# Ultrasensitive human prion detection in cerebrospinal fluids using real-time quaking-induced conversion

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**ABSTRACT**

The development of technologies for the *in vitro* amplification of the abnormal conformers of prion protein ($PrP^{Sc}$) has generated the potential for a novel diagnostic assay for prion disease. Previously, we developed a new $PrP^{Sc}$ amplification assay designated quaking-induced conversion (QUIC), which involves intermittent, automated shaking of the substrate, soluble recombinant PrP. We further improved the rapidity and practicality of this method by combining it with thioflavin T fluorescence to monitor the amyloid fibril formation. This assay, termed "real-time QUIC (RT-QUIC)", allows within 48 h, the detection of ≥1 fg of $PrP^{Sc}$ in diluted Creutzfeldt-Jakob disease (CJD) brain homogenate. Moreover, we assessed the technique first in a series of Japanese subjects, and then in a blind study of 30 cerebrospinal fluid specimens from Australia, which achieved greater than 80% sensitivity and 100% specificity. These findings indicate the promising enhanced diagnostic capacity of RT-QUIC in the ante-mortem evaluation of suspected CJD.

Transmissible spongiform encephalopathies or prion diseases are characteristically associated with the accumulation of $PrP^{Sc}$ in the central nervous system through auto-catalytic conversion of normal cellular PrP ($PrP^{c}$) into replicate misfolded isomers[1,2]. Despite occasional reported exceptions[3,4], $PrP^{Sc}$ remains the best characterized and most reliable marker of prion disease.

Definitive ante-mortem confirmation of CJD requires the detection of $PrP^{Sc}$ in patient biopsy specimens, the practice of which is discouraged because it is both invasive and poses risks to health care personnel. Recently, however, *in vitro* $PrP^{Sc}$ amplification techniques, including protein misfolding cyclic amplification (PMCA)[5-7], the amyloid seeding assay[8], as well as QUIC have been reported to enable the direct and highly sensitive detection of $PrP^{Sc}$ in various tissues, including cerebrospinal fluid (CSF). QUIC assays involve the use of soluble recombinant PrP (rPrP-sen) as a substrate, which is seeded with $PrP^{Sc}$, and then subjected to intermittent automated shaking. This technique can be performed more easily than the PMCA, which requires repeated sonication. Previous studies have demonstrated that QUIC assays correctly discriminated between normal and scrapie-infected CSF samples in both hamster and sheep prion disease models[9,10]. However, ultrasensitive $PrP^{Sc}$ detection in CSF from CJD subjects has not yet been accomplished. Accordingly, we further refined the QUIC

assay to improve its sensitivity and practicability, and then applied the technique in a blind pilot study to detect $PrP^{Sc}$ in CJD-CSF specimens.

Given that a correlation between protease-resistant rPrP aggregate (rPrP-res) levels and thioflavin T (ThT) fluorescence had been shown previously[7], we sought to determine the relative kinetics of rPrP-res formation by monitoring levels of ThT fluorescence in the QUIC assay. This was intended to minimize the time needed to detect rPrP-res. We first tested whether $PrP^{Sc}$-dependent rPrP-res ($rPrP\text{-}res^{(Sc)}$) formation could be induced using a microplate reader with intermittent shaking. Human rPrP-sen (rHuPrP-sen) and a $10^{-7}$ dilution of CJD (molecular subtype MM1) brain homogenate (BH) were used as the substrate and "seed", respectively. We conducted QUIC reactions at various concentrations (0, 0.25, 0.5 and 1.0 M) of guanidine-HCl (GdnHCl), because it has been demonstrated that GdnHCl greatly enhances conversion of PrP-sen to PrP-res in cell-free conversion reactions. Unexpectedly, positive $PrP^{Sc}$-dependent ThT fluorescence was observed within 24 h, both in the presence and absence of GdnHCl (**Fig. 1a**). In contrast, the negative control reactions without seed and in the absence of GdnHCl resulted in no increase in ThT fluorescence over 24 h; however, de novo formation of rPrP-res ($rPrP\text{-}res^{(spon)}$) was rapidly induced in the presence of GdnHCl when shaking was added (**Fig. 1a,b**). These results indicate that

shaking accelerates $PrP^{Sc}$-dependent rPrP-$res^{(Sc)}$ formation even without GdnHCl (**Supplementary Fig. 1**), albeit with a lower peak of fluorescence.

Shaking is thought to cause partial unfolding of a portion of the rPrP-sen by increasing the air-water interface[11]. Moreover, shaking enhances the interaction between rPrP-sen and $PrP^{Sc}$, and promote the fragmentation of rPrP-res polymers[12]. It is generally accepted that the energetic barrier of seed-dependent fibril formation and elongation is lower than that of spontaneous fibril formation, which first requires nucleation as the rate-limiting step[13]. The partial unfolding of rPrP-sen by shaking appears to be more heterogeneous than that facilitated by a denaturant such as GdnHCl, perhaps because the air-water interfaces created by shaking are unequally distributed in solution. The addition of GdnHCl to QUIC reactions leads to an increase in the nucleation rate, and increased spontaneous fibril formation. The early appearance of rPrP-$res^{(spon)}$ decreases the specificity of QUIC, because ThT fluorescence cannot distinguish between rPrP-$res^{(Sc)}$ and rPrP-$res^{(spon)}$. Therefore, we chose not to use GdnHCl in subsequent analyses.

