Project Title: Validation of a non-invasive diagnostic test for prion diseases using tear fluid
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Table of Contents

Summary of Proposed Research

Real-time quaking-induced conversion (RT-QuIC) is a highly sensitive and specific diagnostic test that detects misfolded prion proteins (PrPSc), which are associated with prion diseases such as Creutzfeldt-Jakob disease (CJD). The test amplifies the seeding activity of PrPSc through a template-induced conversion process, enabling its detection. RT-QuIC was originally established for cerebrospinal fluid (CSF) and has since become a routine diagnostic tool, demonstrating impressive results with a sensitivity of 80-89% and near 100% specificity. Recently, RT-QuIC has been adapted to detect prion proteins in other biological fluids, including tear fluid (TF), which could offer new possibilities for non-invasive diagnostic methods and longitudinal disease monitoring, particularly for evaluating therapeutic interventions.

The objective of this study is to validate RT-QuIC for detecting prion proteins in TF from patients with various prion diseases. Using a prospective cohort of patients with sporadic and inherited prion diseases, as well as non-prion disease controls, the study will address several key objectives: (1) evaluating the stability of TF samples under different conditions, (2) validating the diagnostic accuracy of RT-QuIC in identifying prion diseases such as sporadic CJD (sCJD), fatal familial insomnia (FFI), and Gerstmann-Sträussler-Scheinker (GSS) syndrome, (3) assessing disease progression through longitudinal TF RT-QuIC analysis, and (4) exploring the potential of RT-QuIC to detect early markers of prion diseases in asymptomatic PRNP mutation carriers.

The RT-QuIC protocol was refined for optimal sensitivity using recombinant prion protein substrates, including a mutant version like E200K, in both CSF and TF samples. We collected additional samples and significantly increased the patient sample size. Next, we evaluated the stability of TF samples under different conditions and defined the minimal amounts required. Afterward, we validated the diagnostic accuracy of TF RT-QuIC in prion diseases. For TF samples, the diagnostic sensitivity was 84% for sCJD and 78% for genetic prion diseases, with only 1 out of 184 control samples testing positive.

In conclusion, our results highlight the potential of RT-QuIC for diagnosing prion diseases in tear fluid, offering a less invasive, highly sensitive, and specific method for early disease detection and monitoring. The improved sensitivity with the FL Hu E200K substrate further enhances the test's diagnostic capabilities, paving the way for early diagnosis and the evaluation of therapeutic trials in prion diseases.

Progress Report:

1.Pre-analytic Studies:

The full-length human E200K substrate was selected for the TF RT-QuIC assay due to its superior sensitivity in detecting prion diseases in CSF and its reliable signal response characteristics. Comparative analysis of TF samples seeded with sCJD revealed that the E200K substrate produced distinct seeding response curves, while the chimeric hamster-sheep substrate showed no measurable activity even after 150 hours of testing (Fig. 1A). This underscores the E200K substrate's efficiency for the TF RT-QuIC.

To examine the stability of prion seeding activity in tear fluid, samples from four prion disease patients were divided into aliquots and stored under two conditions: -80°C and room temperature (RT) for five days. Analysis of the RT-QuIC kinetic parameters, including lag-phase and AUC, demonstrated no significant differences between the storage conditions. These findings confirm that prion seeding activity in TF remains stable for up to five days at RT, making the assay suitable for routine clinical workflows (Fig. 1B).

Furthermore, consistent positive RT-QuIC results were obtained when using the E200K substrate with tear fluid samples collected on Schirmer Tear Test strips that had a minimum wetting length of 15 mm. This corresponds to an estimated tear fluid volume of 10-20 µL, which is sufficient for reliable diagnostic outcomes (Fig. 1C-D). These results validate the practicality and robustness of the assay in detecting prion diseases using minimally invasive sampling methods.

