Video Article

Assessing Transmissible Spongiform Encephalopathy Species Barriers with an *In Vitro* Prion Protein Conversion Assay

Christopher J. Johnson[1], Christina M. Carlson[2], Aaron R. Morawski[3], Alyson Manthei[4], Neil R. Cashman[5]

[1]USGS National Wildlife Health Center

[2]Department of Soil Science, University of Wisconsin–Madison

[3]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health

[4]Merial Veterinary Scholars Program, School of Veterinary Medicine, University of Wisconsin–Madison

[5]Department of Neurology, University of British Columbia

Correspondence to: Christopher J. Johnson at cjjohnson@usgs.gov

URL: https://www.jove.com/video/52522
DOI: doi:10.3791/52522

Keywords: Medicine, Issue 97, Prion, species barrier, conversion, immunoblotting, transmissible spongiform encephalopathy, interspecies transmission

Date Published: 3/10/2015

Citation: Johnson, C.J., Carlson, C.M., Morawski, A.R., Manthei, A., Cashman, N.R. Assessing Transmissible Spongiform Encephalopathy Species Barriers with an *In Vitro* Prion Protein Conversion Assay. *J. Vis. Exp.* (97), e52522, doi:10.3791/52522 (2015).

Abstract

Studies to understanding interspecies transmission of transmissible spongiform encephalopathies (TSEs, prion diseases) are challenging in that they typically rely upon lengthy and costly *in vivo* animal challenge studies. A number of *in vitro* assays have been developed to aid in measuring prion species barriers, thereby reducing animal use and providing quicker results than animal bioassays. Here, we present the protocol for a rapid *in vitro* prion conversion assay called the conversion efficiency ratio (CER) assay. In this assay cellular prion protein (PrP^C) from an uninfected host brain is denatured at both pH 7.4 and 3.5 to produce two substrates. When the pH 7.4 substrate is incubated with TSE agent, the amount of PrP^C that converts to a proteinase K (PK)-resistant state is modulated by the original host's species barrier to the TSE agent. In contrast, PrP^C in the pH 3.5 substrate is misfolded by any TSE agent. By comparing the amount of PK-resistant prion protein in the two substrates, an assessment of the host's species barrier can be made. We show that the CER assay correctly predicts known prion species barriers of laboratory mice and, as an example, show some preliminary results suggesting that bobcats (*Lynx rufus*) may be susceptible to white-tailed deer (*Odocoileus virginianus*) chronic wasting disease agent.

Video Link

The video component of this article can be found at https://www.jove.com/video/52522/

Introduction

Transmissible spongiform encephalopathies (TSEs, prion diseases) are a group of fatal neurodegenerative diseases with extended incubation periods that affect a variety of animals and humans. The putative etiological agent of TSEs is comprised of a misfolded isomer of the host prion protein (PrP) that is capable of self-propagation by template-driven conversion of the normal cellular form of PrP (PrP^C) into an infectious, disease-associated form (PrP^{TSE}) that accumulates in central nervous system tissues of the infected host[1]. Infectious mammalian prions generally transmit from host-to-host in a species-specific manner, which has given rise to the concept of the "TSE species barrier" limiting interspecies transmission events[2]. The biological determinants of the prion species barrier are not well understood. Amino acid sequence similarity between the infectious PrP^{TSE} and the host PrP^C can strongly influence whether conversion takes place[3-5], but remains insufficient to explain all prion transmission events observed *in vivo*[6,7].

Thus, characterization of TSE species barriers has largely relied upon animal challenge studies: exposing naïve animals from a given species to prions from another and measuring the resulting incubation time to disease onset and attack rate as indicators of transmission efficiency. Mice expressing PrP^C from heterologous species are also used for this type of study[8]. The costs associated with transgenic mouse production and protracted prion bioassays, as well as ethical considerations of animal use, are obstacles to experimental investigation of TSE species barriers. Assessment of the human species barrier to TSEs relies on mice engineered to express human PrP^C. These mice require long incubation periods to succumb to human TSEs or modifications to the human PrP^C molecule for rapid disease onset[9]. Incubation periods for non-human TSEs in these mice may extend beyond the normal mouse lifespan making interpretation of negative results challenging. Non-human primates have also been used as proxies for studying human species barriers, but these studies are fraught with the same challenges as other types of animal experimentation and non-human primates may not precisely recapitulate disease as it proceeds in the human host.

Animal bioassays remain the "gold standard" method for measuring the susceptibility of a species to a TSE, but the obstacles, costs and ethics of these live animal studies have compelled investigation into alternatives. A number of *in vitro* assays, based on assessing the conversion of host PrP^C to a proteinase K (PK)-resistant state (PrP^{res}) when seeded by PrP^{TSE}, have been developed and used to investigate TSE species barriers[10-12]. Examples of *in vitro* assays include cell-free conversion assays, protein misfolding cyclic amplification (PMCA), and the conversion

efficiency ratio (CER) assay[10-14]. While none of these assays take into account peripheral factors involved in species barriers after natural infection, all can be useful to identify potentially susceptible hosts for TSEs.

