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Detection of ultra-low levels of pathologic prion protein in scrapie infected hamster brain homogenates using real-time immuno-PCR☆

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Abstract

Pathologic prion protein (PrP^{Sc}), implicated in transmissible spongiform encephalopathies, is detected by antibody-based tests or bioassays to confirm the diagnosis of prion diseases. Presently, the Western blot or an ELISA is officially used to screen the brain stem in cattle for the presence of PrP^{Sc}. The immuno-polymerase chain reaction (IPCR), a technique whereby the exponential amplification ability of PCR is coupled to the detection of proteins by antibodies in an ELISA format, was applied in a modified real-time IPCR method to detect ultra-low levels of prion protein. Using IPCR, recombinant hamster PrP^{C} was consistently detected at 1 fg/mL and proteinase K (PK)-digested scrapie infected hamster brain homogenates diluted to 10^{-8} (approximately 10–100 infectious units) was detected with a semi-quantitative dose response. This level of detection is 1 million-fold more sensitive than the levels detected by Western blot or ELISA and poises IPCR as a method capable of detecting PrP^{Sc} in the pre-clinical phase of infection. Further, the data indicate that unless complete PK digestion of PrP^{C} in biological materials is verified, ultrasensitive assays such as IPCR may inaccurately classify a sample as positive.

Keywords: BSE; Immuno-PCR; IPCR; Real-time IPCR; Prion; PrP^{C}; PrP^{Sc}; Scrapie; TSE

1. Introduction

Transmissible spongiform encephalopathies (TSEs) in mammals are mediated by an abnormal conformer of the normal prion protein (PrP^{C}). The abnormal conformer, termed PrP-scrapie (PrP^{Sc}), is characterized by insolubility, a high proportion of beta-pleated sheet tertiary structure, and resistance to degradation by PK (Prusiner, 1991). In humans, the clinical forms of TSE include Kuru, Creutzfeld–Jacob disease (CJD), Gerstmann–Straussler–Scheinker syndrome (GSS) and fatal familial insomnia (FFI). In animals, a variety of TSEs have been described, including scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle (for reviews, see Prusiner, 1991). In 1996, a variant of CJD (vCJD) was described, which occurs in young adults, has atypical clinical features and neuropathology (Will et al., 1996), and is transmitted by the consumption of tissue from cattle contaminated by the BSE infectious agent (Bruce et al., 1997; Scott et al., 1999; Collinge, 1999).

Prion diseases are typically identified during the clinical stages of infection when it is present in high quantities in the brain. During the clinical stage of vCJD, PrP^{Sc} has been detected in a variety of tissues including CSF, spleen, tonsils, lymph glands, retina, proximal optic nerve, rectum, adrenal gland, thymus, and muscle (Hill et al., 1997; Hilton et al., 1998; Bieschke et al., 2000; Bruce et al., 2001; Wadsworth et al., 2001; Glatzel et al., 2003), and in the appendix during the pre-clinical stage of infection (Hilton et al., 1998). One study attempted the detection of PrP^{Sc} in blood using immunocompetitive capillary electrophoresis (ICCE), but it

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was determined that the method was unsatisfactory for use as a screening test in human TSE (Cervenakova et al., 2003a).

It has been shown that infectivity is present in the blood of infected animals, both during and preceding symptomatic disease. Animals that are injected intracranially (IC) or intravenously (IV) with blood from an infected animal or human-derived vCJD and GSS will develop disease (Casaccia et al., 1989; Brown et al., 1999; Taylor et al., 2000; Houston et al., 2000; Hunter et al., 2002; Cervenakova et al., 2003b). The presence of infectivity by experimentally induced infections in the absence of detectable PrP^{Sc} in blood is taken to imply that the pathogenic prion protein exists below the level of detection of current serological methods. Thus, the inability of current methods to detect ultra-low levels of PrP^{Sc} in blood (or other biologic samples) is the most likely limitation for the development of effective ante-mortem screening tests for the TSEs. In humans, this possibility has recently been supported by two transfusion-associated infections from individuals who later developed vCJD (Llewelyn et al., 2004; Peden et al., 2004).

