

# Evaluation of the effect of various main elements on the PrP<sup>Sc</sup> detection by real-time quaking-induced conversion assay

KANG XIAO<sup>1,2\*</sup>, QI SHI<sup>1,2\*</sup>, WEI ZHOU<sup>1,2</sup>, CHEN GAO<sup>1,2</sup>, CAO CHEN<sup>1,2</sup>,  
BAO-YUN ZHANG<sup>1,2</sup>, JING WANG<sup>1,2</sup> and XIAO-PING DONG<sup>1,2</sup>

<sup>1</sup>State Key Laboratory for Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases (Zhejiang University), Hangzhou, Zhejiang 310003; <sup>2</sup>National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, P.R. China

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**Abstract.** In order to definitively diagnosis sporadic Creutzfeldt-Jakob disease (sCJD), brain tissue is currently required. Therefore, there is a great need for tests that can detect sCJD in body fluids or other types of tissues. Different variables, including the amount of recombinant cellular prion protein (rPrP<sup>C</sup>), salt, cleaning surfactants and thioflavin T (ThT), in human cerebrospinal fluid (CSF) were evaluated. The reagent concentrations of 1X PBS, 170 mM NaCl, 1 mM EDTA, 0.01 mM ThT and 0.001% SDS, and the amounts of 10  $\mu$ g rPrP<sup>C</sup> and 10  $\mu$ l CSF were considered to be optimal for the real-time quaking-induced conversion (RT-QuIC) assay. Using these conditions, the RT-QuIC assay for prion protein (PrP<sup>Sc</sup>) detection was observed to be sensitive to 10<sup>-8</sup> diluted brain homogenates of hamsters infected with the 263K scrapie strain. Furthermore, CSF samples from 70 probable sCJD cases and 48 non-CJD cases were preliminarily screened. A substantial proportion of sCJD samples (57.14%) tested positive by RT-QuIC, with a short lag phase (<50 h post-reaction) and high peak ThT values (>25,000 relative fluorescence units). By contrast, only a small number of non-CJD samples displayed weakly positive results, and these were detected at a later stage (>50 h post-reaction) and had much lower ThT values. In conclusion, the RT-QuIC assay in CSF samples reported in the present study may provide a useful pre-mortem tool for the diagnosis of sCJD, particularly in China where postmortem examination is rarely conducted.

## Introduction

Prion disease, or transmissible spongiform encephalopathy, refers to a group of fatal neurodegenerative disorders reported in humans and animals (1). Prion diseases in humans include Creutzfeldt-Jakob disease (CJD), fatal familial insomnia and Gerstmann-Sträussler-Scheinker syndrome. The most common form of human prion disease is sporadic CJD (sCJD), with a worldwide incidence of ~1 case per million individuals annually (2,3). Typically, sCJD presents with rapidly progressing ataxia, dementia and myoclonus. The clinical duration of the disease is usually <2 years, with the majority of patients succumbing to the disease within 6 months.

A definitive diagnosis of sCJD requires neuropathological or immunochemical detection of the prion protein (PrP<sup>Sc</sup>) (4). PrP<sup>Sc</sup> is partially protease-resistant and can induce its normal cellular isoform (PrP<sup>C</sup>), to undergo a conformational change. This occurs in a self-propagating manner through a seeded aggregation process, resulting in accumulation of PrP<sup>Sc</sup> throughout the brain tissues, together with accompanying spongiform alterations, neuronal loss and gliosis (1,5,6). Other than brain biopsy, there is currently no other disease-specific pre-mortem diagnostic test for sCJD (7). Diagnosis of probable sCJD is based on abnormal findings as determined by clinical examinations and laboratory tests. Abnormal test results include periodic sharp wave complexes on electroencephalogram, and altered signals on brain magnetic resonance image and/or positive detection of 14-3-3 protein in the cerebrospinal fluid (CSF) (8). Postmortem examinations are rarely performed in China due to cultural traditions (9). Therefore, methods that permit the accurate diagnosis of sCJD are required.

Numerous studies have attempted to identify biomarkers in CSF samples for the diagnosis of human prion diseases. Proteins that have showed diagnostic values include 14-3-3, tau, S100 and neuron specific enolase (10-13). However, only a positive test result for 14-3-3 protein in the CSF using western blotting is included in the diagnostic criteria for sCJD (14,15). The detection of PrP<sup>Sc</sup> in the CSF of patients with sCJD and other types of human prion diseases is difficult with routine testing methods, even using the sensitive protein-misfolding cyclic amplification (PMCA) technique.

