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Altered rPrP substrate structures and their influence on real-time quaking induced conversion reactions

Keding Cheng[a,b,*], Angela Sloan[a], Brooks Waitt[a], Robert Vendramelli[a], Debra Godal[a], Sharon L.R. Simon[a], Joe O'Neil[c], Michael Carpenter[a,d], Dave Jackson[e], Jane Eastlake[e], Gary Mallinson[e], J. David Knox[a,d,**]

[a] National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada
[b] Department of Human Anatomy and Cell Sciences, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada
[c] Department of Chemistry, University of Manitoba, Winnipeg, Manitoba, Canada
[d] Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada
[e] Bristol Institute for Transfusion Sciences (BITS), National Blood Service, Bristol, UK

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ABSTRACT

Background: Bacterially-produced recombinant prion protein (rPrP) has traditionally been used for *in vitro* fibrillation assays and reagent development for prion disease research. In recent years, it has also been used as a substrate for real-time quaking-induced conversion (RT-QuIC), a very sensitive method of detecting the presence of the misfolded, disease-associated isoform of the prion protein (PrP^d). Multi-centre trials have demonstrated that RT-QuIC is a suitably reliable and robust technique for clinical practice; however, in the absence of a commercial supplier of rPrP as a substrate for RT-QuIC, laboratories have been required to independently generate this key component of the assay. No harmonized method for producing the protein has been agreed upon, in part due to the variety of substrates that have been applied in RT-QuIC.

Methods: This study examines the effects of two different rPrP refolding protocols on the production, QuIC performance, and structure characteristics of two constructs of rPrP commonly used in QuIC: full length hamster and a sheep-hamster chimeric rPrP.

Results: Under the described conditions, the best performing substrate was the chimeric sheep-hamster rPrP produced by shorter guanidine-HCl exposure and faster gradient elution.

Conclusions: The observation that different rPrP production protocols influence QuIC performance indicates that caution should be exercised when comparing inter-laboratory QuIC results.

1. Introduction

The accumulation of misfolded prion protein (PrP^d) is widely regarded as the cause of prion diseases, such as sporadic Creutzfeldt–Jakob disease (sCJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. These diseases, producing unique spongiform changes in the brain, progress rapidly from first neurological symptoms to death [1,2]. Traditionally, prion diseases are suspected in ante-mortem patients using a combination of clinical symptoms, electroencephalography, magnetic resonance imaging, and laboratory tests of surrogate protein biomarkers in cerebrospinal fluid (CSF) [3,4]. Nonetheless, definitive diagnosis generally requires immunohistochemistry of post-mortem brain tissue [1,2]. To address the lack of a sensitive and specific ante-mortem diagnostic test for prion diseases, the ability of PrP^d to induce the conversion of cellular PrP (PrP^c) into a misfolded form has been exploited in recent years. In real-time quaking-induced conversion (RT-QuIC), diseased brain homogenate (BH) or CSF are used to “seed” the reaction and recombinant prion protein (rPrP) is used as the “substrate” [5–8]. Patient samples (often CSF) containing sufficient amounts of PrP^d will convert the rPrP into a misfolded form. The integration of the fluorescence dye thioflavin T (ThT) into the newly generated PrP^d aggregates changes the dye's emission spectrum, which can then be monitored in real time [5]. RT-QuIC using human CSF as the seed has demonstrated comparable specificity to post-mortem diagnosis [7,9].

While hamster rPrP is currently the most widely-used substrate for the RT-QuIC assay [6,8,9], sheep-hamster chimera rPrP has also been used [10–12]. In order to compare and contrast the suitability of the

* Corresponding author. National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada.
** Corresponding author. National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada.
E-mail addresses: Keding.Cheng@phac-aspc.gc.ca (K. Cheng), David.Knox@phac-aspc.gc.ca (J.D. Knox).

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two RT-QuIC substrates for eventual use in diagnostic ante-mortem prion disease testing, both sheep-hamster chimera and full-length hamster rPrP were expressed and purified in this study. The performance of these substrates was then determined in RT-QuIC assays using the harvested products of previous RT-QuIC reactions as seeds (product as seed, PAS), as well as CSF from sCJD positive and negative patients. This study represents the first side-by-side comparison and optimization of the most commonly used rPrP substrates for RT-QuIC assay.

2. Materials and methods

2.1. General supplies

All chemicals used in this study were of the highest grade commercially available. All individual reagents were passed through a 0.22 μm filter after preparation. Bacterial media and RT-QuIC reagents were prepared by the media department of Canadian Science Centre for Human and Animal Health in Winnipeg, Manitoba, Canada. *E. coli* Rosetta (DE3) clones expressing full-length (23–231) hamster rPrP were kindly provided by Dr. Byron Caughey's lab (Rocky Mountain Laboratory, Hamilton, Montana, USA) [6]. The use of human specimens from the CJD biobank housed at the National Microbiology Laboratory of Canada was reviewed and approved by the Health Canada's Research Ethics Board (HC-PHAC REB 2014-0033).

2.2. Transformation and culture of pET41-chimeric rPrP expressing cells

The expression vector pET41-PrP, encoding sheep-hamster chimeric PrP (Syrian hamster residues 23 to 137 [GI:191429] followed by sheep residues 141 to 234 of the R154 Q171 polymorph [GI:59596090]), with and without a C-terminal 6-His-tag, were designed, synthesized, and ligated into the pET41a(+) vector (Novagen, USA) at restriction enzyme sites *Nde*1 and *Not*1 by GenScript, and sequence verified (Supplementary Text 1). One μl containing 0.8 ng or 8 ng plasmid prepared in 10 mM Tris-EDTA buffer, pH 7.5 was added to 20 μl competent Rosetta (DE3) cells and left on ice for 30 min. The cells were heat-shocked at 42 °C for 30 s and placed on ice for approximately 10 s before the addition of 250 μL S.O.C (super optimal broth with catabolite repression) media (Invitrogen). The cells were then shaken at 250 RPM at 37 °C in horizontally oriented tubes for 30 min, spread on Luria-Bertani (LB) agar plates containing 100 μg/ml kanamycin, and incubated at 37 °C overnight. Discrete colonies were picked with a pipette tip and inoculated into 2 ml LB-Miller broth containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol and shaken at 250 RPM for further growth for five more hours.

