Prions propagate as multiple strains in a wide variety of mammalian species. The detection of all such strains by a ultrasensitive assay such as Real Time Quaking-induced Conversion (RT-QuIC) would facilitate prion disease diag

surveillance and research. Previous studies have shown that bank voles, and transgenic mice expressing bank vol are susceptible to most, if not all, types of prions. Here we show that bacterially expressed recombinant bank vole (residues 23-230) is an effective substrate for the sensitive RT-QuIC detection of all of the different prion types that so far – a total of 28 from humans, cattle, sheep, cervids and rodents, including several that have previously been RT-QuIC or Protein Misfolding Cyclic Amplification. Furthermore, comparison of the relative abilities of different prion positive RT-QuIC reactions with bank vole and not other recombinant prion proteins allowed discrimination of prion classical and atypical L-type bovine spongiform encephalopathy, classical and atypical Nor98 scrapie in sheep, and variant Creutzfeldt-Jakob disease in humans. Comparison of protease-resistant RT-QuIC conversion products also discrimination and suggested the existence of several distinct classes of prion templates among the many strains t

related to this prion type.

This study provides a basis for wide-ranging prion detection and strain discrimination.
Prion diseases, or transmissible spongiform encephalopathies, are neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI) and sporadic fatal human, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and chronic wasting disease (CWD) in cervids. Prion origin of prion diseases can be infectious, genetic or sporadic. Many prion diseases also have subtypes or strains distinguished based on the PRNP (prion protein) genotype, transmission characteristics, clinical manifestations, lesion profiles and/or biochemical properties of the disease-associated forms of prion protein (PrP<sup>D</sup>) [1–9]. PrP<sup>D</sup>, or PrP<sup>Sen</sup>, is the predominant molecular component of the infectious agent, or prion, which propagates itself by inducing the host’s normal protease-sensitive prion protein, PrP<sup>PC</sup> or PrP<sup>Sen</sup>, into additional PrP<sup>D</sup>. This propagation mechanism involves seeding, or templated, polymerization in which the given PrP<sup>D</sup> conformation is imposed upon normally monomeric molecules as they are recruited into growing PrP<sup>D</sup> multimers [3,4].

PrP<sup>D</sup> usually includes forms called PrP<sup>Res</sup> that, unlike the normal PrP<sup>Sen</sup>, are partially resistant to digestion by protease. The banding pattern of PrP<sup>Res</sup> in immunooblots can vary distinctly depending on the prion strain, host species or genotype. With most prion diseases the predominant 21-32-kDa variably glycosylated PrP<sup>Res</sup> fragments observed extend from ragged N-termini between residues ~80–96 to the GPI-anchored C-terminus (e.g., at residue 231 in human sequence). In contrast, the PrP<sup>Res</sup> associated with sheep Nor98 scrapie and human GSS linked to the P102L, F198S, A117V and A117E mutations include much smaller 6–14 kDa bands [10–12]. These bands are internal fragments with ragged N- and C-termini between residues ~80–160 [13]. In cases of P102L GSS, brain tissue from some individuals can also give 21–32 kDa PrP<sup>R</sup>, the 7–8 kDa bands, while others give the 21–32 kDa PrP<sup>Res</sup> bands but lack the 7–8 kDa bands. Hereafter, we will refer to the former cases as GSS P102L<sup>+</sup> and the latter as GSS P102L<sup>−</sup>.

A major challenge for the prion disease field is the development of sufficiently practical and sensitive tests for routine detection and strain discrimination in medicine, agriculture, wildlife management and research. The Real Time Quaking Conversion (RT-QuIC) assay, which is based on prion-seeded fibrillization of recombinant prion protein (rPrP<sup>Sen</sup>), is highly specific and ultra-sensitive for detection of multiple human and animal prion diseases [14–20]. Moreover, like the seeding assay [21], RT-QuIC is more practical than comparably ultra-sensitive assays by being relatively rapid and well plate format with fluorescence readout [14,16]. Appropriate combinations of prion type and rPrP<sup>Sen</sup> substrate are important in the performance of various RT-QuIC assays [14,18–20,22–25]. For several types of prion disease, however, effective rPrP<sup>Sen</sup> substrate has been identified; these types include human GSS arising from P102L<sup>+</sup>, F198S, A117V and A117E mutations and the atypical sheep scrapie strain Nor98. Moreover, no single substrate has yet been shown to be effective across all prion variants of humans, cattle, sheep, cervids and rodents.