To further optimize the conditions, we examined the effects of pH, as well as the concentrations of rHuPrP-sen and salt on QUIC reactions in GdnHCl-free conditions with shaking (**Fig 1c–e** and **Supplementary Fig. 2**). After assessment, we successfully

established a method for the real-time monitoring of the kinetics of rPrP fibril formation seeded with CJD-BH (see **Supplementary Methods** online), without the generation of rPrP-res$^{(spon)}$, and designated the assay "real-time QUIC (RT-QUIC)".

To determine the minimum amount of $PrP^{Sc}$ detectable by the RT-QUIC, we diluted CJD-BH (MM1 and MM2) serially with artificial CSF (A-CSF) and used these dilutions to seed the reactions. Increased $PrP^{Sc}$-dependent ThT fluorescence was seen within 48h in more than half the replicates of CJD-BH, with dilutions ranging from $10^{-5}$ to $10^{-9}$ (**Fig. 1f** and **Supplementary Table 1**). With $10^{-10}$ BH dilutions we observed a marginally lower rate of positive reactions and the $10^{-11}$ dilutions of the CJD-BHs produced no ThT fluorescence response. The negative controls seeded with $10^{-5}$ and $10^{-7}$ dilutions of non-CJD BH or A-CSF alone (no seed) did not produce an increase in the fluorescence. The $10^{-9}$ dilutions of MM1 and MM2-CJD-BH contained approximately 0.8 and 1.9 fg of $PrP^{Sc}$, respectively, according to our estimation (data not shown). Consequently, the results indicate that this assay consistently enables us to detect ≥ around 1 fg of $PrP^{Sc}$ in the diluted CJD-BHs within 48 h. Moreover, the fact that there was no rPrP-res$^{(spon)}$ formation under the conditions employed implies a reduced and acceptable risk of false-positive reactions. Whether the RT-QUIC has the same sensitivity to CJD-BH with 129MV or VV as 129MM remains to be determined.

CSF is routinely used in the evaluation of CNS disorders, and presumably contains more $PrP^{Sc}$ and fewer impurities than blood. This prompted us to compare the RT-QUIC seeding activity in CSF samples from CJD- and non-CJD subjects. For the pilot study, we initially tested CJD-CSF samples from 18 definite cases of CJD in Japan (**Table 1**) and 35 non-CJD controls from subjects with other neurodegenerative diseases (**Supplementary Table 2**). We saw minimal ThT fluorescence increase in the controls, with no false positives in the assay. In contrast, increased $PrP^{Sc}$-dependent fluorescence was seen in at least one of four replicates in 15/18 (83.3%) of the CJD-CSF samples (**Table 1** and **Supplementary Table 3**).

To further confirm the very promising reliability of RT-QUIC, we conducted a blind trial using 30 CSF samples from the Australian National CJD Registry and 155 CSF samples, containing 25 probable cases of CJD and 130 cases of other neurological diseases, obtained in Japan. In the Australian samples, we were able to detect $PrP^{Sc}$ in 14/16 (87.5%) definite CJD-CSF samples, as opposed to 0/14 of the non-CJD controls (**Table 1**, **Supplementary Fig. 3** and **Supplementary Table 3**). It should be noted that 3/4 129VV and 2/2 129MV cases were positive by the RT-QUIC. These results indicate that RT-QUIC has the ability to discriminate CJD-CSF samples that include 129MM, MV and VV cases from non-CJD CSF samples. In addition, none of the 130 Japanese

cases of other neurological diseases was positive, further confirming the reliability of this assay (**Supplementary Table 4**). Collectively, the RT-QUIC assays showed more than 80% sensitivity and 100% specificity. The sensitivity was equivalent, and the specificity greatly exceeded that of 14-3-3[14,15], a non-specific marker of rapid neuronal damage (**Supplementary Table 3**).

Although we have never experienced a false-positive reaction among the hundreds of non-CJD neurodegenerative disease samples we have so far tested, it remains possible that certain conditions may evoke a positive reaction, and further studies will be required to eliminate this possibility. Furthermore, scrupulous attention to the conditions of the assay is essential to avoid false-positives in the clinical setting. Nevertheless, we believe that the ultrasensitive detection of $PrP^{Sc}$ in CSF by RT-QUIC represents a valuable novel means for the early, rapid and specific diagnosis of CJD.

**ACKNOWLEDGMENTS**

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**AUTHOR CONTRIBUTIONS**

R.A. designed the project, performed experiments, and wrote the manuscript. K.Satoh, K.Sano, T.F., N.Y., D.I., T.M., T.N. and H.Y. performed experiments. K.Satoh, S.S., M.Y., H.M., T.K., G.K., A.McG. and S.J.C. contributed to the collection of human specimens, and provided information about subjects. N.N. supervised the project. K.Satoh, K.Sano, A.McG., S.J.C. and N.N. helped with the editing of the manuscript.

