

Standards for the assay of Creutzfeldt–Jakob disease specimens

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Assays for the agent of Creutzfeldt–Jakob disease (CJD) include measurement of infectivity in different animal systems, such as wild-type or transgenic mice, and detection of PrP^{Sc} by different methods and formats. The various assays could be best calibrated against each other by use of uniform readily available materials, and samples of four human brains, two from sporadic CJD patients, one from a variant CJD patient and one from a non-CJD patient, have been prepared as 10% homogenates dispensed in 2000 vials each for this purpose. Results of *in vitro* methods, particularly immunoblot assays, were compared in the first collaborative study described here. While dilution end-points varied, the minimum detectable volume was surprisingly uniform for most assays and differences in technical procedure, other than the sample volume tested, had no detectable systematic effect. The two specimens from sporadic CJD cases contained both type 1 and type 2 prion proteins in approximately equal proportions. The materials have been given the status of reference reagents by the World Health Organization and are available for further study and assessment of other *in vitro* or *in vivo* assay procedures.

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INTRODUCTION

Creutzfeldt–Jakob disease (CJD) is a transmissible spongiform encephalopathy (TSE) (Prusiner, 1997), a class of disease characterized by slow, progressive, non-inflammatory neurodegeneration with a long pre-clinical period before

symptoms develop. In the course of the disease, a normal host protein (cellular prion protein; PrP^C) is deposited in an abnormal conformation (scrapie-associated prion protein; PrP^{Sc}), which is believed by many to be the infectious agent itself (Prusiner, 1991). Infection can be transmitted to susceptible species including wild-type mice with varying efficiency, depending in part on the amino acid sequence of the host PrP; the barrier to infection can be overcome to some degree by constructing transgenic mice carrying PrP

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from a different species against a background in which the murine endogenous PrP gene has been ablated. Assays to detect and quantify the TSE agents are currently based on titrations of infectivity in susceptible animals or on the detection of PrP^{Sc} and differ in technical details so that they might be expected to differ in sensitivity. Thus, it is difficult to compare the results obtained by different methods, and assessments of the amount of PrP^{Sc} present at any one time, or the relationship with infectivity, are impossible to compare between laboratories. Uniform reference preparations have been used in a variety of biological systems to provide a means of calibrating assay results for comparative purposes. Thus, the results of an assay of an unknown preparation can be expressed in terms of the results obtained with the same assay with a reference preparation to give a relative potency. This has proved to be an effective way of reducing variation among laboratories and calibrating one assay against another.

CJD is classified into sporadic, iatrogenic, familial and variant forms, depending on causation (Prusiner, 1997). In addition, a number of different forms are recognized based on clinical and histopathological presentation, and different molecular forms of PrP^{Sc} have been recognized, depending on the size and properties of the residual fragments after treatment with proteinase K, including the cleavage site and the glycosylation profile (Parchi *et al.*, 1996; Wadsworth *et al.*, 1999). The differences observed imply a difference in the conformation of the protein linked to the strain of CJD involved. Finally, the human PrP gene has a polymorphism at codon 129, which may encode either methionine or valine; the genotype modulates the expression of the disease (Palmer *et al.*, 1991). Met/Met homozygotes are over-represented in cases of sporadic CJD and all cases of variant (v) CJD to date have been of this genotype. All of these factors interact to some extent and it is possible that standard preparations suitable for one particular type will not be suitable for others.

This report describes materials for use in the calibration of results of assays of PrP^{Sc} in preparations of CJD brains of the Met/Met genotype. This type was chosen as vCJD is of major concern and has thus far always been of this type. However, at least two molecular types of PrP^{Sc} in cases of sporadic CJD are recognized in this genotype. Specimens of sporadic and vCJD were therefore sought. Under the auspices of the World Health Organization (WHO), the materials were homogenized and distributed in identical aliquots in ampoules. The preparations have been characterized in the preliminary study reported here, dealing in the first instance with *in vitro* assays of PrP^{Sc} content. The objective was to measure PrP^{Sc} content in some commonly used assays that might differ in sensitivity and therefore benefit from the use of a calibrating reference material. No attempt was made to include all assay methods and formats, the study being intended to demonstrate the value of the reference materials and to characterize them in part, rather than to assess the assays

used to examine them. The design of the study was discussed and agreed by the WHO Working Group on International Reference Materials for the Diagnosis and Study of TSEs, whose membership is given in the Acknowledgements.

