Video Article

Protein Misfolding Cyclic Amplification of Prions

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Abstract

Prions are infectious agents that cause the inevitably fatal transmissible spongiform encephalopathy (TSE) in animals and humans[9,18]. The prion protein has two distinct isoforms, the non-infectious host-encoded protein (PrP^C) and the infectious protein (PrP^{Sc}), an abnormally-folded isoform of PrP^C [8].

One of the challenges of working with prion agents is the long incubation period prior to the development of clinical signs following host inoculation[13]. This traditionally mandated long and expensive animal bioassay studies. Furthermore, the biochemical and biophysical properties of PrP^{Sc} are poorly characterized due to their unusual conformation and aggregation states.

PrP^{Sc} can seed the conversion of PrP^C to PrP^{Sc} *in vitro*[14]. PMCA is an *in vitro* technique that takes advantage of this ability using sonication and incubation cycles to produce large amounts of PrP^{Sc}, at an accelerated rate, from a system containing excess amounts of PrP^C and minute amounts of the PrP^{Sc} seed[19]. This technique has proven to effectively recapitulate the species and strain specificity of PrP^{Sc} conversion from PrP^C, to emulate prion strain interference, and to amplify very low levels of PrP^{Sc} from infected tissues, fluids, and environmental samples[6,7,16,23].

This paper details the PMCA protocol, including recommendations for minimizing contamination, generating consistent results, and quantifying those results. We also discuss several PMCA applications, including generation and characterization of infectious prion strains, prion strain interference, and the detection of prions in the environment.

Video Link

The video component of this article can be found at https://www.jove.com/video/4075/

Protocol

1. Preparing the Equipment

1. Use a Misonix 3000 or Misonix 4000 sonicator (Farmingdale, NY) connected to a Thermo Electron Neslab EX-7 water bath (Newington, NH) to keep a constant temperature of 37 °C. Sonicate the samples in 200 µl thin-walled PCR tube strips with domed caps obtained from Thermo Scientific (Waltham, MA).
2. A brand new sonicator requires a "break-in" period of continuous operation[9]. A two-month break-in period consists of a 40-sec sonication burst and 10-minute incubation cycles with the amplitude level set to 10. One should observe erosion throughout the surface of the titanium microplate horn (**Figure 1**, panel B). The circulating water will look white turbid due to erosion of the titanium microplate horn. Replace this water daily.
3. The amplification of PrP^{Sc} will vary throughout the microplate horn. The best PrP^{Sc} amplification efficiency can be obtained within a radius of 5 cm from the center of the microplate horn (See **Figure 1**, panel C).
4. One round of PMCA consists 144 cycles. Each cycle consists of 5 sec sonication and 10 min incubation. This will roughly entail to 24 hr per round of PMCA.
5. The power output of sonication, displayed on the sonicator's control panel, varies with the number of PCR tubes present in the sonicator's rack and the temperature of the circulating water from the water bath. Ensure that the water is equilibrated at 37 °C, and do not fill more than 30% of the sonicator's tube rack (**Figure 1**, panel C). Spread the PCR tubes through the microplate horn. Have at least one row of empty space between PCR tube strips and allow no more than four tubes connected together per strip (**Figure 1**, panel C).
6. The strength of sonication is critical for a successful prion strain amplification. While sonicating, adjust the amplitude to have a power output ranging between 155 ~ 170 watts. In our laboratory the power level is set to six and seven for the Misonix 3000 and Misonix 4000, respectively.

7. Use distilled water in the water bath to avoid accumulating hard water stains. Replace this water weekly. During the incubation and sonication cycles, water condensation will occur inside the microplate horn lid. To avoid condensation, attach a small aquarium heat pad to the top of the sonicator's enclosure box as shown in **Figure 1**, panel A.