One potential rPrP<sup>Sen</sup> substrate that has not been described for RT-QuIC assays is based on the bank vole seques [26], and transgenic mice that express bank vole (BV) PrP<sup>Sen</sup> [27], are susceptible to an unusually wide range of different species. Furthermore, PrP<sup>Sen</sup> in bank vole brain tissue homogenates is a broadly reactive, but not universal, highly sensitive protein misfolding cyclic amplification (PMCA) assay for prions [28]. Here we have tested the s recombinant bank vole PrP<sup>Sen</sup> (BV rPrP<sup>Sen</sup>), when expressed in E. coli and purified, as an RT-QuIC substrate. We found that BV rPrP<sup>Sen</sup> is a universally effective substrate for multiple prion strains from multiple species, and, most notably, for prions for which no effective substrate has been available. Furthermore, we have found that BV rPrP<sup>Sen</sup>-based RT-QuIC strain-dependent PK-resistant products in a manner that should further facilitate prion strain discrimination.

Results

Lack of detection of GSS and atypical scrapie subtypes associated with 6–14 kDa PrP<sup>Res</sup> fragments using previously described rPrP<sup>Sen</sup> constructs

Most mammalian PrP<sup>Res</sup> types with predominant 21–32 kDa PrP<sup>Res</sup> bands can seed Thioflavin T-positive (ThT) aggregates in RT-QuIC reactions [17,22,29–32] using at least one of the following substrates: Syrian golden hamster rPrP<sup>Res</sup> [14,30,31], Syrian golden hamster rPrP<sup>Sen</sup> 23–231 [18], human rPrP<sup>Sen</sup> 23–231 [16], murine rPrP<sup>Sen</sup> 23–231 [24],...
sheep chimeric rPrPSEN 23–231 [19,22]. For example, detection of human P102L GSS brain tissue using hamster
tissue is shown in Fig 1. However, to date, no detection of RT-QuIC seeding activity has been reported using these rPrPSEN with cases of human GSS or sheep scrapie that give prominent low molecular weight PrPRES fragments in immunoblotting. Specifically, these cases include human GSS with the F198S, A117V or H187R mutations and sheep scrapie types giving 6–14 kDa PrPRES fragments [10–13], and the human P102L-GSS with an ~8 kDa PrPRES fragment [10]. Our inability to detect these prion types is exemplified in Fig 1 using 10–3 brain tissue dilutions of human GSS P102L* and sheep Nor98 scrapie with the hamster rPrPSEN 90–231. In contrast, P102L GSS (without the ~8-kDa fragment) gave positive reactions with 1,000,000-fold smaller amounts of brain tissue.

### Fig 1. RT-QuIC detection of GSS P102L and lack of detection for GSS F198S, P102L* and sheep atypical Nor98 scrapie using hamster rPrPSEN 90–231.

Serial dilutions (10^-6 to 10^-9) of GSS P102L brain tissue dilutions were used to seed quadruplicate RT-QuIC reactions (red lines) with hamster 90–231 rPrPSEN as the substrate, 300mM NaCl, 0.002% SDS. The same rPrPSEN and RT-QuIC conditions listed above were used in reactions seeded with the designated brain tissue dilutions of GSS human patients with F198S (green line) or P102L* (gray line) PRNP point mutations, Alzheimer’s disease (AD, blue line), sheep without prion disease (blue line) or with Nor98 scrapie (orange line). Average ThT fluorescence readings from replicate wells for each sample were plotted as a function of time. Results are representative of similar findings from at least 10 independent experiments using hamster 90–231, hamster 23–231, human or hamster-sheep chimeric rPrPSEN substrates. doi:10.1371/journal.ppat.1004983.g001