METHODS

Preparation of brain samples. Samples of grey-matter-enriched frontal cortex from four brains were provided by the CJD Surveillance Unit in Edinburgh, who retain the relevant clinical information. Samples of approximately 100 g were provided from: one normal non-CJD brain; one sporadic CJD brain, nominally assigned to type 1 (sp1CJD) (Parchi *et al.*, 1996); one sporadic CJD brain, nominally type 2 (sp2CJD); and one vCJD brain. Histopathological examinations of the three CJD brains were reported to be consistent with the preliminary PrP^{Sc} types and codon 129 genotype. All four brains were from patients homozygous for Met at codon 129 in the prion protein (*PRNP*) gene; all cases of vCJD to date have been of this allelic form. (A fifth sample from the brain of a sporadic CJD subject who was heterozygous for Met/Val at codon 129 of the *PRNP* gene was later supplied by Professor H. Budka, Vienna, and was also prepared and distributed in ampoules, but this did not form part of the study reported here.) The disease had been confirmed histologically in all cases. Samples of the three brains with CJD had previously been shown to contain PrP^{Sc} and, at least in the case of the vCJD case, to transmit disease while being of low toxicity when inoculated into animals (J. Ironside, personal communication). The specimens were transported to the Institute for Animal Health, Compton, UK, where they were homogenized in 0.25 M unbuffered sucrose using a programmable Camlab Omni-Mixer set at 4000 r.p.m. for six bursts of 30 s to give a final volume of 1 litre of 10% homogenate. A new grinder was used for each individual brain to avoid the possibility of cross contamination. Concern had been expressed in discussion over the homogeneity of laboratory preparations and the uniformity of the aliquoted preparations is an essential element in their use. Uninfected bovine brain homogenized by this method was therefore analysed in preliminary experiments to provide an indication of the quality of the preparations and showed a very uniform distribution of particle size by ultracentrifugation (data not shown). The suspension was kept on ice on a magnetic stirrer and dispensed in 0.5 ml aliquots into 2000 1.2 ml cryovials (Nalgene), flash-frozen in liquid nitrogen and stored at -86 °C for subsequent use. The bacterial bioburden was 200–600 colony-forming units ml⁻¹.

Design of study. Participants were requested to use routine in-house methods to titrate the amounts of PrP^{Sc} in the preparations four times from separate vials, using 10-fold serial dilutions for the first run to establish approximate end-points and 3-fold serial dilutions for the remainder. The highest dilution containing detectable PrP^{Sc} was reported and raw data were returned with technical details of the method employed.

RESULTS

Immunoblot assays

Thirteen laboratories initially agreed to participate in the collaborative study in which the materials were to be studied by immunoblotting using in-house procedures. Two of the laboratories involved in the immunoblotting studies stopped after preliminary characterization for reasons described below, two analysed the samples by methods

other than immunoblotting and six others returned data. No results were returned by the remaining three laboratories. The procedures followed by those laboratories returning data are summarized in Table 1. They involved treatment of the samples with proteinase K at either 50 or 100 $\mu\text{g ml}^{-1}$ for 30–60 min and detection of undigested PrP, identified as PrP^{27–30} (the protease cleavage product of PrP^{S^c}), after electrophoresis by immunoblotting using a specific monoclonal antibody, either 3F4 (Kascsak *et al.*, 1987) or 6H4 (Korth *et al.*, 1997). The sample volume loaded ranged from 6 to 40 μl , but, because of the differences in pre-treatment and dilution or concentration, the equivalent volume of brain homogenate that would have been loaded from a hypothetical undiluted preparation varied from 3 to 100 μl as shown in Table 1. The other major difference in reported technique was in the dilution of the samples. One laboratory diluted the sample in buffer before digestion, two diluted the sample in normal brain before digestion and three diluted the sample in buffer after digestion.

The end-point dilutions of assays of the preparations, defined as the last dilution giving a positive signal, are summarized in Table 2, with the geometric mean titres. The variation in the titre of a given preparation within a laboratory was of the order of 3-fold, consistent with the dilution series used, except for Laboratory 1 with the first assay, which employed 10-fold dilution steps, and Laboratory 3 where the range was up to 27-fold. The range of end-point titres between laboratories was much higher, of the order of 100-fold.

