN SR and Prionics®-Check LIA SR. Enfer used the same aliquots for preparation of samples for testing of the Enfer V2 and Enfer V3. The CRL received atypical scrapie results from the manufacturers on 17th February 2009.

All samples were also tested by Bio-Rad Western Blot and some were tested by Bio-Rad TeSeE™ (see Annex 6 for results). Two tissue samples (OH0134 & OH0137) originally included within the blind panel sent to the two manufacturers had anomolous results. Sample OH0134 had an inconclusive result and sample OH0137 had a negative result when analysed on the Bio-Rad Western Blot. These tissue samples were originally selected for inclusion within the extended study panel as they were IHC positive for atypical scrapie.

The resultant data sets were analysed by the CRL. The data from the follow–up atypical scrapie study are presented alongside the atypical scrapie analytical sensitivity data for both manufacturers (Prionics and Enfer).

3.4.1 Bio-Rad, Marnes-la-Coquette, France

3.4.1.1. Bio-Rad TeSeE™ Short Assay Protocol Test & Bio-Rad TeSeE™ Sheep / Goat-Test Principle

Please refer to Section 3.2.1.1 for protocol details.

3.4.1.2. Interpretation and validation of results

Please refer to Section 3.2.1.2 for result interpretation and validation.

3.2.1.3. Period of Assessment

CRL representatives despatched the analytical samples to Bio-Rad at their laboratory in Marnes-la-Coquette, France on Monday 11th November 2008. The testing of these samples took place on 14th November 2008, results were emailed back to the CRL on Monday 17th November 2008.

3.2.1.4. Rapid Test Kit information

TeSeE™ Sheep/Goat Purification kit Lot number 8F0017 (Expiry date: 05/03/2009) and TeSeE™ Sheep/Goat Detection kit Lot Number 8F0017 (Expiry date: 05/15/2009) was used throughout the testing. The TeSeE™ SAP Purification kit Lot number 8G0064 (Expiry date: 07/15/2009) and TeSeE™ SAP Detection kit Lot Number 8F0022 (Expiry date: 05/15/2009) was used throughout the testing of atypical scrapie samples. The current manufacturer user instructions Version Rev. A.4 - 12/2006 were used throughout the testing period.

3.2.1.5. Problem Samples and Testing Issues

One sample (Sample OH0120, 1/128 dilution of positive tissue homogenate) was tested with TeSeE™ Sheep/Goat kit, the interpretation of results was doubtful and theoretically (i.e. according to the manufacturers kit instructions) the samples required retesting in duplicate. This step was not undertaken as there was insufficient material.The results were recorded as negative but annotated accordingly (*) in Table 3.36.

3.2.1.6. Bio-Rad TeSeE™ SAP Test Analytical Sensitivity Results

Results shown in Table 3.35 indicate that the Bio-Rad TeSeE™ SAP kit detected replicate positive atypical samples down to a dilution of 1/128. The remaining dilutions were consistently negative.

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	POS	2/2
1/4	Positive	POS	2/2
1/8	Weak Positive	POS	2/2
1/16	Weak Positive	POS	2/2
1/32	Negative	POS	2/2
1/64	Negative	POS	2/2
1/128	Negative	POS	2/2
1/256	Negative	Neg	0/2
1/512	Negative	Neg	0/2
1/1024	Negative	Neg	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	Neg	2/2
Negative	Negative	Neg	2/2

Table 3.35 Bio-Rad TeSeE™SAP Protocol CRL prepared Atypical Scrapie dilution series

3.2.1.7. Bio-Rad TeSeE™ Sheep/GoatTest Analytical Sensitivity Results

Results shown in Table 3.36 indicate that the Bio-Rad TeSeE™ Sheep/ Goat kit detected replicate positive atypical samples down to a dilution of 1/128. The remaining dilutions were consistently negative. The interpretation of results for Sample OH120 was doubtful and theoretically the samples required retesting in duplicate.

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	POS	2/2
1/4	Positive	POS	2/2
1/8	Weak Positive	POS	2/2
1/16	Weak Positive	POS	2/2
1/32	Negative	POS	2/2
1/64	Negative	POS	2/2
1/128	Negative	POS	1*/2
1/256	Negative	Neg	0/2
1/512	Negative	Neg	0/2
1/1024	Negative	Neg	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	Neg	2/2
Negative	Negative	Neg	2/2

*** Manufacturer comment for negative sample:**
'Doubtful. Should be retested in duplicate'

Table 3.36 Bio-Rad TeSeE™ Sheep/Goat Protocol CRL prepared Atypical Scrapie dilution series

3.4.2 Enfer TSE Kit V2 & V3, Enfer Scientific Limited, Naas, Co. Kildare, Ireland

3.4.2.1. Enfer TSE Version 2 & Version 3 Test Kit -Test Principle

Please refer to Section 3.2.2.1 for protocol details.

3.4.2.2. Interpretation and validation of results for Enfer TSE Version 2 and Enfer TSE Version 3

Please refer to Section 3.2.2.2 for protocol details.

3.4.2.3. Period of Assessment

CRL representatives despatched the analytical samples to Enfer Scientific at their laboratory in Naas, Ireland on Monday 11th November 2008. The testing of these samples took place on Monday 17th November 2008, results were emailed back to the CRL on Wednesday 19th November 2008.

A further study was carried out on a panel of neat tissue samples. The testing of these samples took place on 11th February 2009, results were emailed back to the CRL on Wednesday 17th February 2009.

3.4.2.4. Rapid Test Kit information

Enfer TSE Version 2 kit Lot number K08I08A (Manuf. Date 2008/05/29, Exp. Date 2008/11/29) and Enfer TSE Version 3 kit Lot number K09I08A (Manuf. Date 2008/09/09, Exp. Date 2009/01/07) were used throughout the testing. The current manufacturer user instructions for Version 2 (C104J06GB September 2007) or Version 3 (C026L72GB May 2008) were used throughout the testing period.

For the further study, Enfer TSE Version 2 kit Lot number K15L08A (Exp. Date 01/06/2009) and Enfer TSE Version 3 kit Lot number K01L08A (Exp. Date 29/10/2009) were used throughout the testing. The current manufacturer user instructions for Version 2 (C114J06GB October 2008) or Version 3 (C036L72GB October 2008) were used throughout the testing period.

3.4.2.5. Problem Samples and Testing Issues

There were no repeated samples or problem samples reported.

3.4.2.6. Enfer TSE Version 2 Test Analytical Sensitivity Results

Results shown in Table 3.37 indicate that the Enfer V2 kit failed to detect any positive atypical scrapie samples as part of a dilution series prepared by the CRL.