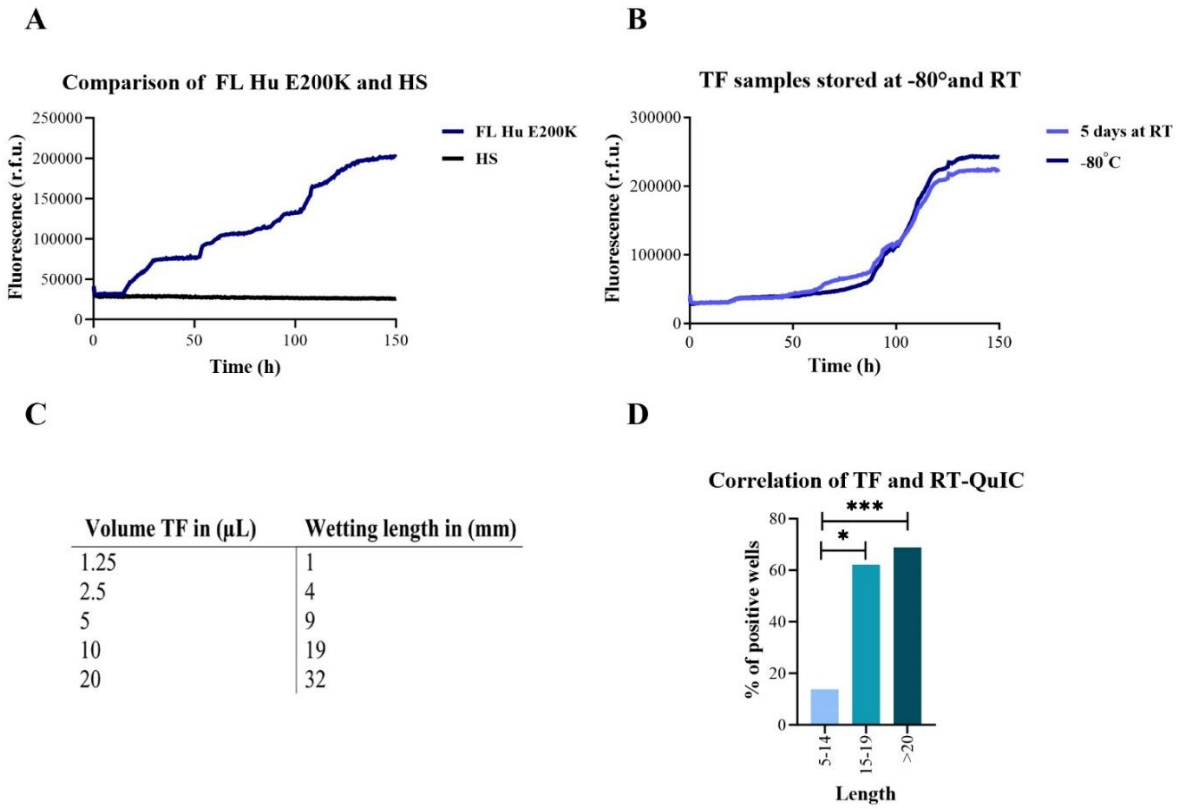

Fig. 1: TF RT-QuIC Seeding Kinetics, Stability, and Pre-Analytical Conditions
(A) RT-QuIC kinetic curves of sCJD tear fluid (TF) samples (n=4) using HS substrate (black) and FL Hu E200K substrate (blue). The HS substrate showed no detectable seeding activity over 150 hours.
(B) Stability test of sCJD TF samples (n=4) with FL Hu E200K substrate after storage at -80°C (dark blue) and at room temperature for 5 days (light blue). Both conditions showed comparable RT-QuIC responses, confirming assay reliability.
(C) Correlation between Schirmer Tear Test strip wetting length and TF volume (µL).
*(D) Positive RT-QuIC wells as a function of wetting length from Schirmer strips (n=20). A minimum wetting length of 15–25 mm is recommended for accurate diagnostics. Statistical analysis: one-way ANOVA with Tukey's post hoc test (*p < 0.05, ***p < 0.001).*

Effect of Gender, Age, and Codon 129 MV Genotype

The influence of gender, age, and codon 129 MV genotype on the outcomes of the TF seeding assay was systematically evaluated to determine if these factors affect diagnostic performance. Analysis of key seeding parameters, including lag-phase and AUC, revealed no statistically significant differences between male and female participants (Fig. 2A1-3).
Similarly, age did not appear to impact assay results, as no variations were observed between individuals younger than 50 years and those aged 50 or older (Fig.2 B1-3; C1-3).

