The real-time quaking-induced conversion assay for detection of human prion disease and study of other protein misfolding diseases

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The development and adaption of *in vitro* misfolded protein amplification systems has been a major innovation in the detection of abnormally folded prion protein scrapie (PrP^{Sc}) in human brain and cerebrospinal fluid (CSF) samples. Herein, we describe a fast and efficient protein amplification technique, real-time quaking-induced conversion (RT-QuIC), for the detection of a PrP^{Sc} seed in human brain and CSF. In contrast to other *in vitro* misfolded protein amplification assays—such as protein misfolding cyclic amplification (PMCA)—which are based on sonication, the RT-QuIC technique is based on prion seed–induced misfolding and aggregation of recombinant prion protein substrate, accelerated by alternating cycles of shaking and rest in fluorescence plate readers. A single RT-QuIC assay typically analyzes up to 32 samples in triplicate, using a 96-well-plate format. From sample preparation to analysis of results, the protocol takes ~87 h to complete. In addition to diagnostics, this technique has substantial generic analytical applications, including drug screening, prion strain discrimination, biohazard screening (e.g., to reduce transmission risk related to prion diseases) and the study of protein misfolding; in addition, it can potentially be used for the investigation of other protein misfolding diseases such as Alzheimer's and Parkinson's disease.

INTRODUCTION

Transmissible spongiform encephalopathies or prion diseases, such as Creutzfeldt–Jakob disease (CJD), are fatal neurodegenerative disorders characterized by the accumulation of abnormally folded PrP^{Sc} in the brain. In humans, sporadic CJD (sCJD) is the most common prion disease, followed by genetic CJD (gCJD) and transmitted CJD (iatrogenic CJD and variant CJD). For diagnosis of sCJD, elevated levels of certain biomarkers for neurodegeneration (such as 14-3-3, total tau, calcium-binding protein B (S-100B), neuron-specific enolase, desmoplakin, and α-synuclein)[1–8], in the CSF in combination with a detailed clinical examination, magnetic resonance imaging (MRI) and electroencephalogram, are important[9,10], although currently a definite diagnosis of CJD requires confirmation at autopsy[11]. However, the development of *in vitro* prion protein conversion assays, such as RT-QuIC, to detect misfolded prion protein has, to our knowledge, for the first time led to a test with a specificity close to 100% (refs. 12–15) and with high reproducibility across different laboratories[12,16], arguably making postmortem autopsies less essential.

Development of the protocol

To date, several *in vitro* protein conversion systems, which are directly based on the conversion of cellular prion protein (PrPC) to a conformationally altered isoform, PrP scrapie (PrPSc), and aggregation of a PrP-amyloid, have been developed. These include PMCA[17], the amyloid seeding assay[18], QuIC[19] and RT-QuIC[14,19,20]. These assays are comparable to a PCR for misfolded proteins, exploiting the seeded/template-induced assembly and conversion of PrP^{C} directly to ordered PrP^{Sc} aggregates, thereby amplifying miniscule amounts of PrP^{Sc} to a detectable level[14,17,20,21]. Although initial aggregation assays used brain material as the source of PrP^{C} substrate[17], these have subsequently been improved by the use of bacterially synthesized recombinant PrP^{C} (recPrP^{C}) substrate[19,22].

In RT-QuIC reactions, small amounts of misfolded PrP^{Sc} act as a seed, recruiting single recPrP^{C} substrate molecules and inducing their conversion by integrating them into a growing amyloid aggregate concomitant with a conformational change of the substrate to a seeding-competent state (**Fig. 1**). Atarashi *et al.*[14] were the first to use a QuIC assay to demonstrate the capacity of PrP^{Sc} from human CSF samples to seed conversion of recPrP^{C}. During RT-QuIC, the samples are subjected to cycles of vigorous shaking, which presumably fragment the PrP^{Sc} aggregates into additional reactive seeds for conversion. With each cycle, consisting of incubation and shaking steps, the amyloid reaction product can increase exponentially (**Fig. 1**). The PrP^{Sc}-seeded conversion reaction product is enriched in β-strand secondary structure, as seen in amyloid, which enhances the fluorescence of the thioflavin T (Th-T) dye upon binding. Hence, nascent PrP^{Sc} can be monitored in real time using a temperature-controlled shaking fluorescence plate reader. The amplitude and kinetics of fluorescence enhancement can be used to evaluate relative seeding activities in test samples. The quantitative parameters were defined as lag phase (time to reach 10,000 r.f.u. (relative fluorescence units)), area under the curve (AUC) and maximal signal intensity (**Fig. 1**). Proteinase K–resistant PrP (PrP^{res}), generated after amplification by RT-QuIC, can be detected by western blotting[13,14].

The adaptation of the RT-QuIC assay to a 96-well-plate format[14] has the advantage over previous *in vitro* amplification systems of facilitating the automation of the technical process, thus enabling the easy detection of PrP aggregates by Th-T; these aggregates are considered to be noninfectious. Analysis of multiple reactions (96-well plates) also allows the measurement of up to 32 different samples in triplicate in the same run. In our 96-well-plate-based RT-QuIC assay[12], the signal response depends in part on the amount of PrP^{Sc} seed. With the cutoff threshold set at 10,000 r.f.u., the typical detection limit for postmortem sCJD brain tissue for the assay conditions described here is a dilution of $\approx 10^{-8}$, whereas for CSF it is a dilution of $\approx 10^{-1.2}$ (**Figs. 2a–e** and **3a–e**, **Supplementary Tables 1** and **2**). Higher dilutions reveal mean positivity rates below 50%, even though a positive fluorescence signal can be still detected in some reactions (**Figs. 2e** and **3e**). Control samples (taken from patients without prion disease) show, independently of the dilution, no PrP-seeding reaction (maximal signal below cutoff) in the brain or in CSF (**Figs. 2f** and **3f**).

Applications of the method

Being able to analyze RT-QuIC reactions in 96-well plates in an automated assay permits the high-throughput analyses of samples and increases the potential for numerous other applications in scientific and industrial fields[23,24]. Moreover, RT-QuIC assays have been developed for most prion strains and many human and animal tissues and body fluids[15,20,25–28], which makes this method applicable for diagnostics, biological science, prescreening for potential therapeutics and testing of materials for contamination with infectious prions. The following paragraphs illustrate examples of the manifold applications of the RT-QuIC assays.