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	**Neg**	0/2
14	Positive	**Neg**	0/2
1/8	Weak Positive	**Neg**	0/2
1/16	Weak Positive	**Neg**	0/2
1/32	Negative	**Neg**	0/2
1/64	Negative	**Neg**	0/2
1/128	Negative	**Neg**	0/2
1/256	Negative	**Neg**	0/2
1/512	Negative	**Neg**	0/2
1/1024	Negative	**Neg**	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	**Neg**	2/2
Negative	Negative	**Neg**	2/2

Table 3.37 Enfer V2 CRL pre-prepared dilution series

Results shown in Table 3.38 indicate that the Enfer V2 kit failed to detect any positive atypical scrapie samples as part of a neat tissue panel prepared by the CRL. The Enfer V2 kit detected all positive classical scrapie samples included as part of the neat tissue panel prepared by the CRL.

Sample Ref.	Dilution	Expected Result based on IHC results	Area of brain sampled	CRL Western Blot Results 12.2.09	Manufacturer Test Result	Total Number of Positive Replicates
OH0140	Neat Tissue	Atypical Scrapie Positive	Cerebellum	POS	Neg	0/2
OH0139	Neat Tissue	Atypical Scrapie Positive	Cerebellum	POS	Neg	0/2
OH138	Neat Tissue	Atypical Scrapie Positive	Rostral medulla	POS	Neg	0/2
OH0137	Neat Tissue	Atypical Scrapie Positive	Cerebellum	Neg	Neg	0/2
OH0136	Neat Tissue	Atypical Scrapie Positive	Cerebellum	POS	Neg	0/2
OH0135	Neat Tissue	Atypical Scrapie Positive	Cerebellum	POS	Neg	0/2
OH0134	Neat Tissue	Atypical Scrapie Positive	Rostral medulla	Inconclusive	Neg	0/2
OH0133	Neat Tissue	Classical Scrapie Positive	Cerebellum & Rostral medulla	POS	POS	2/2
OH0132	Neat Tissue	Classical Scrapie Positive	Cerebellum & Rostral medulla	POS	POS	2/2
Sample Ref.	**Dilution**	**Expected Result**	**Area of brain sampled**	**CRL Western Blot Results 12.2.09**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
OH0129	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	Neg	Neg	2/2
OH0130	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	Neg	Neg	2/2
OH0131	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	Neg	Neg	2/2

Table 3.38 Enfer V2 CRL pre-prepared neat tissue sample study

3.4.2.7. Enfer TSE Version 3 Test Analytical Sensitivity Results

Results shown in Table 3.38 indicate that the Enfer V3 kit failed to detect any positive atypical scrapie samples as part of a dilution series prepared by the CRL.

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	Neg	0/2
1/4	Positive	Neg	0/2
1/8	Weak Positive	Neg	0/2
1/16	Weak Positive	Neg	0/2
1/32	Negative	Neg	0/2
1/64	Negative	Neg	0/2
1/128	Negative	Neg	0/2
1/256	Negative	Neg	0/2
1/512	Negative	Neg	0/2
1/1024	Negative	Neg	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	Neg	2/2
Negative	Negative	Neg	2/2

Table 3.39 Enfer V3 CRL prepared dilution series

Results shown in Table 3.40 indicate that the Enfer V3 kit failed to detect any positive atypical scrapie samples as part of a neat tissue panel prepared by the CRL. The Enfer V3 kit detected all positive classical scrapie samples included as part of the neat tissue panel prepared by the CRL.

Sample Ref.	Dilution	Expected Result based on IHC results	Area of brain sampled	CRL Western Blot Results 12.2.09	Manufacturer Test Result	Total Number of Positive Replicates
OH0140	Neat Tissue	Atypical Scrapie Positive	Cerebellum	POS	Neg	0/2
OH0139	Neat Tissue	Atypical Scrapie Positive	Cerebellum	POS	Neg	0/2
OH138	Neat Tissue	Atypical Scrapie Positive	Rostral medulla	POS	Neg	0/2
OH0137	Neat Tissue	Atypical Scrapie Positive	Cerebellum	Neg	Neg	0/2
OH0136	Neat Tissue	Atypical Scrapie Positive	Cerebellum	POS	Neg	0/2
OH0135	Neat Tissue	Atypical Scrapie Positive	Cerebellum	POS	Neg	0/2
OH0134	Neat Tissue	Atypical Scrapie Positive	Rostral medulla	Inconclusive	Neg	0/2
OH0133	Neat Tissue	Classical Scrapie Positive	Cerebellum & Rostral medulla	POS	POS	2/2
OH0132	Neat Tissue	Classical Scrapie Positive	Cerebellum & Rostral medulla	POS	POS	2/2
Sample Ref.	**Dilution**	**Expected Result**	**Area of brain sampled**	**CRL Western Blot Results 12.2.09**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
OH0129	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	Neg	Neg	2/2
OH0130	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	Neg	Neg	2/2
OH0131	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	Neg	Neg	2/2

Table 3.40 Enfer V3 CRL prepared dilution series

3.4.3 IDEXX Laboratories, Maine, USA

3.4.3.1. IDEXX HerdChek Standard, Short and Ultrashort Assay-Test Principle

Please refer to Section 3.2.3.1 for protocol details.

3.4.3.2. Interpretation and validation of results

Please refer to Section 3.2.3.2 for protocol details.

3.4.3.3. Period of Assessment

IDEXX representatives undertook this evaluation at the Molecular Pathogenesis Group facilities (MPG4) based at the Veterinary Laboratories. The testing of atypical scrapie samples took place on 13th November 2008. Results were emailed back to the CRL on Monday 17th November 2008.

3.4.3.4. Rapid Test Kit information

Kit Lot number 99-08600 FD165 (Expiry Date; 2 June 2009), was used throughout the testing. The current manufacturer user instructions 06-08519-05 Version #05 were used throughout the testing period. The ‘’CRB-CC’’ conjugate concentrate, for small ruminant brain tissue, was used for all testing.

3.4.3.5. Problem Samples and Testing Issues

The manufacturers did not report any problems with samples or testing for this part of the study.

3.4.3.6. IDEXX HerdChek Standard Protocol (Scrapie Conjugate) - Analytical Sensitivity Results

Results shown in Table 3.41 indicate that the IDEXX HerdChek Standard Protocol detected replicate positive atypical samples down to a dilution of 1/16. The remaining dilutions were consistently negative. One of the two duplicate samples representative of the 1/1 dilution was recorded as negative by the manufacturer, the the second replicate for the 1/2 dilution was correctly identified as positive. The raw data indicate that this sample had a high negative average OD value (0.216) lying just below the negative/positive threshold (0.230).

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	POS	1/2*
1/4	Positive	POS	2/2
1/8	Weak Positive	POS	2/2
1/16	Weak Positive	POS	2/2
1/32	Negative	Neg	0/2
1/64	Negative	Neg	0/2
1/128	Negative	Neg	0/2
1/256	Negative	Neg	0/2
1/512	Negative	Neg	0/2
1/1024	Negative	Neg	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	Neg	2/2
Negative	Negative	Neg	2/2

***1/2 replicates scored a high negative OD value**

Table 3.41 IDEXX Standard Protocol CRL pre-prepared dilution series

3.4.3.7. IDEXX HerdChek Short Protocol (Scrapie Conjugate) - Analytical Sensitivity Results

Results shown in Table 3.42 indicate that the IDEXX HerdChek Short kitProtocol Test detected replicate positive atypical samples down to a dilution of 1/64. At a dilution of 1/64 only one of two replicate sample dilutions was positive. The remaining samples in the dilution series were consistently negative.