*Correspondence to:* Dr Qi Shi or Dr Xiao-Ping Dong, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 155 Chang-Bai Road, Beijing 102206, P.R. China  
E-mail: shiqi76@126.com  
E-mail: dongxp238@sina.com

\*Contributed equally

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Recently, a novel technique known as real-time quaking-induced conversion (RT-QuIC) has been developed that is based on amyloid fibril formation, a characteristic feature of prion proteins (16-18). A number of studies have identified that RT-QuIC has good sensitivity and specificity for the diagnosis of sCJD using human CSF samples (16,17). In the present study, a number of experimental variables that may influence RT-QuIC assay were evaluated. Using the identified optimal conditions, the capacity of the RT-QuIC assay to detect PrP<sup>Sc</sup> in the brain homogenates of 263K-infected hamsters and in the CSF samples of probable sCJD patients was also evaluated.

## Materials and methods

**Ethics statement.** The present study was approved by the Ethical Committee of the National Institute for Viral Disease Control and Prevention (Beijing, China) under the protocol 2009ZX10004-101, including the use of brain samples of hamsters infected with the scrapie strain 263K and CSF samples from probable sCJD patients and non-CJD patients. Informed consent was obtained from all patients prior to participation.

**Expression and purification of the hamster recombinant PrP (rPrP) protein.** Hamster rPrP<sup>C</sup> protein (residues 23-231; GenBank accession no. K02234) was prepared according to the method described by Wilham *et al* (18) with a few modifications. Briefly, the DNA sequence of hamster PrP<sup>C</sup> was ligated into the pRSET vector (cat. no. V35120; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and then the recombinant plasmid was transformed into BL21 (DE3)pLysS competent cells (cat. no. C1500; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). We expressed rPrP<sup>C</sup> in 1 liter terrific broth medium (cat. no. 71491; Novagen; Merck KGaA, Darmstadt, Germany). Next, rPrP<sup>C</sup> protein was denatured with guanidine-HCl and purified by chromatography using Ni-NTA Superflow resin (cat. no. 30430; Qiagen, Hilden, Germany) in an XK 16/40 column (GE Healthcare Life Sciences, Little Chalfont, UK) at a flow rate of 1.9 ml/min. The concentration of rPrP<sup>C</sup> was adjusted to ~500 µg/ml as determined by the bicinchoninic acid reagent (cat. no. 71285; EMD Millipore, Billerica, MA, USA). The purity of the prepared rPrP<sup>C</sup> was evaluated by 15% SDS-PAGE and western blot analysis. Briefly, proteins were transferred onto a nitrocellulose filter membrane (cat. no. 10600001; GE Healthcare Life Sciences), which was then blocked with 5% skim milk in tris-buffered saline with 0.1% Tween-20 containing 0.1% Tween-20 for 1 h followed by incubation with the primary antibodies against 3F4 (cat. no. MAB1562-K; EMD Millipore) and β-actin (HX1827, Beijing Huaxing Bochuang Gene Technology, Co., Ltd., Beijing, China) overnight at 4°C. Subsequently, a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat. no. 31430; Thermo Fisher Scientific, Inc.) was incubated at 37°C for 2 h. Both primary and secondary antibodies were used at a dilution of 1:5,000. The results were scanned with Bio-Rad Molecular Imager (Bio-Rad Laboratories, Inc., Hercules, California, USA) and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Preparation of brain homogenate (BH) samples.** A total of 10 3-week-old female Syrian golden hamsters (45.8±1.3 g; cat. no. 501; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were intracerebrally inoculated with 5 µl hamster-adapted scrapie agents 263K (19,20). Animals were housed with free access to food and water under a humidity between 40-70%, temperature between 20-26°C and a 12 h light/dark cycle. The incubation time of the 263K-infected hamsters was 70.5±4.93 days (21,22). Animals were sacrificed using ether and exsanguinated, and then the brains were surgically removed from each hamster. Subsequently, 10% BHs were prepared in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.5) according to a previously described protocol (21,22). In order to set up a PrP<sup>Sc</sup> panel for RT-QuIC, serially diluted (from 10<sup>-3</sup> to 10<sup>-9</sup>) Sc-BH samples were prepared. Proteinase K-resistant PrP signals were detectable in Sc-BH samples of 10<sup>-3</sup> using routine western blotting.