Human prion disease diagnostics in CSF and in nasal brushings. The RT-QuIC assay was first established as a test for human prion disease diagnostics using CSF by *Atarashi et al.*[14]. The recent experience of different groups underlines the reproducibility of RT-QuIC across various CSF storage conditions with remarkable sensitivity and specificity, indicating that RT-QuIC is a robust diagnostic method[12,13,25,29]. Currently, although the RT-QuIC assay exhibits excellent specificity, it remains contentious whether this test can be used for a 'definite' diagnosis of prion disease, for which confirmation by neuropathological examination is still generally required. Given the sensitivity of most reported RT-QuIC assays, a negative test result cannot generally be interpreted as ruling out a CJD diagnosis. Future studies need to assess the potential of the method to detect all forms of CJD, including variant, genetic and atypical cases. To minimize 'gray-zone' and/or equivocal results but maintain specificity, we recommend testing each sample at least in triplicate and defining a sample as positive when >50% of the replicate wells show a PrP seeding reaction within 80 h. The evidence for this has been published recently[12].

Another RT-QuIC application is the detection of PrP^{Sc} in the olfactory neuroepithelium of prion disease patients[15]. RT-QuIC assays seeded with dilutions of nasal brushing samples were positive in 42 of 43 sCJD patients (cumulatively from two reports) and negative in 43 of 43 control donors, indicating a sensitivity of 97% and a specificity of 100% (refs. 15,30). Testing nasal brushings elicited stronger and faster RT-QuIC responses (higher seeding signal and shorter lag phase) as compared with corresponding CSF samples from the same patients. Moreover, as compared with lumbar punctures, nasal sampling provides an alternative and less invasive tool for collecting diagnostic specimens.

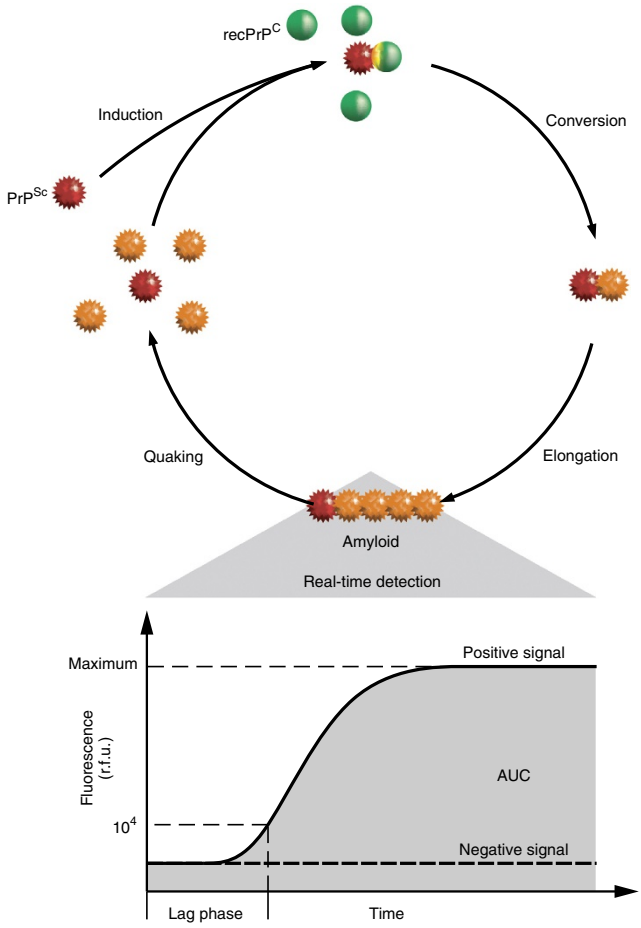

Figure 1 | Schematic diagram of PrP seed amplification by RT-QuIC assay. PrP^{Sc} seed derived from brain or CSF and $recPrP^{C}$ (substrate) is mixed and incubated, promoting the misfolding and incorporation of $recPrP^{C}$, and thus generating polymers of misfolded β strand–rich $recPrP^{C}$ as found in amyloid. The upper part shows how RT-QuIC exploits aspects of the prion replication mechanism. PrP^{Sc} is used as a seed (prickly dark red balls), which elongates through incorporation of $recPrP^{C}$ substrate (green balls). A conformational change is induced in the substrate, faithfully adopting some characteristics of the initial seed, including PK resistance, β-strand secondary structure and the ability to seed PrP amyloid formation (prickly orange balls). The growth of the generated β strand–rich PrP polymers can be detected in real time by a fluorescent dye. Through periods of shaking (quaking) and rest, these β strand–rich PrP polymers can potentially fragment, generating more seeds, in turn inducing an exponential amplification of $recPrP^{C}$ conversion. Signal quantification occurs according to the seeding parameters of interest, such as lag phase (time to reach 10,000 r.f.u.), area under the curve (AUC) and maximal signal intensity.

Prion strain typing. In sCJD, different types of PrP^{Sc} (type 1 or 2) and codon 129 genotypes of the prion protein gene (*PRNP*) determine at least six different molecular sCJD subtypes[9,31] that show distinct clinicopathological phenotypes and transmission characteristics[32]. A CSF study specifically tested the effects of molecular subtypes of prion disease on the RT-QuIC response[33] and found that the type of prion disease (sporadic or genetic), the *PRNP* mutation (E200K, D178N), codon 129 genotype and PrP^{Sc}