2.3. Confirmation of rPrP-containing colonies by PCR

One μl from each 2 ml starting culture above was added to PCR Master Mix (BioRad). Recombinant protein constructs were amplified using T7 forward (5′-TAATACGACTCACTATAGGG-3′) and reverse (5′-TAGTTATTGCTCAGCGGTGG-3′) primers (Invitrogen) under the following conditions: denaturing at 98 °C for 2 min, then 38 cycles of denaturation at 98 °C for 30 s, annealing at 52 °C for 15 s, and extension at 72 °C for 45 s. A final extension was carried out at 72 °C for 5 min and the products kept at 4 °C until electrophoresis on a 1.25% agarose gel for size estimation. The amplicons of appropriate length were purified and confirmed by sequence analysis.

2.4. Overexpression of pET41-rPrP

Positive clones were further cultured in 30 ml of LB-Miller broth media containing antibiotics with constant shaking until OD_{600} reached 0.6–0.9. Cultures were then diluted 50-fold by the media containing either 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Novagen) or Overnight Express Autoinduction System 1 (OEA; Novagen; used as per manufacturer's instructions), and grown overnight for 18 h at 37 °C, or 28 h at 30 °C. After centrifugation at 2600 × *g* for 20 min, the resulting cell paste was either subjected to three cycles of freezing and thawing using liquid nitrogen and then frozen at −80 °C to be processed later, or directly underwent Bugbuster (Novagen) treatment for collection of inclusion bodies. In order to optimize the use of Bugbuster, the cell paste weight was taken (difference between the weight of a 50 ml tube with the cell paste and the weight of an empty 50 ml tube).

2.5. Confirmation of rPrP expression by western blotting and mass spectrometry

Cultures underwent analyses by SDS-PAGE and mass spectrometry (MS) to confirm rPrP expression. Ten μl of induced and non-induced cultures were mixed with 10 μl 4× SDS-PAGE sample buffer (Invitrogen) and heated for 5 min at 99 °C. Samples were loaded onto two Criterion XT 4–12% Bis-Tris gels (Bio-Rad). Western blotting with PrP specific antibody 3F4 (Covance, USA) was used on one gel and the other gel was stained with SimplyBlue SafeStain (Invitrogen) for MS analysis. Bands of interest were excised and de-stained with 40% acetonitrile (ACN) in 50 mM ammonium bicarbonate (AB; Sigma). The gel pieces were dehydrated with 100% ACN, and digested overnight with MS-grade trypsin (Thermo-Fisher) at a final concentration of 10 ng/μl in 50 mM AB. Tryptic peptides were solubilized upon the addition of 40% ACN in 0.1% formic acid (FA; Fluka) onto the gel pieces with gentle shaking for 10 min, followed by additional dehydration of the gel pieces with 100% ACN. Peptide extracts were vacuum-dried, dissolved in 0.1% FA, and detected and sequenced by liquid chromatography-tandem MS (LC-MS/MS) with an Orbitrap MS system (ThermoFisher).

2.6. Collection of rPrP inclusion bodies

Cell paste was added to Bugbuster Master Mix (Novagen) at 10 ml Master Mix per 2 g of cell paste weight. The paste was disintegrated with a tissue homogenizer with a tip probe (Omni International) at medium speed, lysed on a platform rotator for 20 min, centrifuged at 13,000 × *g* for 20 min at 4 °C, and the supernatant discarded. Bugbuster Master Mix was again added and the cycle repeated. The resulting inclusion body pellet was subsequently washed twice with 0.1× Bugbuster, centrifuged at 13,000 × *g* for 20 min at 4 °C, and the supernatant discarded.

2.7. Refolding of rPrP

Two methods (method A and method B) similar to the respectively reported techniques [8,13] were employed and the key points are summarized in supplementary Table 1. In both methods, inclusion bodies were dissolved in 8 M guanidine-hydrochloride (GuHCl; MP Biomedicals) in 100 mM sodium phosphate (Sigma) buffer and 10 mM Tris (Sigma), pH 8.0 and homogenized using the tissue homogenizer. All the buffer components are the same between method A and B, and the major difference is the amount of time that the proteins remain in GuHCl. In method A, solubilized inclusion body proteins were exposed to GuHCl for 5 h prior to refolding, while refolding itself (the removal of GuHCl) occurred over a 12 h period. In method B, solubilized inclusion body proteins were exposed to GuHCl for 70 min prior to refolding. Refolding occurred over a 3 h period.

2.8. Elution and collection of rPrP

After refolding, the column was disconnected from the HPLC system and connected to an FPLC instrument (GE Healthcare AKTA) pre-equilibrated with 100 mM phosphate buffer (pH 8.0). In order to elute sufficient chimeric protein for subsequent experiments an isocratic elution profile was used in method A. The column was eluted with

500 mM imidazole (Sigma) in 100 mM sodium phosphate, pH 5.8 at a flow rate of 2.5 mL/min into same volume of ice-cold dialysis buffer (10 mM sodium phosphate, pH 5.8). The elution was monitored with ultra violet (UV) light and all fractions above 800 mAU (milli Absorbance Unit) were pooled together. Method B utilized a gradient elution profile at a flow rate of 2.5 mL/min and the imidazole concentration reached 500 mM in 30 min. Multiple fractions were also collected and pooled.