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	POS	2/2
1/4	Positive	POS	2/2
1/8	Weak Positive	POS	2/2
1/16	Weak Positive	POS	2/2
1/32	Negative	POS	2/2
1/64	Negative	POS	1/2
1/128	Negative	Neg	0/2
1/256	Negative	Neg	0/2
1/512	Negative	Neg	0/2
1/1024	Negative	Neg	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	Neg	2/2
Negative	Negative	Neg	2/2

Table 3.42 IDEXX Short Protocol CRL pre-prepared dilution series

3.4.3.8. IDEXX HerdChek Ultrashort Protocol (Scrapie Conjugate)-Analytical Sensitivity Results

Results shown in Table 3.43 indicate that the IDEXX HerdChek Standard kitProtocol Test detected replicate positive atypical samples down to a dilution of 1/16. The remaining dilutions were consistently negative.

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	POS	2/2
1/4	Positive	POS	2/2
1/8	Weak Positive	POS	2/2
1/16	Weak Positive	POS	2/2
1/32	Negative	Neg	0/2
1/64	Negative	Neg	0/2
1/128	Negative	Neg	0/2
1/256	Negative	Neg	0/2
1/512	Negative	Neg	0/2
1/1024	Negative	Neg	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	Neg	2/2
Negative	Negative	Neg	2/2

Table 3.43 IDEXX Ultrashort Protocol CRL pre-prepared dilution series

3.4.4. Prionics AG, Schlieren-Zurich, Switzerland

3.4.4.1. Prionics®-Check WESTERN SR - Test Principle & Interpretation/Validation of results

Please refer to Section 3.2.4.1.

All CRL samples were prepared for testing using the standard approach outlined in the current IFU.

3.4.4.2. Prionics®-Check LIA SR - Test Principle & Interpretation/Validation of results

Please refer to Section 3.2.4.2.
All CRL samples were prepared for testing using the standard approach outlined in the current IFU.

3.4.4.3. Period of Assessment

CRL representatives despatched the analytical sample to Prionics AG at their site in Schlieren-Zurich, Switzerland on Monday 11th November 2008. The testing of atypical scrapie samples took place on Monday 17th November 2008, results were emailed back to the CRL on Tuesday 18th November 2008.

A further study was carried out on a panel of neat tissue samples. The testing of these samples took place on 11th February 2009, results were emailed back to the CRL on Wednesday 17th February 2009.

3.4.4.4. Rapid Test Kit information

Prionics®-Check LIA SR Lot No. M080303A and Prionics®-Check WESTERN SR Lot. No.W080808B were used throughout the testing. The current manufacturer user instructions for Prionics®–Check WESTERN SR were Version 2.2 e and Prionics®-Check LIA SR were Version 1.7e used throughout the testing period.

For the further study, Prionics®-Check LIA SR Lot No. M080911A (Expiry date: 14/06/2009) and Prionics®-Check WESTERN SR Lot. No. U080509 (Expiry date: 20/05/2009) were used throughout the testing. The current manufacturer user instructions for Prionics®–Check WESTERN SR were Version 2.2 e and Prionics®-Check LIA SR were Version 1.7e used throughout the testing period.

3.4.4.5. Problem Samples and Testing Issues

During the first visit to Prionics, the manufacturers prepared a common set of samples for testing of the different tests in the study (Prionics®-Check WESTERN SR and Prionics®-Check LIA SR). No problem samples and testing issues were reported in the initial study. For the further study, Prionics representatives reported a mixing of two samples at the test preparation stage of the Western Blot process potentially leading to contamination of the test samples. These samples are identified in results tables for this particular study.

3.4.4.6. Prionics®-Check WESTERN SR - Analytical Sensitivity Results

Results shown in Table 3.44 indicate that the Prionics®-Check WESTERN SR detected 2 positive atypical scrapie samples as part of a dilution series prepared by the CRL. Prionics representatives reported positive signals for 1/2 and 2/2 CRL negative control replicates. It is the considered opinion of the CRL that interpretation of the blots was challenging and determination of positive signals was not possible.

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	POS	1/2
1/4	Positive	Neg	0/2
1/8	Weak Positive	POS	1/2
1/16	Weak Positive	Neg	0/2
1/32	Negative	Neg	0/2
1/64	Negative	Neg	0/2
1/128	Negative	Neg	0/2
1/256	Negative	Neg	0/2
1/512	Negative	Neg	0/2
1/1024	Negative	Neg	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	POS	0/2
Negative	Negative	POS	1/2

Table 3.44 Prionics®-Check WESTERN SR CRL pre-prepared dilution series

Results shown in Table 3.45 indicate that the Prionics®-Check WESTERN SR detected 3 positive results from a panel of 5 confirmed atypical scrapie positive samples prepared in duplicate by the CRL. Prionics representatives reported a positive signal for one of a duplicate pair of CRL negative control samples and explained that there had been a mix-up and potential contamination of samples at the test preparation stage of the process, which may have accounted for this anomalous result. The Prionics®-Check WESTERN SR detected all positive classical scrapie samples included as part of the neat tissue panel prepared by the CRL.

Sample Ref.	Dilution	Expected Result based on IHC results	Area of brain sampled	CRL Western Blot Results 12.2.09	Manufacturer Test Result	Total Number of Positive Replicates
OH0140	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**POS**	**POS**	2/2
OH0139	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**POS**	**POS**	1/2
OH138	Neat Tissue	Atypical Scrapie Positive	Rostral medulla	**POS**	**Neg**	0/2
OH0137	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**Neg**	**Neg**	0/2
OH0136	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**POS**	**POS**	2/2
OH0135	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**POS**	**Neg**	0/2
OH0134	Neat Tissue	Atypical Scrapie Positive	Rostral medulla	**Inconclusive**	**Neg**	0/2
OH0133	Neat Tissue	Classical Scrapie Positive	Cerebellum & Rostral medulla	**POS**	**POS**	2/2*
OH0132	Neat Tissue	Classical Scrapie Positive	Cerebellum & Rostral medulla	**POS**	**POS**	2/2
Sample Ref.	**Dilution**	**Expected Result**	**Area of brain sampled**	**CRL Western Blot Results 12.2.09**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
OH0129	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	**Neg**	**Neg**	2/2
OH0130	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	**Neg**	**POS**	1/2*
OH0131	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	**Neg**	**Neg**	2/2

***The marked samples were accidently mixed at the time of test preparation, leading to potential contamination of tissue for analysis**

Table 3.45 Prionics®-Check WESTERN SR CRL pre-prepared neat tissue sample panel

3.4.4.7. Prionics®-Check LIA SR - Analytical Sensitivity Results

Results shown in Table 3.46 indicate that the Prionics®-Check LIA SR failed to detect any positive atypical scrapie samples as part of a sequential dilutions prepared by the CRL.