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**Table 1** Clinical data and RT-QUIC reactions seeded with CSF samples

CSF from 18 definite CJD subjects in Japan

| No. | Age(yr)/Sex | CJD type | Molecular subtype[a] | 14-3-3 (γ-isoform)[b] | RT- QUIC[c] |
|---|---|---|---|---|---|
| C1 | 68/M | sCJD | MM 1 | + | + (3/4) |
| C2 | 66/F | sCJD | MM 1 | + | + (2/4) |
| C3 | 71/F | sCJD | MM 1 | + | + (1/4) |
| C4 | 57/F | sCJD | MM 2 | + | + (2/4) |
| C5 | 70/M | sCJD | MM 2 | + | + (2/4) |
| C6 | 66/M | sCJD | MM 2 | + | + (2/4) |
| C7 | 60/F | sCJD | MM 2 | – | – (0/4) |
| C8 | 73/F | sCJD | MM 2 | + | + (4/4) |
| C9 | 74/M | sCJD | MM 2 | + | – (0/4) |
| C10 | 79/F | sCJD | MM 2 | – | + (3/4) |
| C11 | 65/F | sCJD | MM 2 | + | + (4/4) |
| C12 | 69/M | sCJD | MM 2 | + | – (0/4) |
| C13 | 69/F | sCJD | MM 2 | + | + (3/4) |
| C14 | 54/F | sCJD | MM 2 | + | + (3/4) |
| C15 | 76/F | sCJD | MM 2 | – | + (2/4) |
| C16 | 68/M | sCJD | MM 2 | – | + (4/4) |
| C17 | 58/F | iCJD | MM 1 | + | + (3/4) |
| C18 | 79/F | iCJD | MM 1 | – | + (3/4) |

Blind trial using 30 CSF samples in Australia

| No. | Age(yr)/Sex | Diagnosis[d] | Codon129 | profile of $PrP^{Sc}$ | 14-3-3 (all isoforms)[e] | Real-time QUIC[g] |
|---|---|---|---|---|---|---|
| A1 | 53/F | PN/MC | | | – | – (0/4) |
| A2 | 59/F | PN/ MC | | | – | – (0/4) |
| A3 | 85/M | AD | | | – | – (0/4) |
| A4 | 60/F | ICD | | | + | – (0/4) |
| A5 | 83/M | AD | | | + | – (0/4) |
| A6 | 73/M | sCJD | VV | 2 | + | + (2/4) |
| A7 | 67/F | sCJD | MM | 1 | + | + (4/4) |

| | | | | | | |
|---|---|---|---|---|---|---|
| A8 | 82/F | sCJD | MM | 1 | + | + (2/4) |
| A9 | 67/M | sCJD | MV | 1 | – | + (2/4) |
| A10 | 50/M | sCJD | MM | 1 | + | + (3/4) |
| A11 | 66/M | PN/MC | | | – | – (0/4) |
| A12 | 61/M | PN/MC | | | – | – (0/4) |
| A13 | 84/F | sCJD | MM | 1 | + | + (3/4) |
| A14 | 76/M | sCJD | MM | 1 | + | + (2/4) |
| A15 | 69/M | sCJD | MV | 1 | + | + (4/4) |
| A16 | 67/M | AD | | | – | – (0/4) |
| A17 | 75/F | PN/MC | | | + | – (0/4) |
| A18 | 93/M | DLB/PD | | | – | – (0/4) |
| A19 | 67/F | sCJD | ND | 2 | + | + (2/4) |
| A20 | 53/M | DLB/PD | | | – | – (0/4) |
| A21 | 71/F | sCJD | VV | 2 | + | – (0/4) |
| A22 | 62/F | sCJD | MM | 2 | – | – (0/4) |
| A23 | 90/M | sCJD | VV | ND | + | + (1/4) |
| A24 | 61/F | DLB/PD | | | – | – (0/4) |
| A25 | 74/M | sCJD | MM | ND | ±[f] | + (2/4) |
| A26 | 74/F | AD | | | – | – (0/4) |
| A27 | 68/F | sCJD | ND | ND | + | + (2/4) |
| A28 | 69/F | sCJD | VV | 2 | + | + (1/4) |
| A29 | 82/M | DLB/PD | | | + | – (0/4) |
| A30 | 70/F | sCJD | ND | 2 | + | + (4/4) |