2.9. Filtration, dialysis and re-filtration of rPrP

Eluate was filtered through a 0.22 μm syringe filter (pre-washed with dialysis buffer) into dialysis tubing with a 7 kDa molecular weight cutoff (Thermo Scientific) that had been dampened in milli-Q water. In method A, the eluate was dialyzed at room temperature for 26 h in 3 steps, with buffer changes each time: dialysis tubing was placed in a 4-L beaker of fresh dialysis buffer for 4 h, 16 h overnight, and 6 h the following morning. In method B, the eluate was dialyzed at 4 °C for 20 h in 3 steps: 1 h, 15 h overnight, and 4 h the following morning. Dialyzed rPrP was filtered once more using a 0.22 μm syringe filter in order to eliminate potential precipitates [6,8,13]. The filtrate protein concentration was estimated using Qubit 2.0 kit (Invitrogen), divided into 300 μg aliquots, and stored at −80 °C.

2.10. RT-QuIC assay with product as seeds (PAS) and patient CSF

In contrast to previous publications, the substrate was not further filtered with a 100 K spin column [6,8–11] because there was little difference before and after filtration (data not shown). Literature has shown that human CSF and human BH [6,9] exhibit different dynamics in RT-QuIC reactions and need to be tested at different volumes and dilutions. To address the limited availability of patient CSF (which is the most commonly used body fluid for sCJD diagnosis with QuIC platforms) [9–11,14,15], commercial artificial CSF (Harvard Apparatus, USA) was used as negative control. Positive controls were PAS, harvested reaction products of a confirmed sCJD positive RT-QuIC reactions when hamster rPrP was used as the substrate. PAS was diluted 100-fold with the artificial CSF before use. All CSF analyses were performed in triplicate in black 96-well optical-bottom plates (ThermoFisher) in 100 μl reaction volume. Each well had 15 μl CSF, diluted PAS, or artificial CSF added to 85 μl RT-QuIC buffer with a final concentration of 300 mM NaCl, 10 μM EDTA, 10 μM ThT, and 0.1 mg/ml rPrP in 10 mM phosphate buffer (pH 5.8). Sealing tape was applied and the plate incubated with intermittent shaking (90 s double orbital shaking at 900 rpm, 30 s rest) for 90 h at 42 °C in a FLUOstar Omega plate reader (Mandel Canada). A fluorescence reading at an excitation wavelength of 450 nm and emission wavelength of 480 nm was taken by the FLUOstar Omega plate reader every 44 min [15].

2.11. Circular dichroism (CD) spectropolarimetry

CD spectra were recorded on a Jasco J-810 spectropolarimeter in 0.01 cm path length cylindrical quartz cuvettes between 180 and 260 nm. All measurements were carried out using 100 μg/ml rPrP in 10 mM sodium phosphate, pH 5.8 at 25 °C. Spectra were recorded at a scan rate of 10 nm/min with a data pitch of 0.1 nm and a response time of 4 s. The CD intensity and wavelength of the spectropolarimeter were calibrated using solutions of *d*-10-camphorsulphonic acid. Mean Residue Ellipticities (10^{-3} deg.cm^2.dmole^{-1}) were calculated using the equation: $[\theta]_M = (M_r)\ (\theta)/(10)(l)(c)(n)$ where M_r is 23,471.8 g per mole for sheep-hamster rPrP and 24,098.8 g per mole for hamster rPrP, l is the cell path length, θ is the measured ellipticity in millidegrees, c is the protein concentration in g/L, and n = 210 (amino acids number) for hamster-sheep rPrP and n = 219 for hamster rPrP. CD spectra were deconvoluted using the CDSSTR algorithm at DICHROWEB [16–19].

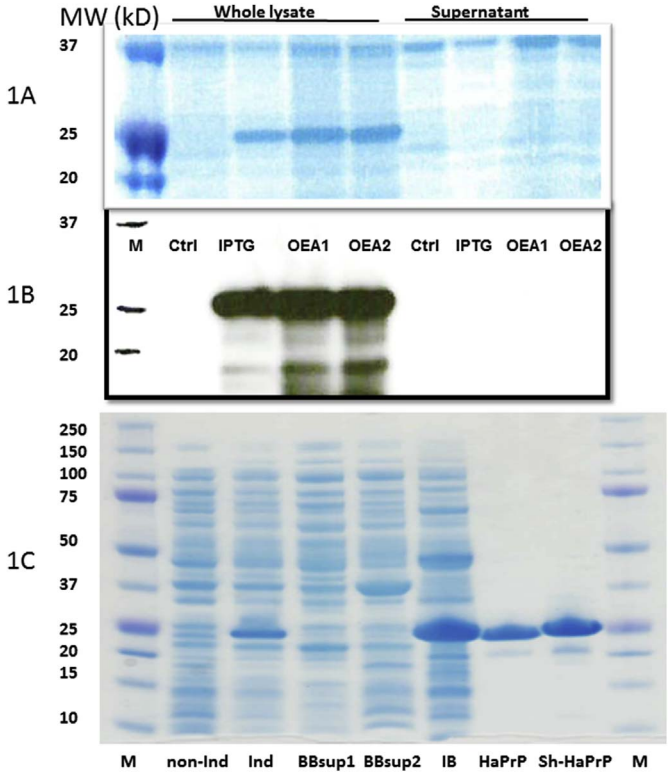

Fig. 1. Gel-staining and western blotting of pET41 culture expressing sheep-hamster rPrP. Cultured cells were sonicated, and pellets and supernatants run on duplicate SDS-PAGE gel for SimplyBlue staining (1A) and western blotting with anti-PrP antibody 3F4 (1B). M, molecular weight (MW) marker; Ctrl, non-induced cell culture control (ctrl); IPTG, culture induced using IPTG; OEA, culture induced using the Overnight Express Autoinduction (OEA) System. IPTG induced and OEA1 represent the same clone, while OEA2 represents a different clone. Fig. 1C further shows SDS-PAGE of pET41-rPrP-expressing *E. coli* culture components. Non-Ind, non-induced hamster rPrP culture; Ind, hamster rPrP culture induced with OEA System; BBsup1, supernatant after first-round treatment of hamster rPrP culture with Bugbuster; BBsup2, supernatant after second-round treatment of hamster rPrP culture with Bugbuster; IB, inclusion bodies containing hamster rPrP; HaPrP, purified and dialyzed hamster rPrP; Sh-HaPrP, purified and dialyzed untagged sheep-hamster rPrP. The experiment has been repeated once.