Dilution	Expected Result	Manufacturer Test Result	Total Number of Positive replicates
1/2	Positive	**Neg**	0/2
1/4	Positive	**Neg**	0/2
1/8	Weak Positive	**Neg**	0/2
1/16	Weak Positive	**Neg**	0/2
1/32	Negative	**Neg**	0/2
1/64	Negative	**Neg**	0/2
1/128	Negative	**Neg**	0/2
1/256	Negative	**Neg**	0/2
1/512	Negative	**Neg**	0/2
1/1024	Negative	**Neg**	0/2
Dilution	**Expected Result**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
Negative	Negative	**Neg**	2/2
Negative	Negative	**Neg**	2/2

Table 3.46 Prionics®-Check LIA SR CRL pre-prepared dilution series

Results shown in Table 3.47 indicate that the Prionics®-Check LIA SR failed to detect any positive atypical scrapie samples prepared by the CRL. Prionics representatives reported a positive signal for one of a duplicate pair of CRL negative control samples and explained that there had been a mix-up and potential contamination of samples at the test preparation stage of the process, which may have accounted for this anomalous result. The Prionics®-Check LIA SR detected all positive classical scrapie samples included as part of the neat tissue panel prepared by the CRL.

Sample Ref.	Dilution	Expected Result based on IHC results	Area of brain sampled	CRL Western Blot Results 12.2.09	Manufacturer Test Result	Total Number of Positive Replicates
OH0140	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**POS**	**Neg**	0/2
OH0139	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**POS**	**Neg**	0/2
OH138	Neat Tissue	Atypical Scrapie Positive	Rostral medulla	**POS**	**Neg**	0/2
OH0137	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**Neg**	**Neg**	0/2
OH0136	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**POS**	**Neg**	0/2
OH0135	Neat Tissue	Atypical Scrapie Positive	Cerebellum	**POS**	**Neg**	0/2
OH0134	Neat Tissue	Atypical Scrapie Positive	Rostral medulla	**Inconclusive**	**Neg**	0/2
OH0133	Neat Tissue	Classical Scrapie Positive	Cerebellum & Rostral medulla	**POS**	**POS**	2/2*
OH0132	Neat Tissue	Classical Scrapie Positive	Cerebellum & Rostral medulla	**POS**	**POS**	2/2
Sample Ref.	**Dilution**	**Expected Result**	**Area of brain sampled**	**CRL Western Blot Results 12.2.09**	**Manufacturer Test Result**	**Total Number of *Negative* Replicates**
OH0129	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	**Neg**	**Neg**	2/2
OH0130	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	**Neg**	**POS**	1/2*
OH0131	Neat Tissue	Scrapie Negative	Cerebellum & Rostral medulla	**Neg**	**Neg**	2/2

***The marked samples were accidently mixed at the time of test preparation, leading to potential contamination of tissue for analysis**

Table 3.47 Prionics®-Check LIA SR CRL pre-prepared Neat Tissue sample panel

3.5. Statistical Analysis of Bovine and Ovine Test Results

Tables 3.44 and 3.45 show statistical analyses data for bovine and ovine test results for all manufacturers' tests. The data analyses are presented for each pool of CRL and manufacturer prepared dilution series. A 50 % end-point calculation for each set of tests and two-way analyses of variance with sample and test as main effects were carried out.

The statistical analyses report (Annex 7) indicates that Tables 3.48 and 3.49 show the 50% end-points expressed as –log2 (dilution). Higher values indicate a better performance, for example, 1/64=6, 1/128=7 etc. The table values can easily be converted back to dilutions by raising 2 to their power. For example, 2 raised to the power 6.5 is 90.5 and therefore 6.5 corresponds to a 1/90.5 dilution.

There were highly significant ($p<0.001$) differences between the tests and the results of all pairwise comparisons by the Tukey HSD test are indicated by the superscripts in the tables. Means that share a common superscript are not significantly different at the 5% level.

Test	Preparation	Bovine Sample Pool			
		A	B	C	Mean*
AJ Roboscreen BetaPrion®	CRL Set	8.5	8.5	8.3	8.43 ^{fg}
	Manuf. Set	--	--	--	--
Bio-Rad TeSeE™ SAP	CRL Set	5.3	6.1	5.5	5.63 ^{a}
	Manuf. Set	8.1	8.7	8	8.27 ^{ef}
Enfer Version 2	CRL Set	6.1	7.1	6.5	6.57 ^{b}
	Manuf. Set	7.5	7.7	7.3	7.50 ^{cde}
Enfer Version3	CRL Set	6.3	6.3	6.3	6.30 ^{ab}
	Manuf. Set	6.7	7.3	6.5	6.83 ^{bc}
IDEXX HerdChek Standard Protocol	CRL Set	9.7	10.1	9.5	9.77 ^{h}
	Manuf. Set	--	--	--	--
IDEXX HerdChek Short Protocol	CRL Set	9.9	9.9	9.9	9.90 ^{h}
	Manuf. Set	--	--	--	--
IDEXX HerdChek Ultra Short Protocol	CRL Set	9.7	9.9	9.5	9.70 ^{h}
	Manuf. Set	--	--	--	--
Roche PrionScreen	CRL Set	7.5	7.5	7.5	7.50 ^{cde}
	Manuf. Set	--	--	--	--
Prionics®-Check WESTERN	CRL Set	8.3	8.5	7.5	8.08 ^{ef}
	Manuf. Set	9.3	9.3	8.9	9.17 ^{gh}
Prionics®-Check LIA	CRL Set	--	--	--	--
	Manuf. Set	6.3	7.5	7.5	7.10 ^{bcd}
Prionics®-Check PrioSTRIP	CRL Set	--	--	--	--
	Manuf. Set	7.5	8.1	7.5	7.70 ^{def}

***** Means that share a common superscript are not significantly different at the 5% level.

Table 3.48. 50% end-point dilutions (-log2) for the bovine sample pools.

Test	Preparation	Ovine Sample pool			
		X	Y	Z	Mean*
Bio-Rad TeSeE™ SAP	CRL Set	6.5	6.7	8.5	7.23 [cd]
	Manuf. Set	6.5	7.3	9.5	7.77 [cd]
Bio-Rad TeSeE™ Sheep/Goat	CRL Set	9.3	9.3	10.7	9.77 [f]
	Manuf. Set	8.9	9.5	11.5	9.97 [f]
Enfer Version2	CRL Set	7.3	7.5	8.7	7.83 [cd]
	Manuf. Set	8.5	8.3	10.5	9.10 [ef]
Enfer Version3	CRL Set	7.1	7.5	8.5	7.70 [cd]
	Manuf. Set	7.7	7.7	9.5	8.30 [de]
IDEXX HerdChek Standard Protocol	CRL Set	8.9	9.5	10.9	9.77 [f]
	Manuf. Set	---	---	---	--
IDEXX HerdChek Short Protocol	CRL Set	8.7	9.5	10.5	9.57 [f]
	Manuf. Set	---	---	---	--
IDEXX HerdChek Ultra Short Protocol	CRL Set	8.5	9.3	10.5	9.43 [ef]
	Manuf. Set	---	---	---	--
Prionics®-Check WESTERN SR	CRL Set	5.1	5.5	6.7	5.77 [b]
	Manuf. Set	5.7	7.5	7.7	6.97 [c]
Prionics®-Check LIA SR	CRL Set	3.1	3.5	5.3	3.97 [a]
	Manuf. Set	3.3	4.9	6.5	4.90 [ab]

* Means that share a common superscript are not significantly different at the 5% level.

