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# Estimating prion concentration in fluids and tissues by quantitative PMCA

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## Abstract

Prions, the proteinaceous infectious agent responsible for prion diseases, can be detected with high sensitivity by protein misfolding cyclic amplification (PMCA) technology. Here we describe a quantitative PMCA procedure to calculate the concentration of very low levels of prions in biological samples. Using this procedure, we determined the quantities of misfolded prion protein ($PrP^{Sc}$) in brain, spleen, blood and urine of scrapie-affected hamsters.

Prion diseases are transmissible neurodegenerative disorders affecting humans and various animals[1]. The infectious agent, prion, is composed by a misfolded form of the prion protein ($PrP^{Sc}$) that propagates in the absence of nucleic acid. The unprecedented nature of the infectious agent and the recent appearance of new forms of transmissible prion diseases with devastating consequences, pose a considerable risk for human health[1]. One important objective in prion research is to detect and quantify small amounts of $PrP^{Sc}$ present in various samples, for both research and practical applications[2,3]. Quantification of prions will allow estimation of the concentration of infectious material in diverse samples, which will be important for risk assessment and implementation of regulatory measures to prevent prion spreading.

Prions accumulate in large quantities in the brain, but small amounts of $PrP^{Sc}$ exist in many tissues and biological fluids even at early stages of the presymptomatic period[1–4]. $PrP^{Sc}$ can be detected with high sensitivity by PMCA[5]. PMCA is a cyclical process, conceptually analogous to PCR, that takes advantage of the nucleation-dependent prion conversion to



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**AUTHOR CONTRIBUTIONS**

B.C. performed most of the experiments, analyzed the data and prepared the figures; R.M. helped with experimental design and data analysis; M.A.B. performed the PMCA experiments in blood; and C.S. developed the hypothesis, designed and analyzed all data, wrote the manuscript and supervised the project.

**COMPETING FINANCIAL INTERESTS**

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accelerate the process by using ultrasound waves to multiply the number of replication nuclei[5]. Prion replication can be started with the equivalent of one molecule of $PrP^{Sc}$ (ref. 6). This high level of sensitivity has enabled detection $PrP^{Sc}$ in blood and urine of experimentally inoculated animals[7–11].

Here we establish a quantitative PMCA (qPMCA) procedure to estimate the $PrP^{Sc}$ concentration in various samples. The method is based on the observation that there is a direct relationship between the quantity of $PrP^{Sc}$ in a given sample and the number of PMCA cycles and rounds necessary for its detection. To establish the method and minimize variability owing to the presence of other components in the tissue or fluid sample, we first partially purified $PrP^{Sc}$ using a procedure involving precipitation in the presence of sarkosyl, as described previously[12]. Using this protocol, more than 90% $PrP^{Sc}$ is recovered and corresponds to full-length $PrP^{Sc}$. To estimate the $PrP^{Sc}$ concentration in our stock solution, we deglycosylated partially purified $PrP^{Sc}$ and determined its concentration by comparison to the signal for known quantities of recombinant prion protein via western blotting and enzyme-linked immunosorbent assay (ELISA) (data not shown). Then we spiked various amounts of partially purified $PrP^{Sc}$ ($1 \times 10^{-8}$ to $1 \times 10^{-19}$ g) into normal hamster brain homogenate and subjected the samples to serial rounds of 144 PMCA cycles. We determined the number of PMCA rounds required to produce a $PrP^{Sc}$ signal detectable by western blot. For the signal to be considered 'positive' it had to be at least five times greater than the densitometric signal of the background. We then plotted the quantity of $PrP^{Sc}$ added to the tube versus the number of PMCA rounds needed for detection (Fig. 1). By extrapolating the number of PMCA rounds required to detect an unknown sample, we could estimate the quantity of $PrP^{Sc}$ originally present in the sample.