Here we present the protocol for the CER assay, in which two denatured PrP^{C} substrates derived from normal brain homogenates are used in bench top prion conversion reactions (**Figure 1**)[13]. PrP^{C} in the substrate denatured at pH 7.4 can only be converted to PrP^{res} by PrP^{TSE} seeded into the reaction in the absence of a species barrier[14]. In contrast, denaturation of PrP^{C} in the other substrate at pH 3.5 allows it to be converted to PrP^{res} following incubation with PrP^{TSE} from *any* species and serves as a control for conversion. The ratio of conversion of PrP^{C} to PrP^{res} in pH 7.4 substrate relative to that of the pH 3.5 substrate provides a measure of the species barrier. We have found that the CER assay predicts known species barriers of laboratory mice to various TSEs and have used the assay in efforts to predict the species barrier of numerous mammalian species, including bighorn sheep, to chronic wasting disease (CWD) and other TSEs[14,15]. Investigators interested in a tool allowing rapid screening of TSE species barriers or assessment of PrP^{C}-to-PrP^{res} conversion will find this methodology useful.

Protocol

Animal work conducted at the USGS National Wildlife Health Center was performed in accordance with the NIH Office of Laboratory Animal Welfare guidelines and under institutional animal care and use committee protocol #EP080716. Tissues from hunter-harvested animals were gifts from the Wisconsin or Michigan Departments of Natural Resources.

1. Solution Preparation

NOTE: The solutions listed below are required for preparation of the CER assay substrate in **Section 2** below. Prepare each of these solutions from higher concentration stock solutions; recommended concentrations for stock solutions will be given in parentheses after each respective chemical name listed. Prepare all solutions fresh upon substrate preparation and store at 4 °C until used.

1. For lysis buffer, prepare a solution of the following in 10 mM Tris, pH 7.5 (1 M Tris, pH 7.5 stock solution recommended): 100 mM sodium chloride (NaCl, 1 M stock solution), 10 mM ethylenediaminetetraacetic acid (EDTA, 500 mM EDTA, pH 8.0 stock solution), 0.5% NP-40 (10% stock solution), 0.5% deoxycholate (DOC, 10% stock solution).
2. For conversion buffer, prepare a solution of 0.05% sodium dodecyl sulfate (SDS, 10% stock solution) and 0.5% Triton X-100 (10% stock solution) in 1 phosphate buffered saline (PBS, 10, pH 7.4 stock solution).
3. For chaotropic solutions, prepare two 3 M solutions of guanidine hydrochloride (GdnHCl, 8 M stock) in 1x PBS (10x stock solution). Adjust the pH of one of the solutions to 3.5 ± 0.05 using concentrated hydrochloric acid (HCl). Check that the pH of the second solution remains at pH 7.4 ± 0.05 and adjust using concentrated HCl or sodium hydroxide (NaOH) if necessary.

2. Prepare CER Assay Substrate Pairs

1. Obtain healthy, non-TSE-infected brain tissue from species of interest and prepare a 10% weight-per-volume (w/v) homogenate in lysis buffer using any of the following methods: dounce, bead-mill, or mortar and pestle homogenization. Further refine resulting homogenates by subjecting them to syringe-and-needle homogenization, using needles of increasing gauge until substrate can be aspirated and expelled with ease through a 27 G syringe needle.
    1. For substrates prepared from rodents and other small mammals, homogenize whole brain(s); for substrates prepared from larger mammals, use obex (brainstem) tissue to prepare homogenates. When selecting the quantity of brain homogenate, prepare no more than 50% of the capacity of the centrifuge used for protein precipitation in step 2.6.
    2. If the quality of acquired brain tissue is questionable, assess PrP^{C} levels by SDS-PAGE[16] and immunoblot[17] prior to substrate preparation.
2. Divide brain homogenate into two equal volumes in separate labelled plastic conical tubes. Add GdnHCl (3 M solutions in 1 PBS) at either pH 3.5 or 7.4 to each tube in a 1:1 volume ratio to brain homogenate.
3. Check the pH values of the two solutions. Adjust the pH of the pH 3.5 substrate to pH ± 0.05 using concentrated HCl. Ensure the pH of the other substrate remains at pH 7.4 ± 0.05 and adjust pH if necessary with HCl or NaOH as needed. Avoid overshooting pH values by judicious addition of acid or base.
4. Rotate solutions on an end-over-end mixer at room temperature (RT) for 5 hr.
5. Precipitate protein by adding four volumes of methanol to the substrate solutions in each conical tube, vortex to mix well, and incubate at -20 °C for 16-18 hr.
6. Sediment samples by centrifugation at 13,000 × g for 30 min at 4 °C. Carefully decant and discard the supernatants and allow methanol to evaporate from the pellets. Use cotton-tipped applicators to assist in absorbing methanol clung to the inside of the tube. Do not allow pellets to over-dry and once methanol is no longer visible, proceed to the next step.
7. Resuspend protein pellets in conversion buffer. Use a quantity of conversion buffer equal to the amount of brain homogenate starting material dispensed into each tube in step 2.2.
   NOTE: It is critical that the conversion buffer used to resuspend protein pellets from both pH 3.5 and 7.4-treated substrate preparations is the solution defined in step 1.2 above (which contains low levels of detergents and a physiological pH).
8. Briefly sonicate substrate solutions in a cuphorn sonicator (10 sec at ~30% maximal power), aliquot into 0.5 - 1.0 ml volumes in labeled 1.5 ml microcentrifuge tubes. Perform a quality control immunoblot (step 2.9) on an aliquot of each substrate. Store other aliquots at -80 °C until ready to perform CER assay (Section 4).
9. Perform quality control on CER substrate pairs prior to use (**Figure 2**). These steps ensure that CER substrates will provide optimal results in conversion studies.
    1. Ensure comparable amounts of PrP^{C} in the two substrates. Compare PrP levels in 10-25 µl of each substrate by SDS-PAGE[16] and immunoblotting[17]. If protein levels in the pH 7.4 and 3.5 substrates are within ~10% of each other, the substrate pairs are appropriate for use in CER studies.