2. Preparing the Samples

1. All procedures involving animals were approved by the Creighton University Institutional Animal Care and Use Committee and comply with the Guide for the Care and Use of Laboratory Animals. Before any tissue collection, the animal must be anesthetized and transcardially perfused using ice-cold phosphate buffered saline solution with 5 mM EDTA at pH 7.4 to remove blood from the brain and avoid the inhibition of PMCA reaction by blood[7]. Collect animal tissues rapidly using aseptic technique and have dedicated dissection tools for each prion strain. After dissection, place the tissue in a 1.5 ml microcentrifuge tube, flash freeze it on dry ice, and store it at -80 °C until homogenization.
2. To prepare the PMCA substrate (PrP^{C}) solution, homogenize an uninfected hamster brain (UN BH) to a 10% weight/volume (w/v) solution with ice cold conversion buffer (see part 3) using a Tenbroeck tissue grinder. While homogenizing, be careful not to get air inside the grinder to avoid excessive foaming of the solution. Clarify the solution by centrifuging at 500 x g for 30 sec and aliquot the supernatant in 1.5 ml microcentrifuge tubes. Store at -80 °C until further use.
3. To prepare the PMCA seed (PrP^{Sc}) solution, homogenize an infectious material (brain, spleen, lymph nodes, other tissues or contaminated soil) to a 10% w/v solution with ice cold water and aliquot the solution in 0.6 ml microcentrifuge tubes. Store at -80 °C until further use.

3. Preparing Conversion Buffer

1. Weigh 0.5093 g of Triton X-100 in a 50 ml volumetric flask (result is a 1% final concentration of Triton X-100) (Sigma-Aldrich GmbH; Steinheim, Germany).
2. Add one Complete Protease Inhibitor Tablet (Roche Diagnostics GmbH; Mannheim, Germany).
3. Add 0.0931 g of EDTA (this will result in a 5 mM final concentration of EDTA) (Mallinckrodt Baker Inc.; Phillipsburg, NJ).
4. Adjust the pH to 7.4 with 1N NaOH.
5. Adjust volume to 50 ml with Dulbecco's Phosphate Buffered Saline (DPBS) (Mediatech Inc.; Manassas, VA).
6. Filter the solution through a 0.45 µm nitrocellulose membrane with a vacuum filtration apparatus. The conversion buffer can be stored at 4 °C for no more than one month.

4. Amplification of Prion Strains Using PMCA

1. Dilute the PMCA seed into the PMCA substrate at a ratio of 1:20 (*i.e.* mix 5 µl of the PMCA seed and 95 µl of the PMCA substrate) into a PCR tube. Remove and store a 15 µl aliquot at -80 °C for unsonicated control; this unsonicated control will be used to quantify the PrP^{Sc} amplification via proteinase K (PK) digestion followed by Western blot (WB) analysis. An additional unsonicated control could be generated by placing a second 15 µl aliquot at 37 °C for the duration of the PMCA reaction. A minimum of three repetitions is required for statistical purposes.
2. Subject the remainder of the sample (70 µl) to one round of PMCA as depicted on **Figure 1**, Panel A. Shake tubes every 5 hr to avoid water condensation on the domed cap of the PCR tube.
3. The sample's tubes should be spun down and gently vortexed (being careful that the solution do not go into the domed cap) before opening after any procedure to ensure homogeneity and avoid potential contamination.
4. For serial PMCA (sPMCA) of short incubation period strains (*i.e.* HY TME, HaCWD, or 263K), dilute the sonicated material at a ratio of 1:20 by transferring 5 µl of the sonicated sample from round one into 95 µl of fresh uninfected brain homogenate (**Figure 2**, Panel B).
5. For sPMCA of long incubation period strains (*i.e.* DY TME, 139H, 22CH, 22AH, or ME7H), dilute the sonicated material at a ratio of 1:1 by transferring 50 µl of the sonicated sample from round one into 50 µl of fresh uninfected brain homogenate (**Figure 2**, Panel B).
6. Remove two 15 µl aliquots from the previously diluted sonicated mixture (from steps 4.4 or 4.5) and store one of them at -80 °C and the other at 37 °C (unsonicated control solutions for PMCA round two). Subject the remnants to a PMCA round two.
7. This procedure can be repeated indefinitely. In our laboratory, we have performed up to fifteen PMCA rounds, with the strain properties remaining unchanged.
8. PK digest the samples. For a typical WB, mix 5 µl of PK 80 µg/ml to 5 µl of sample (this solution will have a final PK concentration of 40 µg/ml) and incubate at 37 °C for one hour. Add 10 µl of gel loading buffer. Boil this solution for 10 min. Let the solution cool down and load 10 µl into the gel.
9. To calculate the success of amplification perform a WB analysis on the PK-digested samples to estimate the amount of amplified PrP^{Sc} by comparing them to either, the unsonicated controls[20,21] **Figure 2**, Panel C or to known amounts of PK-undigested recombinant PrP.