Detection of GSS F198S and A117V prion seeding activity using BV rPrPSEN

We then tested bank vole rPrPSEN residues 23–230 (BV rPrPSEN) as a substrate to detect seeding activity of two human subtypes that have not been detectable previously by RT-QuIC, namely F198S- and A117V-GSS. Concurrently, we varied two parameters that we have shown to be influential, namely the concentrations of NaCl [14] and Sodium Dodecyl Sulfate (SDS) [15]. Each reaction was seeded with 10^-4 dilutions of frontal cortex brain tissue from confirmed GSS cases carrying either the F198S or A117V mutation of the prion protein gene. We found that our standard concentrations of SDS (0.002%) in combination with either 130 or 300mM NaCl failed to allow a distinction in lag phase between prion positive and uninfected brain homogenate (BH) seeded reactions (Fig 2A and 2B). Lowering the SDS concentration to 0.001% with either 130 or 300mM NaCl improved this distinction between prion positive and uninfected BH seeded reactions (Fig 2C and 2D). However, using final concentrations of 300 mM NaCl and 0.001% SDS, provided much shorter lag phases in reactions seeded with the two GSS subtypes than with the cerebral ischemia negative control (Fig 2D). These results indicated that under these latter RT-QuIC conditions BV rPrPSEN seeding activity associated with PrP^D conformers that had not otherwise been detectable by RT-QuIC or PMCA prion amplification techniques [33].

### Fig 2. Detection of GSS P102L, F198S and A117V PrP^D types by RT-QuIC using BV rPrPSEN, 300mM NaCl and 0.001% SDS.

Quadruplicate RT-QuIC reactions were seeded with 10^-4 dilutions of human frontal cortex brain tissue from GSS the P102L (red lines), F198S (green lines), or A117V (magenta lines) PRNP mutation. Negative control reaction with 10^-4 dilutions of frontal cortex brain tissue from a cerebral ischemia patient (blue lines). A final SDS concentration...
0.002% (A and B) or 0.001% (C and D) in combination with 130 mM (A and C) or 300 mM (B and D) NaCl were used with BV rPrPSEN. Similar results were seen in three independent experiments. Traces from representative RT-QuIC experiments were the average of four replicate wells.

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Next, we assessed the sensitivity of this new RT-QuIC assay for detecting GSS-associated prion seeding activity. Reactions were seeded with 10⁻⁴ to 10⁻⁹ dilutions of brain tissue from GSS patients carrying the P102L, P102L*, A117V, F198S and H187R mutation of the prion gene (Fig 3A–3E). A reaction time cutoff of 50h was chosen because in more than 20 independent experiments seeded with negative control Alzheimer’s disease (AD) or cerebral ischemia brain homogenates, no positive reactions were observed until after 55h (in rare wells). We detected GSS P102L, P102L*, A117V and F198S and H187R prion seeding activity in as little as 10⁻⁹, 10⁻⁴, 10⁻⁸, 10⁻⁸ and 10⁻⁶ dilutions of brain (frontal cortex) tissue dilutions, respectively.

![Image](https://via.placeholder.com/150)

**Fig 3.** RT-QuIC sensitivity for detection of human GSS P102L, P102L*, A117V, F198S, and H187R seeding activity using BV rPrPSEN.

The designated dilutions of frontal cortex brain tissue from the designated GSS P102L (A), P102L* (B), A117V (and H187R (E) patients were used to seed RT-QuIC reactions with 0.001% SDS and 300mM NaCl. Negative control reactions were seeded with Alzheimer’s disease (AD) brain tissue (A-E, blue lines). Representative data from one of three independent experiments is shown as the average of fluorescence values from four replicate wells.

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Detection of 28 different prion types/strains of human, sheep, cattle, deer, elk, mouse and hamster using BV rPrPSEN

After finding that BV rPrPSEN supported RT-QuIC detection of prion seeding activity from previously undetectable types of GSS, we tested whether BV rPrPSEN could be used to detect other types of prion diseases. We tested 28 different types of prion tissue from humans, sheep, mouse, hamster, cattle, elk, and deer (Tables 1 and 2) and found that all of them gave stronger positive ThT fluorescence responses than a variety of uninfected negative control brain specimens (Fig 4) were the five prion types that have not been detectable by RT-QuIC under other conditions, namely human GSS F198S, A117V, H187R, and P102L* and sheep Nor98 scrapie (Fig 4, red traces). These results indicated that under these conditions, BV rPrPSEN is the most broadly prion-seeded RT-QuIC substrate described to date.