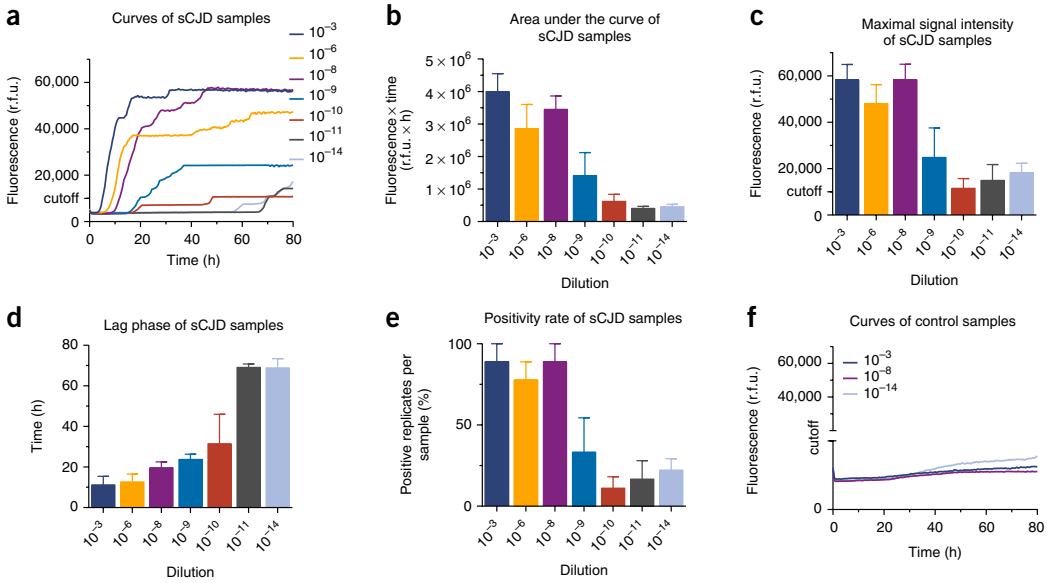

Figure 2 | Influence of serial dilution of brain homogenates on the RT-QuIC response. Serial dilution of brain homogenates, before RT-QuIC analysis, results in a decrease of the RT-QuIC response in sCJD samples. (**a**–**e**) Brain homogenates (10% (wt/vol)) from sCJD (MM1) patients ($n = 6$) and from (**f**) control patients ($n = 8$) (proven free of prion disease, such as those diagnosed with hypoxic encephalopathy, posterior infarct, cerebral amyloid angiopathy or basal ganglion infarct) were diluted in the range of 10^{-3} to 10^{-14}. Diluted samples were analyzed in triplicate by RT-QuIC. Statistical parameters quantifying the RT-QuIC response, such as AUC (**b**), maximal signal intensity (**c**) and time until the signal reached the cutoff (lag phase (**d**)), were determined per experimental group (dilution). (**a**) The curves depict the mean kinetics of the RT-QuIC response of six sCJD samples per experimental group (dilution). Fluorescence signal was measured every 30 min. Mean values were determined per sample and used to calculate total mean per experimental group (dilution) per measurement (time point). Total means were connected by linear interpolation, displaying an averaging line per experimental group (dilution). The cutoff is indicated at 10,000 r.f.u. This diagram shows aggregated data; therefore, no individual information about AUC, maximal signal intensity or lag phase can be extracted. (**b**–**d**) The RT-QuIC responses of sCJD samples were quantified by calculating statistical parameters such as area under the curve, maximal signal intensity and lag phase. Mean values were determined per sample and used to calculate total mean per experimental group (dilution) and s.e.m. Bar graphs show total mean + s.e.m. per experimental group (dilution). Cutoff is indicated at 10,000 r.f.u. (**e**) The validity of the RT-QuIC responses from sCJD samples was determined by calculating the percentage of positive replicates per sample. Mean values were determined per sample and used to calculate total mean per experimental group (dilution) and (s.e.m. Bar graphs show total mean + s.e.m. per experimental group (dilution). Samples diluted 10^{-9} to 10^{-14} showed a mean positivity rate of <50%. (**f**) The curves depict the mean kinetics of the RT-QuIC response from control samples per experimental group (dilution). Fluorescence signal was measured every 30 min. Mean values were determined per sample and used to calculate total mean per experimental group (dilution) per measurement (time point). Total means were connected by linear interpolation, displaying an averaging line per experimental group (dilution). The cutoff is indicated at 10,000 r.f.u. This diagram shows no seeding of PrP. Therefore, seeding parameters such as AUC, maximal signal intensity and lag phase were not available. The study conformed to the Code of Ethics of the World Medical Association and informed consent was given by all study participants or their legal next of kin, and the study was approved by the local ethics committee in Göttingen, Germany (no. 24/8/12). MM1, methionine/methionine 1.

type affected RT-QuIC responses; e.g., in gCJD forms, samples from E200K mutation carriers showed the strongest effect on the observed outcome variables. Recent studies by Orrú *et al.* using multiple recPrP^{C} substrates also revealed a correlation of RT-QuIC responses with different prion strains[24,28,34].

Therapeutic drug prescreening. As PrP^{Sc} is the main causative agent of prion diseases[35,36], most potentially therapeutic substances target PrP^{Sc}, the conversion process of PrP^{C} to PrP^{Sc} or PrP^{Sc} aggregation. The RT-QuIC assay can be used to compare the efficiency of antiprion compounds *in vitro* by using PrP^{Sc} from sCJD brain tissue as seed, providing a screening assay that mimics disease-relevant conditions[23]. However, unlike PMCA, RT-QuIC assays do not fully recapitulate infectious PrP^{Sc} replication[37], and thus it may be possible that some inhibitors of PrP^{Sc} formation might be missed.

However, not all substances that inhibit the conversion process of PrP in the RT-QuIC assay are potential therapeutics of prion disease; thus, we propose that substances known to interfere with Th-T, or with other chemicals from the reaction buffer, to change the reaction conditions (such as a change of the pH or digestion of the rec PrP substrate) or to show abnormal RT-QuIC seeding kinetics should be excluded[23].

In addition, the use of suitable controls without prion disease is very important in order to exclude the possibility that false-positive reactions in the RT-QuIC assay could result from the replication of a noninfectious amyloid state.

Testing of materials for contamination with infectious prions. Prions have a particularly high tolerance to inactivation and can retain infectivity even after undergoing routine sterilization processes[38,39]. Avoiding contamination of reusable surgical instruments and medical devices can therefore be challenging. The detection of prion infectivity by bioassay may take up to 2 weeks in cell-based infectivity assays[40] or even up to 20 weeks when a rapid mouse bioassay is applied[41]. In contrast, the application of an *in vitro* amplification assay (such as PMCA or RT-QuIC) for assessing prion infectivity is less time-consuming and very sensitive[20,42,43]. In this way, the efficiency of existing decontamination procedures can be estimated, and novel substances/procedures for disinfection can be developed.