NOTE: The PrP immunoblots should not show evidence of extensive PrP^C degradation. The PrP immunoreactivity should be mainly >20 kDa. Bands less than this molecular mass can be degradation products. Banding patterns should be roughly equivalent between substrate pairs.

2. As the PrP^C in both substrates should be PK sensitive, treat 10-25 µl of each substrate with PK at a final concentration of 100 µg/ml and digest samples at 1,000 rpm at 37 °C for 1 hr in the thermo-shaker. Assess any remaining PrP signal by SDS-PAGE[16] and immunoblotting[17]. Substrates with PrP^{res} are not appropriate for use in CER studies.

3. Prepare TSE Agent Seeds

1. Obtain TSE-infected brain tissue from species of interest and prepare a 10% (w/v) homogenate in 1x PBS pH 7.4 using the methods listed in step 2.1 above.
   NOTE: Seeds could also be produced from other TSE-infected tissues outside the central nervous system, however, efficiency of conversion of brain derived substrates by seeds derived from TSE-infected peripheral tissues has not yet been experimentally assessed in the published literature.
2. Aliquot resulting homogenate(s) into 100 - 300 µl volumes in 0.5 ml microcentrifuge tubes and store at -80 °C until ready to perform the CER assay.

4. CER Assay

NOTE: The CER assay involves addition of a relatively small amount of PrP^{TSE} (provided in the "seed") from one species into a relative excess of PrP^C (provided in the uninfected brain "substrate") from another species that has been partially denatured at either pH 3.5 or 7.4. Following an extended shaking incubation period, template-driven conversion of PrP^C by PrP^{TSE} in each reaction is assessed by the densitometric signal of PrP^{res} remaining after PK digestion and immunoblot detection. Comparing PrP^{res} levels in the substrates previously denatured at pH 3.5 *vs.* 7.4 provides a measure of the species barrier. An experimental overview of this assay procedure is provided in **Figure 1**.

1. Slowly thaw uninfected CER substrate pairs (assay requires equal volumes of substrates previously denatured at both pH 3.5 and 7.4) and TSE-infected seed solutions by placing them on top of a bed of ice (approximately 1 hr thaw time).
2. Once fully thawed, aspirate and then expel each substrate solution through a 27 G syringe needle several times to re-homogenize it. Briefly sonicate each TSE-infected seed solution for 10 sec at ~30% maximal power. Keep all solutions on ice while preparing conversion reactions.
3. Label 5 individual low binding, thin-walled PCR tubes per TSE agent being tested.
4. Prepare conversion reactions in these tubes by adding 5 µl of 10% TSE-infected seed solution to 95 µl of (a) CER substrate prepared at pH 7.4 and (b) CER substrate prepared at pH 3.5.
   1. For each experimental sample, prepare parallel reactions in CER substrate pairs previously denatured at both pH 3.5 and 7.4.
   2. Optimize ratios of TSE agent seed to uninfected brain substrate through empirical determination. Common seed:substrate ratios employed maintain a total reaction volume of 100 µl and include 1:99 µl, 2:98 µl and 5:95 µl. For most applications, the 5:95 µl seed:substrate volume ratio is appropriate and is what is presented in this protocol.
5. Prepare three control samples per TSE agent being tested as follows: (a) add 5 µl of 10% TSE-infected seed solution to 95 µl conversion buffer as a control for input PrP^{res}; add 5 µl conversion buffer to 95 µl substrate prepared at (b) pH 3.5 and (c) pH 7.4 as controls for non-specific, non-templated conversion.
6. Briefly vortex each sample in its closed PCR tube to mix seed and substrate well. Follow with a momentary pulse in a low-speed bench top mini-centrifuge to remove any volume of the reaction mixture trapped in the PCR tube lid.
7. Load samples in individually-labeled PCR tubes into a bench top thermo-shaker equipped to accept PCR tubes. Shake samples at 1,000 rpm at 37 °C for 24 hr.
8. Following shaking, add sarkosyl to a final concentration of 2% (w/v) and PK to a final concentration of 100 µg/ml. Digest samples at 1,000 rpm at 37 °C for 1 hr in the thermo-shaker.
   NOTE: Addition of sarkosyl to samples post-conversion aids in PK digestion of PrP^C. False positive signals in unseeded samples (Step 4.5) are virtually eliminated by addition of sarkosyl.
9. Add SDS-PAGE sample buffer, heat samples to 95°C for 5 minutes and resolve remaining PrP^{res} in each sample by SDS-PAGE[16] followed by immunoblotting[17].
   NOTE: After addition of sample buffer and heating, samples may be immediately processed or samples can be stored at -20 °C or -80 °C prior to immunoblotting. All data presented here were generated using the Novex NuPAGE gel and transfer systems. Individual samples from the CER assay were treated with sample buffer and reducing agent according to manufacturer's instructions. Samples were subsequently heated at 95 °C for 5 min and 30 µl of each sample was loaded into a 12% bis-tris precast gels.
10. Calculate the conversion efficiency ratio (CER) as a measure of species barrier. Measure levels of PrP^{res} by densitometry[17] and divide the total density of the pH 7.4 sample by that of the pH 3.5 sample. Multiply by 100 to produce a percentage.
11. Compile data from independent experimental replicates to generate a mean CER ± standard deviation for a species with a given TSE agent.