Table 3.49. 50% end-point dilutions (-log2) for the ovine sample pools.

A full version of the statistical analyses report in available in Annex 7.

3.6. Analytical Sensitivity Study - Results Summary

Three composite tables of results are presented summarising all results for all tests included in the study (Tables 3.50, 3.51, 3.52, 3.53). Additionally all summary test data are presented for analytical dilutions prepared by the test manufacturers and the CRL. Several manufacturers opted to undertake testing only of CRL pre-prepared sample dilution series. Therefore the test results presented demonstrate test performance on CRL prepared sample dilution series only.

Specifically for Prionics data, results for CRL samples have been realigned in the summary tables to account for initial adjustments made by the manufacturers during preparation of the dilution series.

Table 3.50 Results Summary for BSE Rapid Test Kits Evaluation

		MANUFACTURERS APPROVED TEST KIT																					
		AJ Roboscreen§ BetaPrion®		Bio-Rad TeSeE™ SAP		Enfer V2		Enfer V3		IDEXX§ HerdChek Standard Protocol		IDEXX§ HerdChek Short Protocol		IDEXX§ HerdChek Ultra Short Protocol		Roche§ PrionScreen		Prionics®-Check WESTERN†		Prionics®-Check LIA		Prionics®-Check PrioSTRIP	
		CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set**	CRL Set	Manuf. Set
	Neat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/2	-
Pool A	**1/2**	2/2	-	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	-	2/2	2/2	Unable to obtain meanin-gful data for this test	2/2	2/2	2/2
	1/4	2/2	-	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	-	7/7	2/2		2/2	5/5	2/2
	1/8	5/5	-	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	2/2	5/5		5/5	5/5	5/5
	1/16	5/5	-	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	6/6	5/5		5/5	5/5	5/5
	1/32	5/5	-	4/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	6/6	5/5		5/5	5/5	5/5
	1/64	5/5	-	0/5	5/5	3/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	5/5	5/5		5/5	4/5	5/5
	1/128	5/5	-	0/5	5/5	0/5	5/5	0/5	1/5	5/5	-	5/5	-	5/5	-	5/5	-	4/4	5/5		3/5	5/5	5/5
	1/256	5/5	-	0/5	3/5***	0/5	0/5	0/5	0/5	5/5	-	5/5	-	5/5	-	0/5	-	3/4	5/5		1/5	2/5	0/5
	1/512	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	5/5	-	5/5	-	5/5	-	0/5	-	0/6	4/5		0/5	2/5	0/5
	1/1024	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	1/5	-	2/5	-	1/5	-	0/5	-	0/6	0/5		0/5	0/5	0/5
	1/2048	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	-	0/4	0/5		0/5	0/5	0/5
	1/4096	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	-	0/2	0/5		0/5	-	0/5
	Neat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	2/2	-
Pool B	**1/2**	2/2	-	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	-	2/2	2/2		2/2	2/2	2/2
	1/4	2/2	-	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	-	7/7	2/2		2/2	5/5	2/2
	1/8	5/5	-	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	3/3	5/5		5/5	3/5	5/5
	1/16	5/5	-	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	5/5	5/5		5/5	5/5	5/5
	1/32	5/5	-	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	6/6	5/5		5/5	5/5	5/5
	1/64	5/5	-	3/5***	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	3/3	5/5		5/5	5/5	5/5
	1/128	5/5	-	0/5	5/5	3/5	5/5	0/5	4/5	5/5	-	5/5	-	5/5	-	5/5	-	7/7	5/5		4/5	5/5	5/5
	1/256	5/5	-	0/5	5/5	0/5	1/5	0/5	0/5	5/5	-	5/5	-	5/5	-	0/5	-	4/4	5/5		1/5	3/5	3/5

	1/512	0/5	-	0/5	1/5***	0/5	0/5	0/5	0/5	5/5	-	5/5	-	5/5	-	0/5	-	1/4	4/5		0/5	4/5	0/5
	1/1024	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	3/5	-	2/5	-	2/5	-	0/5	-	0/5	0/5		0/5	0/5	0/5
	1/2048	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	-	0/5	0/5		0/5	1/5	0/5
	1/4096	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	-	0/3	0/5		0/5	-	0/5
	Neat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1/1	-		-	2/2	-
Pool C	**1/2**	2/2	-	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	-	2/2	2/2		2/2	2/2	2/2
	1/4	2/2	-	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	-	3/3	2/2		2/2	5/5	2/2
	1/8	5/5	-	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	6/6	5/5		5/5	5/5	5/5
	1/16	5/5	-	5/5	4/4*	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	5/5	5/5		5/5	5/5	5/5
	1/32	5/5	-	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	4/4	5/5		5/5	5/5	5/5
	1/64	5/5	-	0/5	5/5	4/5	5/5	4/5	5/5	5/5	-	5/5	-	5/5	-	5/5	-	6/6	5/5		5/5	5/5	5/5
	1/128	5/5	-	0/5	5/5	1/5	4/5	0/5	0/5	5/5	-	5/5	-	5/5	-	5/5	-	5/5	5/5		3/5	5/5	5/5
	1/256	4/5	-	0/5	0/5***	0/5	0/5	0/5	0/5	5/5	-	5/5	-	5/5	-	0/5	-	2/3	4/5		2/5	1/5	0/5
	1/512	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	5/5	-	5/5	-	5/5	-	0/5	-	0/5	3/5		0/5	3/5	0/5
	1/1024	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	2/5	-	0/5	-	0/5	-	0/8	0/5		0/5	1/5	0/5
	1/2048	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	-	0/3	0/5		0/5	0/5	0/5
	1/4096	0/5	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	-	0/3	0/5		0/5	-	0/5
Pool D	**Negative Tissue**	54/54	-	54/54	54/54	54/54	54/54	54/54	54/54	54/54	-	54/54	-	54/54	-	54/54	-	54/54	54/54		53/54	47/54	53/54

*Replicate 5 not tested as-sample mixed up in error

** Manufacturers set with modifications due to preparation differences

*** Results recorded as requiring cautious interpretationl according to manufacturers IFU

† The sampling number of replicates listed for the Prionics Western Blot tests is different to the number listed for all other tests due to variation in sample preparation during visit 1 and visit 2.