Other protein misfolding diseases. A characteristic feature of the major neurodegenerative diseases is the progressive accumulation of protein aggregates in a self-propagating manner, with a topographical pattern characteristic of each disease. Although the spread of misfolded protein was initially thought to have a crucial role only in prion diseases, recent studies have identified similar characteristics for amyloid-beta, tau and α-synuclein in various other models[44–46]. The term 'prion-like' protein propagation in neurodegeneration is now widely used to address analogous mechanisms, which might have a role in Alzheimer's disease and α-synucleinopathies, such as Parkinson's disease. The RT-QuIC technique therefore also has considerable diagnostic and analytical potential with respect to other misfolded proteins (with biomarker potential), such as amyloid-beta, tau and α-synuclein[46,47].

Comparison with other methods

In comparison with the PCMA, there are technical variances between assays. PCMA is based on cyclical sonication of the reaction tube, consisting of an incubation and a sonication step at 37 °C (refs. 17,48,49). Tubes close to the horn's wall might show lower amplification efficiency compared with those in the center[50], indicating that quaking might be easier to control. There are also a few variations regarding the ingredients of the conversion/reaction buffers. The biological tissues in which human prion seeding activity has been detected by RT-QuIC include CSF, nasal fluid or brain[14,51], whereas PMCA can be used to detect PrP^{Sc} also in blood, urine, spleen, milk, oral secretions or liver[27,52–55]. A more sensitive modification of the RT-QuIC, the enhanced QuIC (eQuIC), could, however, be used to detect prion seeds in samples with a very low amount of PrP^{Sc} (e.g., urine) or in samples containing compounds inhibitory for the seeding reaction, such as blood plasma[25,27,52–55]. This includes a preanalytical immunoprecipitation step with beads coated with a PrP^{Sc}-specific antibody (e.g., 15B3).

RT-QuIC uses bacterially synthesized $recPrP^{C}$ as a substrate for the conversion and aggregation of PrP. In contrast, PMCA usually uses brain homogenate as a source of PrP^{C} substrate. In brain-homogenate-based PMCA reactions, PrP^{Sc} is faithfully replicated in the presence of necessary cofactors, giving an infectious product[56]. However, in RT-QuIC assays, the amplified $recPrP^{C}$ reaction product (PrP^{res}) is seeded by, but not conformationally identical to, PrP^{Sc} and lacks infectivity[28]. This is an advantage for prion disease diagnostics because CSF-RT-QuIC requires no high biosecurity level and has little potential for cross-contamination. A further advantage of RT-QuIC analysis compared with PMCA is the multiwell format, which enables the analysis of up to 96 samples in the same experiment and the ease of signal quantification (detection of Th-T in a fluorescence reader, whereas PMCA requires a PK digestion and semiquantitative detection of PrP^{Sc} in a western blot).

Limitations

One of the most important preanalytical issues is that contamination with blood cells, such as erythrocytes, may result in a false-negative RT-QuIC response. During the lumbar puncture, between 5 and 10% of CSF samples are contaminated with blood cells, which may disturb the PrP seeding activity. A blood cell contamination higher than 1,250 cells/μl inhibits