3. Results

3.1. Recombinant PrP is stably expressed

Rosetta (DE3) cells were transformed by the pET41-rPrP construct. Strongly stained gel bands were observed after induction by either IPTG or the Overnight Express Autoinduction System reagents (Fig. 1A and B). The rPrP existed mainly in cellular inclusion bodies after expression in pET41 vector (Fig. 1C). MS analysis of the major gel bands confirmed rPrP expression, despite limited trypsin cleavage sites and the resultant partial protein sequence coverage from in-gel peptide extracts (Supplementary Fig. 1).

To purify the expressed proteins, two protein production optimization methods were initially checked at the exploratory stage of this project: culturing cells at low temperature (15 °C) [20], or adding a His tag to increase the ion-metal chromatography column binding [21,22] were found not being able to give sufficient amount of soluble rPrP for RT-QuIC application due to either an inhibition of protein production at low temperature or a promotion of auto-aggregation on the column with His tags. As a result, induction of untagged rPrP expression at either 37 °C overnight recommended by the Rocky Mountain Laboratory (RML) [6], or 30 °C for 28 h recommended by the Bristol Institute for Transfusion Sciences (BITS) [8] were used. These approaches made the transformed cell grow well and gave similar yield of cell paste for extracting inclusion bodies (Supplementary Table 2), and the prion

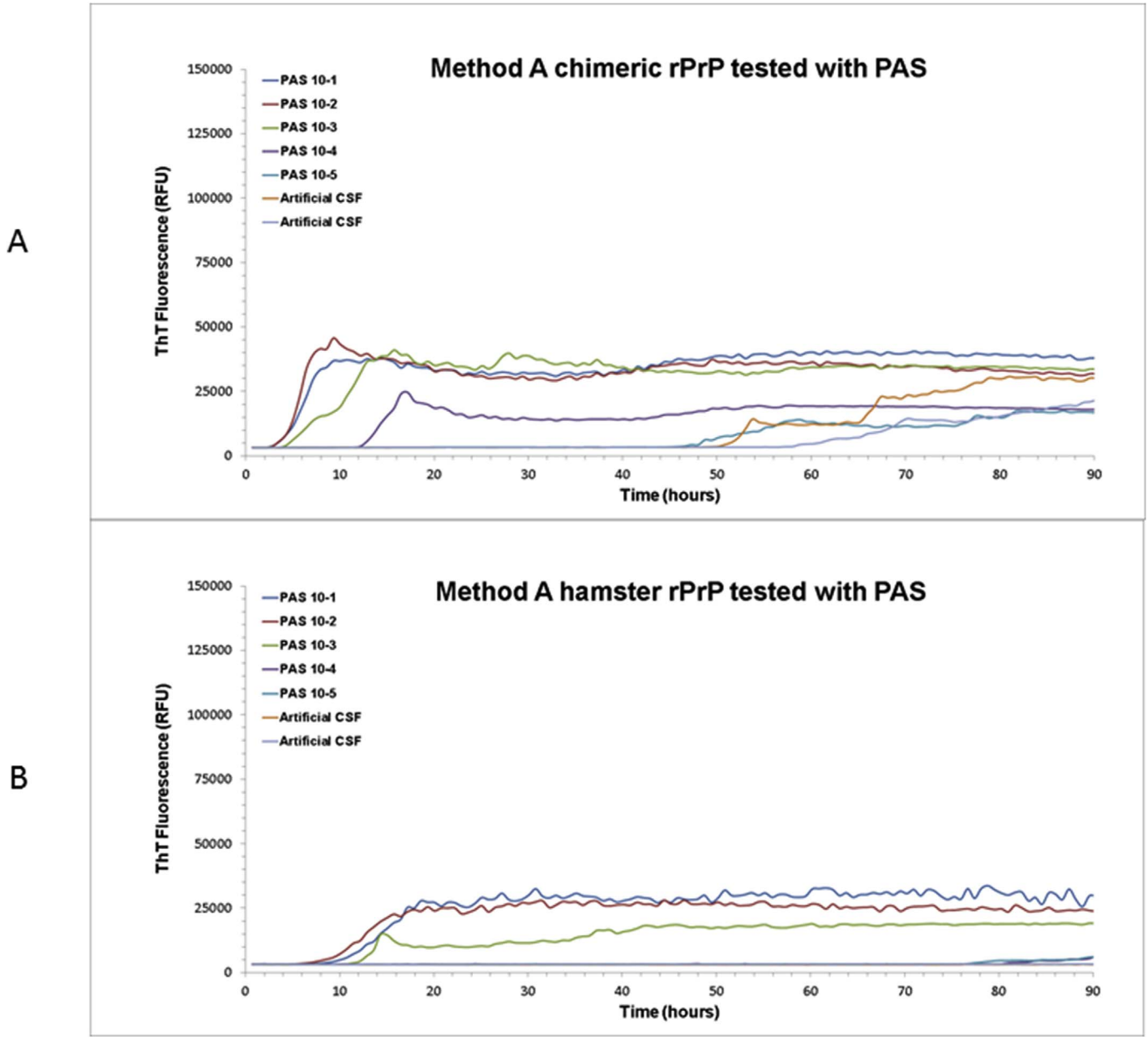

Fig. 2. RT-QuIC performed using chimeric (A) and hamster (B) rPrP substrates under method A with PAS as seeds. Data includes 10^{-1} through 10^{-5} PAS dilutions in artificial CSF and two neat artificial CSF negatives for each substrate. The experiment has been repeated twice.