![Image](https://via.placeholder.com/150)

**Fig 4.** RT-QuIC detection of 28 types of prion seeds from 5 different species using new BV rPrPSEN substrate.

RT-QuIC reactions were seeded with 10⁻⁴ brain tissue dilutions of the indicated human and animal prion types in 300mM NaCl and 0.001% SDS. Equivalent dilutions of species- and brain region-matched samples from uninfected individuals were used as specificity controls (green). Prion types that have been detected previously by RT-QuIC substrate are indicated in black, whereas those that have only been detectable using our selected set of conditions PrPSEN are indicated in red. The traces show the average fluorescence from four replicate wells. Similar data were from a minimum of three independent experiments with each prion type.
Prion strain-type-dependent RT-QuIC products from reactions using BV rPrP\textsuperscript{Sen}

Prion strain-dependence has not been observed previously in the immunoblot banding profile of PK-treated recombinant (rPrP\textsuperscript{Res}) products of RT-QuIC reactions. However, using BV rPrP\textsuperscript{Sen} we observed consistently distinct products of reactions seeded with different types of human prions (Fig 5 and Table 1). The observed banding patterns could be on the type of seed: GSS cases (F198S, A117V, H187R) with the ~8–14 Kda protease-resistant bands and sFl gav
major ~10kDa band and a ~6-9kDa band; the GSS (P102L), gCJD (E200K, V210I, six octarepeat insertion), and t/iCJD cases with ~21–32 kDa PrP^Res bands gave multiple bands with a major ~12 kDa band and multiple minor bands ~6–10 kDa; variant CJD, GSS (P102L*) and FFI (D178N) cases gave a single predominant band at ~10 kDa; and some cases gave two bands between ~10–12 kDa, while in other cases gave a predominant band at ~10 kDa. Repeated analyses (>4) of individual sCJD cases indicated that they consistently seeded the formation of only one or the other of the two products. This observation provided evidence that the different sCJD-seeded PrP^Res products were dictated by differential templating activity in the tissue samples rather than stochastic events during the RT-QuIC reaction. Additionally, since immunoblots used an antiserum to the C-terminus of PrP, the fragments likely differed primarily at their N-termini.

**Fig 5. Western blot of BV rPrP^Res products from RT-QuIC reactions seeded with various human prion types.**

Reaction products were digested with 10μg/mL of PK. Immunoblots were probed with the C-terminal antibody R20 (hamster PrP epitope residues 218–231). Molecular mass (MM) is indicated in kilodaltons. Immunoblots are representative of one biological replicate (n = total biological replicates tested) each giving rPrP^Res banding profiles similar to that/those shown. Samples for which cerebellum, frontal cortex, basal ganglia and thalamus from the same patient were analyzed. Two sets of RT-QuIC bank vole rPrP^Res products were generated from a given prion type and independently subjected to immunoblotting analysis.

We further compared the BV rPrP^Res products of reactions seeded with different rodent, bovine, cervine and ovine (Table 2). As with the human prion seeds, we observed distinct strain-dependent BV rPrP^Res banding profiles from seeded with different prion types. Mouse 22L scrapie-seeded BV rPrP^Res products consistently showed a ~10 and resistant band, whereas BV rPrP^Res products from reactions seeded with Chandler, ME7, 87V and anchorless 22L scrapie displayed a predominant ~10 kDa band (Fig 6A). The lack of the GPI anchor in the 22L GPI− scrapie seed RT-QuIC product that was distinct from the wild-type GPI-anchored 22L scrapie. Additionally, closely related hamster (Hyper and 263K; Fig 6B) showed similar BV rPrP^Res banding profiles (~10 and ~12 kDa PK-resistant bands) which from the Drowsy-seeded BV rPrP^Res products (primarily a ~10 kDa band; Fig 6B). Deer and elk CWD-seeded reactions each gave ~8, 9, 10, and 12 kDa bands, but differed in the relative intensities of the top two bands between the two (Fig 6C). Distinct strain-dependent BV rPrP^Res banding profiles were observed between classical (C-BSE) and atypical (L-BSE: ~9, 10, and 12 kDa bands, respectively; Fig 6C), as well as between classical and atypical Nor98 sheep scrapie (~10, and 12 kDa bands, respectively; Fig 6D). Collectively, these immunoblotting results suggested that certain prion diseases can be discriminated in part based on analysis of the rPrP^Res products of BV rPrP^Sen-based RT-QuIC reactions seeded with samples from PRNP VRQ/VRQ and ARQ/ARQ sheep (not designated). The Nor98-seeded reaction
seeded with samples from ARR/ARR, ARQ/AHQ and ARQ/ARQ sheep. RT-QuIC reactions and immunoblotting each of these types of prions were performed at least twice with similar results. The banding profiles shown are of multiple (n) independently tested biological replicates.
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Detection and discrimination of classical and atypical BSE using BV and hamster rPrPSen substrates