Presently, there are five BSE testing kits that are approved by the Commission of the European Communities for the testing of bovine brain material in Europe (Moynaugh and Schimmel, 1999; European Commission, 2003), and include the Bio-Rad TeSeE (formerly Platelia™ BSE test; Bio-Rad Laboratories, Hercules, CA); the Enfer TSE Test (Enfer Scientific, Newbridge, Ireland); the InPro CDI-5 Test (InPro Biotechnology Inc., San Francisco, CA); and the Prionics®-Check WESTERN and Prionics®-Check LIA (Prionics AG, Schlieren, Switzerland). These test kits utilize either the Western blot or a variation of a standard ELISA (i.e., enhanced chemiluminescent or sandwich immunoassay) for detection of PrP^{Sc} from the obex region of the bovine spinal cord. Results are reported as qualitative by Western blot or semi-quantitative by ELISA (i.e., positive if above a non-specific background threshold or negative if below this threshold). These tests, by nature of their methodologies, lack exquisite sensitivity. The highest sensitivities for these tests are 30 pg/mL, 1 ng/mL, and a limit of a 10^3 dilution of BSE infected brain homogenate for the Prionics®-Check LIA test (Biffiger et al., 2002), the InPro CDI-5 test (Safar et al., 2002), and the Bio-Rad TeSeE® Detection Kit (Grassi et al., 2001), respectively.

The immuno-polymerase chain reaction (IPCR) is a method that combines the specificity of immunologic detection methods with the exponential amplification of PCR. First developed by Sano et al. (1992), it has shown the detection of as few as 10–580 molecules of analyte, corresponding to a 10,000-fold enhancement in sensitivity over standard serological methods for several target antigens (Case et al., 1999; Chang and Huang, 1997; Sano et al., 1992). Recently, IPCR has been applied for the detection of PrP^{Sc} (Gofflot et al., 2004), but the sensitivity of detection of that method was 10x that of ELISA and thus did not make it a candidate for the pre-clinical detection of pathogenic prion protein in blood.

We have designed a modified real-time IPCR method for the ultra-low detection of PrP^{Sc} after PK digestion to remove PrP^{C}. Our objectives were two-fold: (1) to assess the maximum sensitivity of IPCR for the detection of recombinant hamster PrP^{C} and PrP^{Sc} from scrapie infected hamster brain homogenates; and (2) to determine the increase in sensitivity attained by IPCR when compared with the ELISA for the estimation of infectious units detectable in scrapie infected hamster brain homogenates. We demonstrate that IPCR is a method that shows unmatched sensitivity for detection of PrP^{Sc} when compared to other methods. Further, we demonstrate that when using a highly sensitive assay to detect PrP^{Sc} (such as IPCR), complete PK digestion is critical to avoid detection of residual PrP^{C}.

2. Materials and methods

2.1. Hamster brain homogenates

Normal and scrapie hamster brain homogenates (10% homogenates titered at 10^9 IU/mL, strain 263 K) were obtained from Robert Rohwer (Molecular Neurovirology Laboratory, Veterans Affairs Medical Center, Baltimore, MD) and recombinant hamster PrP^{C} was obtained from Prionics AG (Schlieren, Switzerland). Serial dilutions of untreated and PK-digested normal and scrapie infected hamster brain pellets (reconstituted to the original volume of 400 μL) were made in 0.5% Triton-X/PBS and used for both ELISA and IPCR analyses.