protein-encoded octapeptide repeat region alone bound to the Ni-NTA columns sufficiently well [23–25] to ensure chimeric and hamster rPrP were purified in soluble forms for RT-QuIC tests. Although isocratic elution gave higher yield in comparison with gradient elution (Supplementary Fig. 2A and B) under method A, and approximately 5 mg of chimeric rPrP was obtained from 1 L of original cell culture, about 20 mg of chimeric rPrP could be produced under method B from 1 L of cell culture (Supplementary Table 2). Using either an isocratic or gradient elution had a less profound effect on the yield of hamster rPrP, either with method A or B (Supplementary Fig. 3A and B). Boiling of post-elution Ni-NTA resin from method A followed by SDS-PAGE analysis demonstrated that there were still large amounts of chimeric or hamster rPrP bound to the column even after isocratic elution with imidazole and EDTA (Supplementary Fig. 4). Conversely, both hamster and chimeric rPrP generated by method B were eluted easily by gradient elution and resulted in a yield for both types of rPrP (about 20 mg per liter of culture, Supplementary Table 2) enough to do RT-QuIC many times.

3.2. Chimeric rPrP exhibits a shorter lag phase and higher maximal fluorescence than hamster rPrP in RT-QuIC reactions

Using dilutions of PAS as seed, repeated RT-QuIC tests showed that the chimeric rPrP reactions had a shorter lag phase and higher maximal fluorescence reading relative to the reactions using hamster rPrP as substrate. Chimeric rPrP from method A demonstrated a higher sensitivity than hamster rPrP (10^{-4} versus 10^{-3} dilutions for PAS, Fig. 2A and B). With method B, both substrates produced positive reactions at a 10^{-4} dilution of PAS representing an increase in the sensitivity of the hamster rPrP, while the sensitivity of the chimeric rPrP remained unchanged. Noticeable kinetic differences were the exponential rise in fluorescence observed in the chimeric rPrP reactions to very high levels shortly after the end of the lag phase followed by a gradual decline for the remainder of the QuIC period. Interestingly, the maximal fluorescence observed was positively correlated with the amount of seed utilized in the reactions. No positive reaction was observed from the artificial CSF under method B from either rPrP (Fig. 3A and B). Two