**CSF samples.** A total of 70 CSF samples from probable sCJD (63.3±8.8 years old, male/female ratio: 1.12) and 48 CSF samples from non-CJD patients (55.1±14.5 years old, male/female ratio: 1.28) were included in the current study. Informed consent was obtained from all patients prior to participation. All the samples were collected at the CJD surveillance network of hospitals without any additional treatments and sent to the CJD Surveillance Center during Jan to Dec in 2015. The diagnosis of probable sCJD was conducted according to the diagnostic criteria for CJD issued by the World Health Organization and the surveillance document for CJD issued by Chinese Center for Disease Control and Prevention (Beijing, China) (9). Non-CJD cases included patients whose clinical manifestations and examinations did not fulfill the diagnostic criteria for human prion disease even after follow-up, or patients who had other diagnoses. The samples were collected from the CSF Bank in the China CJD Surveillance Center attached to China CDC (Beijing, China), and 200 µl from each CSF sample was obtained. All enrolled CSF samples were free of blood contamination. Routine CSF biochemistry assays of those specimens, including cell count, glucose level and total protein, were all within the normal ranges.

**Evaluation of the effect of various main elements on the reactivity of RT-QuIC.** To identify any factors that affect the RT-QuIC assay for detecting PrP<sup>Sc</sup>, a set of variables were independently evaluated. The basic elements in the working buffer were 1X PBS, 85-340 mM NaCl, 0.5-2 mM EDTA, 10-80 µM thioflavin T (ThT) and 5-20 µg rPrP<sup>C</sup>. These concentrations were selected based on published data and our previous experiments (16,23). In addition, 10<sup>-5</sup> diluted BH from 263K-infected hamster at the terminal stage (Sc-BH) and BH from age-matched normal hamsters (Nor-BH) were also prepared as the positive and negative controls, respectively.

**RT-QuIC assay.** RT-QuIC was conducted in a black 96-well, optical-bottomed plate (Nunc 265301; Thermo Fisher Scientific, Inc.) on a BMG FLUOstar microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). Next, 1 µl diluted BH or 5-30 µl CSF sample was mixed with 1X PBS,

170 mM NaCl, 1 mM EDTA, 0.01 mM ThT, 0.001% SDS and 10  $\mu$ g rPrP<sup>C</sup> in a final volume of 100  $\mu$ l. Each sample was assayed in triplicated. Each reaction contained the following control groups: Blank (reaction buffer only), positive (Sc-BH) and negative (Nor-BH). The working conditions were as follow: Temperature, 50°C; vibration speed, 1.996 x g; vibration/incubation time, 90/30 sec; total reaction time, 90 h. ThT fluorescence (excitation wavelength, 450 nm; emission wavelength, 480 nm) was automatically measured every 30 min and expressed as relative fluorescence units (rfu). The cutoff value was set as the mean value of the negative controls plus 3 times the standard deviation. A sample was considered to be positive when  $\geq 2$  wells revealed positive reaction curves.

## Results

**Determination of appropriate rPrP<sup>C</sup> concentration.** Different amounts (5, 10, 15 and 20  $\mu$ g) of purified rPrP<sup>C</sup>, serving as the substrate, were subjected to RT-QuIC. Using Sc-BH as the seed, which induces the fibrillation of rPrP<sup>C</sup>, increases in the ThT fluorescence curves were observed in the reactions of 10, 15 and 20  $\mu$ g rPrP<sup>C</sup>, which began to increase at ~30 h post-reaction, while only a slight elevation of the ThT value was detected in the 5  $\mu$ g rPrP<sup>C</sup> reaction (Fig. 1). However, a positive increase of ThT value was also recorded in part of duplication of 20  $\mu$ g rPrP<sup>C</sup> using Nor-BH as the seed, indicating a false positive reaction. Based on these data, 10  $\mu$ g rPrP<sup>C</sup> was suggested to be the optimal working amount as the substrate in RT-QuIC.

**Determination of appropriate NaCl concentration.** Different concentrations of NaCl (0, 85, 170, 255 and 340 mM) were examined. In the condition of 10<sup>-5</sup> diluted Sc-BH, four preparations containing NaCl demonstrated positive reaction. The RT-QuIC reactivity was revealed to be NaCl dose-dependent. Along with the increase in the amount of NaCl, the positive conversion time post-reaction was decreased and the peak values of ThT values increased. However, increased ThT curves were also observed in the preparations of negative control containing 255 and 340 mM NaCl. This suggested that a high concentration of NaCl was able to increase the reactivity of RT-QuIC, as well as increase the possibility of false positive (Fig. 2). Thus, the NaCl concentration of 170 mM was selected for subsequent experiments.

**Determination of appropriate EDTA concentration.** Different concentration of EDTA (0, 0.5, 1, 1.5 and 2 mM) were also tested in the RT-QuIC assay containing 10<sup>-5</sup> diluted Sc-BH. After 34 h, the reaction curve with 1 mM EDTA began to increase, followed by the curves of the 1.5 and 2 mM EDTA reactions. At a relatively late stage, the reactions with 0 and 0.5 mM EDTA exhibited weak positive results. These data illustrated that certain concentrations of EDTA (particularly 1 mM) help the reactivity of RT-QuIC assay, whereas high concentrations of EDTA inhibit the assay (Fig. 3).

