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.

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2 Panel members: Olivier Andreoletti, Herbert Budka, Sava Buncic, John D Collins, John Griffin, Tine Hald, Arie Hendrik Havelaar, James Hope, Günter Klein, James McLauchlin, Winy Messens, Christine Müller-Graf, Christophe Nguyen-The, Birgit Noerrung, Luisa Peixe, Miguel Prieto Maradona, Antonia Ricci, John Sofos, John Threlfall, Ivar Vågsholm, Emmanuel Vanopdenbosch. One member of the Panel did not participate in part of the discussion on the subject referred to above because of potential conflicts of interest identified in accordance with the EFSA policy on declarations of interests. Correspondence: biohaz@efsa.europa.eu

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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.

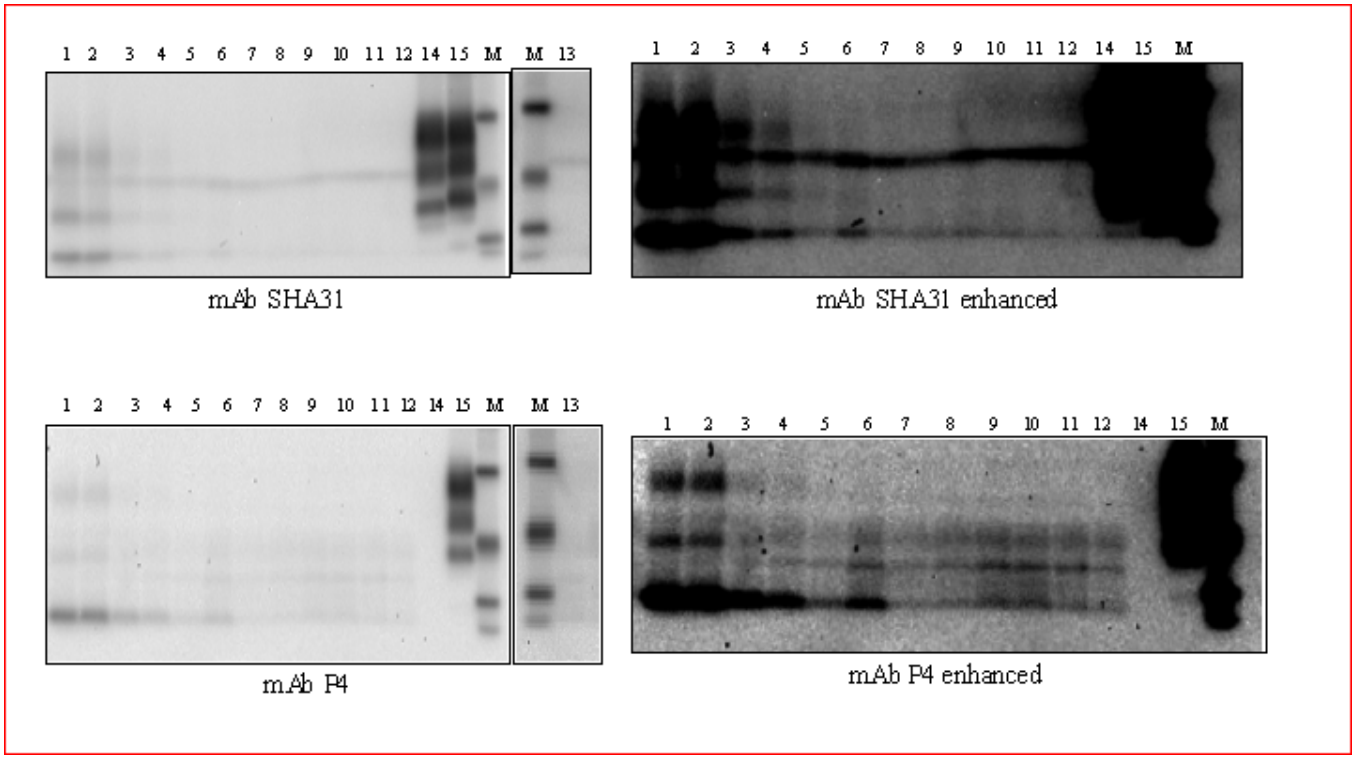

Figure 1: Results of the Western Blot performed on post-homogenate samples (21 November 2008).