2.2. Proteinase K digestion

Proteinase K (PK) digestion (50 μg/mL) was performed as described by Kang et al. (2003). Briefly, 200 μL of 10% brain homogenate was mixed with 100 μL of 8 M guanidine hydrochloride (GdHCl) and 500 μL of PBS was added for a final concentration of 1 M GdHCl. The solution was incubated at room temperature for 10 min with agitation, and then centrifuged at 13,800 × *g* for 10 min. The pellet was resuspended in 100 μL of 8 M GdHCl and 700 μL of PBS. Four hundred microliters of this solution was incubated with or without PK (50 μg/mL) for 30 min at 37 °C. After PK digestion, 1.6 mL of methanol was added to the mixture and the mixture was incubated at −20 °C for 1 h. The sample was centrifuged at 15,800 × *g* for 15 min and the supernatant was removed from the pellet. The supernatant and pellet (which contains the majority of PrP^{Sc}) were both analyzed by Western blot.

2.3. ELISA

The ELISA method was used for the analysis of recombinant hamster PrP^{C}, normal, and scrapie infected hamster brain homogenates. The prion ELISA, which is also the serologic portion of the IPCR, uses TopYield stripwells (Nalge Nunc Corp., Naperville, IL), as previously described

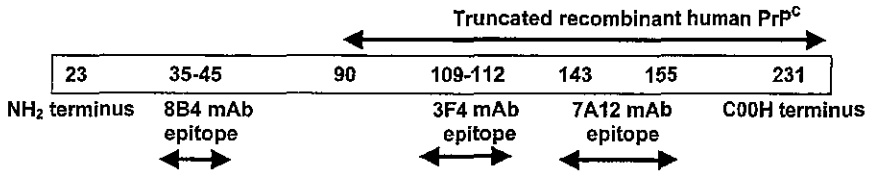

Fig. 1. Schematic representation of human PrP. Numbers represent the amino acid (aa) sequence. The arrowed regions represent the aa recognition sites of the 8B4, 3F4, 7A12 antibodies, and truncated recombinant human PrP^C.

for HIV-1 p24 antigen (Barletta et al., 2004). Briefly, the assay consisted of prion detection using one of two different immobilized capture mouse monoclonal antibodies (provided by Dr. Man Sun Sy, Case Western University, Cleveland, OH; Kang et al., 2003): either 8B4, which recognizes the N-terminal portion of PrP^C and PrP^{Sc} (35–45 aa), or 7A12, which recognizes the central region of PrP^C and PrP^{Sc} (143–155 aa). The specificities of all antibodies used in these studies are illustrated in Fig. 1. Biotinylated mouse monoclonal antibody 3F4 (Kascsak et al., 1987)(Signet Pathology Systems Inc, Dedham, MA) was used as the detector antibody, followed by streptavidin–HRP (KPL; Gaithersburg, MD: 2 μg/mL) and colorimetric detection with TMB substrate (Pierce Co., Rockford, IL) in ELISA. Samples with a mean signal/noise (S/N) of ≥2.0 were considered positive.

2.4. IPCR

The procedure for IPCR was identical to the ELISA procedure except for the addition of 10–100 pg/mL biotinylated reporter DNA (500 bp of DNA sequence with 25% incorporated biotinylated dCTP) after the addition of streptavidin–HRP, which acted as a linker between the biotinylated antibody and the biotinylated DNA. Real-time PCR was then performed directly in TopYield stripwells using the iCycler iQ™ instrument (Bio-Rad Laboratories, Hercules, CA). The principle of the real-time IPCR method with fluorescent probe detection is depicted in Fig. 2. The PCR cycling parameters were an initial 6 min at 96 °C, followed by 20 cycles of 1 min at 95 °C and 2 min at 68 °C. After 20 cycles of PCR, 5 μL from each reaction was aliquoted into new PCR reagents in standard PCR tubes for a second amplification round of 50 cycles. Fluorescence was detected during PCR amplification by hydrolysis of a hybridization probe labeled with a 5′-reporter dye: 6-carboxyfluorescein, and a 3′-Black Hole Quencher dye (Biosearch Technologies Inc, Novato, CA).