We previously reported that classical and atypical L-type BSE strains can be discriminated on the basis of relative reactivities with hamster rPrPSen 90–231 and hamster-sheep chimeric rPrPSen 23–231 substrates [22]. Here we found that rPrPSen can similarly detect both classical and L-type BSE, providing an alternative substrate for discrimination between bovine strains. Specifically, detection of seeding activity with BV rPrPSen (Fig 7) but not with three other rPrPSen substrates that detected only L-type BSE, namely human 23–231, hamster 23–231 or hamster 90–231 [22], can be used to differentiate these bovine prion types.

Fig 7. Detection of Classical (C-BSE) and atypical (L-type BSE) with BV rPrPSen substrate.

RT-QuIC reactions were seeded with $10^{-4}$ brain tissue dilutions of brain stem (C-BSE, blue) or frontal cortex (L type BSE, magenta) from Italian cattle. Negative control reactions (NBH, green) were seeded with $10^{-4}$ dilutions of frontal cortex from uninfected cattle. BV rPrPSen was used as a substrate with 300mM NaCl and 0.001% SDS. RT-QuIC analysis was performed at least twice for each sample with similar results. Results are plotted as the averages from four replicate wells.
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Discrimination of classical and Nor98 sheep scrapie using BV and hamster-sheep chimeric rPrPSen substrates

Having detected Nor98 sheep scrapie with BV rPrPSen, (Fig 4) we tested whether a strategy similar to the one used for C- vs. L-type BSE using different rPrPSen substrates would allow discrimination of Nor98 and classical sheep scrapie from eight sheep with classical scrapie [ARQ/ARQ (n = 6), VRQ/VRQ (n = 2) PrP genotypes, Table 2] were readily detected using the hamster-sheep chimeric rPrPSen 23–231 within ~40hs (Fig 8A). However, brain tissue from eight cases of Nor98 [ARR/AHQ (n = 1), ARQ/ARQ (n = 4), ARQ/AHQ (n = 2) and ARR/ARR (n = 1) genotypes, Table 2] gave no positive responses using the same substrate (Fig 8B). In contrast, consistent with the results in Fig 4, seven of these cases gave positive responses when BV rPrPSen was used in reactions seeded with $10^{-4}$ brain tissue dilutions (Fig 8A–8E, orange lines) and those that were weaker or not detected were positive when seeded with $10^{-3}$ dilutions (Fig 8A–8E, red lines). To compare the sensitivities of the assay for detection of classical and atypical scrapie using these two substrates, we diluted representative brain homogenates from classical and Nor98 scrapie positive sheep (Fig 8G–8J) and tested them using BV and Ha-S rPrPSen. We detected scrapie down to $10^{-6}$ dilutions using Ha-S rPrPSen and down to $10^{-6}$ using BV rPrPSen. Consistent with the data in fluorescence increases were seen in reactions seeded with the same dilutions of a Nor98 atypical scrapie sample rPrPSen. In contrast, parallel reactions with BV rPrPSen gave positive reactions when seeded with Nor98 brain dilution 6–10^{-7}, indicating that BV rPrPSen is ~1,000-fold more sensitive at detecting Nor98 scrapie than is Ha-S rPrPSen. Our results suggest that if an ovine brain sample gives a positive RT-QuIC response with BV rPrPSen, it should give a strong reaction with Ha-S rPrPSen if it contains classical scrapie, but a negative, or at least much weaker, reaction if it contains Nor98 scrapie.
RT-QuIC reactions were seeded with dilutions of cerebellum or cerebral cortex from uninfected, classical or Nor98 scrapie positive sheep. The Nor98 (ARR/AHQ, ARQ/ARQ, ARQ/AHQ and ARR/ARR PRNP genotypes) reaction were seeded with 10^{-4} (orange) brain tissue dilutions. Additional 10^{-3} (red) brain tissue dilutions are also shown for weaker samples. Classical sheep scrapie brain tissue from eight animals (ARQ/ARQ, VRQ/VRQ PRNP genotypes) was used as specificity controls (blue, C and F). Either Ha-S rPrP with 300mM NaCl and 0.002% SDS; A–C) or BV rPrP (300mM NaCl and 0.001% SDS; D–F) were used as substrates. Brain homogenates from classical scrapie positive sheep (green, VRQ/VRQ) and atypical Nor98 scrapie positive sheep (orange, ARR/AHQ) were serially diluted (10^{-1} to 10^{-8}) for QuIC analysis using Ha-S rPrP with 300mM NaCl and 0.002% SDS (G and H) or BV rPrP with 300mM N 0.001% SDS (I and J) substrates. RT-QuIC testing was performed independently twice with similar results. Traces show averages of quadruplicate wells.