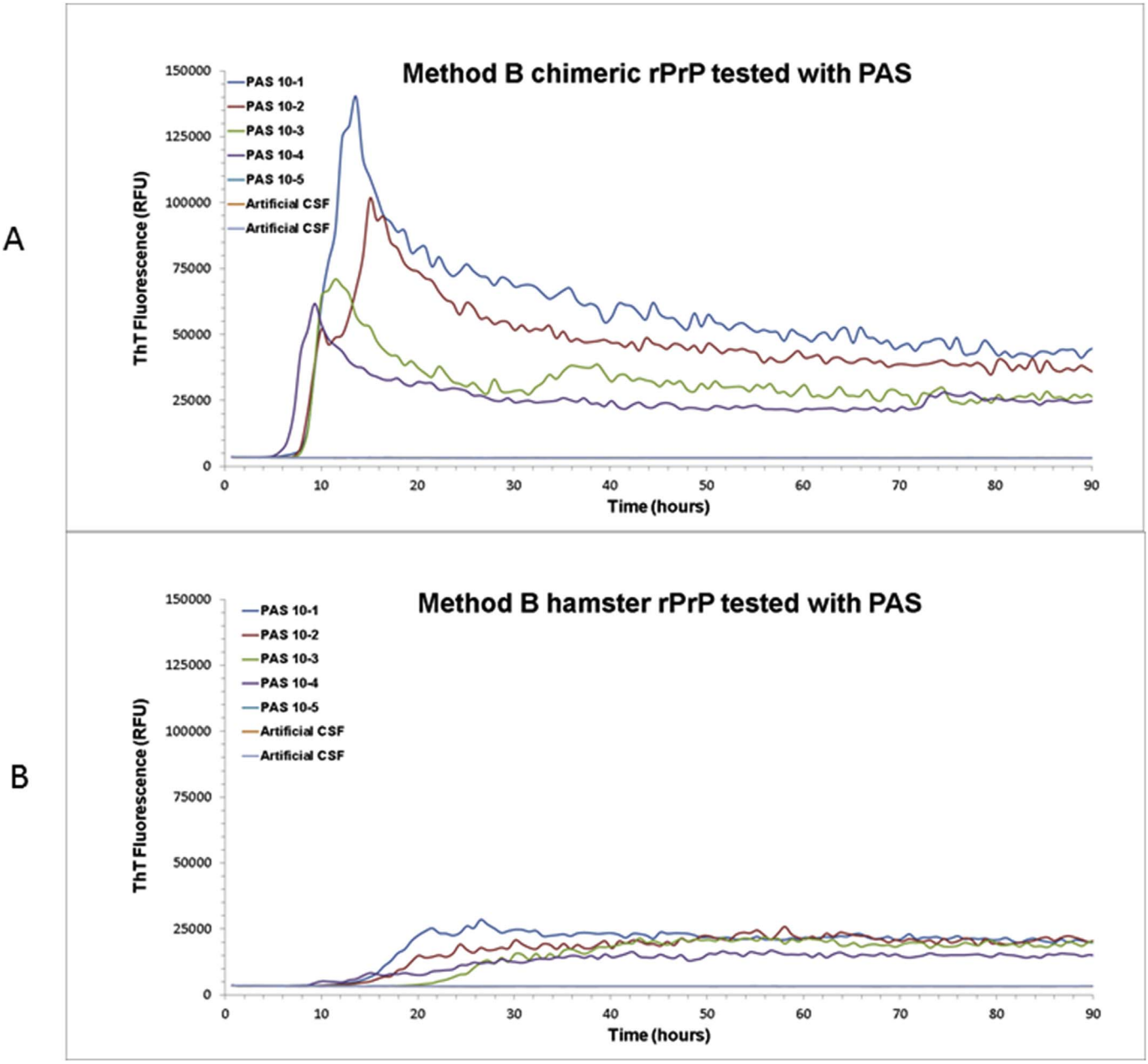

Fig. 3. RT-QuIC performed using chimeric (A) and hamster (B) rPrP substrates under method B with PAS as seeds. Data includes 10^{-1} through 10^{-5} PAS dilutions in artificial CSF and two neat artificial CSF negatives for each substrate. The experiment has been repeated twice.

preparations produced by Method B with isocratic elution and gradient elution showed similar results (Supplementary Fig. 5), and a comparison of hamster substrates produced by BITS and our institute demonstrated very similar RT-QuIC results on 27 non-CJD CSF samples and 25 sCJD samples (Supplementary Fig. 6).

3.3. Chimeric rPrP reacted faster and more intensively than the hamster rPrP with patient CSF

The results of the RT-QuIC experiments using PAS indicated that rPrP substrates produced under Method B were less prone to auto-aggregation in prolonged RT-QuIC reactions. For these reasons we restricted our analysis of clinical CSF specimens as the seed to compare chimeric and hamster rPrP substrates produced by method B. Three confirmed negative and positive CJD CSF samples were tested three times. The performance characteristics of the two substrates observed in the PAS-seeded experiments as positive controls were similar to those demonstrated when the CSF samples were used to seed the reactions. The chimeric rPrP had a shorter lag phase, an exponential rise in fluorescence to very high levels shortly after the end of the lag phase, and then a gradual decline for the remainder of the QuIC period. The lag phases of the hamster rPrP reactions were longer than the chimeric, and the increase in fluorescence more gradual and to lower maximal levels than those observed with the chimeric rPrP with no observed decrease in fluorescence. Both substrates correctly identified the CJD positive and non-CJD samples (Fig. 4A and B).

3.4. Structural similarities and differences were noted by circular dichroism

Circular dichroism measurements were used to illuminate the effects of method A and B on the structure of both the hamster rPrP and chimeric rPrP and related RT-QuIC results. The CD spectra of hamster and chimeric rPrP with method A showed that both protein preparations were predominantly α-helical with positive bands at 192 nm,