**Determination of appropriate ThT final concentration.** Four different final concentrations of ThT (10, 20, 40 and 80  $\mu$ M) were also examined in the RT-QuIC assay containing 10<sup>-5</sup> diluted

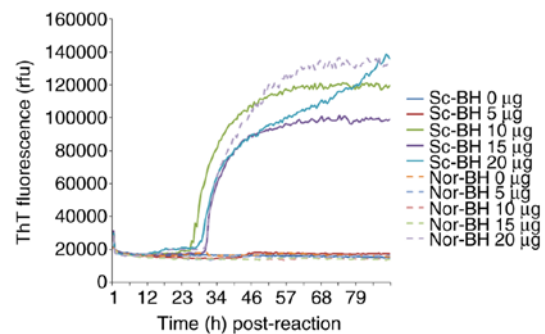


Figure 1. Effects of different rPrP<sup>C</sup> concentrations on the detection capacity of real-time quaking-induced conversion assay. Different amounts of rPrP<sup>C</sup> (0, 5, 10, 15 and 20  $\mu$ g) were added into the samples containing 10<sup>-5</sup> diluted BH from 263K hamsters or normal controls. ThT, thioflavin T; rfu, relative fluorescence units; rPrP<sup>C</sup>, recombinant cellular prion protein; Sc-BH, BH from 263K-infected hamster at the terminal stage; Nor-BH, BH from age-matched normal hamsters; BH, brain homogenate.

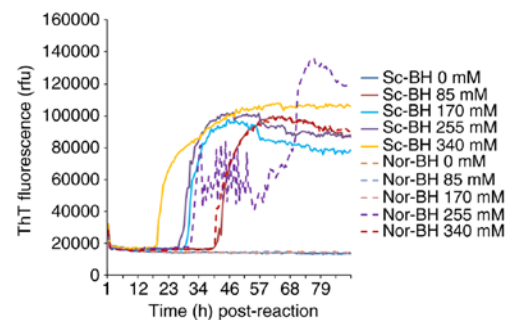


Figure 2. Effects of different concentrations of NaCl on real-time quaking-induced conversion assay. Different concentrations (0, 85, 170, 255 and 340 mM) of NaCl were added into samples containing 10<sup>-5</sup> times diluted BH from 263K hamsters or normal controls. ThT, thioflavin T; rfu, relative fluorescence units; Sc-BH, BH from 263K-infected hamster at the terminal stage; Nor-BH, BH from age-matched normal hamsters; BH, brain homogenate.

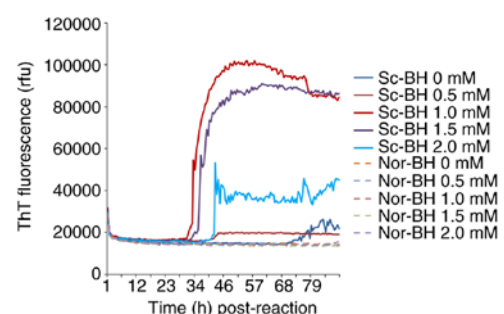


Figure 3. Effects of different concentrations of EDTA on real-time quaking-induced conversion assay. Different concentrations (0, 0.5, 1, 1.5 and 2 mM) of EDTA were added into samples containing 10<sup>-5</sup> diluted BH from 263K hamsters or normal controls. ThT, thioflavin T; rfu, relative fluorescence units; Sc-BH, BH from 263K-infected hamster at the terminal stage; Nor-BH, BH from age-matched normal hamsters; BH, brain homogenate.

Sc-BH. All four preparations of PrP<sup>Sc</sup> exhibited positive curves at approximately 9-12 h post-reaction, and reached similarly high ThT fluorescence values after 30 h of reaction (Fig. 4). However, increased curves were also observed in the negative controls containing 20, 40 and 80  $\mu$ M ThT, although these

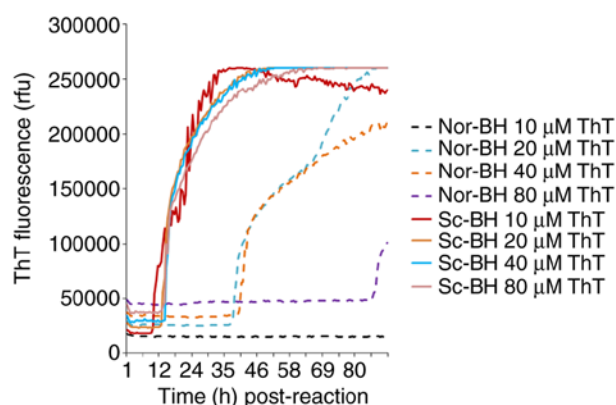


Figure 4. Effects of different concentrations of ThT on real-time quaking-induced conversion assay. Different concentrations (10, 20, 40 and 80  $\mu$ M) of ThT were added into the samples containing  $10^{-5}$  diluted BH from 263K hamsters or normal controls. ThT, thioflavin T; rfu, relative fluorescence units; Sc-BH, BH from 263K-infected hamster at the terminal stage; Nor-BH, BH from age-matched normal hamsters; BH, brain homogenate.