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Detection and discrimination of human sCJD and vCJD using BV and hamster 23–231 rPrP^Sen substrates

To investigate the discrimination of two non-genetic human prion strains, we tested 10^{-4} brain tissue dilutions from cases of Type 1 sCJD (Fig 9A and 9B, Cases a and b, green lines) and two cases of vCJD (Fig 9A and 9B, Cases c and d, orange lines). We used previously described SDS conditions (0.002% final concentration of SDS; [23]) with hamster 23–231: 0.001% SDS with BV rPrP^Sen, both in the presence of 300mM NaCl. We observed rapid amplification of prion seeding activity in the two Type 1 sCJD samples when using either hamster 23–231 or BV rPrP^Sen (Fig 9A and 9B). Our detection of samples with the hamster 23–231 substrate was consistent with previous demonstrations that all sCJD subtypes a with this substrate [23,30,34]. No increase in ThT fluorescence was seen in vCJD-seeded hamster 23–231 rPrP^Sen reactions (Fig 9A). However, in accordance with the results shown in Fig 4, seeding activity was detected in both v using BV rPrP^Sen (Fig 9B). Thus, sporadic and variant CJD sample were discriminated by differential reactivities with hamster 23–231 rPrP^Sen substrates.

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Next we compared the RT-QuIC sensitivities for detection of sCJD and vCJD brain homogenates using hamster or BV rPrP. We performed end-point dilution RT-QuIC analysis of brain tissue from sCJD (Case a) and vCJD (Case c) (Fig 9C-orange lines, respectively). We detected sCJD down to $10^{-8}$-$10^{-9}$ with hamster 23–231 rPrP$_{Sen}$ (Fig 9C) and $10^{-8}$, (Fig 9D). Although sCJD gave slightly slower amplification kinetics with hamster 23–231 rPrP$_{Sen}$ (Fig 9C) compare (Fig 9D), the overall sensitivities using the two substrates were comparable. In contrast, markedly different sensitivities observed with the two substrates in the vCJD-seeded reactions. Specifically, only weak seeding activity was observed in 10$^{-4}$ or 10$^{-5}$ brain dilutions with hamster 23–231 rPrP$_{Sen}$ (representative data in Fig 9E), but fast and sensitive seeding activity down to 10$^{-7}$ brain tissue dilution was observed using BV rPrP$_{Sen}$ (Fig 9F). These results suggest is 100–1,000-fold more sensitive than hamster 23–231 rPrP$_{Sen}$ in detecting vCJD brain derived prion seeding act these findings further support the potential broad applicability of a BV rPrP$_{Sen}$ prion discrimination strategy to a variety of prion types.