5. Avoiding Cross-contamination

Critical steps have to be taken to minimize cross-contamination and the occurrence of false positives due to de *novo* formation of protease resistant products.

1. Use a dedicated set of dissection tools (*i.e.* forceps, tweezers, scissors) for sample collection for each prion strain.
2. Before homogenizing uninfected brains to be used as a PMCA substrate, wipe the pipettes with 1% bleach. Soak the sonicator's PCR tube rack, tissue homogenizers, and PCR tube storage racks with 1% bleach for 10 min and rinse thoroughly with distilled water.
3. Cover the work area with a fresh Versi-Dry Lab Soaker every time a new sample is to be homogenized, a new PMCA experiment is to be prepared, or when aliquoting between sPMCA rounds.
4. Use new gloves each time new samples are handled (especially when transferring aliquots between sPMCA rounds).

5. Use short sonication bursts for each PMCA cycle. Cycles of 5 sec sonication combined with 10 min incubation is sufficient to amplify prions without generating *de novo* PrP^{Sc} [1,20,21,23,24] . As other laboratories have shown, increasing the sonication time, increasing the sonication amplitude, and extending the number of PMCA cycles increases the probability for *de novo* PrP^{Sc} formation[2].

Representative Results

Protein misfolding cyclic amplification (PMCA) is used to amplify PrP^{Sc} *in vitro*[7, 12, 14, 19, 24]. A successful PrP^{Sc} amplification is shown by an increase in band intensity on Western blots of the PK-resistant prion protein (migrating between 19 and 30 kDa for hamster-derived prion strains) as shown in **Figure 3.** The increase in its band intensity after PMCA indicates amplification of the PK-resistant PrP^{Sc} material. Successful amplification of the hamster-derived prion protein, HaCWD and DY TME, is shown in the WB analysis of **Figure 3** by comparing the band intensities before (lanes 5 and 7) and after (lanes 4 and 6) PMCA.

PK digestion of PrP^{Sc} followed by WB analysis shows an upper, middle and lower band corresponding to the di-, mono-, and unglycosylated prion protein, respectively. Some prion strains can be differentiated by their electrophoretic mobility. For instance, there is a two-kDa difference in migration between the HaCWD and DY TME prion strains. (**Figure 3**, lanes 1 and 2, respectively).

A high fidelity PrP^{Sc} amplification is achieved by the PMCA[1, 7, 12, 19, 23, 24]. This high fidelity in PMCA amplification is observed by a similar electrophoretic migration of the PMCA-amplified prion strains (HaCWD and DY TME) when compared to their corresponding seeds (**Figure 3**; lanes 1 & 4 and 2 & 6 for HaCWD and DY TME, respectively).

If no cross-contamination or *de novo* PrP^{Sc} formation occurs, WB analysis of mock control PMCA samples should remain clear after PK digestion (**Figure 3**, lanes 8 and 9).