emerged with relatively long lag phases (Fig. 4). These findings suggest that an increase in ThT concentration does not enhance the sensitivity of RT-QuIC assay and provides false positive results.

**Determination of appropriate SDS concentration.** Different concentrations of SDS were added to reactions containing  $10^{-5}$  diluted Sc-BH, resulting in final concentrations of SDS between 0.1 and 0.00001%. As shown in Fig. 5A, positive reaction curves were observed in the preparations of 0.001, 0.0001 and 0.00001% SDS, among which the reaction using 0.001% SDS exhibited a markedly shorter lag phase and higher ThT value. Positive reactivities were markedly inhibited in the presences of 0.1 and 0.01% SDS, and a false weak positive reaction was observed in the preparation of Nor-BH containing 0.01% of SDS. Furthermore, the effect of 0.001% SDS on the reactivity of different amounts of PrP<sup>Sc</sup> was evaluated. In the conditions of 263K BH diluted for  $10^{-5}$  and  $10^{-7}$  times, the presence of 0.001% SDS caused a evidently quicker increase and higher ThT fluorescence values (Fig. 5B).

**Determination of appropriate CSF amount.** To test the potential influence of human CSF on the reactivity in RT-QuIC assays,  $10^{-5}$  times diluted Sc-BH was added into 30  $\mu$ l CSF collected from a non-CJD patient with major indexes in the CSF biochemistry within the normal range. Compared with the same dilution of Sc-BH only, the lag time and fluorescence peak in the reaction of Sc-BH in CSF were evidently longer and lower, respectively (Fig. 6A). Furthermore,  $10^{-5}$  times diluted Sc-BH was separately mixed with 5, 10 and 30  $\mu$ l CSF samples. The RT-QuIC assay results demonstrated that the reactions with 5 and 10  $\mu$ l CSF displayed similar positive reactivities with a much shorter lag phase and higher fluorescence peak in comparison with that of 30  $\mu$ l CSF (Fig. 6B). Thus, it appears that certain unknown components of human CSF may hamper the RT-QuIC assay; therefore, using relatively large amount of tested CSF in RT-QuIC does not benefit the detecting ability for PrP<sup>Sc</sup>.

**Final experimental conditions.** Based on the aforementioned data, the experimental conditions of RT-QuIC were set-up, and these involved the final concentrations of 1X PBS, 170 mM NaCl, 1 mM EDTA, 0.01 mM ThT and 0.001% SDS with 10  $\mu$ g rPrP<sup>C</sup> and 10  $\mu$ l CSF in a total reaction volume of 100  $\mu$ l. The positive control was  $10^{-5}$  diluted BH collected from 263K-infected hamsters and the negative control was the same dilution of BH obtained from normal hamsters.

**Evaluation of the capacity of the optimized RT-QuIC assay in the detection of PrP<sup>Sc</sup> in brain tissues and CSF samples.** The serially diluted Sc-BHs ( $10^{-3}$  to  $10^{-9}$ ) were separately subjected to the optimized RT-QuIC reactions. As shown in Fig. 7, the ThT fluorescence values of the preparations of blank control and Nor-BH were almost unchanged at 90 h post-reaction. By contrast, the ThT values of the reactions containing relatively high amounts of Sc-BH (between  $10^{-3}$  and  $10^{-6}$ ) increased markedly after lag phases of approximately 9–12 h post-reaction. The preparations of  $10^{-7}$  and  $10^{-8}$  diluted PrP<sup>Sc</sup> BHs exhibited increased ThT values at 37 and 52 h post-reaction, respectively. No positive result was observed in the reaction of  $10^{-9}$  diluted PrP<sup>Sc</sup> BH. Furthermore, the ThT fluorescence in the preparations containing high amounts of PrP<sup>Sc</sup> was evidently higher in comparison with that of reactions containing low amounts of PrP<sup>Sc</sup>. The PrP<sup>Sc</sup> detection threshold of the RT-QuIC assay reported in the present study was observed to be  $10^{-8}$  diluted BH of 263K-infected hamsters.