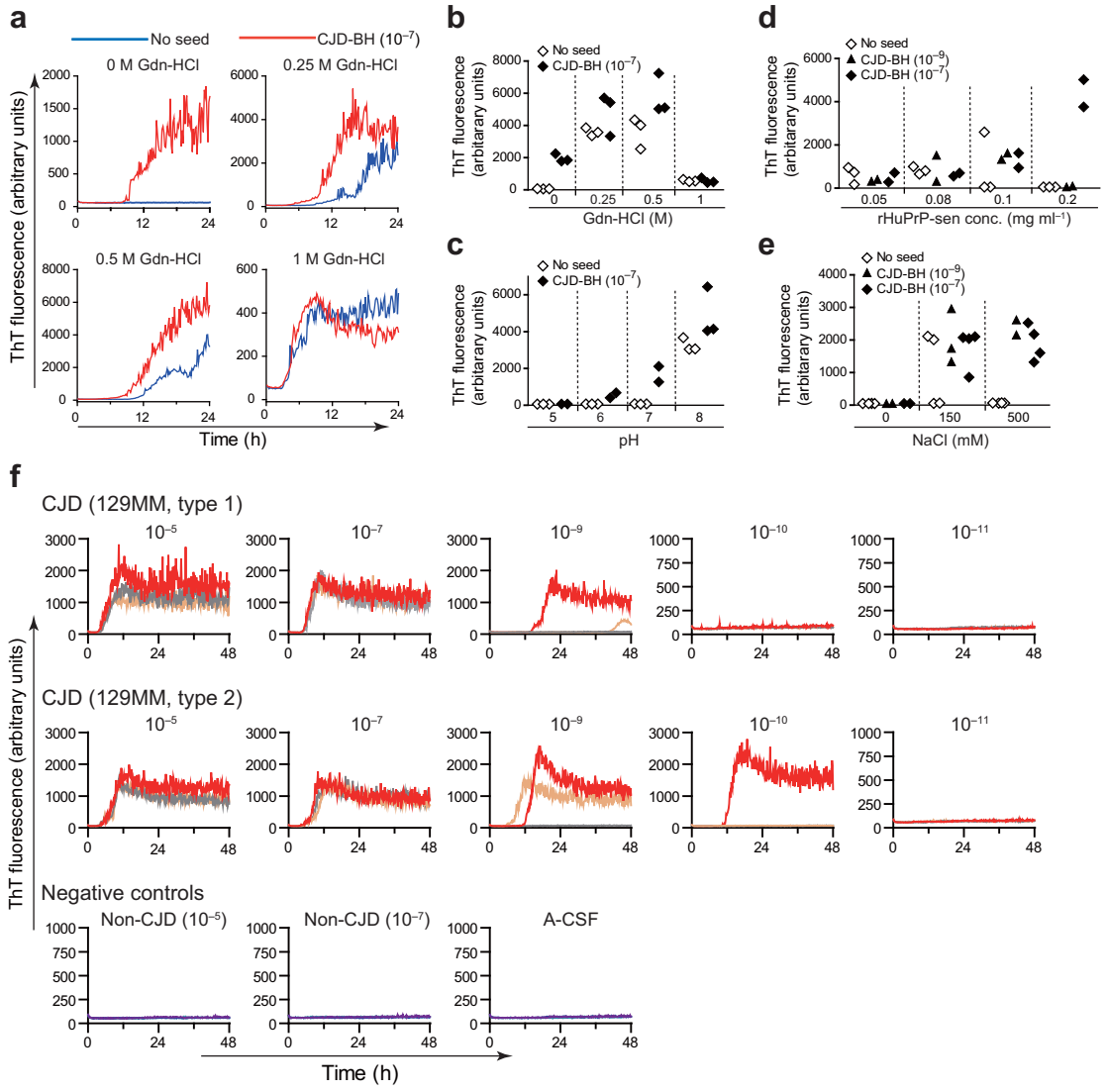
[a]CJD can be divided into six molecular subtypes based on whether methionine (M) or valine (V) is present at codon 129 of the prion protein gene combined with the profile of $PrP^{Sc}$ (type 1 or type 2) as determined by Western blotting[16]. [b]The levels of the γ-isoform of 14-3-3 protein in CSF were determined by Western blotting using polyclonal antibody specific for γ-isoform of 14-3-3 protein[17] (+, positive reaction; –, negative reaction). [c]RT-QUIC was performed as described in **Supplementary Methods**. Positive/number of replicates is shown in parentheses. Samples with at least one positive reaction were defined as +, representing a positive result in the RT-QUIC. [d]The final diagnosis was made by the Australian National CJD Registry. [e]Levels of 14-3-3 protein (all isoforms) in CSF were determined by Western blotting. [f]Additional atypical bands were observed. [g]Kinetics graphs are provided in **Supplementary Figure 3**. Subjects or their families agreed with the aims and significance of our research and gave appropriate informed consent. The investigation protocol was approved by the Ethics Committee of Nagasaki University Hospital (ID: 10042823) and the study was registered with the University Hospital Medical

Information Network (ID: UMIN000003301). sCJD, sporadic Creutzfeldt-Jakob disease; iCJD, iatrogenic Creutzfeldt–Jakob disease; PN/MC, paraneoplastic/metastatic cancer; ICD, inflammatory CNS disorder; DLB/PD, dementia with Lewy bodies/Parkinson disease; ND, not determined.

## FIGURE LEGENDS

**Figure 1** QUIC reactions induce $PrP^{Sc}$-dependent rHuPrP-res formation under GdnHCl-free conditions. (**a,b**) The effect of the indicated concentration of Gdn-HCl on the kinetics of rHuPrP fibril formation with or without $10^{-7}$ dilution of CJD-BH (type 1, 129MM). The reaction buffer contained 150 mM NaCl, 50 mM PIPES pH 7.0, 1 mM EDTA and 10 μM ThT. The concentration of rHuPrP-sen was 0.1 mg $ml^{-1}$. The graphs in **a** depict one representative of triplicates. The maximal fluorescence intensity of each single reaction for 24 h is plotted on **b**. (**c,d,e**) The effect of pH (**c**), the concentrations of rHuPrP-sen (**d**) and the concentration of NaCl (**e**) were tested using the indicated dilutions of CJD-BH (type 1, 129MM) as seeds. Buffers used in **c** were: pH 5; sodium acetate buffer, pH 6; 50 mM MES, pH 7; 50 mM PIPES, pH 8; 50 mM HEPES. The concentration of NaCl in **c** and **d** was 150 mM, the pH of the buffer in **d** and **e** was 7.0 (50 mM PIPES), and the rHuPrP-sen concentration in **c** and **e** was 0.1 mg $ml^{-1}$. Each symbol represents the maximal fluorescence intensity from an individual reaction for 48 h. (**f**) Detection limit of real-time QUIC using the indicated dilutions of CJD-BH (129MM, type 1), CJD-BH (129MM, type 2) as seeds. The indicated dilutions of non-CJD-BH (dissecting aneurysm) or artificial CSF (A-CSF) were used as negative controls. The RT-QUIC reactions were performed as described in **Supplementary**

**Methods**. The colored curves represent the kinetics of ThT fluorescence from an individual reaction seeded with the same dilution of BH. The graphs are representative of two independent experiments, each performed in triplicate.