§ Several manufacturers opted to undertake testing only of CRL prepepared sample dilution series

Negative	Positive for all replicates	Positive for some replicates	No Result

Table 3.50 Results Summary for BSE Rapid Test Kits Evaluation

		MANUFACTURERS APPROVED TEST KIT																	
		Bio-Rad TeSeE™ SAP		Bio-Rad TeSeE™ S/G		Enfer V2		Enfer V3		IDEXX HerdChek Standard Protocol		IDEXX HerdChek Short Protocol		IDEXX HerdChek Ultra Short Protocol		Prionics®-Check WESTERN SR		Prionics®-Check LIA SR	
		CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set	CRL Set	Manuf. Set
Pool W	Negative Tissue	54/54	54/54	54/54	54/54	54/54	54/54	54/54	53/54	54/54	-	54/54	-	54/54	-	54/54	54/54	53/54	54/54
Pool X	Neat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/2	-	2/2	-
	1/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	2/2	2/2	2/2
	1/4	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	5/5	2/2	5/5	2/2
	1/8	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	5/5	3/5	4/5
	1/16	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	4/5	0/5	0/5
	1/32	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	3/5	5/5	0/5	0/5
	1/64	5/5	5/5	5/5	5/5	4/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	0/5	2/5	0/5	0/5
	1/128	0/5	0/5	5/5	5/5	5/5	5/5	3/5	5/5	5/5	-	5/5	-	5/5	-	0/5	0/5	0/5	0/5
	1/256	0/5	0/5	5/5	5/5	0/5	4/5	0/5	1/5	5/5	-	5/5	-	5/5	-	0/5	0/5	0/5	0/5
	1/512	0/5	0/5	4/5	2/5***	0/5	1/5	0/5	0/5	2/5	-	1/5	-	0/5	-	0/5	0/5	0/5	0/5
	1/1024	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	0/5	0/5	0/5
	1/2048	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	0/5	0/5	0/5
	1/4096	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	-	0/5	-	0/5
Pool Y	Neat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/2	-	2/2	-
	1/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	2/2	2/2	2/2
	1/4	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	5/5	2/2	5/5	2/2
	1/8	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	5/5	5/5	5/5
	1/16	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	5/5	0/5	5/5
	1/32	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	4/5	5/5	0/5	2/5
	1/64	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	1/5	5/5	0/5	0/5
	1/128	1/5	4/5***	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	0/5	4/5	0/5	0/5
	1/256	0/5	0/5	5/5	5/5	0/5	4/5	0/5	1/5	5/5	-	5/5	-	5/5	-	0/5	1/5	0/5	0/5

	1/512	0/5	0/5	4/5	5/5	0/5	0/5	0/5	0/5	5/5	-	5/5	-	4/5	-	0/5	0/5	0/5	0/5
	1/1024	0/5	0/5	0/5	0/5***	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	0/5	0/5	0/5
	1/2048	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	0/5	0/5	0/5	0/5
	1/4096	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	-	0/5	-	0/5
Pool Z	**Neat**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/2	-	2/2	-
	1/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	2/2	2/2	2/2	2/2
	1/4	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	5/5	2/2	5/5	2/2
	1/8	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	5/5	5/5	5/5
	1/16	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	5/5	5/5	5/5
	1/32	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	5/5	5/5	3/5	5/5
	1/64	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	4/5	5/5	0/5	5/5
	1/128	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	2/5	5/5	0/5	0/5
	1/256	5/5	5/5	5/5	5/5	4/5	5/5	5/5	5/5	5/5	-	5/5	-	5/5	-	0/5	1/5	0/5	0/5
	1/512	0/5	5/5	5/5	5/5	2/5	5/5	0/5	5/5	5/5	-	5/5	-	5/5	-	0/5	0/5	1/5	0/5
	1/1024	0/5	0/5	5/5	5/5	0/5	5/5	0/5	0/5	5/5	-	5/5	-	5/5	-	0/5	0/5	0/5	0/5
	1/2048	0/5	0/5	1/5***	5/5	0/5	0/5	0/5	0/5	2/5	-	0/5	-	0/5	-	0/5	0/5	0/5	0/5
	1/4096	0/5	0/5	0/5	0/5***	0/5	0/5	0/5	0/5	0/5	-	0/5	-	0/5	-	-	0/5	-	0/5

*** Results recorded as requiring cautious interpretationl according to manufacturers IFU

Negative	Positive for all replicates	Positive for some replicates	No Result

Table 3.51 Results Summary for Classical Scrapie Rapid Test Kits Evaluation

MANUFACTURERS APPROVED TEST KIT RESULTS									
Dilution Factor	Bio-Rad TeSeE™ Sheep/Goat	Bio-Rad TeSeE™ SAP	Enfer V2	Enfer V3	IDEXX HerdChek Standard Protocol	IDEXX HerdChek Short Protocol	IDEXX HerdChek Ultra Short Protocol	Prionics®-Check WESTERN SR§	Prionics®-Check LIA SR
1/2	2/2	2/2	0/2	0/2	1/2**	2/2	2/2	1/2	0/2
1/4	2/2	2/2	0/2	0/2	2/2	2/2	2/2	0/2	0/2
1/8	2/2	2/2	0/2	0/2	2/2	2/2	2/2	1/2	0/2
1/16	2/2	2/2	0/2	0/2	2/2	2/2	2/2	0/2	0/2
1/32	2/2	2/2	0/2	0/2	0/2	2/2	0/2	0/2	0/2
1/64	2/2	2/2	0/2	0/2	0/2	1/2	0/2	0/2	0/2
1/128	2/2	*1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
1/256	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
1/512	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
1/1024	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Negative 1	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
Negative 2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2

* Manufacturer comment for negative sample: 'Doubtful. Should be retested in duplicate'

** 1 replicate reading at 1/1 dilution was recorded by IDEXX as a high negative value, the other sample was recorded as positive

§ WB results were challenging to interpret and CRL concluded determination of positive signals was not possible. Results indicated were assigned a result by Prionics

Negative	Positive for both replicates	Positive for one replicate

Table 3.52 Results Summary for Atypical Scrapie Rapid Test Kits Evaluation

MANUFACTURERS APPROVED TEST KIT RESULTS						
Sample Ref.	**Dilution Factor**	**Expected Result**	**Enfer V2**	**Enfer V3**	**Prionics WB SR**	**Prionics LIA SR**
OH0140	Neat Tissue	Atypical Scrapie Positive	0/2	0/2	2/2	0/2
OH0139	Neat Tissue	Atypical Scrapie Positive	0/2	0/2	1/2	0/2
OH0138	Neat Tissue	Atypical Scrapie Positive	0/2	0/2	0/2	0/2
OH0137	Neat Tissue	Atypical Scrapie Negative	0/2	0/2	0/2	0/2
OH0136	Neat Tissue	Atypical Scrapie Positive	0/2	0/2	2/2	0/2
OH0135	Neat Tissue	Atypical Scrapie Positive	0/2	0/2	0/2	0/2
OH0134	Neat Tissue	Atypical Scrapie Inconclusive	0/2	0/2	0/2	0/2
OH0133	Neat Tissue	Classical Scrapie Positive	2/2	2/2	2/2*	2/2*
OH0132	Neat Tissue	Classical Scrapie Positive	2/2	2/2	2/2	2/2
OH0129	Neat Tissue	Scrapie Negative	2/2	2/2	2/2	2/2
OH0130	Neat Tissue	Scrapie Negative	2/2	2/2	1/2*	1/2*
OH0131	Neat Tissue	Scrapie Negative	2/2	2/2	2/2	2/2

***The marked samples were accidently mixed at the time of test preparation, leading to potential contamination of tissue for analysis**

Negative	Positive for both replicates	Positive for one replicate

Table 3.53 Results Summary for Follow-up Atypical Scrapie Rapid Test Kit Evaluation

4. DISCUSSION

The scope of this study was to produce robust analytical sensitivity data for the current EU-approved rapid *post mortem* tests designed to detect BSE, classical and atypical scrapie. The key design principle of this study was to evaluate each test against the same sample sets for the three main types of ruminant TSE: BSE, classical scrapie and atypical scrapie. This experimental design allows an inter-assay comparison of analytical sensitivity which has not been possible before.