To access the efficacy of the optimized RT-QuIC assay in human sCJD CSF samples, CSF samples from 70 patients that fulfilled the diagnostic criteria for probable sCJD and 48 patients that were classified as non-CJD were assayed. In total, 10 or 30  $\mu$ l CSF sample from each patient was separately tested. Using 30  $\mu$ l CSF, 11 (15.71%) samples in the probable sCJD group tested positive, while all tested samples in the non-CJD group were negative. Positive conversion times varied between 8 and 28 h post-reaction, while the peak ThT value varied between 44,000 and 61,000 rfu. Using 10  $\mu$ l CSF, 40 (57.14%) samples of probable sCJD tested positive, whereas 11 samples of non-CJD also exhibited weakly positive results. Further analysis of the positive reaction patterns in the RT-QuIC assay revealed notably different profiles between the groups of probable sCJD (28 samples) and non-CJD (11 samples). As shown in Fig. 8, the majority of the samples from the probable sCJD group tested positive within 50 h post-reaction (27/28 samples), and exhibited peak ThT values of >25,000 rfu (26/28 samples). By contrast, only 1 case of non-CJD tested positive within 50 h post-reaction, and the peak ThT values of all non-CJD case were <25,000 rfu. These data indicate that applying the CSF RT-QuIC assay with the aforementioned experimental conditions was able to detect substantially more probable sCJD cases, with a significantly shorter lag phase (<50 h post-reaction) and higher ThT fluorescence values (>25,000 rfu).

## Discussion

The results of RT-QuIC, which is considered to be a sensitive assay, can be affected by a variety of factors. Under a specific working temperature and vibration speed, the effect of certain variables on the RT-QuIC detection capacity was evaluated



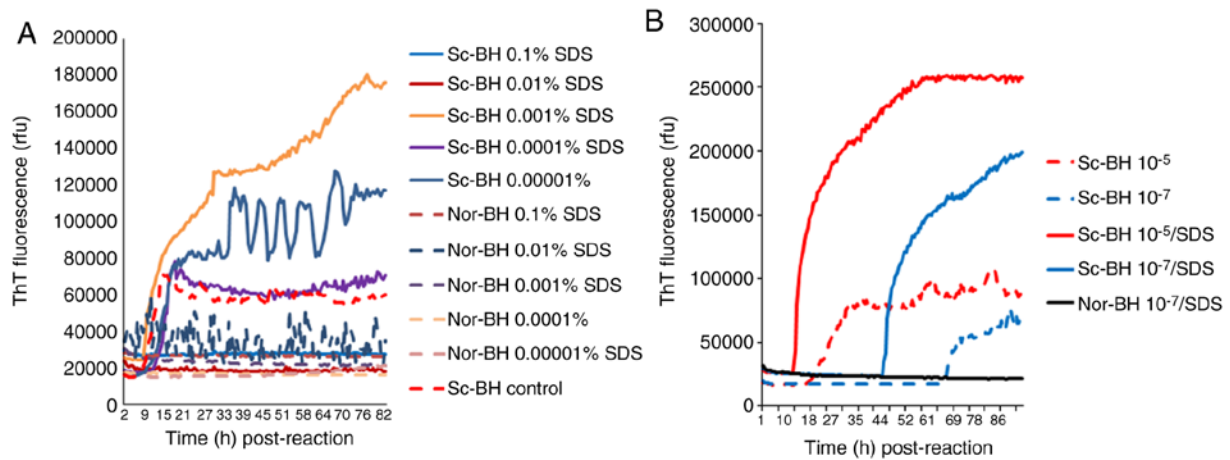


Figure 5. Effects of different concentrations of SDS on real-time quaking-induced conversion assay. (A) Assay of different SDS concentrations in samples containing a fixed amount of PrP<sup>Sc</sup>. Different final concentrations (0.1-0.00001%) of SDS were added into the tested samples containing 10<sup>-5</sup> diluted BH from 263K hamsters or normal controls. (B) Assay of a fixed amount of SDS in samples containing different amounts of PrP<sup>Sc</sup>. Samples containing 10<sup>-5</sup> and 10<sup>-7</sup> diluted 263K hamster BH were mixed with a final concentration of 0.001% SDS. PrP<sup>Sc</sup>, prion protein; ThT, thioflavin T; rfu, relative fluorescence units; Sc-BH, BH from 263K-infected hamster at the terminal stage; Nor-BH, BH from age-matched normal hamsters; BH, brain homogenate.