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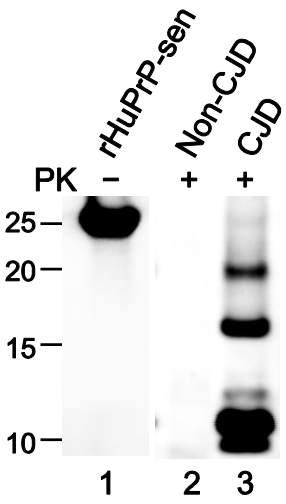
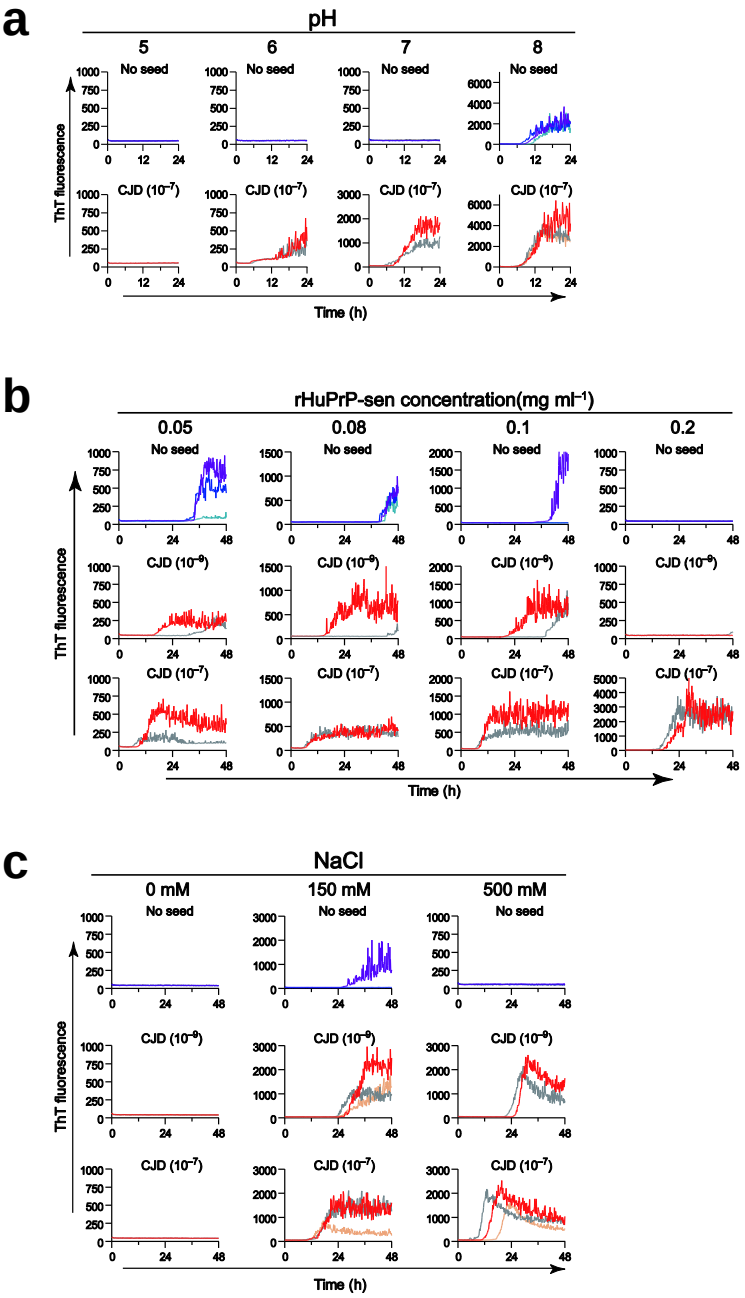
**Supplementary Information**