Representative Results

Successful use of the CER assay is largely dependent upon the quality of the substrate pairs used in conversion reactions. For this reason, following procedures to prepare CER substrate pairs, a quality control immunoblot should be performed (**Figure 2**). For both substrates, assay 10-25 µl by immunoblotting. PrP^C should be easily detectable in each substrate and PrP^C levels must be approximately the same between the two. Immunoreactivity will be mainly visible above 20 kDa, but smaller bands may also be evident. In our experience, the presence of smaller bands is species dependent. If lower molecular weight products are observed, their presence should be confirmed in both substrate pairs.

It is important to ensure that CER substrates are free from endogenous PrP^{res} by treating substrates with PK (**Figure 2**). If PrP^{res} is present, the animal used for substrate preparation may have been TSE-infected or subclinically-infected. Alternatively, the PrP^{C} in the substrate may have misfolded to a PK-resistant state during denaturation or precipitation procedures. Independent from the reason, CER substrate containing PrP^{res} is not acceptable for use in the conversion assay.

Laboratory mice are one of the most frequently used experimental animals in TSE research; as such, their susceptibility to most TSEs is well-characterized[8], providing an *in vivo* benchmark against which to test the fidelity of the CER assay. **Figure 3A** presents results of CER assay conversion of PrP^{C} from mouse (CD1 strain) substrate to PrP^{res} by 1) the RML strain of mouse-passaged scrapie, an agent adapted to the mouse host[18], 2) domestic sheep classical scrapie, an agent that can transmit to mice following a lengthy incubation period[18], and 3) white-tailed deer (*Odocoileus virginianus*) CWD and 263K strain of hamster-passaged scrapie, agents to which mice are minimally or not susceptible[19,20]. Resulting PrP^{res} levels were variable across mouse substrates denatured at pH 7.4 and seeded with the various TSE agents while PrP^{res} was found in all substrates denatured at pH 3.5 and seeded with a TSE agent. Conversion ratios comparing the PrP^{res} levels in the pH 7.4 and 3.5 substrates were independently calculated at least three times and means ± SD are shown in **Figure 3B**. For reactions seeded by RML, conversion ratios between pH 7.4 and 3.5 substrates were approximately 100%. For those seeded by scrapie, conversion in the pH 7.4 substrate was approximately 75% of that in the pH 3.5 substrate. CWD or 263K-induced conversion of the pH 7.4 substrate was minimal. A one-way analysis of variance could not distinguish a difference in the conversion ratios of RML and scrapie or CWD and 263K, however conversion ratios of RML and scrapie were significantly different from those of CWD and 263K.

The CER assay can be adapted for use with human tissues or with animal species where bioassays are too challenging or not ethical and transgenic mouse production is not desired. We have been using this assay to characterize the susceptibility of various wildlife species to CWD. As an example of the use of this assay, we present some preliminary results in **Figure 4**. Brains from hunter-trapped bobcats (*Lynx rufus*) were found to still contain detectable levels of PrP^{C} and PrP breakdown products were not extensive, suggesting that these tissues could be an acceptable source for CER substrate (**Figure 4A**). Substrate pairs generated from bobcat brain showed PrP immunoreactivity of an appropriate molecular weight and did not contain endogenous PrP^{res} (**Figure 4B**). When bobcat CER substrate pairs generated from two separate animals were incubated with the same white-tailed deer CWD agent used in the mouse CER assay described in **Figure 3**, we found substrates prepared at pH 7.4 had levels of PrP^{res} compared to substrates prepared at pH 3.5 (**Figure 4C**), indicative of a minimal species barrier of bobcat PrP^{C} to conversion by CWD. The CER for conversion of white-tailed deer PrP^{C} by the same CWD isolate used to convert bobcat PrP^{C} here, as a positive control, was previously reported as 95.2 ± 18.4% in Morawski *et al.* (2013)[15].