Technical modifications to the original IPCR protocol (Sano et al., 1992) included using 5 U/mL heparin and 0.5 M EDTA in all wash and diluent buffers, additional blocking steps after addition of streptavidin–HRP and before PCR, the use of a reagent in antibody diluents which blocks nonspecific immunologic reactions between antibody molecules (FcR Blocking Reagent, Miltenyi Biotec, Aurora, CA), and two rounds of PCR amplification (an initial 20 cycles followed by 50 cycles in fresh PCR reagents in standard PCR tubes). An automated plate washer with individual probes for each microwell was used after the addition of DNA

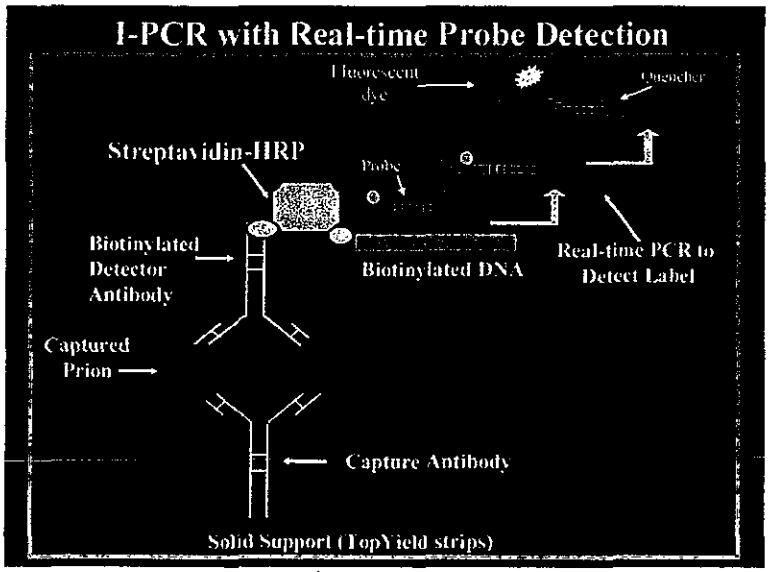

Fig. 2. Diagram of the IPCR technique. Capture anti-PrP antibody, adsorbed to the microwell plate, is used to capture the PrP antigen. Streptavidin–HRP bridges between a biotinylated detector anti-PrP antibody and the biotinylated 500 bp reporter DNA. The reporter DNA is amplified by PCR using a fluorescent probe for real-time analysis.

reporter template to decrease the possibility of (well-to-well) cross-contamination.

To determine the detection limit of IPCR for PrP, we generated an IPCR standard curve using dilutions ranging from 10^4 to 10^{13} molecules of recombinant hamster PrP^C. The specificity of this method was established by screening >2× the number of replicates of negative controls (PK-digested normal hamster brain homogenate) as positive control samples (PK-digested scrapie infected hamster brain homogenates). All samples were tested in three to four replicates and the negative controls were run in replicates of 8–12. The criterion for acceptance of a run was that 75% or greater of the negative controls must fall below the background fluorescent threshold (BFT). Samples were determined to be positive when the relative fluorescence units (RFUs) of at least 50% of the test replicates were above the threshold set for 75% or more of the negative control replicates. Additionally, serial dilutions of recombinant hamster PrP^C standards or scrapie infected hamster brain homogenates had to exhibit a semi-quantitative dose response for the run to be accepted.

The standard curve generated by IPCR (in contrast to a PCR standard curve using template DNA) was used to determine the molecular numbers of PrP^{Sc} detected. The fluorescent threshold was automatically set by the iCycler instrument and was defined as the mean standard deviation of fluorescence in the sample well over baseline cycles (Bio-Rad Laboratories, iCycler iQ Operating Instructions, Hercules, CA). For some runs, the threshold was manually raised above the fluorescence level of the negative controls. Cycle threshold (Ct) was the cycle at which the sample's fluorescence intersected the BFT during continued rounds of amplification.