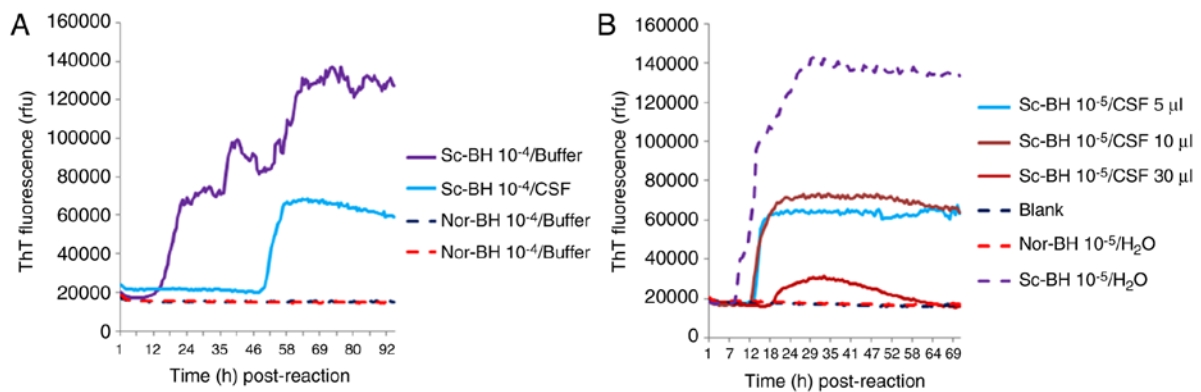


Figure 6. Effects of different volumes of CSF on real-time quaking-induced conversion assay. (A) Examination of reactions with or without human CSF. Samples containing 10<sup>-4</sup> diluted 263K hamster BH were mixed with or without 30 µl CSF. (B) Examination of reactions with different amounts of human CSF (5, 10 and 30 µl) added into the samples containing 10<sup>-5</sup> diluted 263K hamster BH. CSF, cerebrospinal fluid; ThT, thioflavin T; rfu, relative fluorescence units; Sc-BH, BH from 263K-infected hamster at the terminal stage; Nor-BH, BH from age-matched normal hamsters; BH, brain homogenate.

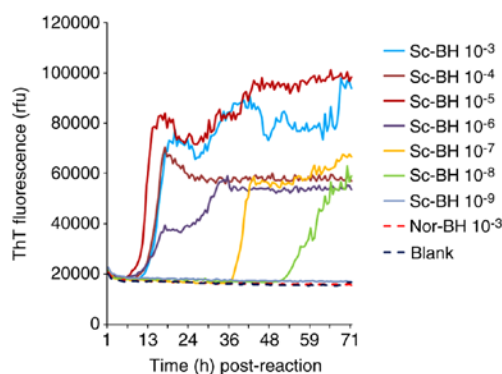


Figure 7. Sensitivity of the real-time quaking-induced conversion assay for detecting PrP<sup>Sc</sup>. Different dilutions (10<sup>-3</sup> to 10<sup>-9</sup>) of 263K hamster BH were examined. Samples of 10<sup>-3</sup> to 10<sup>-6</sup> diluted 263K hamster BH exhibited positive reactions after lag phases of 9-12 h. Samples of 10<sup>-7</sup> and 10<sup>-8</sup> diluted BH displayed positive curves at 37 and 52 h post-reaction. No positive reaction was recorded for the sample containing 10<sup>-9</sup> dilution, as well as those of normal hamster brain or blank control. PrP<sup>Sc</sup>, prion protein; ThT, thioflavin T; rfu, relative fluorescence units; Sc-BH, BH from 263K-infected hamster at the terminal stage; Nor-BH, BH from age-matched normal hamsters; BH, brain homogenate.

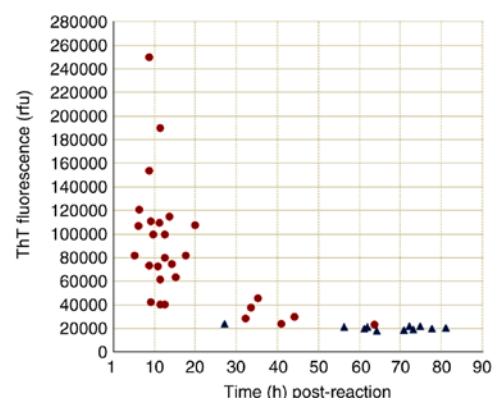


Figure 8. Dot plot of the positive CSF real-time quaking-induced conversion assay results for the probable sCJD and non-CJD groups. A total of 28 CSF samples from probable sCJD cases (red dots) and 11 CSF samples from non-CJD cases (blue triangles) with positive results were included. Among the probable sCJD samples, 27 out of 28 exhibited positive results within 50 h post-reaction, while the peak ThT values of 26 cases were >25,000 rfu. Only 1 non-CJD case was positive within 50 h post-reaction, and the peak ThT values of all the non-CJD cases were <25,000 rfu. CSF, cerebrospinal fluid; CJD, Creutzfeldt-Jakob disease; ThT, thioflavin T; rfu, relative fluorescence units.