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	-	

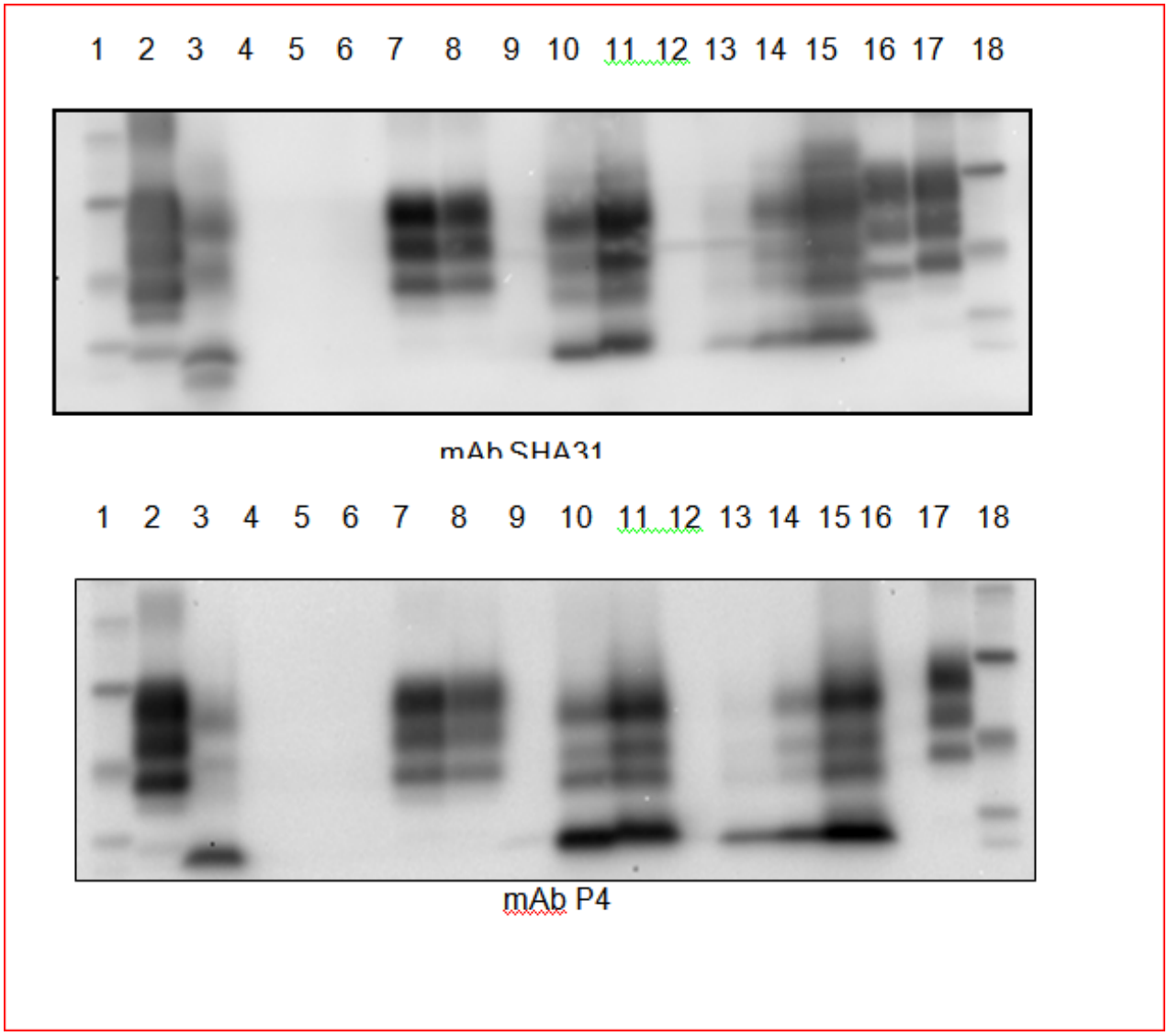

Figure 2: Results of the Western Blot performed on post-homogenate samples (12 February 2009).