3. Results

The PCR portion of the method, optimized for use with IPCR, displayed a correlation coefficient of 0.997, an efficiency of 99.6%, and was able to detect down to one molecule of DNA template in a standard curve (data not shown) (Edelman and Barletta, 2003). The IPCR standard curve, derived from dilutions of recombinant PrP^C, displayed mean Cts of 19.23, 19.02, 25.26, 37.89, 42.86 for 10^{13}, 10^{11}, 10^9, 10^5, and 10^4 molecules of recombinant hamster PrP^C, respectively (Fig. 3a) with a correlation coefficient of 0.961, and an efficiency of 123.3% (Fig. 3b). One femtogram per milliliter (approximately 10^4 molecules/mL) of recombinant hamster PrP^C was consistently detectable when analyzing two to four replicates of each standard dilution, and 100 ag/mL (2600 molecules/mL) was detectable in approximately 50% of replicate experiments (data not shown). Similar levels of sensitivity of detection (i.e., 10^2–10^3 molecules/mL) have been observed using a real-time IPCR method specific for HIV-1 p24 antigen (Barletta et al., 2004). Note that the standards with higher concentrations of

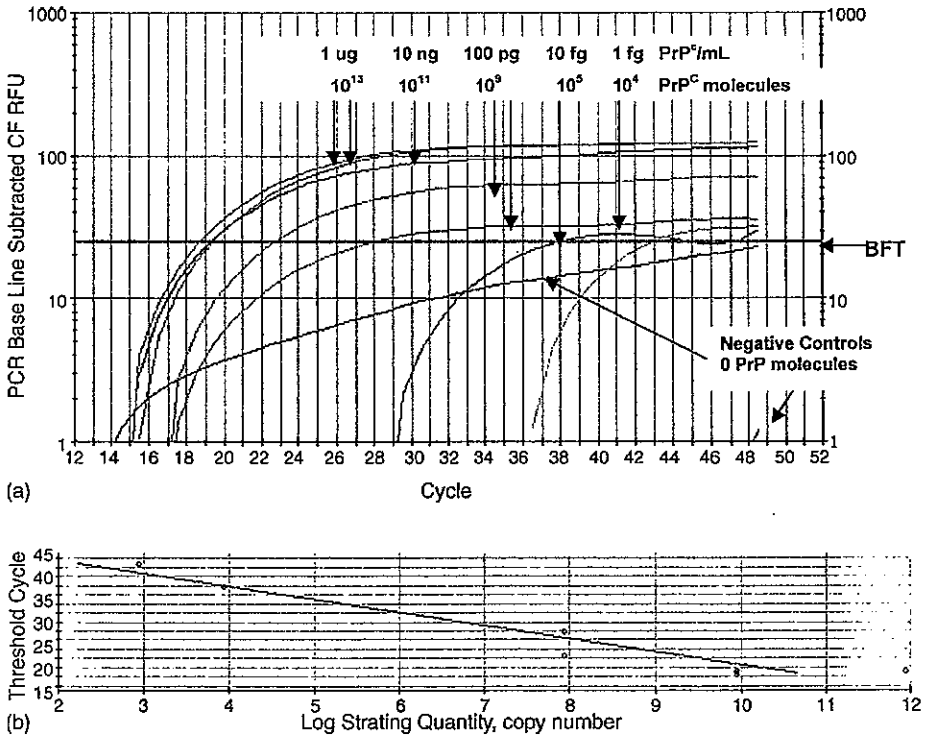

Fig. 3. IPCR standard curve. (a) IPCR was performed on serial dilutions of recombinant hamster PrP^C ranging from 10^{13} to 10^4 molecules for the standard curve. The background fluorescent threshold (BFT) is placed at 25 RFU to exclude one negative control with low levels of fluorescence. (b) The correlation coefficient of the IPCR standard curve with the corresponding regression equation. The mean Ct was derived from two to four replicates.

input recombinant hamster PrP^C (10^{13} and 10^{11} molecules per reaction) are indistinguishable by IPCR and did not display a dose response but instead exhibited nearly identical Cts (i.e., 19.23 and 19.02, respectively), as shown in Fig. 3a.