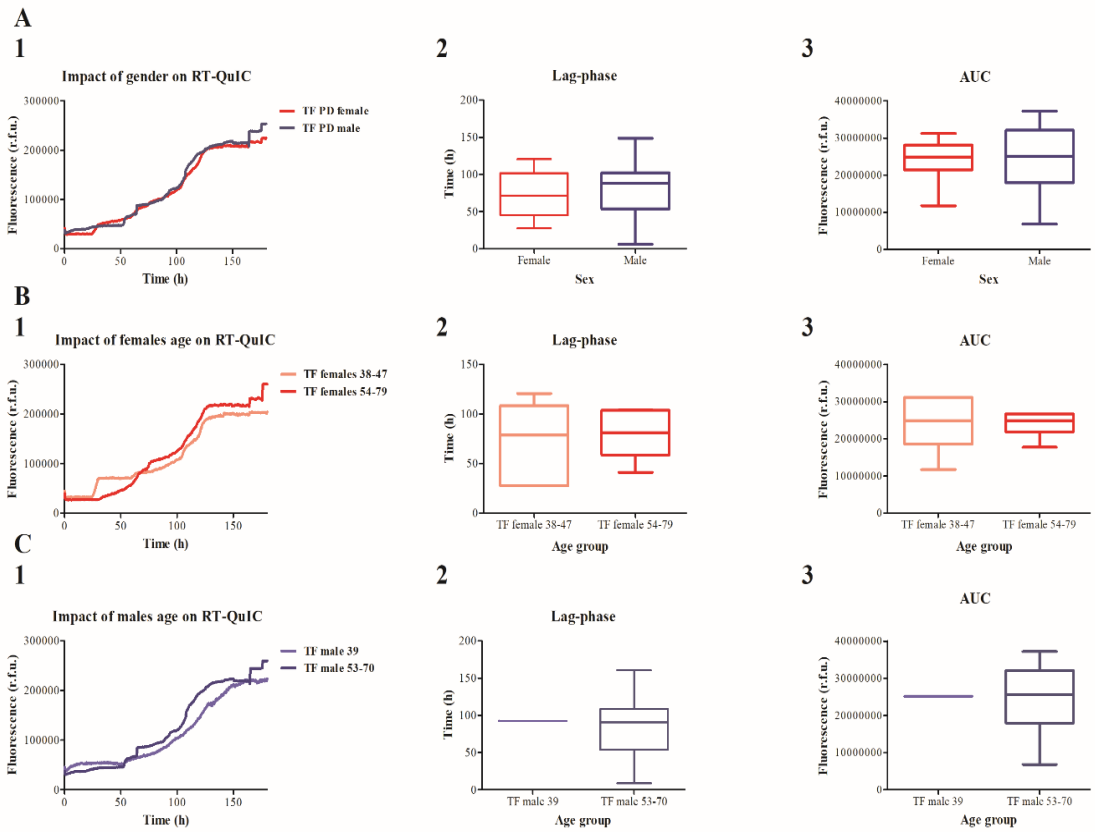

Figure 2: Impact of Age and Gender on TF RT-QuIC Signal Response
The TF RT-QuIC assay's signal response was analyzed across different age groups and genders in individuals with prion diseases. Kinetic curves of positive reactions included both male and female participants aged 38 to 79 years. Fluorescence intensity was compared between female (n=12) and male (n=11) samples (A1), and within age subgroups for males and females under and over 50 years old (B1, C1). No significant differences in signal response were observed based on gender (A) or age (B-C), highlighting the consistency of the TF RT-QuIC assay across these demographic factors.

Next we explored the impact of the PRNP codon 129 MV genotype on the signal response of the TF RT-QuIC. Our analysis showed no measurable effect of the codon 129 genotype on the RT-QuIC assay performance (Fig.3A-C).
These findings underscore the robustness of the TF RT-QuIC assay across diverse demographic and genetic subgroups, suggesting that it can reliably detect prion disease biomarkers without interference from these variables.