The original rapid test evaluations were carried out between 1999 and 2005. They involved assessment of analytical and diagnostic sensitivity criteria, but different sample panels were utilised. This meant that it is difficult to directly compare all the tests. Subsequently DG SANCO requested that the CRL assess analytical sensitivity for all the currently approved TSE rapid tests. It was anticipated that the resulting information would enable the European Commission to mandate EFSA for a scientific evaluation of the report and provide an opinion on the suitability for the currently approved rapid tests to maintain EU approval[14].

The use of finely chopped tissue as a suitable starting matrix for assessment of analytical sensitivity has been accepted as the standard approach, approved by EFSA[14] and previously utilised in studies conducted by IRMM[10]. However, there is scope for criticism because, whilst considerable care is taken, there is a theoretical risk that the material will still not be completely homogenous and therefore aliquots may not be identical, because PrP^{sc} is not evenly distributed in brain tissue[9]. Should this happen, relative sensitivity estimates would be compromised. More homogenous samples can be prepared using the CRL standard method for preparation of homogenates for proficiency testing, but some manufacturers claim that this mode of preparation is not optimal for their tests.

For this reason the CRL provided the opportunity to test both manufacturer-prepared dilution series and CRL–prepared dilution series made from the same starting material. Three independent pools of positive material were prepared for classical BSE and classical scrapie studies. This mitigated against the above risk, as similar trends in results would be expected for all three-dilution series when all the test data are compared. Thus unexpected results would be more obvious. It is also likely that the composition of the pools, in terms of concentration of PrP^{sc}, will affect the final analytical sensitivity results. Therefore it was anticipated that the different pools may have had different lowest detectable dilutions.

Several manufacturers (IDEXX, Roboscreen and Roche) chose to opt out of preparing and testing a manufacturer-prepared dilution series. This decision was made by the manufacturers either because they considered that the production method employed by the CRL for generating test samples had no negative impact on their test performance and/or due to constraints in manufacturer resources. In this scenario the manufacturers tested the CRL pre-prepared series under observation of the CRL.

Two manufacturers (BioRad and Prionics) expressed concern prior to the laboratory phase of the study, that the CRL prepared dilution series would not be optimal for their tests. Enfer also chose to prepare their own dilution series. The protocols for the preparation of each "manufacturer's" dilution series were agreed with the CRL, prior to the lab phase of the study.

Six manufacturers of rapid test kits participated in the study. Overall the study comprised assessment of 16 tests, with 9 tests evaluated for BSE (11 protocols) and 7 tests (9 protocols) evaluated for Classical and Atypical Scrapie.

BSE Tests and Analytical Sensitivity

A comparison of the 9 different approved BSE rapid tests demonstrated a variation in lowest detectable dilutions.

Data for the Bio-Rad TeSeE™SAP test demonstrated that manufacturer produced dilution series had detectable dilution limits down to 1/512 for one particular pool. Testing of the CRL prepared sample set produced less sensitive results, as anticipated by the manufacturer, and this may be because the preparation method was not optimal for the test.

A comparison of results for Enfer V2 and Enfer V3 showed manufacturer-produced dilution series for all pools had similar detectable dilution limits with a signal detected down to 1/256 for one particular pool using the Enfer V2 test. Results were similar for the CRL and manufacturers series, with the manufacturers series generally, but not always being one dilution more sensitive.

The Prionics®-Check WESTERN detected a dilution of 1/512 for one pool. Results were similar for the CRL and manufacturer prepared samples with the manufacturers' series generally, but not always being one dilution more sensitive.

The data set obtained from testing the manufacturer-prepared dilution series on the Prionics®-Check LIA gave detection limits of 1/256.Valid results were not produced for the CRL prepared sample series. Prionics had two opportunities to generate these data. On both occasions the control data produced on the Prionics®-Check LIA had high background values that skewed the threshold cut-off thus influencing all data points both positive and negative from both sample sets. However, the Prionics®-Check LIA produced valid results for the manufacturer-prepared dilution series on the second visit. It is interesting that samples prepared by the CRL using the CRL standard protocol have been used for proficiency testing and been tested by National Reference Laboratories using the Prionics®-Check LIA, apparently without problems. However, this may reflect the concerns of Prionics that the CRL sample set was not optimal for its test.

The Prionics®-Check PrioSTRIP results for manufacturer-produced dilution series had similar detectable dilutions (generally 1/256) for all pools tested. There was also a false positive result. The data for the CRL series cannot be interpreted because there were several false positive results (7/54 negatives tested), again this may reflect Prionics' observation that the CRL samples were not optimal for their test.

Three manufacturers (IDEXX, Roboscreen and Roche) only tested the CRL dilution series. Three protocols were assessed for the Idexx test (standard, short and ultrashort). These produced consistent results, generally detecting dilutions of 1/1024. The Roboscreen BetaPrion test produced consistent results generally detecting down to 1/256. The Roche test produced consistent results detecting dilutions down to 1/128.

Statistical analyses demonstrated that the three IDEXX tests showed high analytical sensitivity (CRL dilution sets) detecting dilutions to 1/1024, and significantly more sensitive than all except Prionics®-Check WESTERN (manufacturers dilution sets). The lowest detection limits (limit of detection) of the other tests can be seen in Table 3.49 expressed as 50% end point dilutions.

Classical Scrapie Tests and Analytical Sensitivity

A comparison of the 7 different approved Classical Scrapie rapid tests demonstrated a variation in lowest detectable dilutions. The BioRad TeSeE SAP produced similar results for the CRL and manufacturers produced dilution series. The range of detection was 1/64-1/512 and was dependent on the pool tested.

The BioRad TeSeE sheep and goat test produced similar results for the CRL and manufacturers dilution series. The range of detection was 1/512-1/2048 and was dependent upon the pool tested.

Overall the Bio-Rad TeSeE™ Sheep/ Goat appeared more sensitive than the TeSeE™ SAP with positive results detected up to two dilutions further than those for TeSeE™ SAP. Similar results were obtained using the manufacturers and CRL prepared samples.