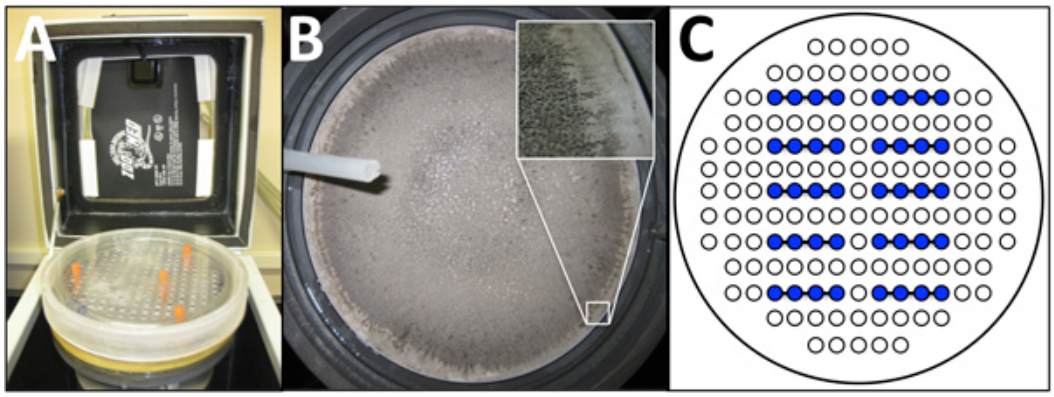

Figure 1. To avoid water condensation, a regular aquarium heat-pad is placed above the lid of the microplate horn (panel A). A brand new sonicator should be allowed a break-in period of continuous sonication for about two months. Panel B shows the erosion on the titanium microplate horn after the break-in period. Panel C shows a possible arrangement of PCR tube strips that would allow an optimal sonication of the samples.

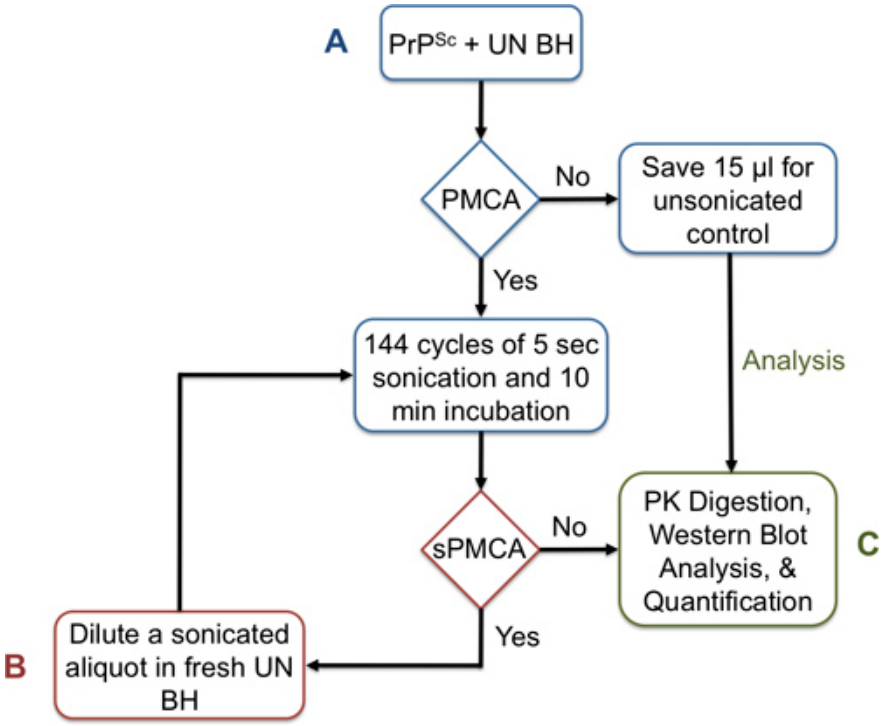

Figure 2. Flow chart for PMCA (panel A), sPMCA (panel B), and WB analysis (panel C). PrP^{Sc} is the PMCA seed and the uninfected brain homogenate (UN BH) is the PMCA substrate. The quantification of the amplified PrP^{Sc} for each PMCA round is obtained by dividing the band intensity after PMCA by its corresponding band intensity before PMCA.