**Contents:**

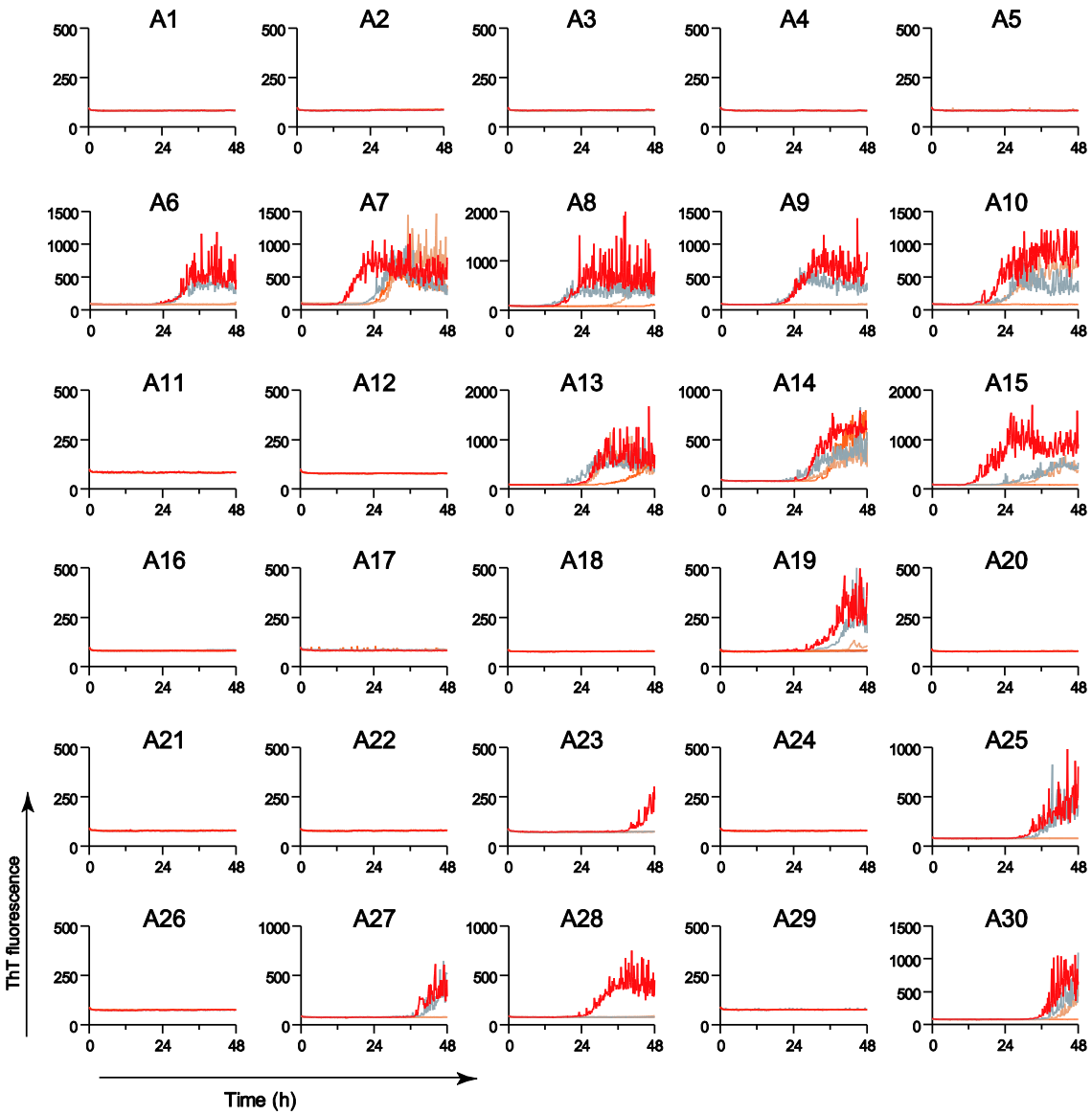



**Supplementary Figure 1** The formation of rPrP-res$^{(Sc)}$ on QUIC reactions seeded with CJD-BH in the absence of GdnHCl. All reaction buffers contained 500 mM NaCl, 50 mM PIPES pH 7.0, 1 mM EDTA and 10 μM ThT. The concentration of rHuPrP-sen was 0.1 mg ml$^{-1}$. After reactions, the samples were digested with 10 μg ml$^{-1}$ of proteinase K (PK). PK-digested samples were immunoblotted using polyclonal anti-PrP antibody R20 (epitope located at human PrP amino acids 218–231)[1]. The QUIC reaction seeded with non-CJD-BH (dissecting aneurysm) produced no rPrP-res (lane 2), whereas the CJD-seeded reaction produced several (20-, 16-, 12-, 11-and 10-kDa) PK-resistant bands (lane3). The banding pattern of rHuPrP-res is similar to that of 263K-seeded rHaPrP-res reported previously[2,3].




**Supplementary Figure 2** The effect of pH, the concentration of rHuPrP-sen and NaCl on QUIC reactions with or without $10^{-7}$ dilutions of sCJD-BH**.** (**a**) All reaction buffers contained 150 mM NaCl, 1 mM EDTA and 10 μM ThT. The concentration of rHuPrP-sen was 0.1 mg ml$^{-1}$. Buffers used were: pH 5, sodium acetate buffer; pH 6, 50 mM MES; pH 7, 50 mM PIPES; pH 8, 50 mM HEPES. Artificial CSF was added to negative controls (no seed). The graphs are representative of two independent experiments, each performed in duplicate or triplicate. The colored curves represent the kinetics of ThT fluorescence from an individual reaction under the same conditions. In QUIC reactions performed at pH 5, 6, 7, and 8, we found that the rate of rPrP-res formation

correlated positively with increases in the pH of the reaction mixture. An early and distinct $PrP^{Sc}$-dependent ThT fluorescence increase was observed at pH 7 within 24 h. There was no emergence of rPrP-res$^{(spon)}$ under these same conditions, indicating that the QUIC exhibits peak sensitivity at pH 7. (**b**) The effect of the concentration of rHuPrP-sen was tested with the indicated dilution ($10^{-9}$ or $10^{-7}$) of CJD-BH (type 1, 129MM) as seeds. All reaction buffers contained 150 mM NaCl, 50 mM PIPES pH 7.0, 1 mM EDTA and 10 μM ThT. Somewhat unexpectedly, we observed that there was an inverse correlation between the rate of rPrP-res formation at pH 7 and the concentration of rHuPrP-sen substrate. Notably, this inverse relationship between the substrate protein concentration and aggregation in a denaturant-free buffer with shaking has also been reported in other proteins[4,5]. In contrast, previous studies using cell-free conversion[6] and rPrP fibril formation[7–9], respectively, in the presence of denaturant or at low pH have shown that the rate of PrP-res formation was directly proportional to the PrP-sen concentration, presumably owing to the homogenous denaturation status of PrP-sen induced by these treatments. (**c**) The effect of the concentration of NaCl. All reaction buffers contained 50 mM PIPES pH 7.0, 1 mM EDTA and 10 μM ThT. The concentration of rHuPrP-sen was 0.1 mg $ml^{-1}$. Assessment of the effect of ionic strength on QUIC reactions showed that salt is needed for rPrP-res formation and the sensitivity of this method was maximal at 500 mM NaCl at pH7. The requirement for salt in rPrP-res formation is consistent with the fact that salt is required for cell-free conversion in the absence of GdnHCl[10] and the maintenance of a protease-resistant $PrP^{Sc}$ conformation[11]. In addition, salt has also been reported to reduce the thermodynamic stability of the rHuPrP-sen[12].