A comparison of results for Enfer V2 and V3 showed manufacturer-produced dilution series for all pools had comparable detectable dilutions for both versions of the test (range for manufacturer prepared samples 1/256-1/1024). Results for manufacturer prepared samples were generally a dilution more sensitive than for the CRL set. Results were not found to be significantly different between Enfer versions 2 and 3.

A comparison of results for Prionics®-Check WESTERN SR showed manufacturer-produced dilution series for all pools had detectable thresholds of as low as 1/256 for one particular pool (range for manufacturer prepared dilutions 1/64-1/256). A comparison of results for Prionics®-Check LIA SR showed manufacturer-produced dilution series had detectable thresholds of 1/64 for one particular pool (range for manufacturer produced series1/8-1/64).

Results for IDEXX HerdChek Standard, Short and Ultrashort assays were comparable and consistent between tests for all pools prepared by the CRL. (range 1/256-1/2048). As for the other tests, there were differences in the lowest dilution which could be detected for each pool. As observed previously for the BSE

evaluation, it would have been constructive to have obtained corresponding data for manufacturer produced samples.

The most analytically sensitive tests were the Bio-Rad TeSeE™ Sheep/Goat, the standard and short protocols, the IDEXX test and Enfer version 2. The least sensitive was the Prionics®-Check LIA SR. Data for all tests is shown as 50% end point dilutions in Table 3.49.

Atypical Scrapie Stability Study

The preparation for the atypical scrapie stability study was challenging as early results from the stability study using the Bio-Rad TeSeE™ Sheep / Goat suggested degradation of atypical scrapie homogenates was occurring over time. These observations were supported by the western blot results (Bio-Rad Western Blot) that required an increase in the development time to show a positive result for the 1/50 aliquot at 4 weeks. However, for the specific purposes of this study the original plan to prepare homogenates of atypical scrapie tissue and distribute to manufacturers to test within a specified time period was utilised.

Atypical Scrapie Tests and Analytical Sensitivity

A comparison of results for the different scrapie rapid tests demonstrated a wide range of lowest detectable dilutions. Specifically, Bio-Rad data for the TeSeE™ SAP and TeSeE™ Sheep/ Goat test showed that positive results were obtained for dilutions down to 1/128. These results were comparable for both Bio-Rad tests. By contrast, Enfer V2 and V3 tests were unable to detect a positive signal in any of the samples. Similarly, no positive signal was detected with the Prionics®-Check LIA SR.

Two positive data points and one false positive data point was reported for the Prionics®-Check WESTERN SR. The Western Blot image submitted to accompany these data points was subjected to close scrutiny by the CRL representatives. It is the considered opinion of the CRL that interpretation of the blots was challenging and determination of positive signals was not possible. Consequently it was not possible to assign a threshold dilution to which the detectable limit could be set for this test.

The IDEXX HerdChek test detected positive signals for several dilutions under all test conditions (Standard, Short and Ultrashort protocols). Specifically, the Short Protocol detected a positive signal down to 1/64 dilution; the other protocols detecting to a 1/16 dilution. One of the two duplicate samples representative of the 1/2 dilution was recorded as negative by the use of the standard protocol. The raw data indicate that this sample had a high negative average OD value.

Consequently, the atypical scrapie analytical sensitivity study indicates that, on the basis of the small sample set, the Bio-Rad tests performed best detecting highly diluted positive atypical scrapie samples. The IDEXX test protocols all detected a positive signal at a 1/16 dilution and using the short protocol down to 1/64. Enfer and Prionics tests failed to detect a positive signal, even with 1/2 dilutions of positive

homogenate. It was not clear why the Enfer and Prionics tests did not detect the atypical samples. As these were prepared by the CRL standard method as tissue/water homogenates it could have been due to the preparation method. In the intervening period further samples had become available. To evaluate this further, a larger panel of undiluted atypical scrapie tissue from different animals was tested using these methods and reagents. Both Enfer tests and the Prionics®-Check LIA SR again failed to detect a positive signal for all atypical samples. The Prionics®-Check WESTERN SR detected a positive signal for 3 of 5 positive atypical scrapie samples. These results were detected with undiluted tissue and as a consequence the data infer that the diagnostic sensitivity of this test is low.

5. GENERAL CONCLUSION

The analytical sensitivity results generated in this study enable specific conclusions to be drawn regarding the performance of the currently approved TSE rapid tests.

Manufacturer	Test Name	Test Target		
		BSE	Classical Scrapie	Atypical Scrapie
AJ Roboscreen	BetaPrion®	√		
Bio-Rad	TeSeE™ (SAP)	√	√	√
Bio-Rad	TeSeE™ Sheep/Goat		√	√
Enfer	TSE v2 Automated	√	√	X
Enfer	TSE v3 Automated	√	√	X
IDEXX	HerdChek- Standard (bovine conjugate)	√		
IDEXX	HerdChek- Short (bovine conjugate)	√		
IDEXX	HerdChek- Ultra Short (bovine conjugate)	√		
IDEXX	HerdChek- Standard (scrapie conjugate)		√	√
IDEXX	HerdChek- Short (scrapie conjugate)		√	√
IDEXX	HerdChek- Ultra Short (scrapie conjugate)		√	√
Prionics®	Prionics®-Check LIA	√		
Prionics®	Prionics®-Check LIA SR		√	X
Prionics®	Prionics®-Check PrioSTRIP	√		
Prionics®	Prionics®- Check Western	√		
Prionics®	Prionics®-WB Check Western SR		√	X*
Roche	Prionscreen	√		

Grey shading	Represents test not approved for listed target
√	Success of test in detection of positive signal
X	Failure of test to detect positive signal
*	Ambiguous results and interpretation by manufacturer

Table 5.01. Summary of Manufacturers Test performance against BSE, Classical and Atypical Scrapie targets.

The BSE results indicate that Prionics®-Check WESTERN, Prionics®-Check LIA test, Prionics®-Check PrioSTRIP, Enfer TSE Version 2, automated, Enfer TSE Version 3, Bio-Rad TeSeE™ SAP test, IDEXX HerdChek BSE- Scrapie Antigen Test Kit (using bovine conjugate), Roboscreen Beta Prion BSE EIA Test Kit and Roche Applied Science PrionScreen detected the appropriate detection target (Table 5.01).

The Classical Scrapie results indicate that Bio-Rad TeSeE™ SAP test, Bio-Rad TeSeE™ Sheep/ Goat test, Enfer TSE version 2, Enfer TSE Version 3, IDEXX HerdChek BSE-Scrapie Antigen Test Kit (using scrapie conjugate) Prionics®-Check WESTERN Small Ruminant test and Prionics®-Check LIA SR detected the appropriate detection target (Table 5.01).

The Atypical Scrapie results indicate that Bio-Rad TeSeE™ test, Bio-Rad TeSeE™ Sheep/Goat test, and IDEXX HerdChek BSE-Scrapie Antigen Test Kit (using scrapie conjugate) detected the appropriate detection target (Table 5.01). The Prionics®-Check WESTERN SR test results indicated low test sensitivity.

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