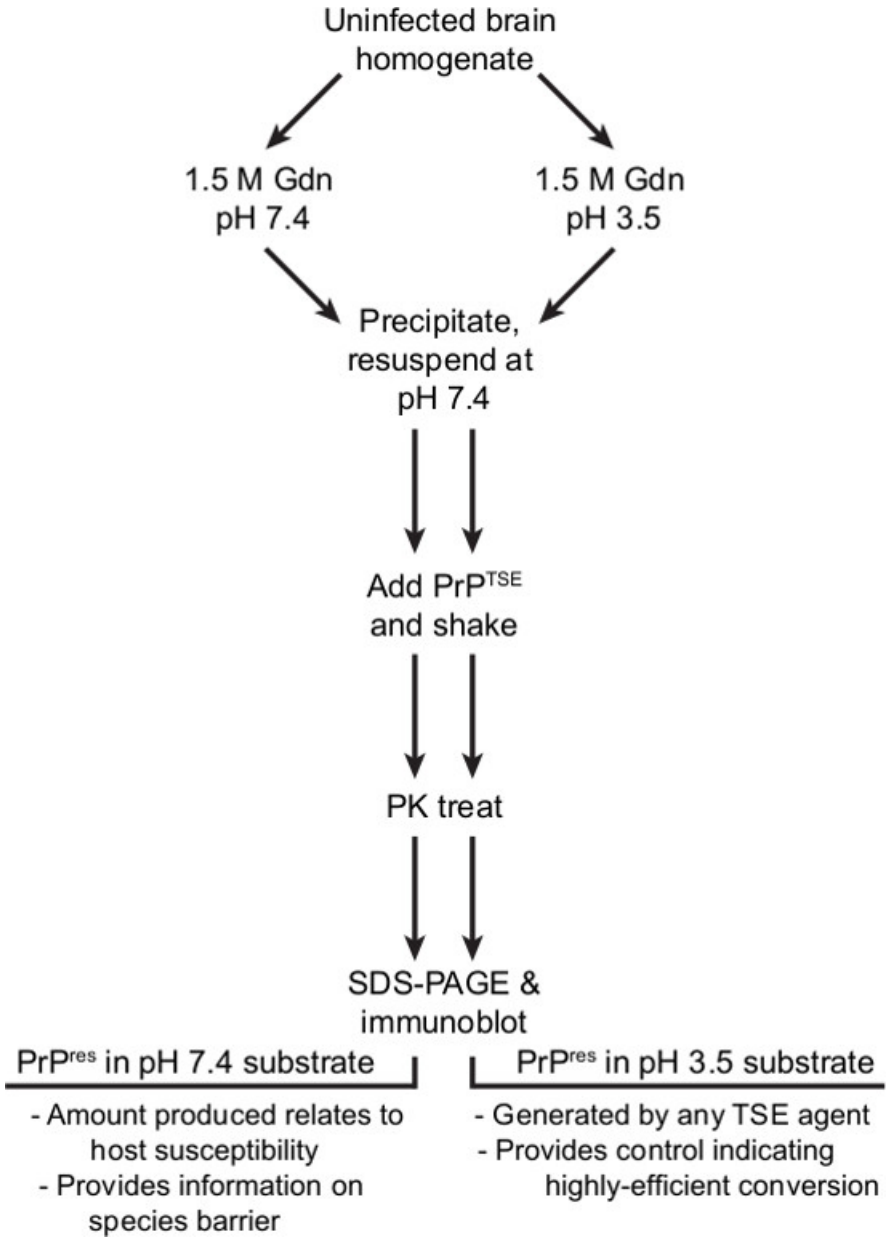

Figure 1: Experimental overview of the CER assay. Two assay substrates are prepared by partially denaturing PrP^{C} in non-prion infected brain tissue with chaotropic solutions at either pH 3.5 or pH 7.4. Substrates are seeded with PrP^{TSE} from prion agent of interest and experimental samples are subjected to 24 hr incubation with shaking to allow PrP^{C} to PrP^{TSE} conversion in both pH 3.5 and 7.4 substrates. Following incubation, conversion is assessed by SDS-PAGE and immunoblotting. Signal densities are read by densitometry and the CER is calculated by the ratio of pH 7.4 to pH 3.5 signal densities for each experimental sample.

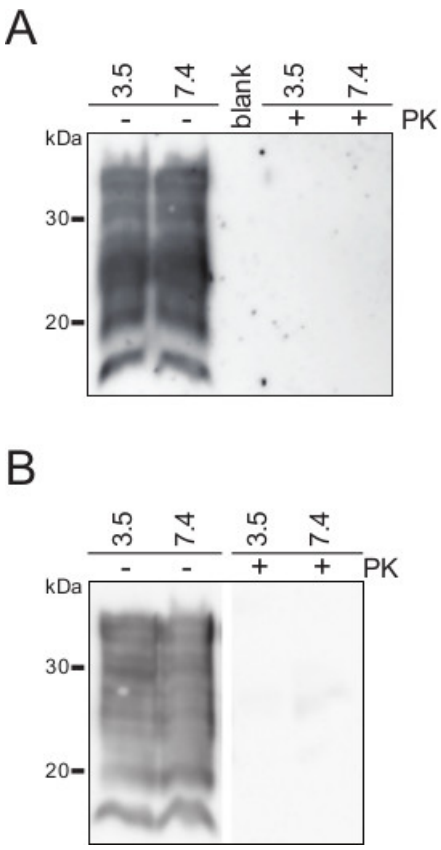

Figure 2: Quality control on CER substrates. (A) Prior to their use in the CER assay, mouse substrates prepared at either pH 7.4 or 3.5 are assayed by immunoblot in the 1) absence of PK treatment to assess PrP^{C} content of each substrate pair (PrP^{C} levels should be equivalent between pH 7.4 and 3.5 substrates for use in CER assay) and 2) presence of PK (100 µg/ml) treatment to test for pre-existing PrP^{res} in tissues used to prepare substrates (any substrates that display pre-existing PrP^{res} are not suitable for use in the CER assay). **(B)** To test for non-specific PrP^{res} formation during the conversion assay, mouse PrP^{C} substrates prepared at either pH 7.4 or 3.5 are seeded with conversion buffer, shaken for 24 hr at 1,000 rpm, 37 °C, and subsequently PK-treated (100 µg/ml) and assayed for PrP^{res} by immunoblotting (right two lanes). Non-shaken, non-PK treated mouse substrates represent total PrP^{C} content in assay reactions (left two lanes). For (B), irrelevant lanes have been cropped for clarity, but each immunoblot panel derives from the same gel, membrane and exposure. Immunoblots in all panels used monoclonal antibody SAF 83.