**Supplementary Figure 3** The kinetics graphs of real-time QUIC reactions using 30 CSF samples from Australia. All reactions were performed in quadruplicate. The colored curves represent the kinetics of the ThT fluorescence from an individual reaction seeded with the same CSF.

**Supplementary Table 1** Detection limit of RT-QUIC seeded with the indicated dilutions of CJD-BH (MM 1), CJD-BH (MM 2) as seeds

| dilution | CJD-BH | | Non-CJD-BH |
|---|---|---|---|
| | MM1 | MM2 | dissecting aneurysm |
| $10^{-5}$ | (3/3, 3/3) | (3/3, 3/3) | (0/3, 0/3) |
| $10^{-7}$ | (3/3, 3/3) | (3/3, 3/3) | ND |
| $10^{-9}$ | (2/3, 3/3) | (2/3, 2/3) | (0/3, 0/3) |
| $10^{-10}$ | (0/3, 1/3) | (1/3, 1/3) | ND |
| $10^{-11}$ | (0/3, 0/3) | (0/3, 0/3) | ND |

The RT-QUIC reactions were performed as described in **Supplementary Methods**. Non-CJD-BH (dissecting aneurysm) seeded reactions were used as negative controls. The results shown are from two independent experiments, each performed in triplicate (positive/total reactions). ND, not determined.

**Supplementary Table 2** Clinical data and RT-QUIC reactions using CSF from 35 non-CJD subjects in Japan

| No. | Age(yr)/Sex | Diagnosis | 14-3-3 (γ-isoform) | RT-QUIC |
|---|---|---|---|---|
| N1 | 52/F | FTLD | – | – (0/4) |
| N2 | 65/F | AD | – | – (0/4) |
| N3 | 72/M | AD | – | – (0/4) |
| N4 | 65/F | AD | – | – (0/4) |
| N5 | 75/F | PML | – | – (0/4) |
| N6 | 62/F | FTLD | – | – (0/4) |
| N7 | 63/M | AD | – | – (0/4) |
| N8 | 65/F | AD | – | – (0/4) |
| N9 | 75/F | HE | – | – (0/4) |
| N10 | 68/F | HE | – | – (0/4) |
| N11 | 60/F | AD | – | – (0/4) |
| N12 | 82/M | Epilepsy | – | – (0/4) |
| N13 | 65/M | AD | – | – (0/4) |
| N14 | 66/M | Lymphoma | – | – (0/4) |
| N15 | 45/M | Lymphoma | + | – (0/4) |
| N16 | 52/M | Schizophrenia | – | – (0/4) |
| N17 | 58/M | Schizophrenia | – | – (0/4) |
| N18 | 32/M | AE | – | – (0/4) |
| N19 | 75/F | NMO | + | – (0/4) |
| N20 | 73/F | CBD | + | – (0/4) |
| N21 | 85/F | AD | + | – (0/4) |
| N22 | 87/F | AD | + | – (0/4) |
| N23 | 72/F | AD | – | – (0/4) |
| N24 | 76/F | AD | – | – (0/4) |
| N25 | 56/M | AD | – | – (0/4) |
| N26 | 83/F | AD | – | – (0/4) |
| N27 | 84/F | AD | – | – (0/4) |
| N28 | 62/F | AD | – | – (0/4) |
| N29 | 82/M | AD | – | – (0/4) |
| N30 | 77/F | AD | – | – (0/4) |

| | | | | |
|---|---|---|---|---|
| N31 | 72/F | AD | – | – (0/4) |
| N32 | 79/M | VaD | – | – (0/4) |
| N33 | 69/F | VaD | – | – (0/4) |
| N34 | 85/F | VaD | – | – (0/4) |
| N35 | 66/M | VaD | – | – (0/4) |
| Number of samples with positive reactions/total number of samples | | | | **0/35** |

FTLD, frontotemporal lobar degeneration; AD, Alzheimer disease; PML, progressive multifocal leukoencephalopathy; HE, Hashimoto encephalopathy; AE, anoxic encephalopathy; NMO, neuromyelitis optica; CBD, corticobasal degeneration; VaD, vascular dementia.

**Supplementary Table 3** Comparison of the sensitivity and specificity of RT-QUIC with those of 14-3-3 protein

| | CSF samples in Japan [Pilot study] | | CSF samples from Australia [Blind study] | |
|---|---|---|---|---|
| | RT-QUIC | 14-3-3 (γ-isoform) | RT-QUIC | 14-3-3 (all isoforms) |
| Sensitivity | 83.3% (15/18) | 72.2% (13/18) | 87.5% (14/16) | 87.5% (14/16) |
| Specificity | 100% (0/35) | 85.7% (5/35) | 100% (0/14) | 71.4% (4/14) |

Positive samples/number of samples is shown in parentheses.