Dilutions of PK-digested normal and scrapie infected hamster brain homogenates were tested by IPCR using either 8B4 or 7A12 as the capture antibody. A semi-quantitative dose response relative to the Ct was observed. Under optimal conditions, the Ct should increase 3.3 cycles for every log decrease in molecular number. When using 8B4 as the capture antibody, multiple (two to four) determinations for the 10^{-4} and 10^{-5} dilutions of PK-digested scrapie infected hamster brain homogenates exhibited a dose response with mean Cts of 23.4 and 27.1, respectively. Note that one of the three replicates of the 10^{-5} dilution was not detected as positive (i.e., was below BFT) (Fig. 4a), and the mean Cts of the 10^{-2} and 10^{-3} dilutions were indistinguishable from the 10^{-4} dilution (e.g., 23.5 and 23.6 versus 23.4, respectively) (data not shown).

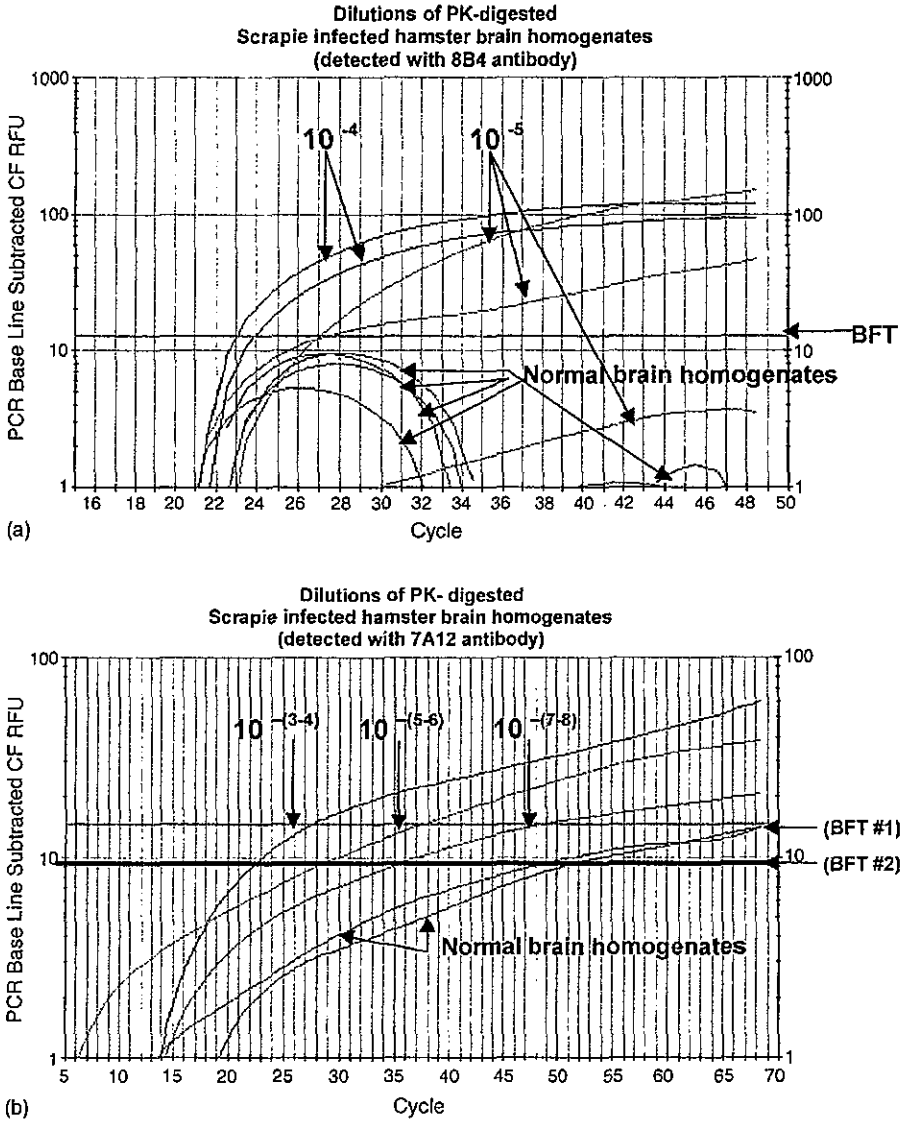

Fig. 4. IPCR of PK-digested normal and scrapie infected hamster brain. A 10% homogenate of normal or scrapie infected hamster brain was digested with 50 μg/mL PK at 37 °C for 30 min. IPCR was performed on serial dilutions (a: 10^{-4} and 10^{-5}; b: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}) of PK-digested scrapie infected hamster brain homogenates using 8B4 (a) or 7A12 (b) as the capture antibody. A 10^{-6} dilution was below the level of detection for 8B4 but a $10^{-(7-8)}$ dilution was detectable by 7A12 indicating that all residual PrP^C had been digested. Single lines shown in the graph are representative of three to four replicates from several experiments where 50% or more of the replicates were above the fluorescent threshold. Eighty percent of all negative controls (eight replicates) were below the fluorescent threshold. Not all replicates of the normal brain homogenates are visible on the graph. The 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions are approximately 7 ng/mL, 0.7 ng/mL, 70 pg/mL, 7 pg/mL, 700 fg/mL, and 70 fg/mL (or 10^5, 10^4, 10^3, 10^2, and 10^1 IU), respectively. BFT #1 or BFT #2: background fluorescent threshold #1 setting versus #2 setting.

Table 1