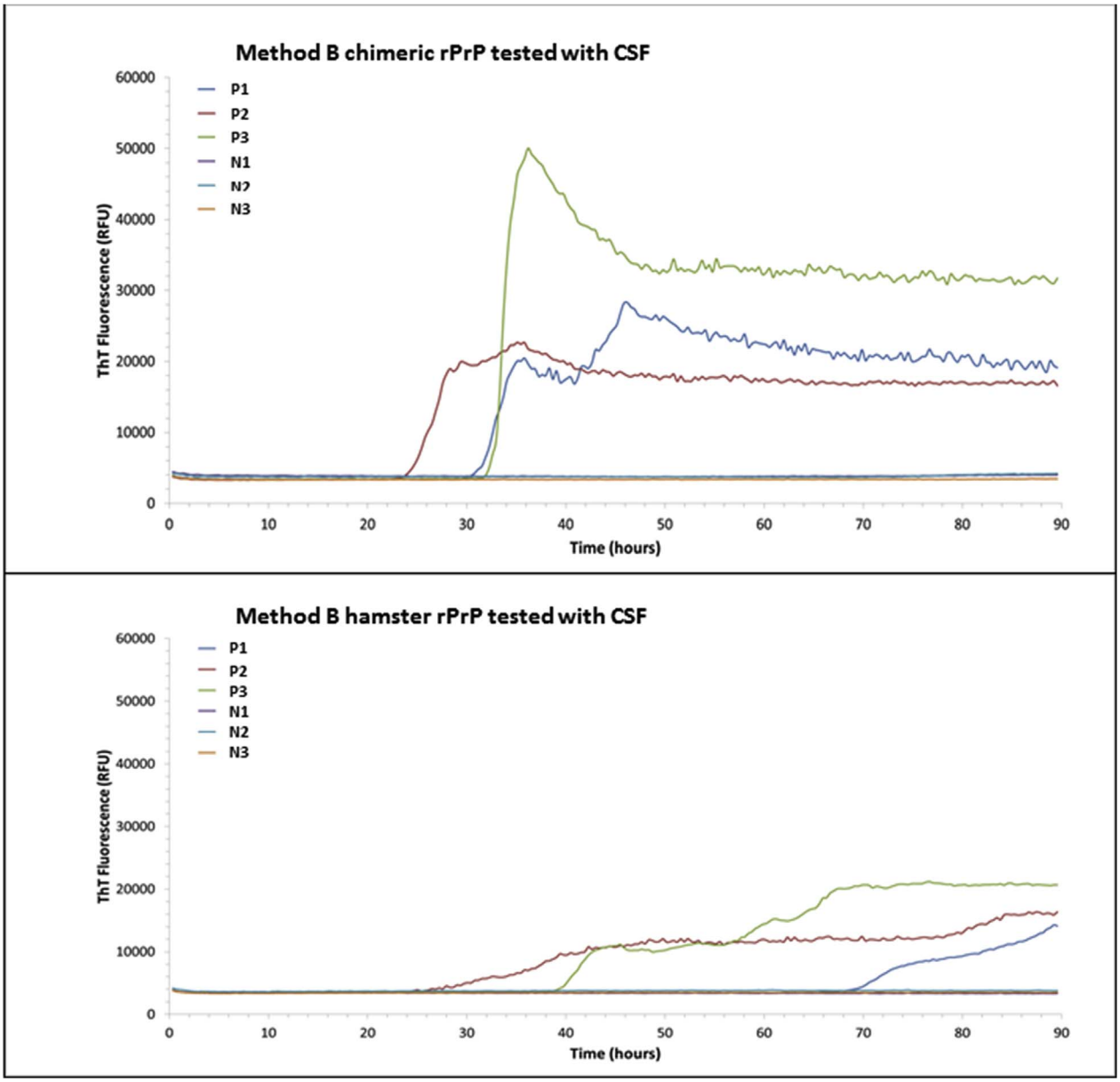

Fig. 4. RT-QuIC performed using chimeric (A) and hamster (B) rPrP substrates under method B using human CSF samples. Data includes three autopsy confirmed CJD samples (P1, P2, P3) and three autopsy confirmed non-CJD samples (N1, N2, N3) for each substrate. The experiment has been repeated twice.

cross-over points at 201 nm, and negative bands at 210 nm and 222 nm. Deconvolution based on protein concentration, wavelength range, and protein quantities and sequences [16–19] showed the CD spectra of the proteins with method A yielded 61% α-helix, 13% β-strand, 10% turns, and 17% unordered for the chimeric rPrP, and 38% alpha helix, 17% β-strand, 19% turns, and 27% unordered for the hamster rPrP. With method B, chimeric rPrP showed a sizeable difference in structure (16% alpha helix, 32% β-strand, 21% turns, and 29% unordered) in comparison with method A. In contrast, method B only had a minor effect on the structure of hamster rPrP which remained largely as α-helical (35% alpha helix, 22% β-strand, 18% turns, 25% unordered). Superimposing the CD spectra demonstrates the effect of the refolding processes on the hamster and chimeric rPrP structures (Fig. 5).

4. Discussion

The purification of many proteins on a Ni-NTA column necessitates the addition of a C-terminal 6-His-tag such that the engineered protein binds tightly to the column in buffers that permit the removal of most other non-specific materials by a general buffer wash. Once the undesired materials have been flushed out from the column the desired protein with 6-His-tag can be eluted with imidazole [21,22]. In the case of prion proteins the four naturally occurring histidine-containing octapeptide repeats (HGGGWGQP) result in strong binding to the column in the absence of any His modification [23–25], negating the need to introduce a His tag. Indeed, method A, designed to result in protein conformations similar to the native state [26–28], resulted in the chimeric rPrP being so firmly bound to the column that isocratic elution was required to elute sufficient quantities of rPrP for subsequent experiments. Even after further elution with 500 mM imidazole and 200 mM EDTA, boiling the resin in SDS prior to gel analysis demonstrated that large amounts of the rPrP still remained in the column. A possible explanation is that the long-refolding process in method A might cause some of the rPrP to precipitate on the affinity column [26–28], making it recalcitrant to elution by imidazole. This will result in a lower production of soluble rPrP as proven by the quantitation of the protein yield although this is enough for multiple RT-QuIC reactions.