The end-points were then calculated in terms of the minimum volume of original 10% brain homogenate yielding a detectable signal, by dividing the volume loaded from an undiluted preparation by the mean end-point dilution. The results are given in Table 3. They ranged from 0.067 to 0.33 μl for sp1CJD, from 0.35 to 2.0 μl for sp2CJD, and from 0.067 to 0.6 μl for vCJD. The variation in the detection limit between laboratories for the different samples was therefore 4.9-fold (sp1CJD), 5.7-fold (spCJD2) and 9.0-fold (vCJD). Apart from the vCJD sample, the variation between laboratories was therefore of the same order as the dilution steps used. The variations in the details of the methods, in particular diluting the sample in normal brain before digestion, appeared to have no effect on the assay sensitivity, defined as the minimum detectable amount of 10% homogenate.

The reciprocal end-point dilutions of the different preparations were expressed in terms of that of the vCJD sample to see whether this would reduce the variation between laboratories. The results are expressed in Table 4. The values ranged from 0.42 to 2.4 (5.7-fold) for sp1CJD and from 0.1 to 0.60 (6.0-fold) for sp2CJD. As the inter-laboratory variation was of the same order as predicted from variation in making the dilutions, the use of a relative potency would not reasonably be expected to reduce it further.

Table 1. Summary of methods used in immunoblot assays

Laboratory	Pre-treatment	Proteinase K treatment	Post-treatment	Vol. loaded (μl)	Equivalent vol. loaded (μl)	Gel concn (%)	Detector antibody
1	Thaw, vortex	50 $\mu\text{g ml}^{-1}$, 37 °C, 1 h	Dilute in 2% SDS, boil 10 min	10	5	16	3F4
2	Dilution in lysis buffer	50 $\mu\text{g ml}^{-1}$, 37 °C, 1 h	Boil in 2% SDS, 10 min	40	20	12	3F4
3	Dilution in 10% brain	50 °C, 30 min*	—*	6	3	12	6H4
4	Thaw, homogenize	100 $\mu\text{g ml}^{-1}$, 37 °C, 1 h	Dilute in 2% SDS, boil in 2% SDS	15	8	14	3F4
5	Thaw, homogenize	50 $\mu\text{g ml}^{-1}$, 37 °C, 1 h	Boil in 2% SDS, 10 min, dilute in 2% SDS	10	5	12	3F4
6	Dilution in normal brain. Pellet 500 μl sample to digest	100 $\mu\text{g ml}^{-1}$, 37 °C, 30 min	Boil in 2% SDS, 10 min	34	100	12	6H4

*A commercial digestion buffer and loading buffer was used.

Table 2. Detectable dilution end-point titres

Laboratories 1–6 used immunoblots as described in Table 1. Laboratory 7 used the CDI method in two formats, (a) and (b). GMT, geometric mean titre.

Laboratory	sp1CJD	GMT	Sp2CJD end-points	GMT	VCJD end-points	GMT
1	10, 30, 10, 100	23	10, 10, 10, 10	10	100, 100, 30, 30	55
2	100, 100, 100, 100	100	10, 10, 10, 10	10	100, 100, 100, 100	100
3	9, 3, 9, 81	12	9, 0, 1, 9	3	3, 3, 3, 27	5
4	100, 100, 100, 300	120	10, 30, 30, 30,	23	100, 100, 100, 300	120
5	10, 11, 36, 11	15	1, 3, 11, 11	4	10, 11, 36, 36	20
6	1000, 300, 1000, 300	550	300, 300, 100, 300	225	300, 1000, 1000, 300	550
7a	1000, 3000, 3000, 3000	2.2×10^3	1000, 3000, 1000, 1000	1.3×10^3	1000, 3000, 3000, 3000	2.2×10^3
7b	30000, 100000, 100000, 300000	9.7×10^4	10000, 10000, 30000, 100000	2.3×10^4	100000, 100000, 300000, 300000	1.7×10^5

Table 3. Minimum detectable volume of homogenates

Laboratories 1–6 used immunoblots. Laboratory 7 used the CDI method in two formats, (a) and (b).

Laboratory	Vol. loaded*	sp1CJD†	sp2CJD†	vCJD†
1	5	0.22	0.50	0.091
2	20	0.20	2.0	0.20
3	3	0.25	1.0	0.60
4	8	0.067	0.35	0.067
5	5	0.33	1.25	0.25
6	100	0.18	0.44	0.18
7a	167	0.076	0.13	0.076
7b	167	0.0017	0.0073	0.00098

*Equivalent volume (µl) of 10% brain homogenate in undiluted sample.

†Equivalent volume (µl) of 10% brain homogenate in last loading track containing detectable PrP.