We used the qPMCA methodology to estimate $PrP^{Sc}$ concentration in various tissues and biological fluids of scrapie-affected hamsters. We collected samples from brain, spleen, blood and urine from five hamsters exhibiting the clinical signs of the disease after intraperitoneal inoculation with strain 263K prions. As described above, we partially purified $PrP^{Sc}$ by sarkosyl precipitation to remove components that may affect PMCA efficiency. After centrifugation, we resuspended the pellets directly in healthy hamster brain homogenate and subjected them to serial rounds of 144 PMCA cycles. From three positive spleen samples, $PrP^{Sc}$ was detectable after two rounds of PMCA for two samples and after the third round for the third sample (Fig. 2). Extrapolation from the calibration curve (Fig. 1b) enabled us to estimate that the average concentration of $PrP^{Sc}$ in the 'symptomatic' spleen was 20 pg $g^{-1}$ of tissue (Table 1). As controls, we subjected various samples of spleen and brain from healthy hamsters to the same procedure. We observed no $PrP^{Sc}$ signal after six rounds of PMCA in any of the control samples (Fig. 2), indicating that under the experimental conditions used, we detected no spontaneous generation of $PrP^{Sc}$.

We also analyzed $PrP^{Sc}$ concentrations in other tissues and fluids (Table 1). As expected, the brain contained the highest amount of $PrP^{Sc}$, which was readily detectable by western blotting without the need for PMCA (data not shown). We separated blood samples into plasma and buffy coat; using qPMCA we calculated a concentration of $PrP^{Sc}$ of 13 and 260 fg $ml^{-1}$ of blood, respectively (Table 1). Finally, we estimated $PrP^{Sc}$ concentration in urine as 0.2 fg $ml^{-1}$. These results indicate that spleen, buffy coat, plasma and urine from

symptomatic hamsters respectively contain approximately $10^6$, $10^8$, $2 \times 10^9$ and $10^{11}$ times less $PrP^{Sc}$ than the brain. These proportions, however, likely change dramatically at different stages of the disease. We are currently using qPMCA to assess the dynamic changes in $PrP^{Sc}$ concentration in various tissues and fluids during the progression of the disease from the initial infection to the onset of the clinical disease. The principle of qPMCA is applicable to prions from any strain or species, but the calibration curve and the dynamic range of detection will likely be different and have to be empirically determined for specific samples.

PMCA has contributed to understanding the mechanism of prion replication, the nature of the infectious agent and the detection of small quantities of $PrP^{Sc}$ in biological samples[13]. qPMCA may have many scientific and practical applications to estimate $PrP^{Sc}$ concentration. The technology may be useful to diagnose disease, develop prion decontamination procedures, identify drugs to prevent the formation and eliminate $PrP^{Sc}$, quantify the extent of prion contamination in medical or environmental materials and may be used to guide regulatory measures to prevent further spread of prion diseases.

## ONLINE METHODS

### Sample preparation

Syrian hamsters were intraperitoneally inoculated with strain 263K prions and monitored for the appearance of clinical signs, using a standard scale, as previously described[14]. When disease was confirmed, hamsters were killed by $CO_2$ inhalation, and brains, spleen and blood were collected. Before killing the hamsters, urine was collected using metabolic cages, as described previously[11]. Brain or spleen homogenates were prepared at 10% (wt/vol) in PBS (pH 7.2) plus Complete cocktail of protease inhibitors (Roche). The samples were clarified by a 45-s, low-speed centrifugation (450*g*). Blood samples were obtained directly from the heart in tubes containing citrate. Plasma and buffy coat were separated by centrifugation in ficoll gradient, as described[7]. The samples of normal brain homogenate used for PMCA substrate were obtained after perfusing hamsters with PBS and 5 mM EDTA. Solutions of 10% normal brain homogenate were made in conversion buffer (PBS without $Ca^{2+}$ and $Mg^{2+}$ with 150 mM NaCl, 1.0% triton X-100 and Complete protease inhibitors). Debris was removed by a 45-s, low-speed centrifugation (450*g*) in an Eppendorf centrifuge.

### $PrP^{Sc}$ partial purification by sarkosyl precipitation

To minimize interferences in PMCA from other components present in tissues or fluids, $PrP^{Sc}$ was partially enriched by sarkosyl precipitation, as previously described[12]. Briefly, samples were incubated with 1 volume of 20% sarkosyl for 10 min at room temperature (22–25 °C) and centrifuged at 100,000*g* for 1 h at 4 °C. Supernatants were discarded and pellets were resuspended into two volumes of 10% sarkosyl. The centrifugation process was repeated, and pellets were resuspended directly in 10% normal brain homogenate prepared in conversion buffer. Following this protocol, $PrP^{Sc}$ was recovered in the pellet fraction with a yield higher than 90%.