Determination of IU, PrP^{Sc} concentration, and number of PrP^{Sc} molecules in serial dilutions of scrapie infected hamster brain homogenates derived from the ELISA and IPCR (mean Cts) using 7A12 antibody

Dilutions of scrapie infected hamster brain homogenates (titered at 10^9 IU/mL)	IPCR method		IU[c] (mL)	ELISA method	
	Mean Ct[a] ± S.E. (IPCR)	Calculated number of PrP^{Sc} molecules/mL[b] (regression equation) (IPCR)		Approximate concentration[d] of PrP^{Sc} (ELISA)	Calculated number of PrP^{Sc} molecules/mL[e] (ELISA)
10^{-2}	ELR[f]	ELR[f]	10^7	70 ng/mL	12×10^{11}
10^{-3} to 10^{-4}	27.8 ± 3.3	3.1×10^7	10^{5-6}	0.7–7 ng/mL	$12 \times 10^{9-10}$
10^{-5} to 10^{-6}	34.9 ± 3.4	1.0×10^5	10^{3-4}	7–70 pg/mL	$12 \times 10^{7-8}$
10^{-7} to 10^{-8}	37.7 ± 5.4	1.0×10^4	10–100	70–700 fg/mL	$12 \times 10^{5-6}$

[a] Mean Ct + S.E. was determined from 9–12 replicates from five experiments.
[b] Calculated from the least squares linear regression fit to data from (a) to produce standard curve.
[c] Infectious unit (IU) is the amount of infectivity that will transmit disease to one animal. Brown (2001) estimates 1 LD_{50} is equivalent to 10 IU.
[d] Calculated from the ELISA test using recombinant hamster PrP^C as a standard and 7A12 as the capture antibody. A 1:100 dilution of the PK-digested scrapie infected hamster brain homogenate is approximately equal to 70 ng/mL recombinant hamster PrP^C.
[e] Calculated from the equation: (PrP^{Sc} concentration[d] (weight/mL)/35,000 mw) × (6×10^{23} molecules)/(mole).
[f] ELR: exceeds linear range of the assay.

We have observed that when using high concentrations of the target molecule (e.g., 10 ng/mL–1 μg/mL of PrP^{Sc} or PrP^C), there is little to no quantitative discrimination between adjacent log dilutions. To compensate for this effect in other experiments, we grouped the mean Cts from two adjacent logfold dilutions of the scrapie infected hamster brain homogenates generated from IPCR (e.g., the mean Cts from the combined (a) 10^{-3} and 10^{-4}; (b) 10^{-5} and 10^{-6}; and (c) 10^{-7} and 10^{-8} logfold dilutions) for a semi-quantitative mode of analysis.