Discussion

The lack of practical and cost-effective tests that are sensitive enough to detect the lowest infectious levels of prion a major impediment in coping with prion diseases. Rapid commercially available immunoassays have allowed post detection of prion infections in high-titered tissues such as brain or lymphoid tissues, but diagnostic specimens that accessible in living hosts, such as blood, CSF and nasal brushings, have much lower prion titers that are undetectable assays. In contrast, RT-QuIC assays have been highly effective in detecting prion seeding activity in such low-titered and are being widely implemented as state-of-the-art diagnostic tests for humans and animals [16,18–20,25,34–37 recent improvements have increased the speed and sensitivity of RT-QuIC assays such that sCJD testing based on samples can now be performed in a matter of hours rather than days [30].

In our experience, the most demanding and costly requirement for RT-QuIC testing is the availability of suitable rPrP$_{Sen}$ Prior to the present study, testing facilities would typically have to produce or procure multiple rPrP$_{Sen}$ sequences for multiple prion types. However, we have now shown that all of the prion diseases that we have tested so far from other mammals can be detected sensitively by using BV rPrP$_{Sen}$ (Fig 4). This provides a useful platform for broad-detection and strain discrimination. Thus, we envision that most initial screening for the presence of a wide variety of prions be performed using BV rPrP$_{Sen}$. Once a prion-infected sample from a given host species is identified, one could discriminate between strains by targeted use of another rPrP$_{Sen}$ substrate that is known to be differentially sensitive to prion strains of that host species (Figs 7–9) and/or by performing immunobLOTS of the PK-resistant RT-QuIC products of the reactions (Figs 5 and 6).

Although we have demonstrated detection of a wide variety of prion types, the relative sensitivities of BV rPrP$_{Sen}$, for brain homogenates of hosts with different prion diseases is presumably dependent on the concentrations of PrP$_{Sen}$ samples. Clearly PrP$_{D}$ concentrations may vary markedly between individuals and different regions of the brain as strain. Furthermore, because PrP$_{D}$ can vary markedly in its properties, e.g. amyloid vs. non-amyloid, protease-sensitive, small vs. large particles, infectious vs. non-infectious, it is probable that the RT-QuIC seeding activity will PrP$_{D}$ between different prion strains and tissue sources. Thus, although we have shown the potential for BV rPrP$_{Sen}$,QuIC to detect and help discriminate prion strains, much additional work with each type of prion and sample type v better establish the quantitative relationships between RT-QuIC seeding activity and the levels of various types of tissues of diagnostic or scientific interest.

Since the inception of prion-seeded cell-free PrP conversion reactions [38], striking sequence- and strain-specific observations that appeared to correlate, at least largely, with transmission barriers and strain phenotypes of prion disease [3,39–41]. Indeed, sequence differences of as little as a single residue between the PrP$_{D}$ seed and PrP$_{Sen}$ substrate PrP$_{Res}$ formation in such cell-free reactions [42], as it can in scrapie-infected cells [43] and in vivo [44]. However, have tended to be less constrained by such sequence differences [14]. We reason that this is due in part to the fac reactions, it is only the C-terminal residues ~160–231 of the substrate molecules that must refold into the PK-resistant [45] to give a positive reaction, i.e., an increase in ThT fluorescence. In contrast, earlier cell-free conversion [38,46 reactions [48] have used the immunoblot-based detection of much larger PK-resistant cores, typically comprised of 231, as a positive readout. Thus, much more extensive packing of more N-proximal residues is required in the latter is in vivo, giving more opportunities for sequence differences between seed and substrate to influence conversion. Despite the lower sequence specificity of RT-QuIC reactions, we and others have observed multiple examples of r that can be converted by some types of prion seeds and not others [22,23]. Therefore, we were surprised to find th be induced to convert to ThT-positive amyloid by every type of prion-associated seed that we have tried so far including several that had never before been detected by RT-QuIC or PMCA. We also did not anticipate that different BV rPrP$_{Res}$ products of RT-QuIC reactions would be seeded with different prion strains from a single host species, never seen such distinct templating with the many other rPrP$_{Sen}$ substrates that we have tested. These findings s, rPrP$_{Sen}$-based RT-QuIC reactions may provide a new means of probing the strain-dependent heterogeneity of pric
activities and conformational templates. However, overall, the RT-QuIC technology has been established largely for purposes of rapid, sensitive prion disease-associated seed detection rather than the in vitro recapitulation of prion such, the RT-QuIC tests have not been developed to reflect prion transmission barriers or strain-specificities. In an availability of BV rPrPSen as an apparently universal RT-QuIC substrate may markedly improve the practicality, effi
effectiveness of detecting and discriminating prions.