### PMCA procedure

PMCA was performed as described previously[13,15]. Briefly, samples were loaded onto 0.2-ml PCR tubes. Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix model 4000), and samples were subjected to cycles of 30 min incubation at 37 °C followed by a 20 s pulse of sonication set at an amplitude of 75. Samples were incubated without shaking immersed in the water of the sonicator bath. Standard PMCA rounds consisted of 144 cycles. After each round of cycles, a 10 μl aliquot of the amplified material was diluted into 90 μl of normal brain homogenate and a new round of PMCA cycles was performed.

### $PrP^{Sc}$ detection

Samples were first digested with 50 μg $ml^{-1}$ of proteinase K at 37 °C for 1 h, and the reaction was stopped by adding NuPAGE LDS sample buffer. The proteins were then fractionated using 4–12% SDS-PAGE, electroblotted into Hybond ECL nitrocellulose membrane and probed with the 3F4 antibody (Covance) (dilution 1:5,000). The immunoreactive bands were visualized by ECL Plus western blotting detection system and quantified by densitometry using a UVP Bioimaging system EC3 apparatus. qPMCA is compatible with any $PrP^{Sc}$ detection procedure (for example, ELISA, conformation-dependent immunoassay and others). In this study, we used the western blot because it is the most standard and widely used assay in the field. Additionally, western blot has the important advantage over other techniques, such as ELISA, in that it can be used to distinguish bona fide $PrP^{Sc}$ ($PrP^{Sc}$ protease-resistant fragment after proteinase K treatment) from incomplete digestion of normal cellular prion protein ($PrP^{C}$) (Fig. 2).

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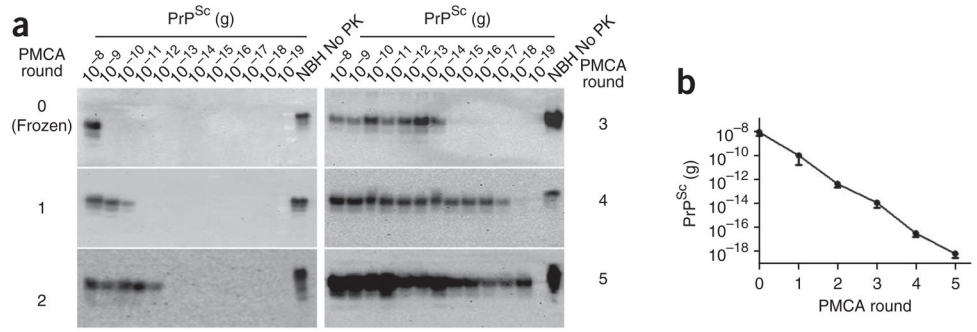


**Figure 1.**

Relationship between $PrP^{Sc}$ concentration and PMCA rounds required for detection. (**a**) Aliquots with the indicated amounts of $PrP^{Sc}$ were subjected to serial rounds of PMCA (144 cycles each) using standard conditions, and $PrP^{Sc}$ was detected by western blotting using the 3F4 antibody. All samples, except the normal brain homogenate (NBH) used as a migration control were digested with proteinase K (PK). (**b**) Western blots from five independent experiments (as the example shown in **a**) were analyzed by densitometry, and the last detectable signal after each PMCA round was plotted, yielding a standard calibration curve to estimate $PrP^{Sc}$ amounts in hamster samples. Error bars, s.e.m. ($n$ = 5).