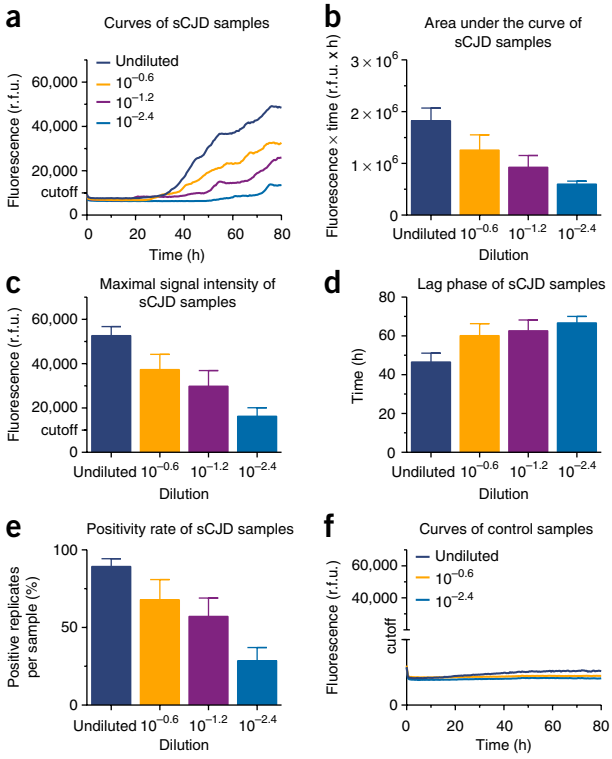

Figure 3 | Influence of serial dilution of CSF on the RT-QuIC response. Serial dilution of CSF, before RT-QuIC analysis, results in a decrease of the RT-QuIC response of sCJD samples. (**a**–**e**) CSF samples from sCJD (MM1) patients ($n = 7$) and from (**f**) control patients ($n = 8$) (without prion disease and with an alternative diagnosis such as headache, psychotic disorder, inflammation or another neurodegenerative disorder) were diluted in the range of $10^{-0.6}$ to $10^{-2.4}$. Diluted samples were analyzed in quadruplicate by RT-QuIC. Statistical parameters quantifying the RT-QuIC response, such as area under the curve (**b**), maximal signal intensity (**c**) and time until the signal reached the cutoff (lag phase (**d**)), were determined per experimental group (dilution). (**a**) The curves depict the mean kinetics of the RT-QuIC response of sCJD samples per experimental group (dilution). Fluorescence signal was measured every 30 min. Mean values were determined per sample and used to calculate total mean per experimental group (dilution) per measurement (time point). Total means were connected by linear interpolation, displaying an averaging line per experimental group (dilution). The cutoff is indicated at 10,000 r.f.u. (**b**–**d**) The RT-QuIC responses of sCJD-samples were quantified by calculating statistical parameters, such as AUC, maximal signal intensity and lag phase. Mean values were determined per sample and used to calculate total mean per experimental group (dilution) and s.e.m. Bar graphs show total mean + s.e.m. per experimental group (dilution). Cutoff is indicated at 10,000 r.f.u. (**e**) The validity of the RT-QuIC responses from sCJD samples was determined by calculating the percentage of positive replicates per sample. Mean values were determined per sample and used to calculate total mean per experimental group (dilution) and s.e.m. Bar graphs show total mean + s.e.m. per experimental group (dilution). Samples diluted $10^{-2.4}$ showed a mean positivity rate of <50%. (**f**) The curves depict the mean kinetics of the RT-QuIC response of control samples. Fluorescence signal was measured every 30 min. Mean values were determined per sample and used to calculate total mean per experimental group (dilution) per measurement (time point). Total means were connected by linear interpolation, displaying an averaging line per experimental group (dilution). The cutoff is indicated at 10,000 r.f.u. This diagram shows no seeding of PrP. Therefore, seeding parameters such as AUC, maximal signal intensity and lag phase were not available. The study conformed to the Code of Ethics of the World Medical Association, and informed consent was given by all study participants or their legal next of kin, and the study being approved by the local ethics committee in Göttingen, Germany (no. 24/8/12).

the RT-QuIC reaction markedly, when all cells are completely hemolyzed by sonication[12].

Other difficulties currently encountered when comparing results of various RT-QuIC assays from different laboratories are the variations in samples, reaction conditions and instruments, and the lack of standardized criteria for defining positive and negative responses. Such criteria will inevitably depend on the goals of the type of testing being performed (e.g., whether to minimize false positives or false negatives) and the idiosyncrasies of individual testing conditions. In the case of using RT-QuIC for human prion disease diagnosis using CSF specimens, further validation studies are required, although the RT-QuIC assay has demonstrated considerable diagnostic utility to date. For the protocol described below, a qualitative cutoff at 10,000 r.f.u. is suggested on the basis of two previous studies[12,33].

The duration of the RT-QuIC assay described below is usually 4 d. However, a recent study, which requires further validation, describes conditions, such as higher temperature (up to 55 °C) and addition of 0.002% (wt/vol) SDS and recPrP^{c} substrate (hamster 90–231), that can reduce assay times to a matter of hours and improve sensitivity[25].

Although not studied exhaustively, another limitation of RT-QuIC assays is that only very few recPrP^{c} substrates, such as bank vole recPrP^{c}, are able to amplify and to detect variant CJD prions. When using hamster 23–231 recPrP^{c}, no PrP variant CJD seeding could be obtained[28].

Experimental design

Protocol workflow. In the RT-QuIC, a PrP^{Sc} seed is incubated with a recPrP^{C} substrate (derived from several species, such as human, hamster, hamster–sheep chimera and bank vole; see further discussion below)[12,13,14,28] and a buffer to induce the conversion of the substrate and to subsequently amplify miniscule amounts of seed up to a detectable limit. Seed (derived from prion-infected human brain or CSF), substrate (recombinant hamster–sheep PrP^{C} (recHaShPrP^{C})) and buffer are prepared (Steps 4–7), combined in a 96-well plate (black 96-well, optical-bottom plates; see Steps 8–12) and incubated in a fluorescence plate reader (FLUOstar OPTIMA) at 42 °C for 80 h with intermittent shaking cycles, consisting of 1 min of double orbital shaking at the highest speed (600 r.p.m.) followed by a 1-min incubation break (Steps 13–17). Converted PrP is monitored in real time by Th-T fluorescent dye analysis. The kinetics of the aggregation process is determined by measuring the Th-T fluorescence signal (450 nm excitation and 480 nm emission) every 30 min. For data analysis, a quantification of statistical parameters, such as lag phase (time to reach the cutoff), AUC and maximal signal intensity, using common software programs is suggested (Steps 18–21).

Source of recPrP^{c}. recPrP^{c} may derive from various species—i.e., human[22], hamster[13], bank vole[24,28]—or, as in the present protocol, from a hamster–sheep chimera, which, in our experience, preferably shows less spontaneous aggregation and has been evaluated for diagnostic purposes over many years. Some evidence for comparability to other PrP substrates (e.g., human recPrP^{c}) can be derived from ring trials, in which the data suggest that various slightly modified test conditions provide similar test sensitivities and positive rates[12,16,25,29]. A recently described bank vole recPrP^{c} has been proven to support the amplification of many different PrP^{Sc} strains, different Gerstmann–Straussler–Scheinker syndrome (GSS) mutations and variant CJD, which cannot be detected with hamster recPrP^{c} (refs. 24,28). We propose that bank vole recPrP^{c} may become interesting for diagnostics in the future; however, an evaluation study on a larger patient cohort is still required.

Validation of the protocol: sensitivity and specificity. This protocol using the hamster–sheep chimera has been extensively validated through the analysis of CSF samples from different patient cohorts (within Europe and Japan) consisting of >500 patients with prion disease and >500 controls. Samples that show a seeding reaction (increase of the Th-T fluorescence signal) in 50% of the replicates are considered to be positive for a prion disease. Negative-control patients (without prion disease) show no seeding activity of PrP in the RT-QuIC assay after 80 h. A test run, in which the positive control remained negative, was considered to be a failed test. Under our conditions, we suggest a cutoff setting at 10,000 r.f.u. (cutoff may vary in different reference laboratories depending on the instrumental settings and the baseline of the reaction) to obtain a specificity of 99% and a sensitivity of 85% for the RT-QuIC assay for all prion diseases[12]. Across different prion diseases, the sensitivity varies from 100% for gCJD, 80% for sCJD and 57% for fatal familial insomnia (FFI) cases[12]. Other studies report a specificity of almost 100% and a sensitivity between 80 and 96% (refs. 13,14,25). For gCJD cases, the following sensitivities for RT-QuIC assays have been reported: FFI (100%), gCJD E200K (87%) and gCJD V210I (100%)[29]. Potential reasons for discrepancies in FFI sensitivities across studies can be sampling errors due to the low number of patients tested (7 versus 22 patients) and methodological differences (e.g., use of different PrP substrates)[12,29].