Other *in vitro* assays

Two other laboratories applied *in vitro* assays to the samples that were not based on immunoblotting. One was the conformation-dependent immunoassay (CDI) (Safar *et al.*, 1998; Bellon *et al.*, 2003), which was used in two formats. In the first format (a), 475 µl samples were added to 1 ml 10% simian brain homogenate to give dilution steps of 0.5 log₁₀. One millilitre of each dilution was treated with proteinase K before precipitation with phosphotungstic acid. The precipitate was resuspended and then split into two aliquots, one of which was denatured with guanidinium hydrochloride, and each aliquot was divided between three glutaraldehyde-activated wells on a microtitre plate. Each well thus received the equivalent of one-third of 500 µl, or 167 µl, of the dilution. Wells were incubated

Table 4. Potency of preparations relative to vCJD preparation

Potencies are expressed as the dilution end-point titre of the preparation divided by the dilution end-point titre of the vCJD preparation measured in the same laboratory. Laboratories 1–6 used immunoblot assays. Laboratory 7 used the CDI method in two formats, (a) and (b).

Laboratory	sp1CJD	sp2CJD
1	0.42	0.18
2	1.00	0.10
3	2.40	0.60
4	1.00	0.19
5	0.75	0.20
6	1.00	0.41
7a	1.00	0.58
7b	0.58	0.13

with labelled 3F4 monoclonal antibody and reactivity assessed by time-resolved fluorescence. This antibody binds native PrP^c but not PrP^{Sc} unless it is denatured. Thus, a dilution containing PrP^{Sc} will give a ratio of denatured to native signals greater than that given by normal brain and an end-point can be determined. The signal was the mean obtained from the three wells. The data are given in Tables 2 and 3 under Laboratory 7a. The end-point titres recorded were 2.2×10^3 , 1.3×10^3 and 2.2×10^3 for sp1CJD, sp2CJD and vCJD, respectively. The equivalent minimum volumes of 10% brain homogenate containing a detectable signal were 0.076 μ l, 0.13 μ l and 0.076 μ l, respectively, in a range similar to that of the immunoblot assays. The results were slightly more comparable to those of the immunoblot assays when expressed as a relative potency (Table 4).

In the second format (b), samples were diluted in 0.5 log₁₀ steps in human plasma containing 2% lauryl sarcosine, after which 1 ml was precipitated with phosphotungstic acid, treated with proteinase K and split into two aliquots as above. The aliquots were treated as for method (a) and divided between three wells in a microtitre plate. The wells were coated with a monoclonal antibody specific for human PrP. Each well thus received the equivalent of one-third of 500 μ l of each dilution, or 167 μ l. The data are given in Tables 2 and 3 under Laboratory 7b. The end-point titres were 9.7×10^4 , 2.3×10^4 and 1.7×10^5 for sp1CJD, sp2CJD and vCJD, respectively (Table 2), equivalent to a minimum detectable volume of 10% brain homogenate of 0.0017, 0.0073 and 0.00098 μ l, respectively, approximately 50-fold lower than any other method. However, the potencies measured by method 7b expressed as the ratio of the end-point dilutions relative to that of the vCJD preparation were similar to those determined for the immunoblot methods (Table 4), suggesting that the use of the vCJD preparation as a calibrant would make assay results comparable.

The other *in vitro* method employed by the eighth laboratory was qualitative in nature in the format used and was based on the extraction of PrP^c by 2 M guanidinium hydrochloride followed by the extraction of PrP^{Sc} by 6 M guanidinium hydrochloride (Barnard *et al.*, 2000). Extracted PrP was assayed by DELFIA (dissociation enhanced lanthanide fluorescence immunoassay) and the results expressed as the ratio of PrP^{Sc} to total PrP. A result in excess of that

of normal brain indicated the presence of PrP^{Sc}. The results from one run are shown in Table 5. Provided sufficient sample was examined, the ratio was independent of dilution. It can be seen that the normal brain gave a figure of 15.5% as a 1:10 or 1:100 dilution of the 10% homogenate, the sp1CJD a mean figure of 50.5% as a 1:10 or 1:100 dilution, sp2CJD a figure of 22.9% at a dilution of 1:10 or 1:100 and vCJD a mean figure of 65.7% up to a dilution of 1:1000. Presumably the data could be reinterpreted to provide a truly quantitative estimate, but the assay was not designed for use in this way. However, it is of interest that the vCJD gave the highest percentage of PrP^{Sc}, closely followed by sp1CJD, and sp2CJD was the least potent, in keeping with the results presented in Tables 2 and 3.