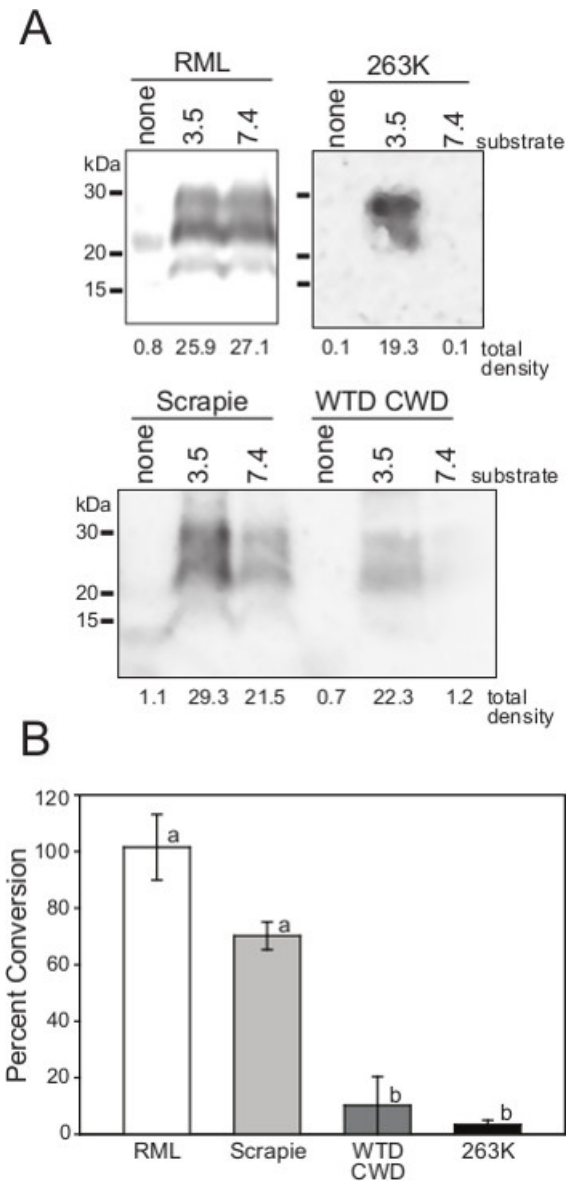

Figure 3: CER assay using laboratory mouse substrate. (A) Mouse CER assay substrate prepared at either pH 7.4 or 3.5 was incubated with RML mouse-adapted scrapie, domestic sheep classical scrapie, white-tailed deer chronic wasting disease (CWD) or 263K strain of hamster-adapted scrapie in the CER assay. Control samples (labeled "none") contained only an equal amount of infectious agent and no mouse substrate. Samples were analyzed for the presence of PK-resistant prion protein (PrP^{res}) by immunoblot with monoclonal antibody SAF 83. Raw densitometric values for each sample are displayed below each lane. **(B)** Bar graph indicating the average ratios (± standard deviation) between pH 7.4 and 3.5 mouse substrates for each infectious agent based on at least 3 independent assay runs. Lower-case letters refer to statistically homogenous subsets (analysis of variance with Tukey-Kramer minimum significance differences method; $p < 0.05$). This figure has been modified from Morawski *et al.* 2013[15].

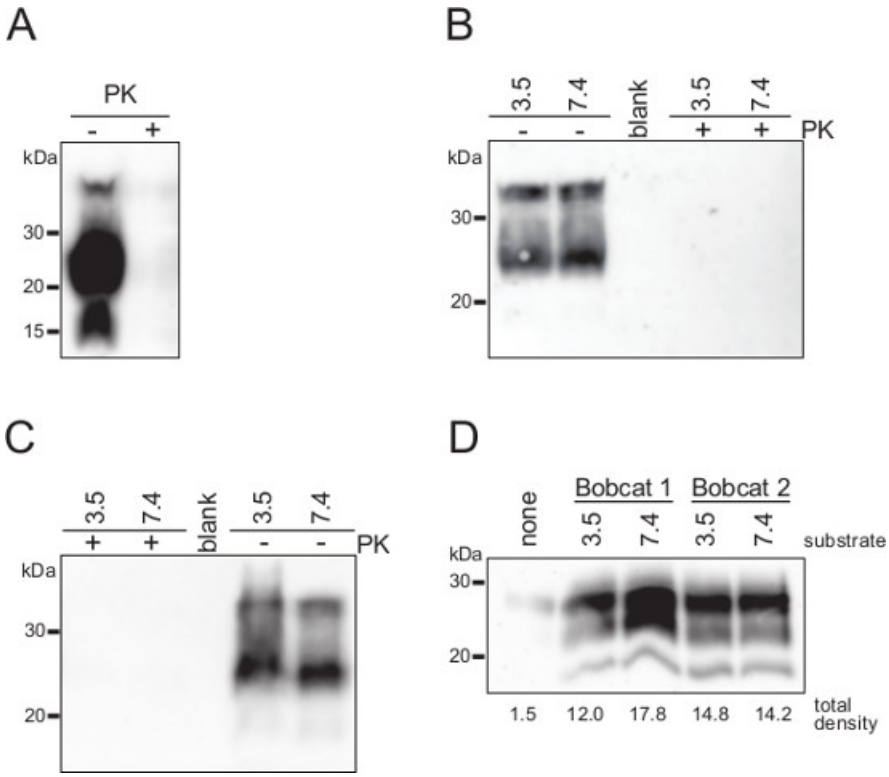

Figure 4: Sample use of CER assay to investigate bobcat susceptibility to CWD. Bobcat brain homogenate **(A)** and CER assay substrate **(B)** was tested by immunoblotting in the absence and presence of PK (100 µg/ml) to assess existing PrP^{C} levels and the presence of pre-existing PrP^{res}, respectively. **(C)** Bobcat substrates were seeded with conversion buffer and assayed in the CER assay to assess non-specific PrP^{res} formation (left two lanes). Non-shaken, non-PK treated bobcat substrates represent total PrP^{C} content in assay reactions (right two lanes). **(D)** Bobcat substrate prepared at either pH 7.4 or 3.5 was incubated with white-tailed deer CWD using the CER assay. The control sample (labeled "none") contained an equal amount of CWD agent but no bobcat substrate. Raw densitometric values for each sample are displayed below each lane. Immunoblots in all panels used monoclonal antibody 3F4, which reacts with the feline prion protein[21].