Replicates and controls. For diagnostics, each sample is generally analyzed in triplicate. A sample is considered as positive for the conversion of a competent amyloid (indicating a prion disease) if two out of three replicates show a Th-T signal higher than the cutoff (10,000 r.f.u.). As controls, samples from patients with (positive control) or without prion disease (negative control) are used. Control samples are prepared as described in the Reagent Setup.

False-positive reactions. A potential problem of *in vitro* amplification assays is the occurrence of false-positive reactions, caused by spontaneous conversion of the recPrP^{C}. Accordingly, our assay components and conditions have been carefully selected to maximize the kinetic difference between PrP^{Sc}-seeded and spontaneous, prion-independent responses. This protocol uses chimeric recPrP^{C}, composed of the Syrian hamster residues 23–137, followed by sheep residues 141–234 (of the R_{154} Q_{171} polymorphism)[26], in conjunction with reaction conditions that greatly retard the spontaneous aggregation of recPrP^{C} and thereby the occurrence of false-positive signals. A number of other combinations of recPrP^{C} substrate and reaction conditions have also been described as being suitable for the detection, and sometimes discrimination, of at least 28 different prions of humans and animals[12–14,20,24].

MATERIALS

REAGENTS

- Seed (brain homogenates or CSF samples from humans with prion diseases) ! CAUTION Informed consent must be obtained from all subjects, according to the relevant national legislation for the use of human biological samples for research. This study conformed to the Code of Ethics of the World Medical Association, and informed consent was given by all study participants or their legal next of kin, and the study was approved by the local ethics committee in Göttingen (no. 24/8/12). ! CAUTION This is a biological hazard. Biocontainment precautions need to be followed, according to the relevant national legislation for the use of biological samples from humans with prion diseases. In this study, precautions were taken related to biosafety level 3. ▲ CRITICAL Stability of the RT-QuIC seed in CSF under short- and long-term storage conditions is very high. CSF can be stored at room temperature or +4 °C for 8 d and at −80 °C for 9 years[12].
- Substrate (recombinant hamster–sheep chimeric PrP^C (recHaShPrP^C)) ▲ CRITICAL Laboratories that do not want to produce their own recHaShPrP^C (as described in **Supplementary Methods**) can request the protein from Thermo Fisher Scientific using the internal cat. no. 7700002 because it is not included in its standard catalog.
- Sodium phosphate dibasic (Sigma-Aldrich, cat. no. S7907)
- Sodium phosphate monobasic (Sigma-Aldrich, cat. no. S8282)
- NaCl (Roth, cat. no. 39571)
- Thioflavin-T (Th-T; Sigma-Aldrich, cat. no. T3516)
- SDS (Sigma-Aldrich, cat. no. L3771)
- Tris–HCl (Sigma-Aldrich, cat. no. T3253)
- 0.5 M EDTA (Fluka Analytical, cat. no. 06390)
- 5 M NaCl (SAFC Biosciences, cat. no. 59222C)
- PBS (Biochrome, cat. no. L 1825)
- 50% (wt/vol) NaOH (Roth, cat. no. 8655.1)

EQUIPMENT

- Centrifuge (Eppendorf, model no. 5415C)
- pH meter (Sartorius, model no. PP-15)
- Vortex (Scientific Industries, model no. Genie 2)
- Scale (Sartorius, model no. TE313S)
- Computer (Dell)
- Excitation filter, 450 nm for FLUOstar OPTIMA (BMG Labtech)
- Emission filter, 480 nm for FLUOstar OPTIMA (BMG Labtech)
- 96-Well Optical-Bottom Plate (Nunc, cat. no. 265301)
- Sealing tape (Nunc, cat. no. 232702)
- Syringe, 5 ml (Ecoject, cat. no. 4676651)
- Filter, Millex-GP, 0.22 µM (Merck-Millipore, cat. no. SLGP033RS)
- Microtubes, 1.5 and 2.0 ml
- Falcon tubes, 15 ml (VWR, cat. no. 734-0452)
- Falcon tubes, 50 ml (Sarstedt, cat. no. 62.547)

REAGENT SETUP

Homogenization buffer This buffer contains 0.1% (wt/vol) SDS prepared in PBS. For a 100-ml preparation, weigh 0.1 g of SDS, add 100 ml of PBS and dissolve the SDS by vortexing for 30 s, using a Vortex Genie 2 at level 10 (or an equivalent). Filter-sterilize the homogenization buffer using a 5-ml syringe and a 0.22-µM filter. Keep the buffer stock at 4 °C in 15-ml Falcon tubes, and discard after 3 months.

5× PBS, pH 6.9, buffer For a 50-ml preparation, weigh 0.67 g of sodium phosphate monobasic, 0.35 g of sodium phosphate dibasic and 1.90 g of NaCl and 48 ml of ddH_2O. Dissolve the substances by vortexing for 30 s, using a Vortex Genie 2 at level 10 (or an equivalent). Adjust the pH to 6.9, using NaOH (50% (wt/vol)) and a PP-15 pH meter (or an equivalent). Dilute 5× PBS, pH 6.9, in 50 ml of ddH_2O and mix by vortexing for 30 s, using a Vortex Genie 2 at level 10 (or an equivalent). Filter-sterilize 5× PBS, pH 6.9, using a 5-ml syringe and a 0.22-µM filter. Keep 5× PBS, pH 6.9, buffer stock at −20 °C in 15-ml Falcon tubes, and discard after 3 months.

Basis mix Prepare basis mix by mixing the following components at the ratio stated below for five reactions: 167 µl of ddH_2O, 100 µl of 5× PBS buffer, pH 6.9, 22 µl of NaCl (5 M) and 1 µl of EDTA (0.5 M). The total volume of basis mix per reaction is 57.0 µl (285 µl for five reactions). Preparing basis mix for multiple reactions in total in one tube is recommended to avoid errors in concentration by pipetting small volumes. Mix by vortexing for 30 s, using a Vortex Genie 2 at level 10 (or an equivalent). Keep the basis mix stock at −20 °C, and discard it after 3 months.