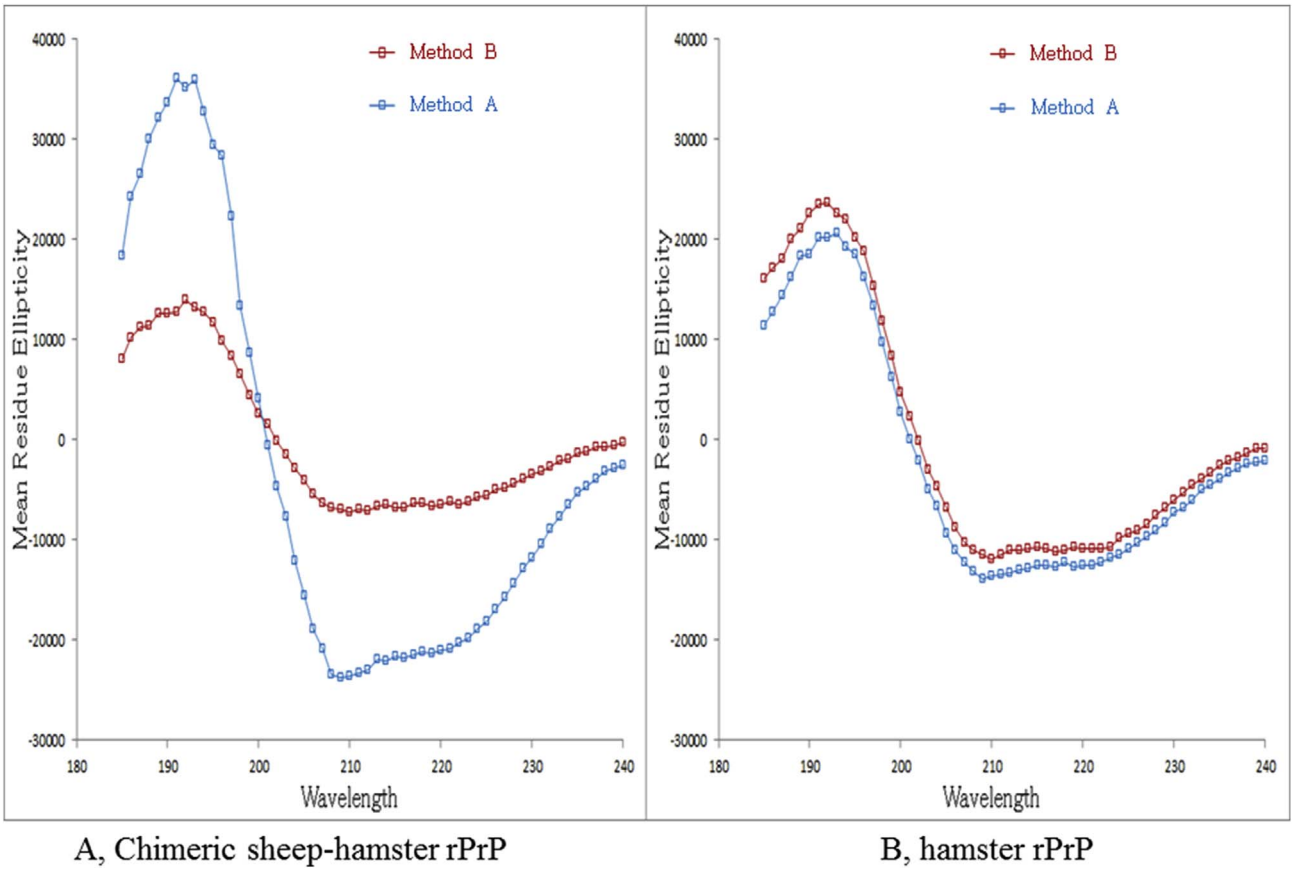

Fig. 5. Circular dichroism measurements of differently refolded rPrP. A, sheep-hamster chimeric rPrP. Blue represents rPrP under method A, and red represents rPrP under method B. B, hamster rPrP, and blue represents rPrP under method A, and red represents rPrP under method B. The experiment has been repeated once. Please see the main text for details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Using an HPLC for part of the protocol and an FPLC for another part in methods described facilitated quality control and consistency of testing results. Technically there is residual denaturing and refolding buffers at the pump heads after refolding if only one system is used. This might interfere with the downstream elution which uses different buffers, unless the column is disconnected and the system re-purged to flush the residual buffers out. In addition, the use of two LC systems speeds up the purification process, because when refolding is finished on one system, the next system for elution is already equilibrated and ready to use.

The different refolding procedures affected the chimeric rPrP to a greater extent than the hamster rPrP. The CD data demonstrated that Method A resulted in the eluted chimeric rPrP and hamster rPrP both being predominantly alpha helical. Interestingly, with method B, where the transition from 6 M GuHCl to 100 mM phosphate buffer occurred in just 3.5 h, the chimeric protein formed a much different mixture of structures relative to what was observed with Method A. This shift in the mixture of structures was accompanied by weaker attachment of the chimeric rPrP to the Ni-NTA resin which enabled more efficient elution from the column using a gradient profile. In absolute terms, the two substrates (hamster and sheep-hamster chimera) under method B were equally sensitive and specific in RT-QuIC, detecting PAS dilutions of 10^{-4}, where neither substrate exhibited auto-aggregation after prolonged reactions. In addition, the chimeric rPrP exhibited a shorter lag phase as well as a much more pronounced increase in fluorescence reading at the end of the lag phase relative to hamster substrate. These observations combined with the absence of commercial sources of rPrP for RT-QuIC indicates that caution should be exercised when comparing inter-laboratory QuIC results with different rPrP preparations. The confirmation that the chimeric rPrP made by method B exhibited a shorter lag phase as well as a much more pronounced increase in fluorescence at the end of the lag phase relative to the hamster substrate in this study highlights the positive attributes of the chimeric rPrP produced by Method B. The faster turnaround time in the absence of false positive results exhibited by the chimeric rPrP produced by Method B makes it attractive as a substrate in both the research and clinical setting. Although more clinical samples are needed to confirm the observations, we feel that the stronger signal and quicker reaction time shown by the chimeric rPrP for RT-QuIC may have some advantages on some samples with low amount of prion aggregates. Chimeric rPrP can be purified with high quantity under method B as well as hamster rPrP for routine RT-QuIC applications.

In this study we did confirm that hamster rPrP provided consistent results regardless of which of the two methods was used. The suggested tolerance of the hamster rPrP performance to different production protocols may in part explain the robust and reliable performance exhibited by RT-QuIC in international studies for which different sources of hamster rPrP preparations with variable methods are used [15].

This study also indicates that the reaction characteristics of patient samples can be effectively predicted by using PAS as the positive control seed. PAS was produced with prion seeds from sporadic CJD patients, so it may be different from prion protein aggregates that were produced *in vitro* under high guanidine concentration [23]. Although more studies are needed to characterize the RT-QuIC-related products, the ability to delay the use of finite and scarce human CSF, and instead use PAS as an essentially endlessly renewable resource for preliminary experiments, quality checks, and functional verifications offers an important advantage. Literature reports have shown that brain homogenate (BH) from post-mortem CJD patients behaves quite differently than CSF [7,9,10], and we strongly recommend using PAS as a mimic of

CSF, instead of BH, for clinical QuIC tests with patient CSF.

The production and function of rPrP has been reported to be influenced by many factors such as pH, temperature, and salt concentrations [13,25]. Similarly, RT-QuIC reactions were also affected by salt and detergent concentrations, reaction time, rPrP types, and seed sources [6–11,13,29]. This is the first thorough study that links different rPrP production methods and their related RT-QuIC reactions with a goal to optimize the rPrP production and refolding procedures to achieve sensitive and specific RT-QuIC reactions for clinical applications. We hope this study will help the standardization of RT-QuIC applications in prion disease diagnosis, and encourage counterparts in this area to test different rPrP substrates for more sensitive and specific diagnosis of prion diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2017.10.007.

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