Discussion

For successful completion of this protocol, attention should be paid to PrP^{C} levels in uninfected brain tissue used for substrate preparation (step 2.1.2) and the seed to substrate ratio for conversion reactions (step 4.4.2). In our experience, brains can be extracted for use as CER substrate after a substantial period post-mortem, as long as PrP^{C} is present by immunoblotting (**Figure 4**). In fact, some autolysis was observed in the brains of the bobcats used for CER studies. Nevertheless, incubation of substrates derived from these brains still resulted in the formation of PrP^{res}. We speculate that, in part, the robustness of the CER assay is derived from the denaturation of CER substrates in 1.5 M GdnHCl, which will inactivate many proteases.

Seed to substrate ratios may need to be empirically determined to obtain acceptable PrP^{res} signal by immunoblotting. Too much or too little PrP^{res} to perform densitometry, high background PrP^{res} levels in control samples lacking substrate and inconsistent conversion ratios are all reasons to optimize seed:substrate ratios. Variations maintain a total reaction volume of 100 µl and include 1:99 µl, 2:98 µl and 5:95 µl. On almost all occasions, we have found that 5:95 µl seed:substrate volume ratio is optimal. No matter what ratio is employed, equal amounts of TSE agent are used and the assay is internally consistent. However, seed concentration of the prion-infected brain homogenate used to template reactions likely affects the outcome of the CER assay in that varying seed titers or dilutions could result in varying levels of PrP^{C}-to-PrP^{res} conversion. This effect has been demonstrated in other *in vitro* prion conversion assays[22] and may complicate the comparison of different prion isolates. To address this in the CER assay, seeds could be titrated in each PrP^{C} substrate prepared at pH 3.5 and 7.4 to identify a range of dilutions that template PrP^{C} conversion. Ideal seed dilutions avoid saturating the solution with excess seed prions and yet provide insight into the sensitivity of the conversion reaction. Alternatively, PrP^{C}-to-PrP^{res} conversion in CER assay reactions could be measured at multiple, regular timepoints throughout the duration of the assay and plotted as rate-of-conversion curves, akin to the readouts for real-time polymerase chain reaction (qPCR)[23] and real-time quaking induced conversion (RT-QuIC)[24] assays. Such readouts could allow more comprehensive interpretations of species barriers and are an interesting focus of future development of this methodology.

In the protocol presented here, we use low binding, thin-walled PCR tubes, but we have also used standard 1.5 ml tubes in a thermo-shaker equipped for this type of tube. No other variations to the protocol are needed to use the larger sized tubes. In our experience, however, we have had more PrP^{res} production and more reproducible results in low-binding PCR tubes compared to 1.5 ml tubes. While explanations for the improved performance of the assay in PCR tubes are only speculative at this time, based on results indicating that the air-water interface is critical for PrP fibrillization[25], we hypothesize that the smaller tubes provide a more optimal surface tension for PrP conversion.

Perhaps the chief limitation to use of the CER assay is in understanding what conversion ratios represent in terms of *in vivo* species barriers determinants, such as disease penetrance or length of incubation period. While currently unknown, translation between *in vitro* and *in vivo*

species barrier factors should become clearer with continued use of the CER assay in testing additional TSE species barriers that have already been established *in vivo* and will help to qualify results in species where bioassay data are not available. An advantage of the CER assay compared to PMCA is the presence of an internal control for PrP conversion, namely the pH 3.5 substrate, which can be converted by any TSE agent. This control is of value when it is unclear which, if any, TSE agents should cause misfolding of an exotic host's PrP^{C}. The inability of a given host substrate to amplify a specific TSE agent in PMCA could be interpreted as evidence of a species barrier or could be due to technical failings. Disadvantages of the CER assay compared to PMCA include limited amplification of PrP^{res}, additional steps in CER substrate preparation and no known infectivity in the PrP^{res} produced by CER reactions. It is to be noted that in a previous study, however, we found the CER assay resulted in a similar pattern of PrP^{res} formation as that of PMCA[15]. The results presented here also indicate that the CER assay correctly predicts the species barriers for transmission of various TSEs to laboratory mice (**Figure 3**) and suggest that bobcats may have susceptibility to CWD (**Figure 4**), in agreement with reports that domestic cats (*Felis catus*) can acquire prion disease after experimental CWD challenge[26,27]. Studies using the CER assay could compliment PrP sequencing efforts to understand wildlife susceptibility to CWD[28].