Th-T solution This solution contains 1 mM Th-T. For a 10-ml preparation of 10 mM Th-T, dissolve 0.036 g of Th-T in 10 ml of ddH_2O and vortex the mixture for 30 s, using a Vortex Genie 2 at level 10 (or an equivalent). Filter-sterilize the 10 mM Th-T solution using a 5-ml syringe and a 0.22-µM filter. Keep the 10 mM Th-T buffer stock in the dark at room temperature in a 15-ml tube, and discard it after 3 months. For a 10-ml preparation of 1 mM Th-T, dilute 1 ml of 10 mM Th-T in 9 ml of ddH_2O. Mix by vortexing for 30 s, using a Vortex Genie 2 at level 10 (or an equivalent). Keep the 1 mM Th-T buffer stock in the dark at room temperature in 2.0-ml tubes, and discard it after 3 months.

Final mix This mix contains 1× PBS, pH 6.9, 170 mM NaCl, 1 mM EDTA, 10 µM Th-T and 0.1 mg/ml recHaShPrP^C. For 1 reaction, mix 57.0 µl of basis mix, 1.0 µl of Th-T (1 mM) and 27.0 µl of recHaShPrP^C (0.37 mg/ml). Freshly prepare the final mix for each experiment, and discard it after use.

CSF sample preparation Prepare the CSF sample at room temperature (~22 °C). Centrifuge the native sample at 720*g* for 10 min to remove cell debris, using a 5415C centrifuge (or an equivalent). Remove the supernatant and discard the pellet. Transfer the supernatant to 1.5-ml tubes. Mix the supernatant for 30 s by using a Vortex Genie 2 at level 10 (or an equivalent). The supernatant can be stored at room temperature or 4 °C for up to 8 d, or at −80 °C for up to 9 years. ! CAUTION This is a biological hazard. Biocontainment precautions need to be followed according to the relevant national legislation for the use of biological samples from humans with prion diseases. In this study, precautions were performed related to biosafety level 3. ! CAUTION Informed consent must be obtained from all subjects, according to the relevant national legislation for the use of human biological samples for research. This study conformed to the Code of Ethics of the World Medical Association, and informed consent was given by all study participants or their legal next of kin, and the study was approved by the local ethics committee in Göttingen (no. 24/8/12). ▲ CRITICAL Blood contamination may cause false-negative results.

Brain sample preparation Prepare brain sample at room temperature. Weigh the native sample using a TE313S scale (or an equivalent), and add 10% (wt/vol) homogenization buffer. Homogenize the sample in homogenization buffer for 30 s, using a Polytron PT1300D (or an equivalent) at highest speed. Centrifuge the homogenized sample at 10,000*g* for 10 min to remove cell debris using a 5415C centrifuge (or an equivalent). Remove the supernatant and discard the pellet. Divide the supernatant into aliquots in 1.5-ml tubes, and vortex them for 30 s to mix adequately using a Vortex Genie 2 at level 10 (or an equivalent). The supernatant can be used immediately, or it can be stored at −80 °C. ! CAUTION Biological hazard. Biocontainment precautions need to be followed, according to the relevant national legislation for the use of biological samples from humans with prion diseases. In this study, precautions were performed related to biosafety level 3. ! CAUTION Informed consent must be obtained from all subjects, according to the relevant national legislation for the use of human biological samples for research. This study conformed to the Code of Ethics of the World Medical Association, and informed consent was given by all study participants or their legal next of kin, and the study was approved by the local ethics committee in Göttingen (no. 24/8/12).

EQUIPMENT SETUP

FLUOstar OPTIMA This RT-QuIC protocol requires the fluorescence reader FLUOstar OPTIMA, a computer and two software programs—i.e., the OPTIMA control program and the OPTIMA data analysis program. Both programs are provided by BMG Labtech. Using the FLUOStar Optima to run the RT-QuIC assay requires two protocols and one script—i.e., the Rocky Mtn Read and Rocky Mtn Shake protocols, and the Rocky Mtn Script. These can be requested from BMG Labtech. To measure the Th-T fluorescence according to this protocol, use two filters for the FLUOstar OPTIMA—i.e., an excitation filter at 450 nm and an emission filter at 480 nm. Both can be bought from the BMG Labtech Company. Enter the following settings into the Rocky Mtn Read protocol, and save the settings: microplate (Nunc 96), optic (bottom optic), excitation filter (450 nm), emission filter (480 nm) and additional shaking (no shaking). Enter the following settings into the Rocky Mtn Shake protocol, and save the settings: shaking mode (double orbital), shaking width (600 r.p.m.), additional shaking (before each cycle) and shaking time (57 s).

PROCEDURE

Operation of the fluorescence reader ● TIMING 15 min

1| Start the FLUOstar OPTIMA fluorescence reader and the computer.

2| Enter the temperature (42 °C) into the OPTIMA control program, and let the machine heat up.

3| Enter the following settings into the Rocky Mtn Script: target temperature (42 °C), cycle time (1,800 s) and total measurement time (80 h).

■ **PAUSE POINT** The fluorescence reader can wait until the start of experiment.

Preparation of recHaShPrP^C ● TIMING 1 h and 15 min

4| Thaw the recHaShPrP^C (0.6 mg/ml) at ≈4 °C.

▲ **CRITICAL STEP** Thawing at higher temperatures may induce aggregation of recHaShPrP^C.

5| Load 500 µl of recHaShPrP^C per 100-kDa filter at room temperature.

6| Centrifuge the loaded filters for 5 min at 1.310*g* at room temperature to remove possible existing aggregates, using a 5415C centrifuge (or an equivalent).

? TROUBLESHOOTING

7| Discard the filters and proceed with recHaShPrP^C filtrate at room temperature.

▲ **CRITICAL STEP** Some recHaShPrP^C is lost during the filtration process because aggregates are removed; the concentration of the filtrate should be ≈0.37 mg/ml.

■ **PAUSE POINT** Filtered recHaShPrP^C can be stored at room temperature for up to 30 min.

Unification and mixing of the sample at room temperature ● TIMING variable; ~2 h

8| Optional: thaw the frozen samples at room temperature.