Characterization of materials

Different forms of CJD have been subclassified based on their histopathological profiles and the biochemical characterization of the PrP^{Sc} deposited. In particular, the ratio of the di- and monoglycosylated forms of proteinase K-digested PrP^{Sc} and the electrophoretic migration of the non-glycosylated form of the digested PrP^{Sc} have been used to classify CJD preparations into different types (Parchi *et al.*, 1996; Collinge *et al.*, 1996). It has been assumed that, except in rare cases, the brain from a case of sporadic CJD has only one type of PrP present, although there are reports of two types in a single brain (Puoti *et al.*, 1999; Kovacs *et al.*, 2002). The samples in this study were chosen on the basis of preliminary analysis of small samples suggesting that one contained type 1 PrP (sp1CJD) and the other type 2 (sp2CJD). However, results returned from Laboratories 1–6, as well as information from the two additional laboratories who carried out preliminary studies, showed that both preparations contained mixtures of type 1 and type 2 PrP^{Sc} according to the nomenclature of Parchi *et al.* (1996). Representative blots from one laboratory are shown in Fig. 1. A double band can clearly be seen in the position of the non-glycosylated fragment for both sp1CJD and sp2CJD, although not for vCJD. The double bands migrated with authentic type 1 and type 2 PrP fragments and were not affected by reduction of disulphide bridges with 2-mercaptoethanol or by digestion of the samples with proteinase K in the presence of EDTA to eliminate divalent cations (M. Head, personal communication). Moreover, studies of extracted DNA by short tandem repeat analysis and for platelet antigen markers both showed that each sample was from a unique individual and not from more than one person (data not shown), ruling out contamination as an explanation. It is therefore likely that the two sporadic CJD brains contained both types of PrP.

DISCUSSION

There is a need for reference materials for the quantitative comparison of the results of diagnostic methods for

Table 5. PrP^{Sc} (%) measured by differential extraction

Dilution	Normal	sp1CJD	sp2CJD	vCJD
10	15.5	49.6	22.9	63.7
100	15.4	51.5	22.9	68.3
1000	1.8	29.8	12.7	65.1
10 000	–	11.9	–	22.5
10 0000	–	–	–	–

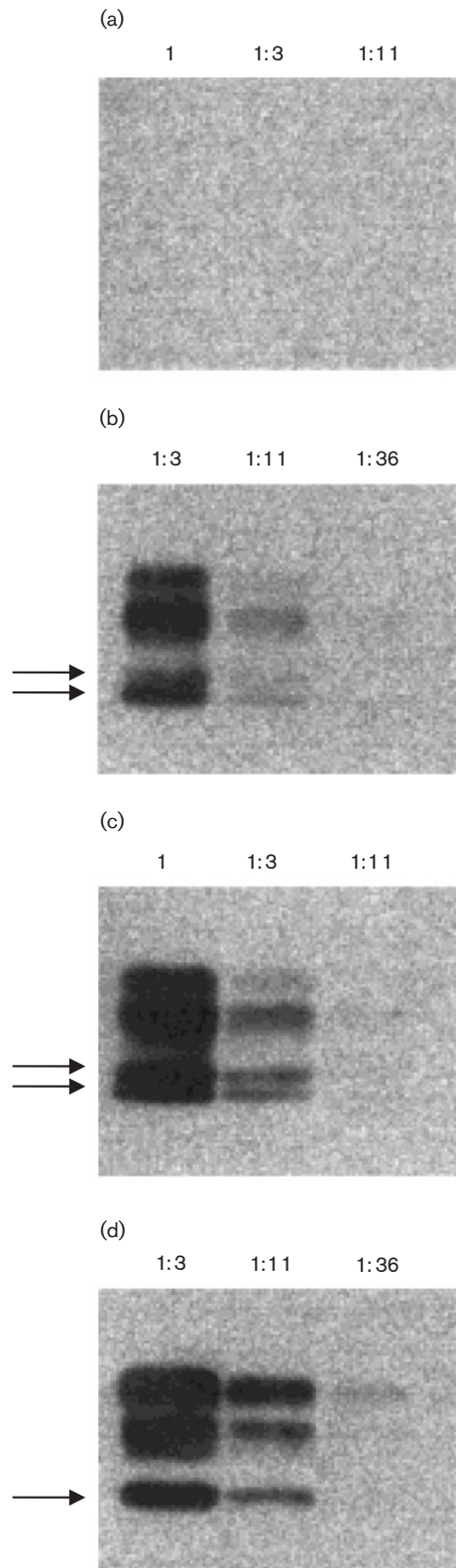


Fig. 1. Immunoblots of preparations resolved after proteinase K treatment using antibody 3F4. (a) Normal brain; (b) sporadic CJD type 1 brain; (c) sporadic CJD type 2 brain; (d) vCJD brain. The non-glycosylated protein gives a doublet band in (b) and (c) but not in (d).