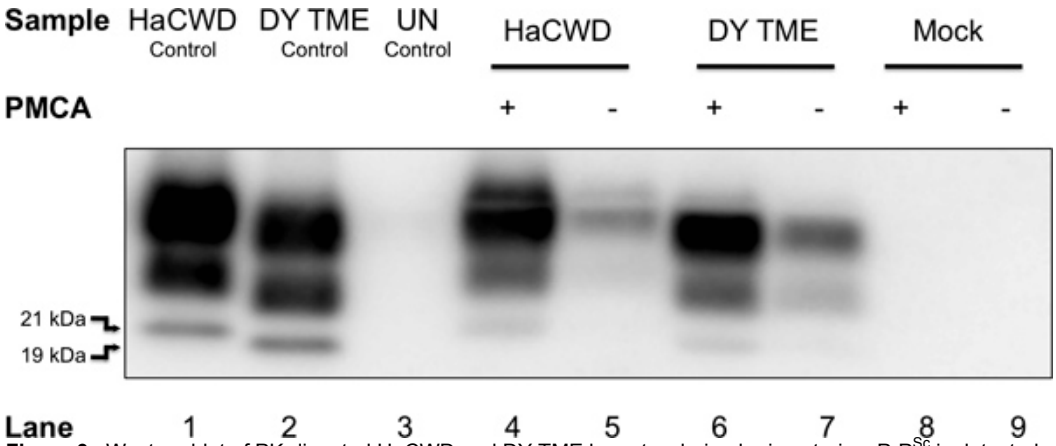

Figure 3. Western blot of PK-digested HaCWD and DY TME hamster-derived prion strains. PrP^{Sc} is detected using 3F4 anti-prion antibody. Analysis of the WB shows the abundance (blot intensity) and electrophoretic mobility of PrP^{Sc}. PMCA reactions were seeded with brain homogenate from hamsters infected with either the HaCWD (lanes 4-5) or DY TME (lanes 6-7) agents. Samples that underwent PMCA (PMCA +) show an increase in the PrP^{Sc} abundance when compared to their unamplified (PMCA -) controls (compare lanes 5 to 4 and 7 to 6). There is a two-kDa difference in the electrophoretic migration between the prion proteins of HaCWD and DY TME (lanes 1-2). This difference in migration is maintained in the PMCA-generated samples using HaCWD and DY TME homogenates (lanes 4 and 6, respectively). PMCA reactions seeded with uninfected brain (mock) homogenate fail to amplify PrP^{Sc} (lane 8). The locations for the 19 and 21 kDa molecular markers are indicated to the left of the panel.

Discussion

Challenges of amplifying infectious prion proteins are the long incubation periods and the expenses of *in vivo* experiments. The PMCA technique is a cost effective means to amplify infectious prion agents. Several laboratories have confirmed the ability of PMCA to accurately amplify prion strains *in vitro* [7, 9, 12, 14, 19,24] .

Prion diseases can be transmitted between species. Bessen and Marsh have effectively inoculated hamsters with transmissible mink encephalopathy, which produced two distinct hamster-derived prion strains[5] . In an elegant study, Telling and co-workers used sPMCA to amplify the mouse-derived prion strain, RML, using transgenic mice expressing cervid PrP^{C} (Tg(CerPrP)1536+/-) as PMCA substrate. A rapid onset of disease was observed after inoculation of Tg(CerPrP)1536+/- with the PMCA-adapted material[12] . Similarly, Kurt *et al.* were able to amplify

chronic wasting disease (CWD), a prion disease in cervids, using non-cervid species PMCA substrate[15]. These data suggest that species barrier in prion diseases can be bypassed *in vitro* via PMCA.

Bessen and Marsh have shown that the hamster's short incubation period strain, HY TME, has a quicker PrP^{Sc} accumulation when compared to its long incubation period counterpart, DY TME[3, 5] . Similarly, this correlation between the incubation period and the accumulation rate is observed after PMCA in several hamster-derived prion strains[1, 23, 24].

The amplification rate is a property intrinsic to each strain. PMCA has been used to quantify this amplification rate. Ayers *et al.* were able to calculate a unitless number, termed amplification coefficient, that represents the rate of amplification for a given hamster-derived prion strain[1] .

Prion strains can interfere with each other when present in the same host. The presence of a long incubation period strain can extend the incubation period or even block the ability of a short incubation period strain to cause disease. This extension in incubation period is termed strain interference. Strain interference has been shown to occur in mice[10] and hamsters[22]. Bartz and co-workers have used PMCA to study prion strain interference in hamsters. Their results show that the PMCA experiments correlate with the findings of a similar experiment *in vivo*[22, 23].

Since there is a relatively low level of PrP^{Sc} outside the central nervous system, prions can only be accurately diagnosed post-mortem via dissection of the brain followed by immunohistochemistry. PMCA can be used as diagnostic tool, since it has shown a great capability of amplifying minute amounts of PrP^{Sc}[7] , even from hamster's urine samples[11].

The prion agent is able to withstand harsh environmental conditions and remain infectious[16,17] . However, the amount of PrP^{Sc} in the environment is too small to be detected using conventional techniques such WB. PMCA has been used to amplify and calculate the approximate amount of PrP^{Sc} the environment, such as soil and water[16, 17, 20, 21] .

Disclosures

No conflicts of interest declared.

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