in the present study. Similarly to a previous study (12), a type of *E. coli*-expressed full-length wild-type hamster rPrP was used as substrate in the RT-QuIC assay reported in the current study. In a certain range of rPrP<sup>C</sup> concentrations, the sensitivity of RT-QuIC exhibits a positive association with the amount of the input rPrP<sup>C</sup>, however, false positive results are also easily inducible. PrP protein is capable of spontaneous fibrillation *in vitro* when incubated at certain conditions, for it was reported that agitation alone induces *de novo* conversion of recombinant prion proteins to  $\beta$ -sheet rich fibrils (24). Using PMCA, a wild-type mouse rPrP protein can be converted into a pathogenic isoform that has the biochemical characteristics of PrP<sup>Sc</sup> *in vitro*, as well as the typical infectivity on experimental rodents (25). Therefore, careful consideration of the rPrP<sup>C</sup> amount and of the normal negative control is important, particularly when using different batches of purified rPrP<sup>C</sup> protein.

Partially denatured rPrP<sup>C</sup> protein may help to form fibrils in RT-QuIC assay. In addition, using specific concentrations of PBS, NaCl, EDTA and SDS in the working buffer benefits the induction of earlier and higher positive reactions in the RT-QuIC assay. Meanwhile, excessive concentrations of salt and cleaning surfactants lead to a false positive result or inhibit the reactivity of RT-QuIC. ThT is a commonly used chemical for diagnosis of the amyloid structure; however, it is not perfectly specific for amyloid (26). The spectroscopic change of ThT may differ largely, depending on the particular protein and experimental conditions. In the present study, it was also observed that a high concentration of ThT induced evidently false positive reactions in the negative control sample. Thus, a careful balance of the use of salt, cleaning surfactants and ThT concentrations in the reaction buffer is essential for ensuring the sensitivity and specificity of RT-QuIC assay.

The current study also demonstrated that the amount of human CSF used can inhibit the RT-QuIC assay. Human CSF samples with normal biochemical profiles significantly reduced the ThT fluorescence intensity and prolonged the lag time until a positive reaction was detected. These results suggested that there were certain unknown factors in human CSF that inhibited the PrP<sup>Sc</sup> amyloid formation in the RT-QuIC assay. An improved RT-QuIC assay sensitivity was detected in reactions using a relatively low amount of human CSF (5 and 10  $\mu$ l) under the experimental conditions of the current study. Further studies identifying and removing any inhibitor(s) in human CSF would improve the sensitivity of CSF RT-QuIC assay for sCJD detection.

Using optimized experimental conditions in the present study, samples containing 10<sup>-8</sup> dilution of 263K hamster BH were successfully detected, which indicated a higher PrP<sup>Sc</sup> detection capacity in comparison with that of routine western blot analysis (10<sup>-3</sup>) and that of PMCA (10<sup>-5</sup>) using 10% BH of normal hamsters as a substrate (27). A number of CSF samples from probable CJD and non-CJD patients were also preliminarily screened in the present study. Approximately 60% of the CSF samples from the probable sCJD patient group tested positive. Notably, the majority of these results occurred within 50 h post-reaction (median, 11.85 h), and had high peak ThT fluorescence values (median, 77,500 rfu). Certain CSF samples from the non-CJD patient group also

exhibited weakly positive reactions, however, these had markedly longer lag phases (median, 70.70 h post-reaction) and evidently reduced peak ThT values (median, 21,000 rfu). On the basis of these data, it is proposed that <50 h post-reaction and/or >25,000 rfu should be used as the cut-off values for positive test results when applying the CSF RT-QuIC assay with the experimental conditions reported in the current study. Certainly, a larger number of samples are required to further validate the suitability of these criteria in the clinical diagnosis of sCJD.

In conclusion, the present study evaluated the factors which may affect the RT-QuIC assay, and confirmed that RT-QuIC was capable of detecting traces of PrP<sup>Sc</sup>. Application of this assay using CSF samples in the present study revealed the potential use as a pre-mortem tool for the diagnosis of sCJD.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Authors' contributions

KX designed the study, acquired the data and prepared the manuscript; QS prepared the manuscript; JW, CG and BYZ assisted in the RT-QuIC assay; CC performed statistical analysis; WZ and QS prepared the samples; XPD, who was the corresponding author, designed the study and revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the National Institute for Viral Disease Control and Prevention (Beijing, China; protocol 2009ZX10004-101). Informed consent was obtained from all patients prior to participation.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no conflict of interest.

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