detecting material containing infectious TSE agents. Various assay approaches are under study, including those based on the detection of PrP^{Sc} or its truncated protease digestion product (PrP²⁷⁻³⁰) by immunological or other methods and infectivity assays in a range of species including wild-type and transgenic mice. Hitherto, different assays have been applied to specimens from the same brain or similar infected tissues, but the preparations described here represent a large number of identical samples, so that the results of one assay can be compared directly with those of another.

The initial characterization of the materials reported here concerned *in vitro* methods, mostly immunoblotting. Differences in sample preparation and the volumes loaded onto the gels resulted in a range of detectable end-point dilutions among laboratories of the order of 100-fold. Within-laboratory reproducibility was of the order of threefold except for Laboratory 3 where the assay was less well controlled at the time of the study. When all results were included and expressed in terms of minimum volume of homogenate containing detectable PrP^{Sc}, the results between laboratories were surprisingly uniform with a range of 4.4- to 9.0-fold depending on the homogenate concerned. This approximates to the dilution steps employed and to the variation within a laboratory. Thus, as might be expected, expressing the results as a potency relative to the vCJD sample did not greatly improve reproducibility, but gave a range of 5.7- and 6.3-fold for the sp1CJD and sp2CJD samples, respectively. It was striking that the details of the assays, specifically dilution before or after digestion in brain or buffer, had no detectable influence on the results obtained.

Data from the other quantitative *in vitro* method, CDI, showed that it was of high sensitivity, but comparable to the most sensitive of the immunoblot methods in terms of minimum detectable volume of brain homogenate when used in the initial format. Differences in sensitivity expressed in terms of end-point dilutions arose chiefly from the sample volume assayed, with the most sensitive able to concentrate dilute preparations in a quantitative manner. However, the modified CDI format, involving dilution in plasma and the use of a monoclonal antibody capture method, gave a signal from 50- to 100-fold less material. The potencies of the sp1CJD and sp2CJD preparations relative to the vCJD preparation were within the same range as for the other assays, however, and the vCJD preparation could be used as a calibrator of potencies.

The semi-quantitative assay method gave results consistent with those of the other methods, with the vCJD and sp1CJD specimens giving a higher PrP^{Sc} content than the sp2CJD material.

The observation that both sp1CJD and sp2CJD preparations contained PrP^{Sc} of both type 1 and type 2 could not be attributed either to accidental mixing of samples from more than one individual or to gel artefacts and is interpreted to mean that the patients' brains actually contained

both types. This may be either because both brains were somehow atypical or because the presence of both types of PrP^{Sc} in a single patient is more common than has been previously thought. Even when small tissue samples (<100 mg) have been assayed, two laboratories have independently reported the presence of type 1 and type 2 PrP^{Sc} in individual spCJD brains (Puoti *et al.*, 1999; Kovacs *et al.*, 2002). The specimens used in this study involved 100 g of each brain, so that material was derived from a larger brain area than is usually examined. The vCJD brain preparation was uniform. While the reproducible behaviour of the sp1CJD and sp2CJD brains in the study suggests that the mixture of types does not present a problem for the types of assays performed here, the suitability of the materials from the sporadic CJD cases as reference preparations for some other purposes might be questioned and that was the reason why two laboratories stopped work after preliminary characterization. This issue will only be settled by further studies using other methods, including infectivity assays that are currently under way or planned. Ultimately the materials described here, with others in development, will be examined by a range of procedures, enabling a correlation between *in vitro* and *in vivo* potencies and providing well-characterized materials by which new procedures, including new infectivity assays in lines of transgenic mice, can be compared quickly. The candidate Biological Reference materials are intended for diagnostic assessment, not as seed materials or for use in spiking studies, and are now available for this purpose. It is intended to expand the available materials to include suspensions of brain tissue from CJD patients with other PrP genotypes and samples of other tissues including lymphoid tissues and blood.

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