When using 7A12 as the capture antibody, the three dilution groups (a, b, and c) of PK-digested scrapie infected hamster brain homogenates exhibited a dose response with mean Cts of 24.9, 37.3, and 45.4, respectively (Fig. 4b, BFT #1). Note that two of the eight replicates of normal hamster brain homogenates exhibited a linear (not exponential) increase in fluorescence. As previously described, a gradually increasing background fluorescence in the negative controls necessitates raising the BFT. In this case, the BFT was raised to 20 RFU (Fig. 4b, BFT #1) which skewed the expected dose response. When using BFT #1, the dose response for the PK-digested scrapie infected hamster brain homogenates was not ideal (i.e., 3.3 cycles per log decrease in molecular number is expected). Rather, in this case, there were approximately 8.1–12.4 cycles between the two groups of log dilutions. However, after lowering the BFT to 10.0 RFU (Fig. 4b, BFT #2), the mean Cts showed improved correlation for the three dilution groups at 22.8, 28.9, and 36.5, respectively (i.e., an increase of approximately 6.1–7.6 cycles between log dilutions). In this case, the Cts of the normal hamster brain homogenates crossed the BFT #2 but exhibited a mean Ct (50.5) that was significantly different (i.e., >14 cycles higher) from the mean Ct for the $10^{-(7-8)}$ dilution of scrapie infected hamster brain homogenate samples) (Fig. 4b). We have also tested a commercially available anti-prion antibody (6H4; Prionics AG, Schlieren, Switzerland) which produces results similar to the 7A12 capture antibody for the IPCR assay (data not shown). Note that 7A12 detects PrP^{Sc} in scrapie infected hamster brain homogenate at higher dilutions ($10^{-(7-8)}$) than 8B4 (which detects PrP^{Sc} at 10^{-5}) dilutions.

The regression equation of the recombinant hamster PrP^C standard curve (Fig. 3b) was used to calculate the PrP^{Sc} log number of molecules (Table 1). These extrapolated numbers of PrP^{Sc} molecules generated from the IPCR standard curve were then compared to the estimated number of PrP^{Sc} molecules generated from the ELISA standard curve using recombinant hamster PrP^C. Using the IPCR regression equation, the mean Cts from several experiments were 27.8, 34.9, and 37.7 for the a, b, and c dilution groups calculated to log 7.5 (antilog = 31,622,777 or 3.1×10^7 molecules), log 5.70 (antilog = 100,000 or 1.0×10^5 molecules), and log 4.0 (antilog = 10,000 or 1.0×10^4 molecules), respectively. The range for these calculated PrP^{Sc} molecular numbers derived from the regression equation of the IPCR standard curve (Fig. 3b) were all within one to two logs of the estimated number of PrP^{Sc} molecules generated from the ELISA test (Table 1).

Finally, the mean Cts correlated well with the estimated prediction of the amount of PrP^{Sc} present in the sample. For example, it has been noted previously that 1 LD_{50} is defined to be equal to approximately 10 IU or 100 fg/mL (Brown et al., 2000). The scrapie infected hamster brain homogenate used was titered at 10^9 IU; therefore, a 10^{-8} dilution of the homogenate would be predicted to equal 10 IU (which is therefore approximately equivalent to 1 LD_{50}, or 100 fg/mL of PrP^{Sc} (according to Brown et al., 2000). When extrapolated from the standard curve of the ELISA test with 7A12 as the capture antibody, a 10^{-8} dilution of the homogenate equaled approximately 70–700 fg/mL, an estimate within 0.7–7 times the predicted concentration (Brown et al., 2000). Brain homogenate sample dilutions, approximate concentrations derived from the ELISA, and calculated PrP^{Sc} molecular numbers are presented in more detail in Table 1.