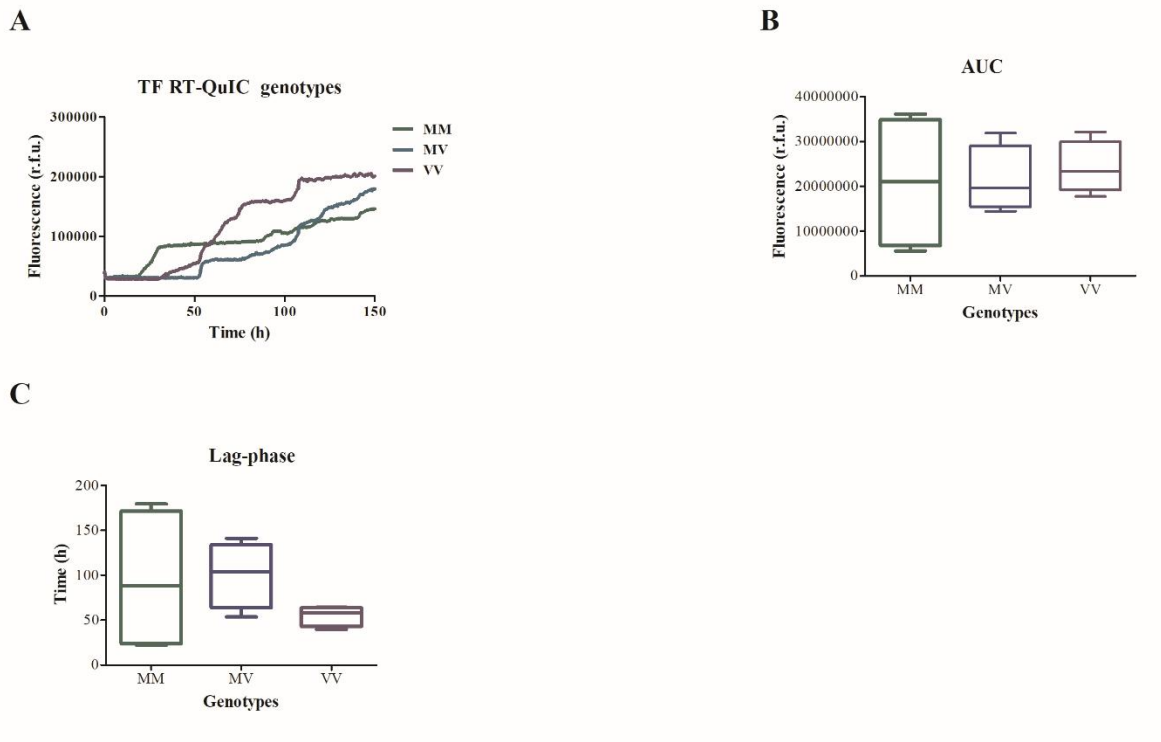

Figure 3. Impact of different sCJD codon 129 MV genotype on the TF RT-QuIC signal response.
A) Comparison of kinetic curves of positive TF RT-QuIC reactions of different sCJD codon 129 MV genotypes (MM, MV, VV). B-C) Applying the quantitative parameters, such as rel. AUC and duration of the lag-phase we obtained no significant differences in signal response in the TF RT-QuIC assay of different sCJD codon 129 MV genotypes.

2. Comparison to CSF RT-QuIC and calculation of the diagnostic accuracy

To compare the seeding activity or PrPSc in CSF and TF, we analysed TF and CSF samples of the same CJD patients via RT-QuIC. The comparative kinetic RT-QuIC curves from CSF and TF are displayed in Figure 4. The AUC was significantly lower ($p < 0.0001$) and the lag-phase was significantly longer ($p<0.01$) in TF samples compared to those seeded with CSF (Fig. 4B-C).

To study the potential effect of the total PrP levels on seeding activity, we used a homebrew assay for PrP based on SIMOA technology and analysed total PrP levels in TF and CSF of the prion diseases patients (n=4) and non-prion disease donors (n=4). In general, we observed a significantly higher amount of the total PrP in the CSF than in TF but no significant difference between prion diseases and non-prion diseases groups ($p=0.18$) (Fig. 4D).