9| Pipette 85 µl of final mix per well into a black 96-well optical-bottom plate.

▲ **CRITICAL STEP** Avoid creating bubbles, as they may cause variable results.

10| Vortex the sample for 30 s to mix adequately, using a Vortex Genie 2 at level 10 (or an equivalent).

11| Pipette 15 µl of sample into each well containing final mix from Step 9.

▲ **CRITICAL STEP** Avoid creating bubbles, as they may cause variable results.

12| Seal the plate with a sealing tape.

▲ **CRITICAL STEP** Make sure that the sealing tape is fixed on the plate without any air bubbles. Air bubbles may connect wells and may cause cross-contamination between different wells during the shaking process.

Running of the experiment ● TIMING 80 h and 15 min

13| Open the plate tray of the FLUOstar OPTIMA, and insert the plate from Step 12 into the plate carrier.

14| Declare used wells in the layout of the Rocky Mnt Read protocol.

15| Click the 'Start' button.

16| Wait until the temperature is at 42 °C, and confirm that the plate is inserted.

17| Run the machine for 80 h.

? TROUBLESHOOTING

■ **PAUSE POINT** After the run is completed, the fluorescence reader is ready for the taking of results; the machine stops executing the shaking and incubating cycles, but holds the temperature. To reduce the run-time of the machine, it is recommended to take the results on the day the run is completed.

Examination of results ● TIMING variable; ~2 h and 15 min

18| Open the OPTIMA data analysis program.

19| Open the latest run and verify the results. Criteria for a successful run are a complete data set (data for every well that should be measured) and valid signals from the controls.
? TROUBLESHOOTING

20| Discard the used black 96-well optical-bottom plate, or freeze it at –20 °C for further analyses of the product. Discard frozen plates after 3 months.
? TROUBLESHOOTING

21| Analyze the data using the OPTIMA data analysis program according to the manufacturer's instructions, or export the data and analyze in another program—e.g., Microsoft Office Excel or GraphPad Prism.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solutions
6	Loss of volume during the filtration process	~30% of the volume is usually lost during the filtration process, due to aggregation of recHaShPrP^C	Take this into account in your preparation of the final buffer mix
17	Unexpected system shutdown	Depending on the computer operating system, the system can shut down and restart for several reasons and, consequently, stop the software executing the fluorescence reader. This can be caused by, e.g., software routines aimed at saving energy or updating the system	Change the software routines to avoid this
19	Failed test (false-negative positive control)	Premature aggregation of recHaShPrP^C Aggregation of recHaShPrP^C may result in a loss of amyloid fibrillation potential	Verify storage and handling conditions RecHaShPrP^C should be stored at –80 °C. Thaw recHaShPrP^C on ice or at 4 °C and do not vortex; gently mix by swinging back and forth
	False-positive result	Bubbles may cause false-positive results	Avoid bubbles in Steps 9 and 11 while pipetting
	False-negative result	Dilution of brain sample might be too high	Choose lower dilutions of the brain sample. Dilutions from 10^{-3} to 10^{-8} usually work
		CSF sample might be contaminated with blood	Verify the absence of blood contamination. Contaminated CSF samples might be rescued by centrifugation at 720*g* for 10 min at room temperature[12]
	There are no data for some wells, and there are data for wells that should not be measured	The plate layout has been changed in the Rocky Mnt Shake protocol, instead of the Rocky Mnt Read protocol	Verify the plate layout in the Rocky Mtn Read protocol
	There are data for all wells that should be measured, but the data do not remotely correspond to anticipated results	The plate was accidently rotated by 180 °, so the original plate layout and orientation of wells no longer fit	Turn the plate layout around by 180°, and verify the position of the plate layout and anticipated results
	Fluorescence curves vary vastly between replicates	The software setting for optic is set to 'top optic'	Change the software setting from 'top optic' to 'bottom optic' in the Rocky Mnt Read protocol
		The filters have been installed in the wrong orientation	Check the orientation of the filters
20	The sealing tape is cracked	The plate is not fixed	Verify this, and if necessary, repair the machine

● TIMING

Steps 1–3, operation of the fluorescence reader: 15 min
Steps 4–7, preparation of recHaShPrP^{C}: 1 h and 15 min
Steps 8–12, unification and mixing of the sample at room temperature: variable; ~2 h to pipette a 96-well plate
Steps 13–17, running of the experiment: 80 h and 15 min
Steps 18–21, examination of results: ~2 h and 15 min; timing for analysis of the data is variable, depending on the experimental setup and analysis software: ~2 h to analyze maximal signal intensity (maximal r.f.u.), AUC and lag phase for a complete 96-well plate and compare between experimental groups

ANTICIPATED RESULTS

This protocol has been used to study the aggregation of misfolded PrP in brain tissue and CSF from prion disease patients[33]. Positive samples showed an increase of seeding activity (>cutoff) within a duration of 80 h (**Figs. 2a** and **3a**). By contrast, negative samples (those without prion disease) revealed no PrP seeding in the brain or in CSF (**Figs. 2f** and **3f**). The seeding efficiency of PrP, indicated by the Th-T signal, correlated with the dilution. Higher dilutions revealed a decrease of PrP seeding, indicated by a lower AUC, lower maximal signal intensity, longer lag phase and a lower positivity rate (**Figs. 2a–e** and **3a–e**; **Supplementary Tables 1** and **2**). In addition to prion disease diagnostics[12], this methodology can be used for a number of different approaches, such as studying protein misfolding in detail, prescreening for compounds inhibiting the PrP conversion, testing for prion contaminations or studying other protein misfolding diseases (e.g., Alzheimer's disease).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS M.S. was involved in study concept and design, and validated the protocol, interpreted data and wrote the manuscript. M.C. performed experiments, analyzed and interpreted data, prepared figures and wrote the manuscript. F.L. provided samples and interpreted data.

D.M.-C. designed **Figure 1**. S.C., R.A., K.S., C.D.O., B.R.G., S.Z. and B.C. critically revised the manuscript. W.J.S.-S. provided samples. I.Z. supervised the study and critically revised the manuscript.

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