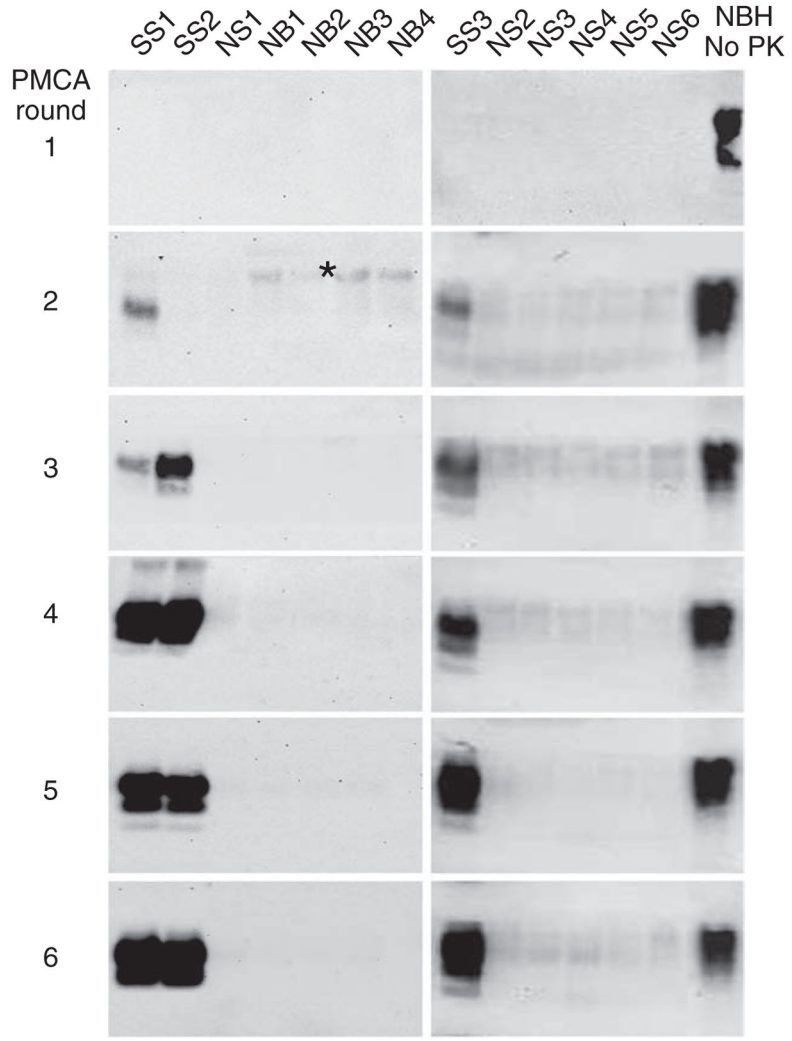


**Figure 2.**

Detection of $PrP^{Sc}$ in spleen of scrapie-affected hamsters. $PrP^{Sc}$ was partially purified from spleen (homogenized in PBS) by sarkosyl precipitation. The equivalent to half of the spleen homogenate was resuspended in normal hamster brain homogenate and subjected to serial PMCA with subsequent detection of $PrP^{Sc}$ by western blot (scrapie spleen, samples SS1–3). Control samples of normal (noninfected) spleen homogenate (samples NS1–6) and brain homogenate (samples NB1–4) were subjected to the same PMCA procedure to assess the rate of spontaneous appearance of $PrP^{Sc}$ reactivity. Normal brain homogenate (NBH) not digested with proteinase K (PK) was used as a migration control. *, incomplete digestion of normal cellular prion protein ($PrP^{C}$) with proteinase K, which sometimes occurs after PMCA and is distinguishable from $PrP^{Sc}$ by the unchanged molecular weight.

## Table 1

$PrP^{Sc}$ concentration in scrapie-affected hamsters

| Source | $PrP^{Sc}$ concentration in tissues (g $g^{-1}$) and fluids (g $ml^{-1}$) |
|---|---|
| Brain | $2.3 \times 10^{-5} \pm 6.8 \times 10^{-6}$ |
| Spleen | $2.0 \times 10^{-11} \pm 1.1 \times 10^{-11}$ |
| Buffy coat | $2.6 \times 10^{-13} \pm 2.4 \times 10^{-13}$ |
| Plasma | $1.3 \times 10^{-14} \pm 1.1 \times 10^{-14}$ |
| Urine | $2.0 \times 10^{-16} \pm 1.7 \times 10^{-16}$ |

$PrP^{Sc}$ concentration in tissues and fluids was estimated by determining the number of PMCA rounds required to detect the signal by western blots and obtaining the concentration from the standard curve. Indicated values are means ± s.e.m. for five hamsters.