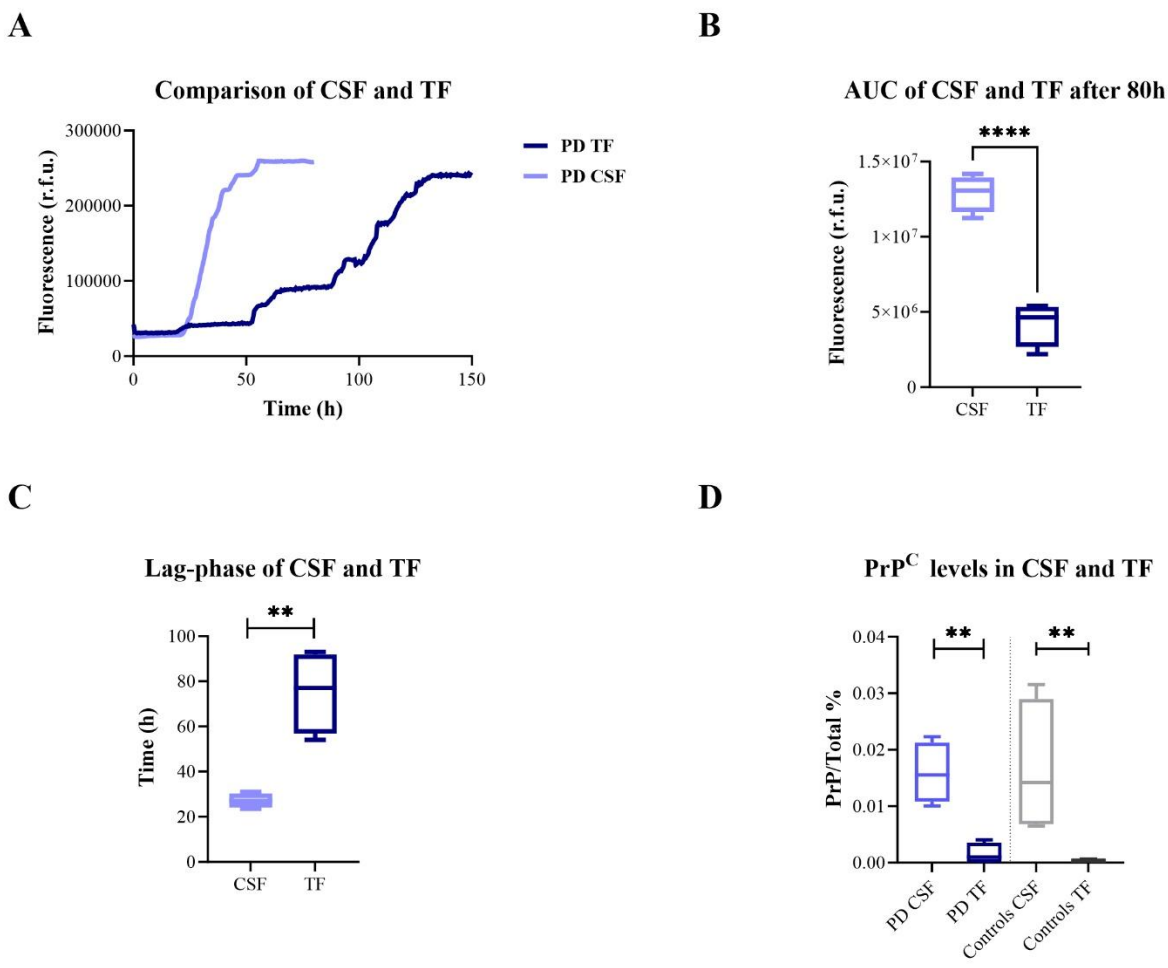

Figure 4: RT-QuIC Performance and PrPC Levels in CSF vs. TF Samples
(A) Fluorescence intensity curves from RT-QuIC analysis comparing CSF (light blue) and TF (dark blue) samples (n=4).
*(B) AUC analysis shows significantly higher fluorescence in CSF than TF (****p < 0.0001).*
*(C) Lag-phase duration is shorter in CSF compared to TF (**p < 0.01).*
*(D) Total PrPC levels (% of total protein) measured via SIMOA were significantly higher in CSF than TF for both patients and controls (**p < 0.01).*

To evaluate the diagnostic performance of the FL Hu E200K recombinant PrP substrate in detecting prion diseases using TF samples, we analyzed data from two combined cohorts (Table 1). These cohorts included patients with sporadic Creutzfeldt-Jakob disease (sCJD), genetic prion diseases, and non-prion disease controls.
In sCJD cases, the FL Hu E200K rec PrP substrate demonstrated an overall sensitivity of 84%. Among patients with genetic prion diseases, the assay achieved a sensitivity of 70% (Table 1).
From a total of 184 non-prion disease controls, only one sample—diagnosed with Alzheimer's disease—yielded a positive result in two out of three replicates, corresponding to a high specificity of 99.5% (Table 1).

A comparative analysis of the diagnostic accuracy between the two prion disease cohorts revealed a comparable rate of positive results for sCJD cases, with sensitivities of 87% and 84%, respectively. This consistency underscores the robustness of the FL Hu E200K rec PrP substrate in detecting prion-associated seeding activity across different sample sets.