The CER assay enables rapid, low-cost, reliable assessment of TSE species barriers *in vitro*. While not a complete replacement for the "gold standard" of animal bioassay, the CER assay can be used as an initial assessment of the existence of a TSE species barrier that may justify or obviate further studies in living animals. For this reason, and because the assay relies only on brain tissue which can be acquired from non-experimental animals, the CER is in keeping with efforts to replace, reduce and refine animal experimental procedures[29]. In addition to assessing species barriers, the CER assay can also provide a simplified platform with which to investigate the mechanisms of the fundamental event defining prion disease biology: the conversion of normal, functional PrP^{C} to a misfolded form.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank John Olsen (Wisconsin Department of Natural Resources), Tom Cooley, Daniel O'Brien and Steve Schmitt (Michigan Department of Natural Resources) and Dr. Daniel Walsh (USGS National Wildlife Health Center) for assistance with tissue acquisition. We also thank the Wisconsin State Laboratory of Hygiene for diagnostic testing services, and Dr. Tonie Rocke and her staff (USGS National Wildlife Health Center) for use of equipment. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

References

1. Colby, D. W., Prions Prusiner, S. B. *Cold Spring Harb Perspect Biol.* **3**, (1), a006833 (2011).
2. Hill, A. F., Collinge, J. Prion strains and species barriers. *Contrib Microbiol.* **11**, 33-49 (2004).
3. Scott, M., *et al.* Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell.* **59**, (5), 847-857 (1989).
4. Prusiner, S. B., *et al.* Transgenetic Studies Implicate Interactions between Homologous Prp Isoforms in Scrapie Prion Replication. *Cell.* **63**, 673-686 (1990).
5. Telling, G. C., *et al.* Prion Propagation in Mice Expressing Human and Chimeric Prp Transgenes Implicates the Interaction of Cellular Prp with Another Protein. *Cell.* **83**, 79-90 (1995).
6. Hill, A. F., *et al.* The same prion strain causes vCJD and BSE. *Nature.* **389**, (6650), 448-450 (1997).
7. Torres, J. M., *et al.* Elements modulating the prion species barrier and its passage consequences. *PLoS One.* **9**, (3), e89722 (2014).
8. Groschup, M. H., Buschmann, A. Rodent models for prion diseases. *Vet Res.* **39**, (4), 32 (2008).
9. Korth, C., *et al.* Abbreviated incubation times for human prions in mice expressing a chimeric mouse-human prion protein transgene. *Proc Natl Acad Sci U S A.* **100**, (8), 4784-4789 (2003).
10. Kocisko, D. A., *et al.* Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. *Proc Natl Acad Sci U S A.* **92**, (9), 3923-3927 (1995).
11. Raymond, G. J., *et al.* Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. *EMBO J.* **19**, (7), 4425-4430 (2000).
12. Fernandez-Borges, N., de Castro, J., Castilla, J. In vitro studies of the transmission barrier. *Prion.* **3**, (4), 220-223 (2009).
13. Zou, W. Q., Cashman, N. R. Acidic pH and detergents enhance in vitro conversion of human brain PrPC to a PrPSc-like form. *J Biol Chem.* **277**, (46), 43942-43947 (2002).
14. Li, L., Coulthart, M. B., Balachandran, A., Chakrabartty, A., Cashman, N. R. Species barriers for chronic wasting disease by in vitro conversion of prion protein. *Biochem Biophys Res Commun.* **364**, (4), 796-800 (2007).
15. Morawski, A. R., Carlson, C. M., Chang, H., Johnson, C. J. In vitro prion protein conversion suggests risk of bighorn sheep (Ovis canadensis) to transmissible spongiform encephalopathies. *BMC Vet Res.* **9**, 157 (2013).
16. *JoVE Science Education Database. Basic Methods in Cellular and Molecular Biology. Separating Protein with SDS-PAGE.* JoVE Cambridge, MA Available from: http://www.jove.com/science-education/5058/separating-protein-with-sds-page (2014).
17. *JoVE Science Education Database. Basic Methods in Cellular and Molecular Biology. The Western Blot.* JoVE Cambridge, MA Available from: http://www.jove.com/science-education/5065/the-western-blot (2014).
18. Chandler, R. L. Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet.* **1**, (7191), 1378-1379 (1961).
19. Browning, S. R., *et al.* Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. *J Virol.* **78**, (23), 13345-13350 (2004).
20. Hadlow, W. J., Race, R. E., Kennedy, R. C. Experimental infection of sheep and goats with transmissible mink encephalopathy virus. *Can J Vet Res.* **51**, (1), 135-144 (1987).

21. Rubenstein, R., *et al.* Immune surveillance and antigen conformation determines humoral immune response to the prion protein immunogen. *J Neurovirol.* **5**, (4), 401-413 (1999).
22. Castilla, J., *et al.* Crossing the species barrier by PrP(Sc) replication in vitro generates unique infectious prions. *Cell.* **134**, (5), 757-768 (2008).
23. Kubista, M., *et al.* The real-time polymerase chain reaction. *Mol Aspects Med.* **27**, 95-125 (2006).
24. Atarashi, R., Sano, K., Satoh, K., Nishida, N. Real-time quaking-induced conversion: a highly sensitive assay for prion detection. *Prion.* **5**, (3), 150-153 (2011).
25. Ladner-Keay, C. L., Griffith, B. J., Wishart, D. S. Shaking Alone Induces De Novo Conversion of Recombinant Prion Proteins to beta-Sheet Rich Oligomers and Fibrils. *PLoS One.* **9**, (6), e98753 (2014).
26. Mathiason, C. K., *et al.* Susceptibility of domestic cats to chronic wasting disease. *J Virol.* **87**, (4), 1947-1956 (2013).
27. Seelig, D. M., *et al.* Lesion Profiling and Subcellular Prion Localization of Cervid Chronic Wasting Disease in Domestic Cats. *Vet Pathol.* (2014).
28. Stewart, P., *et al.* Genetic predictions of prion disease susceptibility in carnivore species based on variability of the prion gene coding region. *PLoS One.* **7**, (12), e50623 (2012).
29. Russell, W. M., Burch, R. I. *The Principles of Humane Experimental Technique. Metheun.* Methuen (1959).