Materials and Methods

Ethics statement

Brain tissue from scrapie-infected mice and hamsters (Table 2) were collected under Protocols 2013–030 and 2011 respectively, that were approved by the Rocky Mountain Laboratories Animal Care and Use Committee. Human brain samples (Table 1) were obtained from the National Prion Disease Pathology Surveillance Center (USA). Brain tissue from humans (Table 1) was obtained from the National Institute for Biological Standards and Controls (UK) repository. No human brain samples were used for this study, but were instead obtained from the existing collections noted above with approval under exemption #1197 from the NIH Office of Human Subjects Research. All human samples were, and remain, at the investigators at Rocky Mountain Laboratories where the RT-QuIC testing was performed.

Protein expression and purification

Recombinant prion protein (rPrPSen) substrates were purified as previously described [49]. Briefly, PrP DNA sequence for Syrian golden hamster (residues 23 to 231; accession no. K02234; or residues 90–231), Bank Vole (residues 2 Methionine at residue 109; accession no. AF367624) or hamster-sheep chimera (Syrian hamster residues 23 to 13 sheep residues 141 to 234 of the R154Q171 polymorph [accession no. AY907689]) prion protein genes were ligated into the pET41 vector (EMD Biosciences). Vectors were transformed into Rosetta (DE3) Escherichia coli and grown in Luria broth/kanamycin and chloramphenicol. Protein expression was induced using the autoinduction system purified from inclusion bodies under denaturing conditions using Ni-nitrilotriacetic acid (NTA) superflow resin (Qiagen) fast protein liquid chromatographer (GE Healthcare Life Sciences). The protein was re-folded on the column using a reduction gradient and eluted using an imidazole gradient as described [49]. The eluted protein was extensively dialyzed against 300 mM sodium phosphate buffer (pH 5.8), filtered (0.22-μm syringe filter [Fisher]) and stored at -80°C. Protein concentration was determined by measuring absorbance at 280 nm.

Brain homogenate preparations

Brain homogenates (BH; 10% w/v, Tables 1 and 2) were prepared as previously described [14] and stored at -80°C analysis BHs were serially diluted in 0.1% SDS (sodium dodecyl sulfate, Sigma)/N2 (Gibco)/PBS as previously reported (25), or 0.002% or 0.001% SDS. NaCl and SDS concentrations were varied where indicated. Aliquots of the reaction mix loaded into each well of a black 96-well plate with a clear bottom (Nunc) and seeded with 2 μL of indicated BH dilutions. The plate was then sealed with a plate sealer film (Nalgene Nunc International) and incubated at 42°C in a BMG FLUOstar Omega plate reader with cycles of 1 min shaking (700 rpm double orbital) and 1 min rest throughout the indicated incubation time. ThT fluorescence measurements (450+/–10 nm excitation and 480+/–10 nm emission; bottom read) were taken every 45 min.

To compensate for minor differences in baselines between fluorescent plate readers and across multiple experiments, were normalized to a percentage of the maximal fluorescence response (260,000 rfu) of the plate readers after subtraction of the baseline, as described [34], and plotted versus reaction time. Reactions were classified as RT-QuIC positive based on those previously described for RT-QuIC analyses of brain specimens [14,34].

Proteinase K (PK) digestion of RT-QuIC products and immunoblotting

RT-QuIC reaction products were collected from the plates by extensive scraping and pipetting and treated with 10 K (PK) for 1 hour at 37°C with 400 rpm orbital shaking. Equal volumes of PK-treated reactions were run on 12% Bis–Tris NuPAGE gels (Invitrogen). Membranes were probed with R20 primary antiserum (hamster epitope: residues 218–231) [52] dilute visualized with the Attaphos AP fluorescent substrate system (Promega) according to the manufacturer's recomme

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Author Contributions

Conceived and designed the experiments: BC CDO BRG. Performed the experiments: CDO BRG. Analyzed the data: BC. Contributed reagents/materials/analysis tools: LDR AGH RN WZ BG PG. Wrote the paper: BC CDO BRG LDR AGH.

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