***Table 1**. Diagnostic accuracies of TF RT-QuIC using the FL Hu E200K rec PrP substrate*

	1st cohort	**2nd cohort**	**Total Sensitivity**
sCJD	13/15 (87%)	14/17 (82%)	27/32 (84%)
Genetic prion diseases	4/5 (80%)	3/5 (60%)	7/10 (70%)
FFI	2/2 (100%)	1/2 (50%)	3/4 (75%)
GSS	1/1 (100%)	2/3 (67%)	3/4 (75%)
T183A	1/2 (50%)	-	1/2 (50%)
Non-prion diseases[3*]	0/68 (0%)	1/116 (99.1%)	**1/184 (99.5%)**

*3 Diagnosis: Healthy individual, immune-mediated disorders, AD, MS, ischemia, Parkinson disease, mixed dementia, VD and others

PUBLICATIONS AND ABSTRACTS

The following abstract was submitted in Translation Science Medicine and is now under consideration.

Advancing prion diagnostics: Novel RT-QuIC substrate enables detection in tear fluid and enhances cerebrospinal fluid sensitivity

Susana Silva Correia[1*], Matthias Schmitz[1*], Peter Hermann, MD[1], Stefan Goebel[1], Jaqueline Gerecke[1], Paul Lingor[2], Fabian Maass[1], Anna-Lisa Fischer[1], Sezgi Canaslan[1], Angela Correia[1], and Inga Zerr[1]

Abstract

The development of in vitro misfolded protein amplification systems, such as the real-time quaking-induced conversion (RT-QuIC) assay, has revolutionized prion disease diagnosis by detecting abnormally folded prion proteins (PrPSc) in tissues like brain and olfactory mucosa, as well as cerebrospinal fluid (CSF). Recently, we achieved a breakthrough by detecting PrPSc in tear fluid (TF), a non-invasive biofluid, warranting further evaluation.

We refined our RT-QuIC protocol to assess seeding conversion efficiency using various recombinant substrates, including human (FL Hu), and mutant versions like E200K and D178N, in both CSF and TF samples. Our study included patients with sporadic and familial prion diseases. FL Hu E200K showed the highest seeding efficiency, with sensitivity increasing from 71% to 92% for sporadic Creutzfeldt-Jakob disease (sCJD) and from 19% to 75% for fatal familial insomnia (FFI).

For tear fluid, diagnostic sensitivity was 84% for sCJD and 78% for genetic prion diseases. Notably, only 1 out of 184 controls without prion disease tested positive. Longitudinal studies showed that later disease stages resulted in stronger signals.

Our study demonstrates that the FL Hu E200K rec PrP substrate improves RT-QuIC sensitivity in CSF diagnostics and allows detection of seeding activity in TF. This robust test opens new avenues for early diagnosis and potential therapeutic trials.

Summary: The refined RT-QuIC protocol with FL Hu E200K improves sensitivity for prion disease detection in CSF and less invasive tear fluids.

Changes in Key Personnel

Dr. Susana Correia is replaced by Dr. Sezgi Canaslan Eyyuboglu, who is working in our group for many years and who can take the tasks from Mrs. Correia.

Paragraph that describes the project in layman's terms.

The real-time quaking-induced conversion (RT-QuIC) assay is a widely used, accurate laboratory test for detecting minuscule amounts of misfolded prion proteins in body fluids. The detection of pathological prion proteins in cerebrospinal fluid (CSF) using the RT-QuIC assay has become a standard method to confirm the clinical diagnosis of prion diseases, such as Creutzfeldt-Jakob disease.

Obtaining CSF through lumbar puncture is an invasive procedure, making it less suitable for follow-up studies and treatment evaluations that require samples from the same patient at different time points.

Due to modifications in the standard RT-QuIC protocol, we can now reliably detect pathological prions in tear fluid (TF), which is collected using a simple paper strip from the patient’s eyes.

In our project, we significantly increased the number of samples and optimized the protocol to achieve optimal reproducibility. Furthermore, we validated the TF-RT-QuIC method by testing a large, well-characterized cohort. This will enable us to implement the assay as a new non-invasive, patient-friendly, and cost-effective diagnostic test. Additionally, we plan to explore the suitability of TF-RT-QuIC for predicting disease onset and progression, which could provide new insights into the mechanisms of prion disease development and assist in the search for medical treatments.