**Supplementary Table 4** Summary of the results from RT-QUIC reactions using 155 CSF samples in Japan under blind conditions

| Diagnosis | RT-QUIC[a] | 14-3-3 (γ-isoform)[b] |
|---|---|---|
| sporadic CJD | 25/25 | 25/25 |
| DAT | 0/122 | 3/122 |
| MELAS | 0/2 | 2/2 |
| limbic encephalitis | 0/2 | 2/2 |
| temporal epilepsy | 0/2 | 2/2 |
| PCD/LEMS | 0/2 | 2/2 |

All cases of sporadic CJD were typical in time-course, clinical features and diffusion-weighed image (DWI) in the MR image, and classified as “probable cases” according to the updated WHO diagnostic criteria[13]. Positive samples/number of samples is shown in parentheses. [a]RT-QUIC was performed as described in **Supplementary Methods**. [b]Levels of the γ-isoform of 14-3-3 protein in CSF were determined by Western blotting. DAT, Dementia of Alzheimer’s type; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes; PCD/LEMS, paraneoplastic cerebellar degeneration/Lambert-Eaton myasthenic syndrome.

**Supplementary Methods**

**Recombinant human PrP expression and purification.** Recombinant PrP equivalent in sequence to residues 23–231 of the human PrP sequence (codon 129M) was expressed, refolded into a soluble form (rHuPrP-sen), and purified essentially as described previously[2]. The concentration of rHuPrP-sen was determined by measuring the absorbance at 280 nm. The purity of the final protein preparations was ≥ 99%, as estimated by SDS-PAGE, immunoblotting and liquid chromatography-mass spectrometry, and analysis by circular dichroism showed the α-helical-rich conformation of rHuPrP-sen (data not shown). After purification, aliquots of the proteins were stored at –80 °C in 10mM phosphate buffer, pH 6.8.

**Real-time QUIC.** We prepared reactions in a 96-well optical black bottom plate (Nunc 265301) to a final total volume of 100 μl. To avoid contamination, we prepared non-infectious materials inside a biological safety cabinet in a prion-free laboratory and used aerosol-resistant tips. Unless indicated otherwise, the final concentrations of reaction buffer components were 500 mM NaCl, 50 mM PIPES pH 7.0, 1 mM EDTA and 10 μM Thioflavin T. We used only freshly-thawed rHuPrP-sen. We observed mild variations in the optimal rHuPrP-sen concentration (0.06–0.1 mg $ml^{-1}$) between the lots

of rHuPrP-sen, but the final sensitivity was approximately the same. Diluted BH or CSF (5 μl well$^{-1}$) was used as seeds for the RT-QUIC reactions. Artificial CSF (A-CSF, 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, 50 mg dl$^{-1}$ glucose, 20 mg dl$^{-1}$ BSA) was added to the negative controls; we refer to these as no-seed controls. Besides the reactions seeded with diluted BH or the CSF samples, we always conducted no-seed controls to confirm that no rPrP-res$^{(spon)}$ formation occurred in the RT-QUIC. Dilutions of the seed-BHs were carried out in A-CSF immediately prior to the reactions. The 96-well plate was covered with sealing tape (Nunc 236366) and incubated at 37 °C in a plate reader (Infinite M200 or F200 fluorescence plate reader; TECAN) with intermittent shaking, consisting of 30 s of circular shaking at the highest speed and no shaking for 30 s, with a 2-min pause to measure the fluorescence. The kinetics of fibril formation was monitored by the bottom reading of the fluorescence intensity every 10 min using 440-nm excitation and 485-nm emission wavelength of monochromators (Infinite M200) or filters (Infinite F200). Although RT-QUIC reactions usually give all-or-none responses, the elevation of fluorescence was relatively slow and the peak was occasionally lower when the dilution of the BHs was very high (e.g. more than $10^{-9}$). In those cases, we regarded the well of a microplate as exhibiting a positive reaction when the fluorescence increase exceeded 200% of

baseline fluorescence of the buffer-only controls over six consecutive readings.

**Subjects.** Type 1, codon 129 MM (MM1) and type 2, codon 129 MM (MM2) CJD brain tissues were obtained from individuals who had received a confirmed diagnosis of CJD at Nagasaki University Hospital. Unfortunately, CJD-BH with codon 129 MV or VV were not available because these polymorphisms are uncommon in Japan[14,15]. Non-CJD brain tissue was obtained from a person with a dissecting aneurysm at Tohoku University Hospital. CJD-CSF samples for the pilot study were collected from 18 definite CJD subjects who were diagnosed according to WHO criteria through the CJD Surveillance Committee in Japan. All of these subjects were homozygous for MM at polymorphic PrP codon 129. Non-CJD-CSF samples for the pilot study were collected from 35 subjects with other diseases at Nagasaki University Hospital, or through the Japan CJD Surveillance Committee. To perform a blind trial of RT-QUIC, 30 CSF samples were sent from the Australian National CJD Registry, without any information other than the sample number. An additional blind test was performed on 155 CSF samples, containing 25 probable cases of CJD, the diagnosis of which had been made in accordance with the updated clinical diagnostic criteria for CJD published in 2009[13], and 130 cases of other neurological diseases, kindly provided by Dr. Tsujihata at

Nagasaki Kita Hospital. Subjects or their families agreed with the aims and significance of our research and gave appropriate informed consent. The investigation protocol was approved by the Ethics Committee of Nagasaki University Hospital (ID: 10042823) and the study was registered with the University Hospital Medical Information Network (ID: UMIN000003301).

**Preparation of brain homogenates.** Brain tissues were homogenized at 10% (w/v) in ice-cold PBS supplemented with a protease inhibitor mixture (Roche) using a multi-bead shocker (Yasui Kikai, Osaka, Japan). After centrifugation at 2,000 g for 2 min, supernatants were collected and frozen at –80 °C until use. Total protein concentrations were determined by the BCA protein assay (Pierce). The $PrP^{Sc}$ concentrations in the BHs were estimated by dot-blot analysis using a reference standard of rHuPrP-sen, as described previously[16].

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